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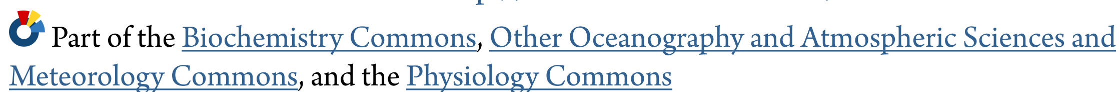
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Biochemical Aspects of the Thermal Sensitivity and Energy Balance of Polar, Tropical and Subtropical Teleosts

by

Eloy Martinez

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
College of Marine Science
University of South Florida

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marine, metabolism, mitochondria, thermal sensitivity, ATP, temperature

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Dedication

I will like to dedicate this dissertation to the people that made it possible. First and foremost, I dedicate this work to my wife and soulmate, Marietta, and our beautiful daughter Amelia, for being the continuous source of motivation and support that made this entire journey possible. I will also like to dedicate this work to my parents, Ana Elsie and Wilfredo, who taught me that the most important reason for living is to pursue one's dreams, which took me to become the first doctor in the family history. I also dedicate this work to my sister Mariela and my awesome nieces and nephews; might this achievement be proof to all of you that everything is possible in life. Lastly, but not the least important, I dedicate this work to my godparents Alfredo and Pilar; it is impossible to express in words how grateful I found myself for having your guidance, love and support since I was brought to this World. To all and each of you; I dedicate my career to you.

Quisiera dedicarles esta disertación doctoral a todas las personas que han hecho la misma posible. Primeramente, le dedico este trabajo a mi esposa y alma gemela Marietta, y nuestra bella hija Amelia, por ser la fuente continua de motivación y apoyo que ha hecho esta jornada enteramente posible. De igual manera, les dedico este trabajo a mis padres Ana Elsie y Wilfredo, quienes persistentemente me demostraron que la razón más importante de vivir reside en la persecución de tus sueños, que hoy me convierten en el primer doctor en la historia de nuestra familia. También le quiero dedicar mi trabajo a mi

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List of Acronyms

ADP	adenosine-5'-diphosphate
ATP	adenosine 5'-triphosphate
State II	Mitochondrial oxygen consumption associated with the addition of NADH and $FADH_2$ generating substrates
State III	Mitochondrial oxygen consumption associated with the addition of ADP in the presence of NADH and $FADH_2$ generating substrates
OXPHOS	Oxidative phosphorylation
LDH	Lactate dehydrogenase
CS	Citrate synthase
MDH	Malate dehydrogenase
ADH	Alcohol dehydrogenase
VO_2	Oxygen consumption rate
ETS	Electron transport system

Abstract

The maintenance of a functional energy balance in ectothermic fauna could be challenging in a thermally disparate environment. Biochemical adaptations at the enzyme and membrane levels allows for a set compensatory mechanism that allow the individual to maintain an energetic surplus, thus allocating energy for growth and reproduction. The present work describes how the energetic machinery in the cell, particularly the mitochondrion, could be affected by temperature changes. More specifically, this work aimed to determine how environmental temperature affects the mitochondria energetic performance of fishes from disparate thermal regimes.

Mitochondrial ATP production efficiency was evaluated in fishes from polar, tropical and subtropical regions. In polar fishes, mitochondria remained functional at temperatures well beyond whole organismal critical temperatures. On the other hand, tropical and subtropical teleosts exhibited a decrease in mitochondrial efficiency at temperatures commonly found during summer seasons. This remarkable variability of mitochondrial thermal sensitivity may restrict the energy allocated for growth and reproduction during the summer months in tropical and subtropical regions. The observed variation in the thermal window of tolerance of mitochondrial function in fishes provides further insight into how the energetic machinery responds to thermal changes, like those associated with warming trends in marine ecosystems. In addition, the reduced efficiency in mitochondrial function among teleosts from latitudinal gradients suggest that warm-

adapted species are close to their upper tolerance range, and further warming trends could severely impact the energy budget of fishes.

Chapter One:

Introduction

Biochemistry of thermal stress

In terms of the absolute temperature of biological systems, both terrestrial and marine ecosystems are considered to be unsuitable for most chemical reactions to occur without a biological catalyst. As a result, evolutionary processes have in turn facilitated the development of biochemical catalysts, in the form of enzymes. In other words, the evolution of enzymes has reduced the activation energy necessary for the feasibility of biochemical reactions in cells at low absolute temperatures.

Enzymes are remarkable biochemical complexes, where a linear sequence of amino acids conform a primary structure, which progressively folds upon formation due to intermolecular interactions. Weak bonds interactions amongst amino acids are responsible for the secondary, tertiary and quaternary structure levels of the enzyme. These weak intermolecular interactions comprise hydrogen bonds, disulfide linkages and hydrophobic interactions, and their formations are crucial to the catalytic properties of the enzyme (Hochachka and Somero 1973). Due to the high specializations of enzymes, their catalytic ability of enzymes could be impinged with minuscule changes in the kinetic energy (i.e. temperature) of the environment, thus affecting the physiological processes depending on their functionality.

Historically, the effects of temperature on the catalytic properties of enzymes have been evaluated for many decades (Arrhenius 1915; Haldane 1931; Kavanau 1950). In most enzymatic reactions, when temperature was altered by 10°C, the rates of enzyme-catalyzed reactions (Q_{10}) were two to three times of the rates found before the change in temperature. This phenomenon was explained in an elegant fashion in the late 19th century by Svante Arrhenius, when he proposed an energy-based explanation to the drastic changes in the catalytic properties of enzymes. In essence, the energetic state of a given population of enzymes in biological systems follow a Maxwell-Boltzmann distribution, comprising a bell-shaped curve distribution where only the fraction of the population of enzymes with kinetic energy equal or higher activation energy are available to react. In other words, only a small fraction of the available enzymes are readily available to react at a given temperature. Furthermore, the fraction of enzymes available for catalysis can change drastically with changes in temperature. Thus, changes in ambient temperature could impose a threat to the balance of biochemical processes in ectothermic fauna.

Metabolic regulation in a dynamic external milieu has been a major challenge for both terrestrial and marine ectotherms. Environmental temperature can alter two main components of the metabolic machinery. First, changes in the kinetic energy of the system (i.e. temperature) will alter the thermodynamic balance of metabolic reactions. This 'rate effect' is evident in enzyme catalyzed reactions, where lower temperature may reduce the number of enzymes with the necessary activation energy, thus reducing the amount of product formation over time. Second, changes in temperature could alter the interaction between weak bonds among molecules, altering the three-dimensional

structure of enzymes. Weak-type bonds such as hydrophobic interactions among amino acids are mostly responsible for the quaternary structure of the enzyme, and could be easily disrupted by temperature changes, rendering the catalytic ability of the enzyme. In response to the thermal effects on rate and weak bond interactions, the biochemistry of ectotherms allows for an elegant set of compensatory strategies, which will be further discussed.

Within the many challenges often posed to ectotherm homeostasis, organismal responses to environmental temperature changes have been one of the most thoroughly studied. Adaptive strategies to temperature occur in various time scales, which provide the organism of both short term, as well as long term biochemical mechanisms to compensate for changes in temperature (Hochachka and Somero 1973). Overall mechanisms of compensation include:

- Changes in the amount of enzymes available to catalyze a given reaction;
- Changes in the amount of modulators (e.g. ATP:ADP:AMP) of enzyme activity;
- And changes in the type of enzyme (i.e. isozyme) available to catalyze the reaction.

Short term responses are often characterized by a quantitative strategy, where biochemical reactions are regulated by the abundance of the enzyme catalyzing the reaction. This has been observed in fishes, where thermal acclimation induced changes in enzyme concentration (Hochachka and Somero 1968; Sidell et al. 1973). Although metabolic compensation have been observed in many fish species, the magnitude and

direction of the compensatory mechanism seems to be tissue-specific (Shaklee et al. 1977) , where changes in the quantitative level of enzymes are often observed within days or weeks of acclimation.

Seasonal changes in enzyme levels have also been found in various marine ectotherms, where researchers have found combinations of compensatory strategies, including the expression of isozymes that are 'better' suited for the new thermal regime, in conjunction with changes in the concentration of other enzymes. In summary, temperature driven compensation revolves in significant changes in the cell's biochemistry over short and long term periods, and involves a variety of strategies, often species and tissue specific.

Compensatory strategies also apply to lipid membranes, which are as thermally sensitive as enzymes complexes. In membranes, compensatory mechanisms are found to be analogous to the those found in enzymes, where quantitative and qualitative alterations of the fatty acid composition and abundance with changes in temperature have been documented (Hazel and Williams 1990). These mechanisms are essential in the maintenance of a homoviscous state, necessary to sustain routine physiological function (Hazel 1995). In the mitochondrion, alteration of the inner membrane viscosity could be highly detrimental to ATP production, as membrane integrity is vital in the formation of the electrochemical gradient necessary for ATP production. Changes in the abundance of polyunsaturated fatty acids (PUFA) have been observed in gill mitochondria of fishes acclimated to various temperatures (Caldwell and Vernberg 1970), where high levels of PUFA's may have resulted in a less viscous inner membrane at lower temperatures. Thus,

homoviscous adaptation of lipid membranes, as well as regulation of enzymes, is central to sustain essential processes, such as ATP production in the mitochondrion.

The biological purpose of metabolic compensation is to reduce energy allocated from metabolism and allocate it as growth (reproductive and/or somatic). In essence, an organism will be able to operate with an energetic surplus over a particular thermal range, delimited by the organism's ability of thermal adaptation. Often, this tolerance range is illustrated as a thermal tolerance polygon (Brett 1952; Brett 1956; Brett and Groves 1979; Eme and Bennett 2009), where the size of the tolerance polygon is a direct reflection of the organism thermal window of tolerance. Within an organism's thermal tolerance window, metabolic adjustments allow for the allocation of energy towards growth (somatic or reproductive). This growth performance is reduced as environmental temperature shifts towards tolerance extremes, thus reducing growth performance.

Current warming trends in marine ecosystems has raised the question whether marine organisms will be able to cope with climate change, as thermal resilience is not a universal characteristic of marine fauna. More specifically, it is of particular relevance how marine fauna inhabiting at the extremes of their tolerance ranges will be affected, and to what extent the energy budget could be adversely affected.

Thermal resilience of Antarctic teleosts

The geographic distributions of marine ectotherms are tightly bound to latitudinal temperature gradients, and their tolerance to fluctuations in environmental temperature is limited to particular ranges (Somero 2005). In Antarctic ectotherms, thermal adaptation to low temperatures has developed with a reduction in tolerance towards higher

temperatures (Somero and DeVries 1967). The ability to carry out metabolic processes at low (<4°C) temperatures is the result of changes in the primary structure of biological macromolecules, leading to conformational changes in enzymes, membranes and other key macromolecules. For example, enzymes of cold adapted ectotherms display a higher catalytic rate constant (K_{cat}) and a lower change in activation enthalpy (ΔE_a) than their tropical counterparts (Hochachka and Somero 2002). It has been shown that changes in as little as a single amino acid (e.g. glycyl residues) of an enzyme's primary structure can confer a higher 'flexibility' and increase the specific activity at lower temperatures by facilitating substrate-enzyme complex formation (Holland et al. 1997; Fields and Somero 1998).

In addition to temperature effects on enzyme-catalyzed processes, membranes are highly susceptible to temperature changes. Temperatures at the low end of a species' range will reduce membrane fluidity, potentially slowing transport processes and compromising membrane integrity with a more crystalline, porous structure. Higher than optimal temperatures result in increased fluidity, also affecting transport processes and the effectiveness of the membrane barrier. Membrane fluidity may be adjusted through homeoviscous adaptation by modifying the concentration of cholesterol and adjustments of the head group and the saturation level of acyl chains in the bilayer (Hazel 1995). Thus, the performance of membrane-based systems like mitochondria will be dependent upon the ability to adapt and preserve its functionality within a temperature range.

One of the most utilized techniques to evaluate mitochondrial thermal sensitivity is the determination of respiratory states of a mitochondrial isolation as a function of temperature (Figure 1.1).

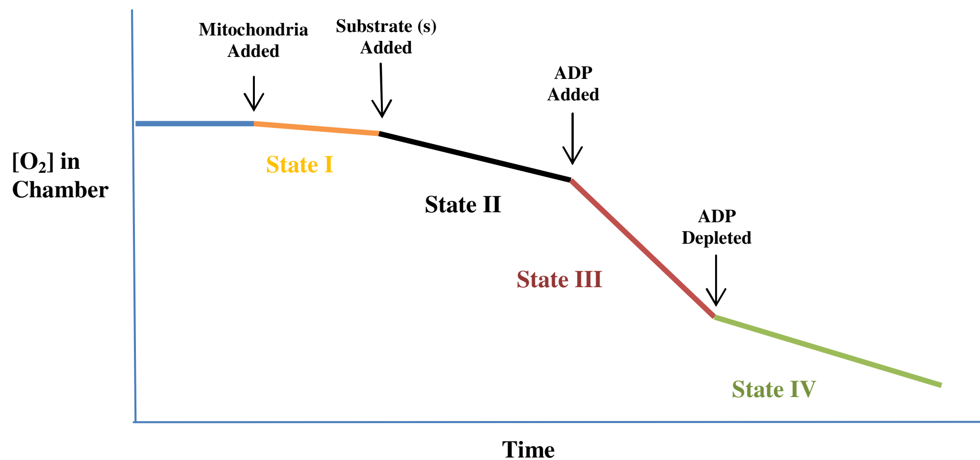

Figure 1.1: Conceptual diagram of the respiration states of mitochondria. Modified after Nicholls and Ferguson (2002).

Briefly, the respiration state I correspond to the oxidation of metabolites present in the tissue after homogenization and the activities of multifunction oxidases. State II respiration rates correspond to the activation of the Electron Transport System (ETS), activating the proton pumps of mitochondrial complexes I III in addition to fueling cytochrome *c* oxidase and transferring electrons to molecular oxygen, forming water. The electrochemical potential formed by pumping protons out into the intermembrane space elicit a proton leak back to the inner matrix, exerting a marginal respiration that is uncoupled from ATP production. A state III rate corresponds to the dissipation of the proton gradient by channeling of protons through the F_0F_1 ATPase after the addition of ADP, thus coupling oxygen consumption with the formation of ATP. After depletion of ADP, mitochondrial respiration is reduced to the uncoupled state IV, which is usually similar to state II. Respiration rate between ATP-coupled (State III) and ATP-uncoupled (State II, IV) states as a function of temperature is compared to determine the coupling efficiency of the Oxidative Phosphorylation (OXPHOS) system.

Thermal sensitivity of mitochondrial function has been evaluated for a number of polar teleosts (Sidell et al. 1973; Weinstein and Somero 1998; Hardewig et al. 1999; Lannig et al. 2005; Perry et al. 2005; Mark et al. 2012). Thermal sensitivity of mitochondrial function has been evaluated for a number of polar teleosts (Sidell et al. 1973; Weinstein and Somero 1998; Hardewig et al. 1999; Lannig et al. 2005; Perry et al. 2005; Mark et al. 2012). If the thermal tolerance of mitochondria is compared to the thermal tolerance window of the whole organism, isolated mitochondria from Antarctic teleosts can function properly in a wider thermal range (Somero and DeVries 1967; Weinstein and Somero 1998). Although the mitochondrial thermal tolerance range is wider than in higher order systems (cell, tissue, individual), understanding the relationship between temperature and energy balance is pivotal to describe how organisms will sustain energetically expensive activities (i.e. reproduction, somatic growth, feeding) under long-term temperature changes, like those associated with global climate change (Somero 2010).

The thermal sensitivity of mitochondria is the result of structural and conformational changes of the inner membrane and its associated proteins, as well as the enzymes associated with the citric acid cycle in the matrix of the organelle. When acclimated to low temperatures, enzymes associated with the oxidative phosphorylation (OXPHOS) system increase their activity levels in temperate and Antarctic zoarcids (Lannig, Storch et al. 2005). When mitochondria from Antarctic species are exposed to temperatures over 10°C, the overall phosphorylation performance declines. Respiratory control ratios (RCR), an indicator of OXPHOS efficiency, decrease as temperature increases due to O_2 consumption associated with proton leakage over the inner

mitochondrial membrane (Lannig et al. 2005; Mark et al. 2006). The temperatures at which oxidation of redox carriers (e.g. NADH, $FADH_2$) uncouple from ADP phosphorylation by the F_0F_1-ATPase depends on the average habitat temperature. The temperature limit at which mitochondrial energy production cannot be sustained is often described by using an Arrhenius plot when comparing respiration rates (Weinstein and Somero 1998; Hardewig et al. 1999; Perry et al. 2005; Mark et al. 2012). A peak in phosphorylation efficiency is often reflected in the Arrhenius breakpoint in temperature (ABT), a breakpoint in the efficiency of the ATP generating machinery of the mitochondrion.

There is a considerable interspecific variability of ABT for Antarctic teleosts, with a range from 9-30.5°C found in the literature (Weinstein and Somero 1998; Hardewig et al. 1999; Perry et al. 2005; Mark et al. 2012). Throughout the second chapter of the present study, the author's goal was to provide additional information on the thermal sensitivity of the OXPHOS system in Antarctic fishes, in addition to discussing relevant differences observed in OXPHOS capacity among the species studied.

Thermal resilience of subtropical and tropical teleosts

Global climate change over the next century is expected to significantly alter marine fish communities at all latitudes. Gradual increases in water temperature have been shown to cause shifts in population centers of many teleost species inhabiting temperate regions (Perry et al. 2005). In tropical regions, increasing water temperatures have been correlated with decreasing abundance and diversity in fishes associated with coral reef assemblages (Jones and Mann 2004; Graham et al. 2006). Thus, increasing

water temperatures associated with climate change are likely to have profound impacts on survival and recruitment of fishes in coral reef communities (Hughes et al. 2003).

Thermal tolerance and sensitivity estimates of fishes have been used to examine potential effects of climate change in marine teleosts (Mora and Ospina 2001; Ospina and Mora 2004). Regional warming trends like those affecting the tropical eastern pacific during el Niño events (Jokiel and Coles 1990) and their relation to coral mortality are evidence of the negative effects of warmer temperatures on tropical reef communities. Observed changes in the community assemblages of corals (Graham et al. 2006) have impacts on associated vertebrate and invertebrate communities.

Thermal stress can occur at a variety of levels of biological organization, from biochemical reaction rates to cellular responses and further to behavioral changes, shaping the community structure of coral reef fishes. Although a wide variety of studies have focused on the physiological effects of temperature in fishes, most of our current knowledge is derived from studies performed on marine teleosts from temperate and polar regions (Somero and DeVries 1967; Hochachka and Somero 1968; Somero and Hochachka 1968; Low et al. 1973; Kultz and Somero 1996; Fields and Somero 1998; Weinstein and Somero 1998; van Dijk et al. 1999; Lucassen et al. 2003; Hardewig et al. 2004; Buckley et al. 2006; Brodte et al. 2008; Hilton et al. 2010), or tropical reef-building corals and invertebrates (Coles et al. 1976; Jokiel and Coles 1990; Gates and Edmunds 1999). To date, the physiological responses of reef- associated and estuarine fishes to thermal insults associated with urban warming and overall global climate change remain unclear. Previous studies have shown that the critical thermal maxima (CTM) for Pacific reef fish species are at least eight degrees Celsius higher than the warmest temperature

found in their natural environment (Mora and Ospina 2001; Pérez et al. 2003). More important, Mora and Ospina (2001) also showed that responses to temperature are species specific, with a significant degree of variability among species living in similar temperatures. Although critical (or lethal) temperatures might not reflect an immediate threat to fishes, it could constitute a significant disadvantage for those species operating close to their thermal maximum in terms of their physiological performance, compromising the energetic surplus allocated for growth and reproduction.

To date, our understanding of how tropical fish communities will react to climate change is limited (Munday et al. 2008). To better understand the effects of thermal stress in tropical fishes at the cellular level, it is in turn, important to understand how temperature affects the ATP-generating processes in the mitochondrion. Although critical thermal maxima provide relevant information on acute thermal effects, there is a clear need to determine sub-lethal effects of temperature on the bioenergetic machinery of coral-reef and estuarine fish species. Various studies have addressed how temperature affects important biochemical characteristics of fishes such as mitochondrial OXPHOS capacity as a function of temperature in polar and temperate regions (Hazel and Williams 1990; Weinstein and Somero 1998; Hilton et al. 2010; Mark et al. 2012). **Unfortunately, studies describing the OXPHOS system in tropical or subtropical marine species have yet to be performed. With this need in mind, a comparative baseline of the OXPHOS system was performed between various tropical and subtropical species in the fourth and fifth chapter, where values obtained for pelagic and benthic estuarine species were presented and discussed in detail.**

Objectives

To understand the response of ectotherms to thermal changes, it is imperative to understand how its energy budget is affected, in addition to how 'thermally flexible' its energetic machinery is. **The author's main goal was to provide a comparative study of how mitochondrial function, specifically OXPHOS capacity, is affected by temperature, and to characterize relevant differences of mitochondrial function among species.** Results shown describe the mitochondrial bioenergetics machinery of various polar, tropical and subtropical teleosts, in addition to multiple-level biochemical analysis of an Antarctic notothenioid.

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Chapter Two:

Mitochondrial Energetics of Benthic and Pelagic Antarctic Teleosts

Introduction

Since the separation of Antarctica from South America, and its subsequent physical isolation by the Antarctic Circumpolar Current 21-22 mya, Antarctic fauna have undergone major adaptive changes to tolerate low environmental temperatures (Eastman 1993). Adaptations to low temperature in Antarctic ectothermic fauna, particularly in fishes, have included a high level of stenothermy (Somero and DeVries 1967). For example, at the whole organism level, the upper incipient lethal temperature of 6°C in the notothenioids *Trematomus bernacchii*, *Trematomus hansoni* and *Trematomus borchgrevinki* is a clear reflection of their stenothermal character (Somero and DeVries 1967). Recently, experiments carried out by Bilyk and DeVries (2011; 2012) have demonstrated that gradual acclimation of Antarctic notothenioids can increase their upper critical temperatures. This new evidence suggests that notothenioids could have a greater potential for adapting to the warming trends observed in the Western Antarctic Peninsula (Vaughan et al. 2003; Turner et al. 2005) than previously thought.

The molecular underpinnings of cold adaptation in notothenioids have received considerable attention in the past 50 years. This includes the description of respiratory

pigments (Tamburrini et al. 1997; Di Prisco et al. 1998) and the characterization of antifreeze glycopeptides (Somero and DeVries 1967; DeVries and Wohlschlag 1969; DeVries 1971). In addition to adaptations that prevent extracellular ice nucleation, various comparative studies investigated the level of cold adaptation of notothenioids, ranging from key enzymatic activities to whole organism oxygen consumption (Wohlschlag 1960; Torres and Somero 1988). More recently, various studies described the thermal-tolerance window of the mitochondrial oxidative phosphorylation (OXPHOS) system in Antarctic teleosts. These studies provide valuable information of the thermal plasticity of the moderately active, benthic species *Trematomus bernacchii* (Weinstein and Somero 1998), *Lepidonotothen nudifrons* (Hardewig et al. 1999a), the less active *Notothenia coriiceps* (Johnston et al. 1994; Mark et al. 2012) and *Notothenia rossii* (Mark et al. 2012). In addition, the thermal sensitivity of ADP-stimulated (state III) respiration based on Arrhenius breakpoints in temperature (ABT) of the red blooded notothenioid *Gobionotothen gibberifrons* and the hemoglobinless *Chaenocephalus aceratus* have shown similar levels of thermal plasticity in notothenioid mitochondria (Urschel and O'Brien 2009). Weinstein and Somero (1998) found an Arrhenius breakpoint in the state III respiration rate at 20.3°C for *Trematomus bernacchii* liver mitochondria, several degrees higher than its upper lethal temperature of around 5°C (Somero and DeVries 1967). Similar Arrhenius plots have been employed to describe the thermal sensitivity of OXPHOS in notothenioids (Hardewig et al. 1999a; Urschel and O'Brien 2008; Mark et al. 2012), where variable breakpoints in state III respiration have been observed between 9°C and 31.5°C.

Although interspecific differences in mitochondrial substrate preferences have been recognized (Johnston et al. 1994), only a few studies describe how substrate combinations affect OXPHOS capacity of fish mitochondria. Mark and coworkers (2012) have described how the mitochondrial complexes I and II contribute to the overall OXPHOS capacity in *Notothenia coriiceps* and *Notothenia rossii*. Moreover, they have shown in the notothenioids *N. coriiceps and N. rossii* that complex-specific contributions to the OXPHOS system are non-additive, which suggest that both complexes should be experimentally activated in order to obtain better estimates of OXPHOS capacity. Previously investigated rates of oxygen flux have used a variety of substrate combinations and inhibitors. Moyes and colleagues (1989) performed a variety of assays in which mitochondrial isolations from *Cyprinus carpio* were exposed to single substrate titrations. Since then, studies in notothenioids combining up to three substrates have been conducted (Weinstein and Somero 1998; Hardewig et al. 1999b; Mark et al. 2012). Recent advances in mitochondrial physiology have provided insights into the use of substrate combinations for maximally engaging electron carriers and substrate transporters for measurements of OXPHOS capacity in mammals (Gnaiger 2010a; Gnaiger 2010b). The activation of both mitochondrial complexes I and II in fish mitochondria by the simultaneous addition of milimolar levels of pyruvate, glutamate, malate and succinate have been proven useful in activating mitochondrial respiration in fishes (Cook et al. 2013). However, studies regarding whether simultaneous activation of complex I and II increases OXPHOS capacity in notothenioids have not been performed.

The two main goals of our studies were a) **to investigate the contributions of NADH-UQ oxidoreductase (Complex I) and succinate dehydrogenase (Complex II)**

activities to respiration rates measured under OXPHOS conditions in liver mitochondria of the benthic notothenioids *Trematomus loennbergii, Notothenia coriiceps* and *Lepidonotothen nudifrons*, and b) **to compare these respiration rates to rates found in the pelagic notothenioid *Pleuragramma antarcticum* and the pelagic myctophid *Gymnoscopelus braueri*.** Furthermore, **we investigated the thermal tolerance window of liver mitochondria from the Antarctic silverfish, *Pleuragramma antarcticum*, to assess any possible 'breaks' in the oxidative phosphorylation system as a function of temperature** and we demonstrate herein a remarkable tolerance of the OXPHOS system of *P. antarcticum* towards warmer temperatures.

Methodology

Specimen collection. Adult specimens of *Pleuragramma antarcticum* and *Gymnoscopelus braueri* were collected in the upper 1000 meters of the water column of the Western Antarctic Peninsula (WAP) shelf from March 25 to April 27, 2010 aboard the RV/IB Nathaniel B. Palmer. Pelagic specimens were collected both day and night using a Multiple Opening and Closing Environmental Sampling System (MOCNESS, 10m^2 mouth area) and a Tucker Trawl (9 m^2 mouth area). Benthic specimens (*Lepidonotothen squamifrons, Trematomus loennbergii*) were collected with an otter trawl. *Notothenia coriiceps* specimens were kindly provided by Dr. Arthur DeVries, which were caught in shallow water (2–3 m deep) by hook and line in the vicinity of Anvers Island (Palmer Station). Both benthic and pelagic specimens were kept in large (~2000 L) flow-through seawater tanks at 0°C (± 2°C) for less than a week, with the exception of *Notothenia coriiceps* which were held in flow through seawater tanks for ~1

month prior to analysis and fed freshly caught *Euphausia superba* every 2-4 days. Specimen density varied from 3-20 individuals per 2000 L tank.

Isolation of liver mitochondria. Fresh livers were excised, weighed to the nearest 0.01 g and processed according to Weinstein and Somero (1998), with minor modifications. Briefly, liver tissue from one or more individuals (~2.0 g of liver tissue) was minced in an ice-cold petri dish, then homogenized in 8 mL of a sucrose-based isolation medium (250 mM sucrose, 1 mM EGTA, 10 mM K_2PO_4, 1% BSA, pH = 7.4, 5°C) using an ice-cold Dounce homogenizer (Kontes, Vineland, NJ) and 5 passes with a loose fitting pestle followed by 2 passes with a tight fitting pestle. The homogenate was distributed among 1.5 mL centrifuge tubes and centrifuged at 650 *g* for 10 min at 4°C to remove cellular debris and undisrupted tissue. The supernatant was collected and again centrifuged at 9,600 *g* for 15 min at 4°C to sediment the mitochondrial fraction. Pellets were washed with isolation medium, resuspended and recollected by centrifugation at 9,600 *g* for 15 min at 4°C two consecutive times. The final pellet was suspended in 300-500 µL of isolation medium and stored on ice until assayed.

Thermal tolerance of *Pleuragramma antarcticum* liver mitochondria. To assess the thermal sensitivity of *P. antarcticum* mitochondria, a high-resolution respirometry system was employed. This system comprises a 2.0 mL water-jacketed respirometric chamber (DW-1, Hansatech Instruments, Norfolk, England) attached to a Clark-type polarographic electrode (C-1, Hansatech Instruments, Norfolk, England). Each respirometric chamber (2 in total) was calibrated at the desired assay temperature in the presence of 500 µL of respiration medium. At each temperature, the background signal was recorded prior to mitochondrial injection. For each run, 20-50 µL of purified

mitochondria was injected into the respirometer chamber containing 500 µL of a 290 mOsm potassium-based respiration medium prepared following Weinstein and Somero (1998), with minor modifications (100 mM KCl, 1% w/v BSA, 2 mM $MgCl_2$, 1 mM EGTA, 25 mM K_2PO_4, 10 mM Tris-HCl, pH = 7.5 at 5ºC). Oxygen consumption was monitored at assay temperatures ranging from 5-35°C. Measurements at habitat temperature (-2 to 2°C) were not possible due to instrument shortcomings. Substrate stocks were carefully prepared according to Lemieux and Gnaiger (2010). To assess the temperature sensitivity of the OXPHOS system, the following substrates were injected consecutively to each chamber: pyruvate, malate, glutamate and succinate. Final substrate concentrations in each chamber were 5 mM pyruvate [P], 2 mM malate [M], 10 mM glutamate [G] and 10 mM succinate [S]. Respiratory states were determined as described in Nicholls and Ferguson (2002). Substrate induced respiration was recorded as state II respiration, and was monitored for approximately 10 minutes. After state II respiration rates stabilized, ADP–stimulated respiration (state III, OXPHOS) was determined after adding 2 mM ADP (10 µL from a 100 mM neutralized ADP stock) into the chamber. State III respiration was recorded until rates stabilized or the chamber became anoxic. ADP concentrations of 2 mM in the chamber were necessary due to a limiting effect of lower ADP in state III observed in earlier experiments, where in some runs state III rates were reduced 33% with ADP concentrations of 1 mM or less at 5ºC (data not shown).

Statistical analysis and Arrhenius breakpoint in temperature (ABT) calculations. State II and state III respiration rates were calculated from the slope in oxygen consumption in each run, and averages were calculated along with their standard error. To understand the effects of temperature on OXPHOS, Arrhenius breakpoints in

temperature for state III respiration rates were calculated using a 2-phase piecewise regression (Nickerson et al. 1989), on averaged respiration rates, where least squares were calculated through 200 iterations (Sigma plot 12.3, Systat Software). Respiratory control ratios (RCR) were calculated via dividing state III respiration rates by state II respiration rates. RCR values were tested for significant differences by employing a one-way ANOVA on ranks, for variable sample sizes. Significance level was set to $P < 0.05$ for all data present in this study. Complex-specific differences in state III respiration were evaluated by employing a one-way ANOVA, $P < 0.05$. In addition, a two-way ANOVA ($P < 0.05$) was performed to describe the differences in state III rates observed between complexes and among species.

Oxidative capacities and complex-specific contributions to the ETS in Antarctic teleosts. To investigate the contribution of specific mitochondrial complexes to overall mitochondrial function, a variety of substrate combinations were tested following the rationale of Gnaiger and coworkers (2010a). Liver samples of *Trematomus loennbergii, Notothenia coriiceps, Lepidonotothen nudifrons* and the myctophid *Gymnoscopelus braueri* were processed as described for *P. antarcticum*. The respirometer was calibrated at 10°C with 500 µL of respiration medium, and 20-50 µL of purified mitochondria were added to each chamber. Each liver sample was assayed at substrate combinations that feed electrons through NADH-UQ oxidoreductase (complex I). Each isolation was independently assayed for complex I employing three substrate combinations; P+M, M+G, P+M+G, at final concentrations reported previously for *P. antarcticum*. This sequence allowed us to determine any additive effects of substrates within complex I. However, no differences among these substrate combinations were

observed and data were pooled for statistical purposes. Assays for succinate dehydrogenase (Complex II) activity were performed after the addition of 10 mM succinate in the presence of 0.5 µM rotenone. In addition, separate experiments were performed where complex I and complex II were activated with a simultaneous addition of P+M+G+S. To ensure a clean chamber between runs, each chamber was thoroughly washed three times with deionized water, three times with 75% ethyl alcohol and three more times with deionized water. Similar to the thermal sensitivity assay, substrate induced respiration was recorded as state II respiration, and was monitored for approximately 10 minutes. After state II respiration rates stabilized, state III respiration was determined after adding 2 mM ADP (10 µL from a 100 mM neutralized ADP stock) into the chamber. State III respiration was recorded until rates stabilized or the chamber became anoxic.

Hepatosomatic Index (HSI). Immediately before the experiment, specimens were weighed to the nearest 0.1 g in order to establish an energy reserve estimate by calculating the hepatosomatic index (HSI) for each species. Liver samples were blotted dry and weighed after excision to the nearest 0.01 g. Hepatosomatic indexes were calculated by dividing total liver weight in grams by the total body mass of the specimen.

Mitochondrial protein quantification. Total protein in each sample was quantified according to Bradford (1976), using the commercially available Better Bradford Commassie Stain Assay (Thermo Scientific, Rockford, IL). Samples were diluted 20:1 in deionized water and absorption values were determined after 10 min incubation with a Cary 1 spectrophotometer at 20°C and $\lambda = 595$ nm. Protein values were

corrected by quantifying the concentration of BSA present in the isolation buffer and subtracting it from the total protein content in the mitochondrial isolation.

Results

Thermal tolerance in *Pleuragramma antarcticum.* Activation of the convergent pathway of electron entry into the ubiquinone pool (Q-junction) of *Pleuragramma antarcticum* was achieved by the addition of saturating ADP levels in the presence of pyruvate, malate, glutamate and succinate. Leak respiration (state II) rates ranged from 6.29 ± 0.37 at 5°C to 15.36 ± 0.57 nmol O_2 min^{-1} mg $protein^{-1}$ at 15°C (n = 8-10 ± SE). The thermal response of a single mitochondrial isolation exemplifies the gradual increase in state II respiration rate observed with increasing temperature (Figure 2.1). The state II respiration rates increased 10-fold from 5.99 ± 0.37 nmol O_2 min^{-1} mg $protein^{-1}$ (n = 12, ± SE) at 5°C to 64.0 ± 6.97 nmol O_2 min^{-1} mg $protein^{-1}$ at 30°C (n = 4, ± SE) (Figure 2.2a). Results from a two-phase regression calculation indicate an Arrhenius breakpoint for state II respiration at 16.7°C and the calculated Q_{10} for state II respiration between 5°C and 15°C was 2.63 ± 0.22 (n = 8, ± SE).

Oxygen consumption in presence of substrates and ADP (OXPHOS, state III) increased with temperatures from 5-20°C (Figure 2.2b). At 5°C, state III respiration rates were lowest at 76.2 ± 4.6 (n = 10, ± SE) nmol O_2 min^{-1} mg $protein^{-1}$. As assay temperature increased, so did state III respiration rates, with a maximal rate registered at 20°C of 169.9 ±16.0 (n = 8, ± SE) nmol O_2 min^{-1} mg $protein^{-1}$. Between 25-35°C, OXPHOS rate decreased to 64.18 (n = 2) nmol O_2 min^{-1} mg $protein^{-1}$ at 35°C. The two-

phase regression showed a breakpoint in state III respiration rate at 18.7°C, and a calculated Q_{10} between 5°C and 15°C of 2.25 ± 0.165 (n = 8, ± SE).

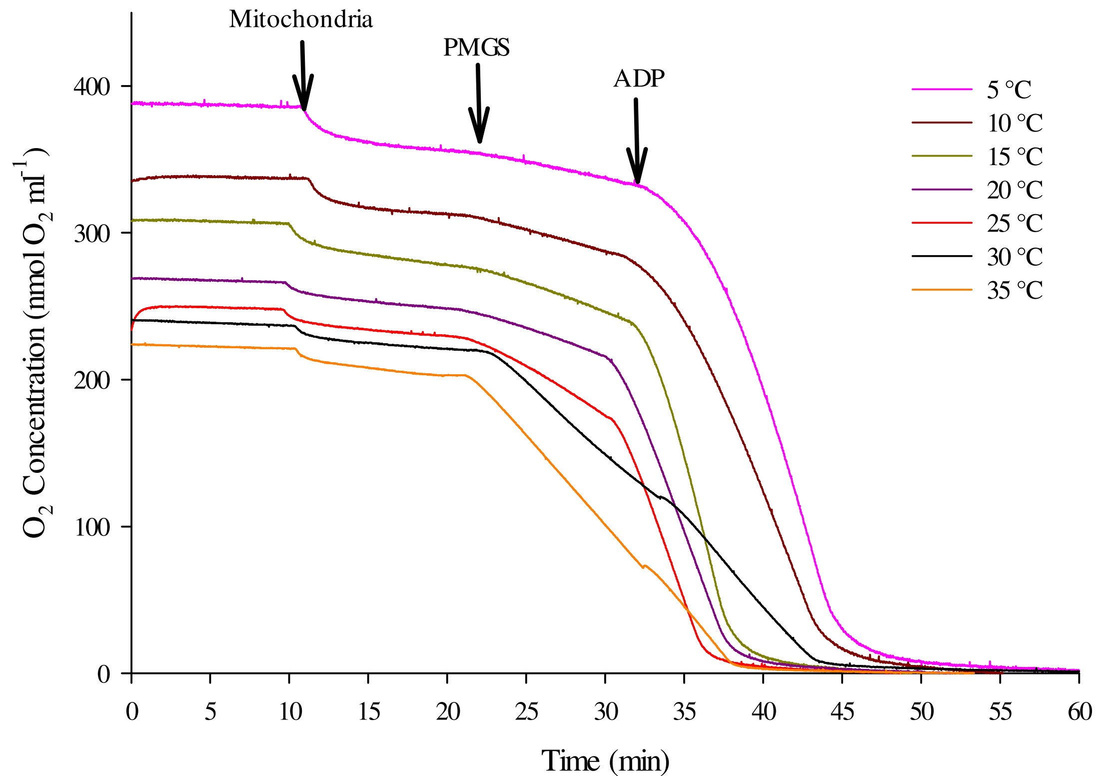

Figure 2.1: Thermal sensitivity of *Pleuragramma antarcticum* liver mitochondria. A representative mitochondria isolation is shown, where consecutive additions of pyruvate (P), malate (M), glutamate (G) and succinate (S) were performed. Note the changes in respiratory activities as a function of temperature.

The respiratory control ratio (RCR) was determined at each temperature, and averages with standard error are shown (Figure 2.3). The oxidation system was highly coupled with the phosphorylation system in *Pleuragramma antarcticum* at temperatures of 5°C and 10°C, where RCR values reached 12.35 ± 0.79 (n = 10, ± SE) and 12.66 ± 1.26 (n = 8, ± SE), respectively.

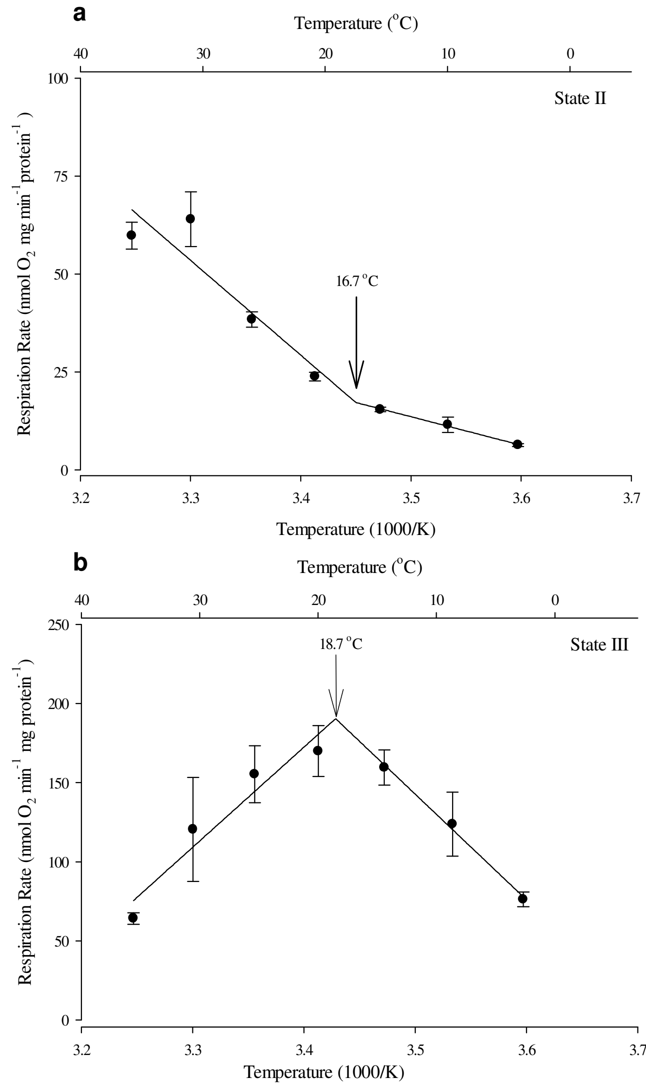

Figure 2.2a, b: Respiratory states as a function of temperature for the pelagic notothenioid *Pleuragramma antarcticum*. Figure **a** represent temperature effects on state II respiration, with a calculated Arrhenius breakpoint at 16.7°C, where leak respiration increases as assay temperature increases. Figure **b** represents state III respiration rates as a function of temperature. Overall state III respiration rates increase with temperature up to 15°C, where respiration rates start to decrease after 18.7°C. (n = 2-14, ± SE). Significance level was set as $P < 0.05$.

These values are among the highest reported for notothenioid liver mitochondria (Hardewig et al. 1999a). No significant differences were found among RCR values obtained between 5°C and 15°C (one-way ANOVA, $P > 0.05$). At temperatures above

15°C, RCR values significantly decrease from 10.36 ± 0.59 (n = 8, ± SE) at 15°C to 7.14 ± 0.58 (n = 8, ± SE) at 20°C (one-way ANOVA, $P < 0.05$).

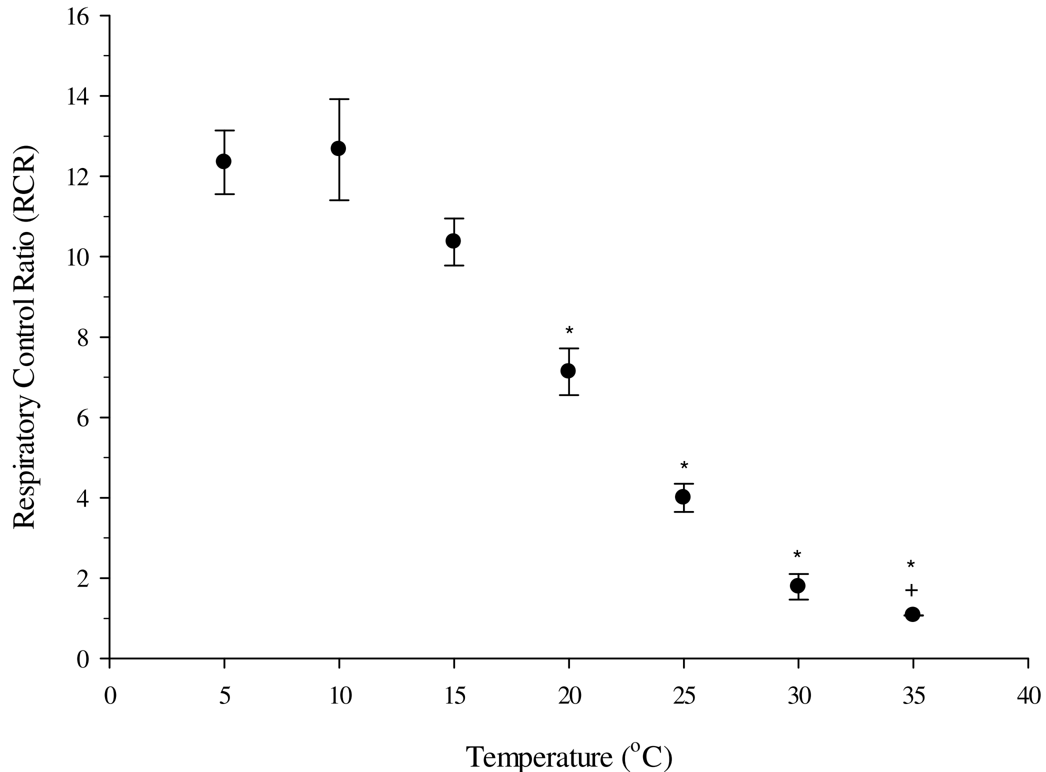

Figure 2.3: *Pleuragramma antarcticum* respiratory control ratio at increasing temperatures. Lower temperatures are characterized by highly coupled respiration. OXPHOS efficiency decreases with increasing temperature, due to an increase in the state II respiration rates (n = 2-14, ± SE). Significance level was set as $P < 0.05$.Asterisks (*) represent significant differences from RCR at 5°C.Plus sign (+) indicates n = 2.

Oxidative capacities and complex-specific contributions to the ETS in Antarctic teleosts. Oxidative capacity of liver mitochondria at 10°C was determined for the notothenioids *Pleuragramma antarcticum, Trematomus loennbergii, Notothenia coriiceps, Lepidonotothen nudifrons* and the myctophid *Gymnoscopelus braueri* (Table 2.1). Benthic notothenioids showed similar state II respiration rates when compared to their pelagic counterparts. However, state III respiration rates were twice as high in pelagic specimens when compared to the state III rates of benthic species (Mann-Whitney

two sample test, $P < 0.001$). Respiratory control ratios calculated at 10°C for benthic and pelagic species ranged from 4-5, with the exception of the pelagic notothenioid *Pleuragramma antarcticum*, which showed comparatively lower state II respiration rates and high rate increases after ADP stimulation which yielded an average respiratory control ratio of 12.66 ± 1.26 (*n* = 8, ± SE).

Table 2.1: Average respiration rates (P+M+G+S) at 10°C, expressed in nmol O_2 min^{-1} mg $protein^{-1}$. Data show significant differences in OXPHOS capacity between benthic notothenioids and the pelagic *Pleuragramma antarcticum* (Mann-Whitney test, $P < 0.001$). State III respiration rates from *P. antarcticum are* at least two times higher than benthic counterparts. Double dagger sign (‡) indicates pelagic species. Significance level was set as $p < 0.05$. Asterisks (*) Significant differences from state III respiration rates found in benthic species. (*n* = 2-12, ± SE). [&]Due to an *n*-value below three, *G. braueri* was not included in the statistical analysis.

Species	**State II**	**State III**	**RCR**
‡*Pleuragramma antarcticum* (*n* = 12)	11.48 ± 6.30	144.09 ± 53.47*	12.66 ± 3.57
‡&*Gymnoscopelus braueri* (*n* = 2)	48.29	206.86*	4.28
Notothenia coriiceps (*n* = 4)	9.97 ± 2.85	50.04 ± 13.37	5.05 ± 0.48
Lepidonothothen squamifrons (*n* = 4)	13.84 ± 0.36	79.00 ± 4.59	5.71 ± 0.03
Trematomus loennbergii (*n* = 3)	15.18 ± 2.94	71.66 ± 17.74	4.78 ±1.24

Interestingly, complex-specific state III respiration rates were strikingly different amongst species (Figure 2.4). No significant differences were found among state III rates obtained by activating complex I with the substrate combinations P+M, M+G and P+M+G within each species (data not shown), thus complex I values represent a pooled average of the aforementioned combinations. In the benthic species *N. coriiceps*, the

activation of complex I by adding saturating levels of ADP in the presence NADH generating substrates yielded 69% of state III respiration rate obtained with simultaneous activation of complex I and II. On the other hand, state III respiration obtained with the activation of complex I in the pelagic *P. antarcticum* yielded 41% of state III rates obtained by simultaneous activation of complexes I and II. It was only in the species *L. squamifrons* where we found similar values of state III rates between the individually activated complexes. Consistently throughout the species range, state III values obtained with the simultaneous activation of both complexes elicited the highest rates observed amongst substrate combinations.

Relative contributions of complex I and II to OXPHOS rates under convergent electron entrance varied significantly among species. By evaluating the relative contributions of complex I over complex II (Figure 2.5), we can show the remarkable variability of complex-specific state III respiration among notothenioids. Similar contributions between complex I and complex II were found in *L. squamifrons* and *T. loennbergii*, where the average ratios were 0.82 ± 0.036 and 0.87 ± 0.015 (n = 4, ± SE), respectively. The highest complex I contribution in state III was found in the pelagic species *G. braueri*, with a complex I-to-complex II ratio (CI/CII)of ~1.62 (n = 2). The highest contribution of complex II was found in *N. coriiceps*, where state III respiration of complex I over complex II was 0.46 ± 0.02 (n = 4, ± SE).

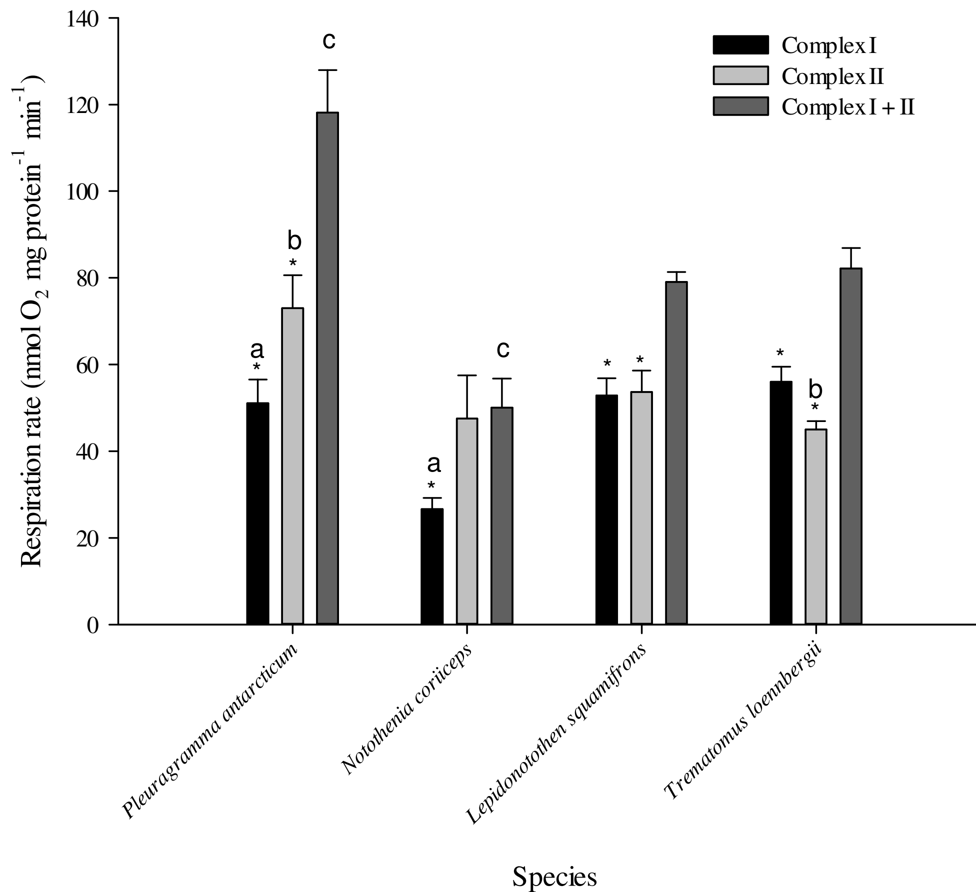

Figure 2.4: Complex-specific, state III respiration rates in liver mitochondria from four Antarctic notothenioids. Complex I respiration was obtained after the addition of saturating levels of ADP in the presence of NADH generating substrates. No difference among the three substrates combinations, pyruvate + malate, malate + glutamate or pyruvate + malate + glutamate, was found and rates were averaged. Complex II rates were obtained by the addition of saturating levels of ADP in the presence of the substrate succinate and the complex I inhibitor rotenone. Simultaneous activation of complexes I and II was achieved by adding saturating levels of ADP to the chamber containing the substrates pyruvate, malate, glutamate and succinate (n = 4-12, ± SE).Values with an asterisk (*) are significant different from rates obtained when both complexes were activated in the same species (one-way ANOVA, P < 0.05). Letters (a-c) indicate statistically significant differences in complex-specific rates among species (two-way ANOVA, P < 0.05).

Hepatosomatic Index. Hepatosomatic indexes are summarized in Table 2.2. Average HSI were variable among species, with *T. loennbergii* displaying the highest HSI obtained of 0.023 ± 0.01 (*n* = 3, ± SE). Pelagic specimens HSI's were variable, where *P. antarcticum* exhibited the lowest HSI (0.011 ± 0.003), and *G. braueri* exhibited and average HSI of 0.0186 (*n* = 2).

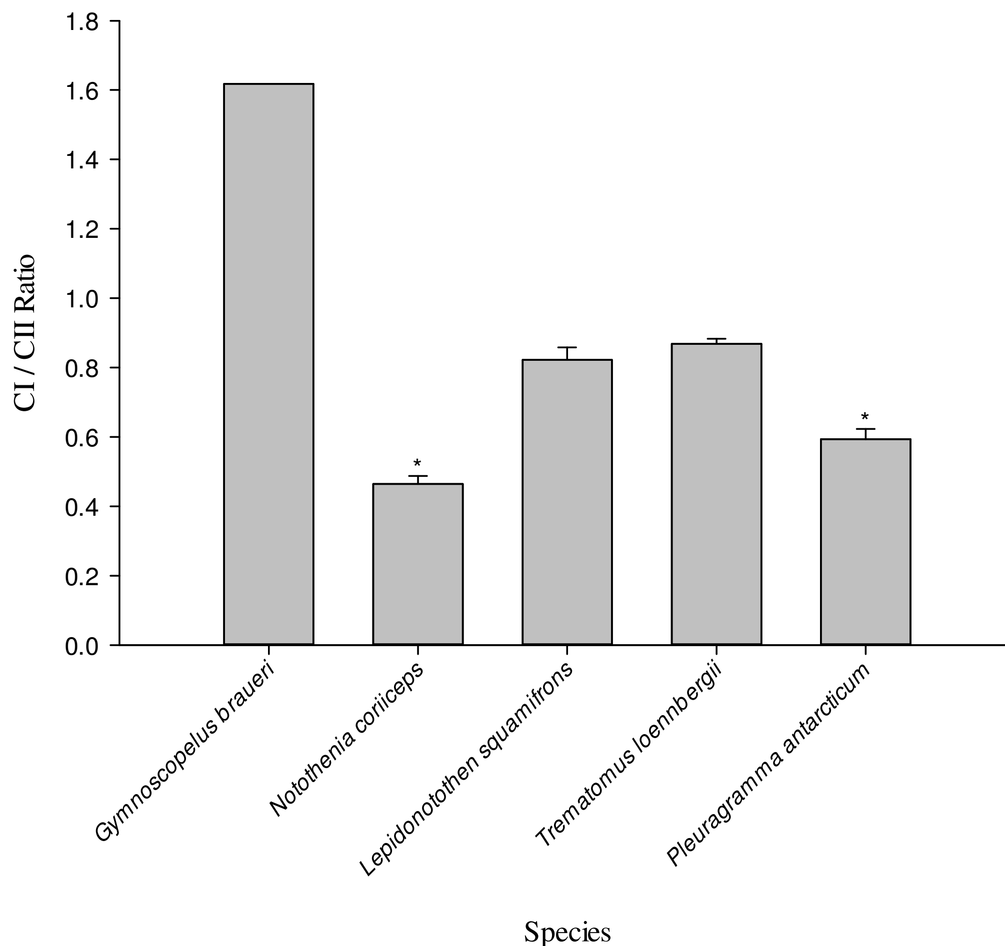

Figure 2.5 : Complex I/complex II ratio of state III respiration in liver mitochondria of Antarctic teleosts, measured independently in the presence of NADH generating substrates (complex I) and in the presence of succinate and the complex I inhibitor rotenone (*n* = 4, ± SE). *G. braueri* was excluded from statistical analysis due to low sample size (*n* = 2). Ratios among species were variable, and statistically significant differences from all other species are indicated by an asterisk (*) (one-way ANOVA, $P < 0.05$).

Table 2.2: Body mass range and hepatosomatic index (HSI) of Antarctic fish species. HSI were calculated as the ratio of liver weight to body weight. It provides an indication on the status of energy reserve of the specimens collected in this study. HSI variability is expressed as standard error.

Species	Body mass range (g)	Mean HSI (±SE)
Pleuragramma antarcticum (*n* = 12)	16.2 – 54.9	0.0108 ±0.003
Gymnoscopelus braueri (*n* = 2)	37.4 – 43.0	0.0186
Notothenia coriiceps (*n* = 4)	732.1 – 830.8	0.0116 ± 0.001
Lepidonothothen squamifrons (*n* = 4)	92.2 – 160.4	0.0153 ±0.005
Trematomus loennbergii (n = 3)	108.3 – 138.6	0.0235±0.005

Discussion

Thermal tolerance in *Pleuragramma antarcticum*. The effects of temperature on the ETS and more globally on the OXHPOS system were characterized in detail for *P. antarcticum*. State II respiration rates obtained by activating complexes I and II with pyruvate, malate, glutamate and succinate were found to increase with assay temperature, with an observable break at 16.7°C in the Arrhenius plot. At temperatures above 15°C, state II respiration rates increased significantly, which resulted in decreasing RCR values. Although the state II breakpoint does not correspond to a transition from positive to negative slope values as found by Weinstein and Somero (1998), it constitutes a breakpoint at which state II respiration increases significantly. Higher leak respiration

rates at increasing temperature are associated with the loss of membrane integrity at these temperatures (Hazel 1995; Hochachka and Somero 2002), which cause a higher proton leak across the inner membrane to the mitochondrial matrix. Moreover, recent findings by Mark and coworkers (2012) indicate a loss of membrane potential during state II over a similar temperature range (0-15ºC) in *Notothenia coriiceps*. This finding is in accordance with our interpretation that the increase in state II respiration observed in *P. antarcticum* may be attributed to an increase in proton permeability of the inner mitochondrial membrane, rather than a decrease in the complex specific activities.

We found that the calculated Arrhenius 'break' temperature (ABT) in state III respiration in *P. antarcticum* is low (18.7°C), compared to a state III ABT of 35°C in the tropical teleost *Sarotheridon mossambica* (Weinstein and Somero 1998). However, results obtained for *P. antarcticum* were found to be similar to the ABT found for the benthic Notothenioid *T. bernacchii* of 20.3°C by Weinstein and Somero (1998). Despite similarities between state III ABT between *Pleuragramma antarcticum* and *Trematomus bernacchii*, Mark and coworkers (2012) found state III ABT's to be between 6 and 9°C in *Notothenia coriiceps* and *Notothenia rossii*, a significant contrast to those ABT's found elsewhere for notothenioids. This remarkable level of variation of the thermal sensitivity of coupled respiration among species could be attributed to a number of factors including the tissue type (e.g. red muscle, cardiac muscle, liver, etc.) and the isolation method among others. Interestingly, a recent study by Urschel and O'Brien (2009) have found a remarkable higher (28.7-31.5°C) state III ABT for mitochondria isolated from heart ventricles of the notothenioids *Gobionotothen gibberifrons*, *Chionodraco rastrospinosus* and *Chaenocephalus aceratus*. High coupling was achieved by utilizing a respiration

assay medium with an osmolarity of 435 mOsm, which comes close to the blood osmolarity of the specimens assayed. Moreover, this and other studies points towards the effect of a hypoosmotic respiration medium in the ABT; buffer osmolarities lower the blood osmotic concentration could reduce state III respiration rates in some fish mitochondria (Ballantyne and Moon 1986; Urschel and O'Brien 2009). Further work on the effects of medium osmolarity should be conducted in various tissue types to confirm this finding, as high coupling at higher osmolarities has been confirmed only for notothenioid cardiac tissue (Urschel and O'Brien 2009).

Leak respiration rates observed when both complexes I and II were activated at temperatures below ABT only constitute about 10% of state III rates obtained at the same temperature. This value is small compared to about 50% of state III with only complex I activated in snapper mitochondria (Cook et al. 2013), and slightly higher than 15% of state III found in permeabilized heart ventricle fibers of triplefin blennies at 15°C (Hilton et al. 2010). However, similar values have been obtained for the notothenioid *L. nudifrons* (10.2% at 0°C), which suggest a low proton leakiness of the inner mitochondrial membrane in notothenioids (Hardewig et al. 1999b). Respiration rates obtained with ADP and uncoupled rates after FCCP additions shows that state III respiration can be up to 89% of the maximum respiratory capacity (data not shown), suggesting that coupled respiration is operating at maximal or close to maximal capacity. These results suggest that the loss of performance observed at temperatures warmer than ABT could be explained by changes in membrane fluidity (Hazel 1995), in addition to a possible decrease in activity of key enzyme complexes involved in the ETS due to the hindering effects of heat on hydrophobic interactions between the inner membrane and

the enzyme complexes involved (O'Brien et al. 1991). However, at temperatures lower than ABT, and supported by the observed steady increase in state III respiration below ABT, we favor an increase in membrane permeability and possibly membrane potential as driving factor for the increase in state II respiration rates. These drastic differences in state II observed in both above and below ABT could be also attributed to an increasing Q_{10} or further activation of uncoupling proteins (UCP) at warmer temperatures (Mark et al. 2006). However, UCP activation should not have contributed majorly to state II rates in our systems due to the addition of high concentrations of BSA which should keep free fatty acid levels low (10.0 g/L BSA). There are several advantages for the expression of the UCP2, which includes the role of a ‘safety valve’, preventing the formation of reactive oxygen species under oxidative stress (Skulachev 1998). Although Hardewig and coworkers (1999a) have suggested an enzyme-mediated leak respiration in *L. nudifrons* due to a high Q_{10} value for state II respiration rates, additional work will be necessary to further confirm this hypothesis.

Highly coupled respiration of *P. antarcticum* is observed well beyond 6-9°C, where critical temperatures have been estimated for other notothenioids (Somero and DeVries 1967; Mark et al. 2012). Therefore, mitochondrial functionality shows a far greater temperature range than the whole organism; the mitochondrial OXPHOS system remains operational at temperatures as high as 15°C. Although the ABT found for state III respiration rate is above 18.7°C, there is a gradual decrease in RCR at temperatures warmer than 10°C. At temperatures at or below 10°C, respiratory control ratios were above 12 and we choose this temperature for our comparative studies among species which minimized performance issues of our respiration system. Similar calculations were

made by Weinstein and Somero (1998), where RCR values of only 4 were observed when *T. bernacchii* liver mitochondria were assayed with pyruvate, malate and succinate combined. High RCR values at low temperatures have also been reported previously for *L. nudifrons* (Hardewig et al. 1999a), contributing to the hypothesis of low leak respiration in mitochondria from Antarctic notothenioids.

Liver mitochondria from *P. antarcticum* were highly coupled at 10°C, especially when compared to tropical fishes. For example, state III respiration rates obtained for *Oreochromis andersoni* at 10°C were less than a third of those obtained for *P. antarcticum* (Johnston et al. 1994). As noted by Hardewig and coworkers (1999a) for *L. nudifrons*, mitochondrial oxidative capacity remains high at temperatures above the lethal limits for whole individuals. Although the stenothermic nature of Antarctic notothenioids has been widely established (Somero and DeVries 1967), our results show that notothenioid mitochondria remain functional at temperatures beyond the thermal limit of the species, which could provide additional insight to recent findings on the ability of Antarctic notothenioids to withstand higher temperature regimes through longer acclimation periods (Bilyk and DeVries 2011).

Oxidative capacities and complex-specific contributions to the ETS in Antarctic teleosts. Additive effects of substrates on ETS activity were evaluated for a series of notothenioids and a pelagic myctophid. State II respiration rates obtained with the activation of complex I were similar among species. Substrate combinations enabling NADH supply to complex I (P+M, M+G, P+M+G) elicited similar state II rates, contributing to less than 50% of state II rates obtained with simultaneous electron entry via NADH and $FADH_2$ (P+M+G+S). Only *P. antarcticum* samples showed a complex I-

specific leak respiration of close to 70% of the state II rate obtained with both complexes activated (PMGS). Electron supply to the ETS via complex II alone by the sole addition of succinate and rotenone yielded similar state II rates as those obtained with the addition of PMGS. These findings suggest a high contribution of electrons from complex II to the Q-junction, or a complex II specific higher proton leak. Although we were unable to measure the mitochondrial membrane potential in our study, it was found by Mark and coworkers (2012) that complex-specific activation can yield to significant differences in membrane potential. Therefore, complex dependent differences in the mitochondrial membrane potential due to independent activations of complex I and complex II may contribute to the variability in leak respiration rates observed in our study.

State III respiration rates obtained by activating individual complexes were variable among species (Figure 2.4). Complex-specific variability among species has also been documented in two other notothenioids (Mark et al. 2012), but the state III rates reported for *N coriiceps* in our study are significantly higher than those found by Mark and coworkers (2012). In addition, our values are in close agreement to the variation in complex specific contributions found in triplefin blennies (Hilton et al. 2010). Furthermore, complex-specific state III rates in figure 2.4 demonstrate that PMGS titration yield consistently higher estimates of state III respiration rates among a variety of species. In previous studies, the use of individual substrates to understand substrate preferences resulted in the use of pyruvate as the major substrate in similar assays (Moyes et al. 1989; Moyes et al. 1992; Johnston et al. 1994). More recent studies utilized titrations including PM to activate complex I, PMS to activate both complexes I and II and others have used rotenone as a complex I inhibitor in the presence of S to activate

solely complex II (Weinstein and Somero 1998; Hardewig et al. 1999a; Lannig et al. 2005). The addition of glutamate and the overall combination of PMGS in a single run has been utilized in snappers recently (Cook et al. 2013), and our study constitutes the first attempt in notothenioids. Recent studies on human muscle mitochondria showed additive effects of those substrates, mainly due to the convergent nature of the ETS (Gnaiger 2009). Any increase in respiration rates observed with glutamate addition to the substrate cocktail may be explained by its participation in the malate-aspartate shuttle, which includes a glutamate-aspartate carrier and 2-oxoglutarate (Gnaiger 2010a). This process provides additional 2-oxoglutarate and NADH, increasing the reducing power of the ETS.

As shown in Table 2.3, maximal OXPHOS activity expressed as state III respiration was similar to that found in the literature for benthic specimens. When extrapolated to 0°C, *N. coriiceps* liver mitochondria exhibited lower state III rates than observed in red muscle tissue of *N. coriiceps* and livers mitochondria of other notothenioids (Johnston et al. 1994; Johnston et al. 1998; Mark et al. 2012). However, comparison of the conspecifics *L. squamifrons* and *L. nudifrons* (Hardewig et al. 1999a) yielded significant differences between state III rates. These differences are expected due to differences in the titration protocol, where values for *L. nudifrons* correspond to state III rates in the presence of only succinate, ADP and the complex I inhibitor rotenone. By looking at the complex-specific contributions to the OXPHOS rate (Figure 2.5), it is possible to elucidate how complex activities vary within a species and how these differences are reflected in the species' maximal OXPHOS capacity. Our work agrees well with the findings by Mark and others (2012), where complex I contributions ranged

from 40-50% of complex II, to about 60-100% in *Notothenia coriiceps* and *Notothenia rossii*, respectively. Similar values have been obtained for tripelfin blennies (Hilton et al. 2010), suggesting an intrinsic variability of complex contributions to the ETS.

Table 2.3: Maximal values of ADP-induced state III respiration reported for Antarctic teleosts in the literature and this study. Only studies that utilized mitochondrial protein for rate standardization are shown. Data with (‡) were standardized to rates at 0°C for comparative purposes by using reported Q_{10} values for *L. nudifrons* (Hardewig et. al., 1999). Q_{10} with an asterisk (*) represent an estimated value.

Species	Lifestyle	Tissue type	Assay Temp (°C)	Substrate/ Concentration (m*M*)	Max Rate (nmol O_2 min^{-1} mg $protein^{-1}$)	Q_{10}	Estimated rate at 0°C	Source
Lepidonotothen nudifrons	Benthopelagic	Liver	0	Succinate/3.3	7.36	2.43	7.36	Hardewig et.al., 1999
Notothenia coriiceps	Benthic	Red muscle	-1.0	Pyruvate/2.5 Malate/0.1	31.0	1.90	31.6	Johnston et. al., 1994
Notothenia coriiceps	Benthic	Liver	3.0	Malate/1.0 Glutamate/1.3	9.0	2.60	7.02	Mark et. al. 2012
Notothenia rossii	Benthic	Liver	3.0	Malate/1.0 Glutamate/1.3	4.4	1.71	2.26	Mark et. al. 2012
Notothenia coriiceps	Benthic	Liver	10.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	50.04	1.9*	26.34	Present Study
Pleuragramma antarcticum	Pelagic	Liver	5.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	76.23	2.25	55.06	Present Study
Pleuragramma antarcticum	Pelagic	Liver	10.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	123.73	2.25	54.99	Present Study
Lepidonotothen squamifrons	Benthopelagic	Liver	10.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	79.00	2.43‡	32.51	Present Study
Gymnoscopelus braueri	Pelagic	Liver	10.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	206.86	2.0*	103.43	Present Study
Trematomus loennbergii	Benthopelagic	Liver	10.0	Pyruvate/5.0 Malate/2.0 Glutamate/10.0 Succinate/10.0	71.66	2.43‡	29.49	Present Study

When state III respiration rates are compared with those rates found elsewhere, the pelagic *Pleuragramma antarcticum* displays OXPHOS rates well above those documented for Antarctic notothenioid liver mitochondria (Weinstein and Somero 1998; Hardewig et al. 1999a). As shown in Table 2.2, *P. antarcticum* exhibit twice the state III respiration rates of benthic counterparts. Citrate synthase activity measured for *G. braueri*, *T. loennbergii*, *T. bernacchii* (Torres and Somero 1988) and *Pleuragramma antarcticum* (Torres, unpublished observations) indicates that the observed high state III respiration rates are not related to significant differences in the expression of enzymes associated with the citric acid cycle. However, this observation does not exclude the possibility of an increase in enzyme activity by allosteric activation or phosphorylation under our assay conditions. Furthermore, large quantities of adipocytes in the muscle tissue of *P. antarcticum*, previously thought to be strictly developed for buoyancy purposes (Devries and Eastman 1978), could point towards a its potential use as a metabolic reservoir (Eastman and DeVries 1989) , thus enhancing the citric acid cycle in lipid-rich tissues. Future studies to investigate the contribution of beta oxidation to OXPHOS would be highly instructive.

Lipid metabolism is known to occur in the liver, where mitochondria are able to metabolize fatty acids under appropriate conditions (Middleton 1978). Fatty acid metabolism involves the cytosolic activation of available fatty acids before they are transported into the mitochondrion, which suggest that differences in state III rates may be explained by a higher coenzyme A concentration in the liver tissue of *P. antarcticum* compared to benthic species. High concentrations of malonyl coenzyme A in the absence of free fatty acids could increase the formation citrate in the presence of pyruvate. This

has been shown in rat soleus muscle, where additions of glucose to skeletal muscle incubations in the presence of insulin increases the level of malonyl CoA, as well as the levels of citrate and malate (Saha et al. 1997). In summary, we consider that the large difference in state III observed between *P. antarcticum* and benthic notothenioids at 10°C could be attributed to varying levels of coenzyme A, which increases cytosolic citrate, increasing the reducing power of the citric acid cycle. However, this has not yet to be experimentally confirmed and a comparative analysis of coenzyme A values among species would be instructive.

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Chapter Three:

Metabolism of the Antarctic silverfish, *Pleuragramma antarcticum,* from the Western Antarctic Peninsula

Introduction

Pleuragramma antarcticum (Boulenger, 1902) is by far the most biomass-dominant notothenioid species in the Antarctic pelagial (Hubold 1984; Donnelly et al. 2004). Its circum-Antarctic distribution along the Antarctic coast, and the lack of other pelagic fishes able to tolerate near freezing waters, makes *P. antarcticum* a keystone species in the Antarctic coastal food web. Feeding habits of this species are primarily based on copepods, amphipods and euphausiids, where species and relative abundance of prey classes will vary according to region, age class and season (DeWitt and Hopkins 1977; Moreno et al. 1986; Reisenbichler 1993).

Variations in dietary composition could reflect changes in the metabolism of fishes, as prey items display variability in caloric content (Reisenbichler 1993). Although routine metabolic rates of notothenioids have been studied for decades (Wohlschlag 1960; Wohlschlag 1963a; Ralph and Everson 1968; Somero et al. 1968; Holeton 1974), such values have yet to be collected in *P. antarcticum*. The evaluation of the energetic requirements of *P. antarcticum* is central to understand how fatty acid present in the

locomotory muscles could serve as energy fuel (Eastman and DeVries 1989), besides the lipid's role as a buoyancy aid (DeVries and Eastman 1978). As a result of the differences in biochemical composition of muscle tissue between *P. antarcticum* and other notothenioids, it is hypothesized that differences in routine metabolism could be expected between benthic notothenioids and *P. antarcticum*. With this in mind, **this study presents the first routine metabolic measurements for *P. antarcticum*, in addition to insightful results of ammonia excretion rates for this species.**

The goals of this study were **to obtain routine metabolic rates of *Pleuragramma antarcticum*,** in order to compare its routine metabolism to those of benthic notothenioids, as well as other mesopelagic fishes. In addition**, ammonia excretion during respiration experiments was quantified to determine significant variability in nitrogen excretion at various year classes.**

Methodology

Specimen collection. *P. antarcticum* specimens were captured in multiple research cruises during in 2001, 2002 and 2010. Fishes from the Antarctic were collected with a 10m^2 MOCNESS during two GLOBEC (Global Ocean Ecosystem Dynamics)-sponsored cruises aboard the R/V Laurence M. Gould and aboard the R/V Nathaniel B. Palmer during austral autumn and winter of 2001 and 2002. Additional specimens were collected during the austral autumn of 2010 aboard the R/V Nathaniel B. Palmer, using a 10 m^2 MOCNESS and a Tucker trawl. Immediately after collection, specimens were transferred to large flow through tanks filled with 0°C seawater. Most specimens were placed in respiration chambers soon after collection.

Respiration measurements. Oxygen-consumption rates were determined following the methods described by Torres and Somero (1988a), with minor modifications. Individuals were placed in a sealed water-jacketed acrylic chamber filled with filtered (0.45 µm pore-size). The rectangular chambers were constructed of Lucite and contained a perforated Lucite false-bottom that isolated the fish from a stirring bar. A low stirring speed was used to minimize disturbance. All experiments took place in the dark, with brief periods of observation in low light. Oxygen partial-pressure was continuously monitored using a Clark polarographic oxygen electrode (Clark 1956). Temperature was maintained at 1.0°C (± 0.1 C) using a circulating refrigerated water-bath, as an individual reduced oxygen levels to intermediate (~80 mm Hg) partial-pressures. Electrodes were calibrated using air- and nitrogen-saturated seawater at the experimental temperature (Childress 1971; Torres et al. 1979). Run time varied from 4-8 h, depending on the specimen size and overall activity. Streptomycin and Neomycin (each 25 mg L^{-1}) were added to the seawater to minimize microbial growth. To control for possible oxygen consumption by microorganisms, an individual was removed after selected runs, its volume was replaced with fresh seawater, and oxygen consumption was again measured for 2 to 4 h. In all cases microbial oxygen consumption was negligibly low (< 5%). Data were recorded using a computer-controlled digital data-logging system. Each oxygen probe was scanned once per minute, its signal averaged over a period of 1 s, and then recorded. Data were reduced by first averaging the 30 recorded values in each 30 min increment of an entire 4 to 8 h experiment, producing between 8 and 16, 30-min points per run. Data obtained during the first hour were discarded due to the high activity of the fish after its introduction into the chamber. All 30-min points thereafter, down to

an oxygen partial-pressure (P_{O_2}) of 80 mm Hg, were averaged to produce a routine rate for each individual. 20 mL of seawater were collected and frozen before and after each run for further ammonia quantification. Fishes were immediately frozen after respirometric assay for further enzyme activity measurements. Due to the physical trauma exerted by collection, respiration rates reported in this study should be regarded as routine metabolism.

Ammonia excretion. Ammonia (NH_3) excreted by each specimen during the respirometric trial was quantified following the methods described by Jones (1991) and modified by Masserini and Fanning (2000). Briefly, frozen water samples collected after each respirometric trial were thawed, diluted with deionized water and analyzed for ammonia with a Technicon® Auto Analyzer II. In general, an ammonia-permeable membrane separates the water sample from a continuous flow of *o*-phthaldialdehyde reagent. This reagent binds to the permeating ammonia, forming a fluorescent product later quantified by the instrument. Ammonia concentrations from the respirometric chamber *before* placing the specimen was subtracted from the ammonia present in the final sample. Corrected ammonia concentrations were divided by the total run time to acquire an estimate of ammonia excretion per individual per hour.

Homogenate preparation and enzyme assay. Epaxial muscle tissue of *P. antarcticum* was excised from frozen specimens, and processed as described in previous studies (Childress and Somero 1979; Torres and Somero 1988a). Briefly, a piece of frozen and skinned epaxial muscle was thawed in ice-cold homogenizing medium containing 50 mM Imidazole/HCl buffer (10mM, pH 7.5 at10°C). Tissues were homogenized by hand in an ice-cold conical glass homogenizer, having ground glass

contact surfaces (Duall, Kontes Glass Corporation). After homogenization, the homogenates were centrifuged at 2,500 *g* for 10min at 4°C to precipitate undissolved tissue. The supernatant fraction was extracted, avoiding the lipid layer formed on top, for enzyme analysis. Although the assay temperature chose for the enzyme assay (10°C) does not correspond to the habitat temperature of *Pleuragramma antarcticum* (-2 to 2°C), previous studies have confirmed that enzyme thermal stability of Antarctic teleosts is not compromised at 10°C (Torres and Somero 1988a; Torres et al. 2012).

Citrate synthase (CS), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzymatic activity was assayed with supernatant fractions of freshly homogenized muscle tissue, following Childress and Somero (1979) with minor modifications (Torres et al. 2012). Alcohol dehydrogenase activities was determined as described primarily in Soubridge and Hochachka (1980), with minor changes (Torres et al. 2012). Activities of key aerobic and glycolytic enzymes were assayed at 10°C in a temperature controlled Varian Cary IE UV/Vis spectrophotometer, coupled with computer based analysis software (CaryWin).

CS activity was assayed in a solution of 42.5mM Imidazole buffer (pH 8.0 at 10°C), 0.2 mM DTNB, 1.5 mM $MgCl_2 \cdot 6H_2O$, and 124 µM acetyl-CoA. To 1 mL of the assay solution, 40µL of homogenate supernatant was added, and the absorbance at 412nm was monitored until reaching a plateau. The enzymatic reaction was initiated by adding 12.5µL of 40mM oxaloacetate, and the increase in absorbance as the reduce acetyl CoA reacts with DTNB was monitored for 4 min.

For L-lactate dehydrogenase (LDH), 10µL of fresh homogenate was added to the assay medium consisting of 80 mM Imidazole buffer, 5.0 mM sodium pyruvate and 0.15 mM NADH. LDH activity was determined by quantifying the decrease in absorbance at 340nm resulting from the oxidation of NADH.

The assay medium for MDH activity contained 200mM Imidazole, 20 mM $MgCl_2$ (pH 8.1 at 20°C) 0.4 mM oxaloacetate, and 150 mM NADH. The reaction was initiated by addition of supernatant and followed by recording the decrease in absorbance at 340 nm due to NADH oxidation.

Alcohol Dehydrogenase (ADH) was assayed according to Shoubridge and Hochachka (1980). Briefly, 10 µL of homogenate supernatant were added to an assay medium containing 100 mM potassium phosphate (pH 7.0 at 10°C), 8.9 mM acetaldehyde, 1.0 mM glutathione (reduced form) and 0.2 mM NADH. The reaction was monitored at 340nm for 1 minute, after the addition of the homogenate, and the slope in the absorbance was utilized as the NADH oxidation.

Results

Oxygen consumption rates of *Pleuragramma antarcticum*. Routine metabolic rates, expressed as the consumption of oxygen (VO_2) were obtained for a total of 11 *P. antarcticum* specimens, ranging in size from 55 – 174 mm Standard Length (SL). As summarized in Figure 3.1, individual oxygen consumption rates correlated positively with mass, where the smallest specimens (1.02 and 1.32 g) oxygen consumption rates averaged 52.17 and 78.03 µL O_2 individual^{-1} Hr^{-1}, respectively. Routine metabolic rates

in larger specimens (28.7 and 35.4 g) were found to be 3,350.40 and 4,720.80 µL O_2 individual^{-1} Hr^{-1}, respectively.

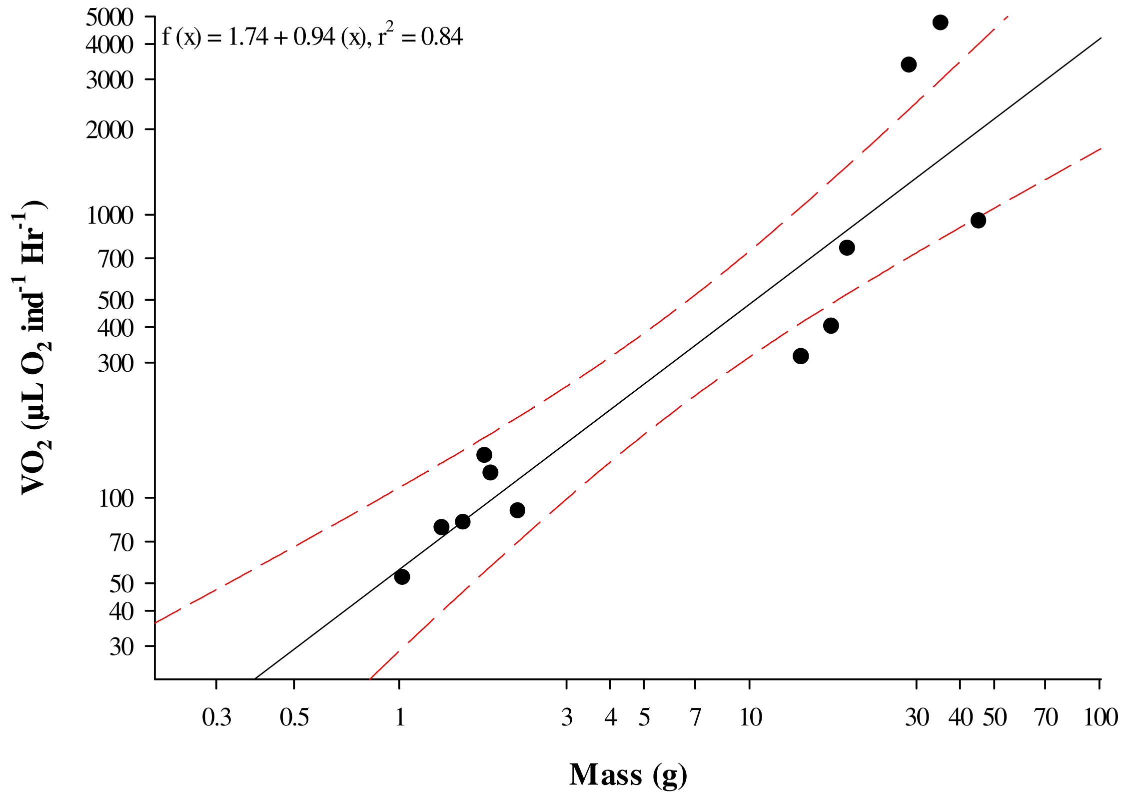

Figure 3.1: Oxygen consumption rates of *Pleuragramma antarcticum*. Linear regression is shown in a solid, black line, along with the 95% confidence interval of the regression (red, dashed lines)

Mass specific oxygen consumption showed to be highly variable across the investigated mass range (Figure 3.2). According to previously published growth curves (Hubold and Tomo 1989; Reisenbichler 1993), respiration values obtained between the earliest year classes 1 and 2 ranged from 40.96 to 79.89 µL O_2 g WM^{-1}Hr^{-1} ($n = 6$). Specimens estimated to be between 6 and 7 year old exhibited variable metabolic rate, ranging between 22.21 and 116 µL O_2 g WM^{-1}Hr^{-1} ($n = 4$). Two specimens ranging from

7 to 8 years of age showed the highest mass specific rates. The oldest individual, estimated to be over 9 years in age, exhibited a respiration rate of 28.02 µL O_2 g WM^{-1} Hr^{-1}.

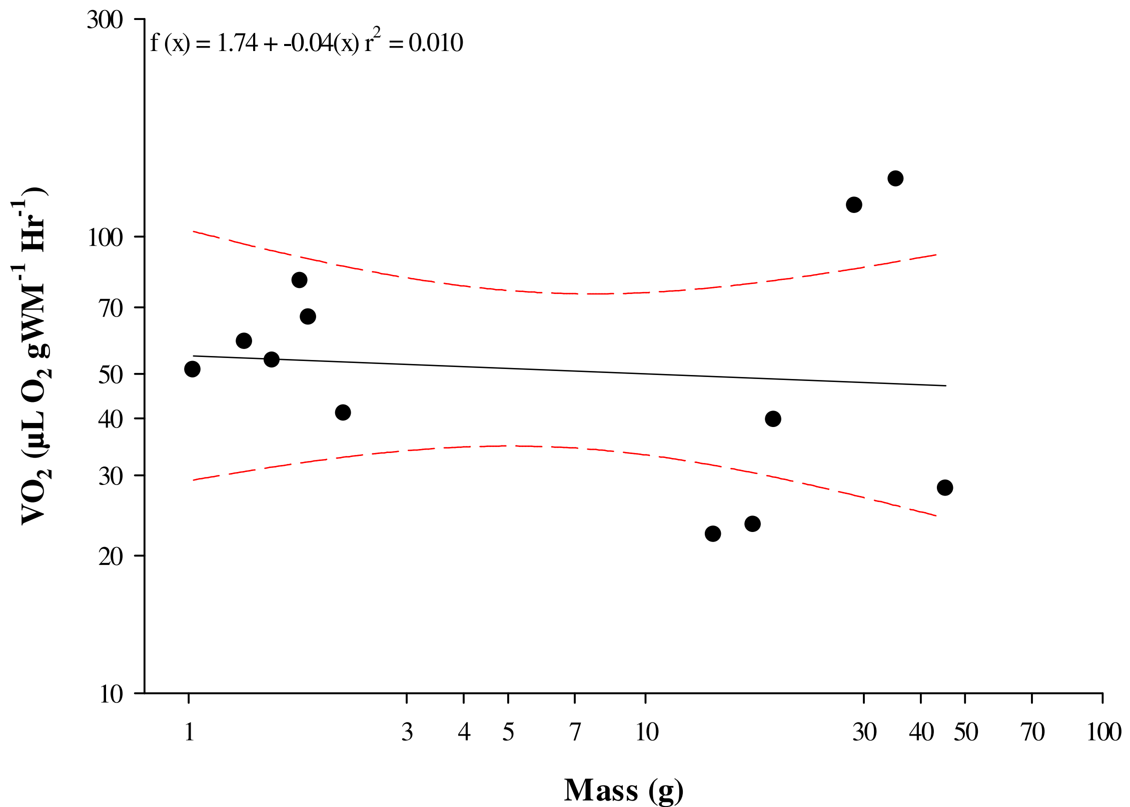

Figure 3.2: Mass specific oxygen consumption of *Pleuragramma antarcticum*. Linear regression line is shown solid line, along with the 95% confidence interval (dashed red lines).

Ammonia excretion of *Pleuragramma antarcticum*. Nitrogenous waste accumulation in the form of ammonia was measured in the chamber water for 11 specimens, ranging from 55-174 mm SL. A positive correlation of ammonia excretion with increasing size was found (Figure 3.3), where excretion values range from 0.25 µmol NH_3 $ind^{-1}Hr^{-1}$ in smaller specimens to 3.25µmol NH_3 $ind^{-1}Hr^{-1}$ in adult specimens.

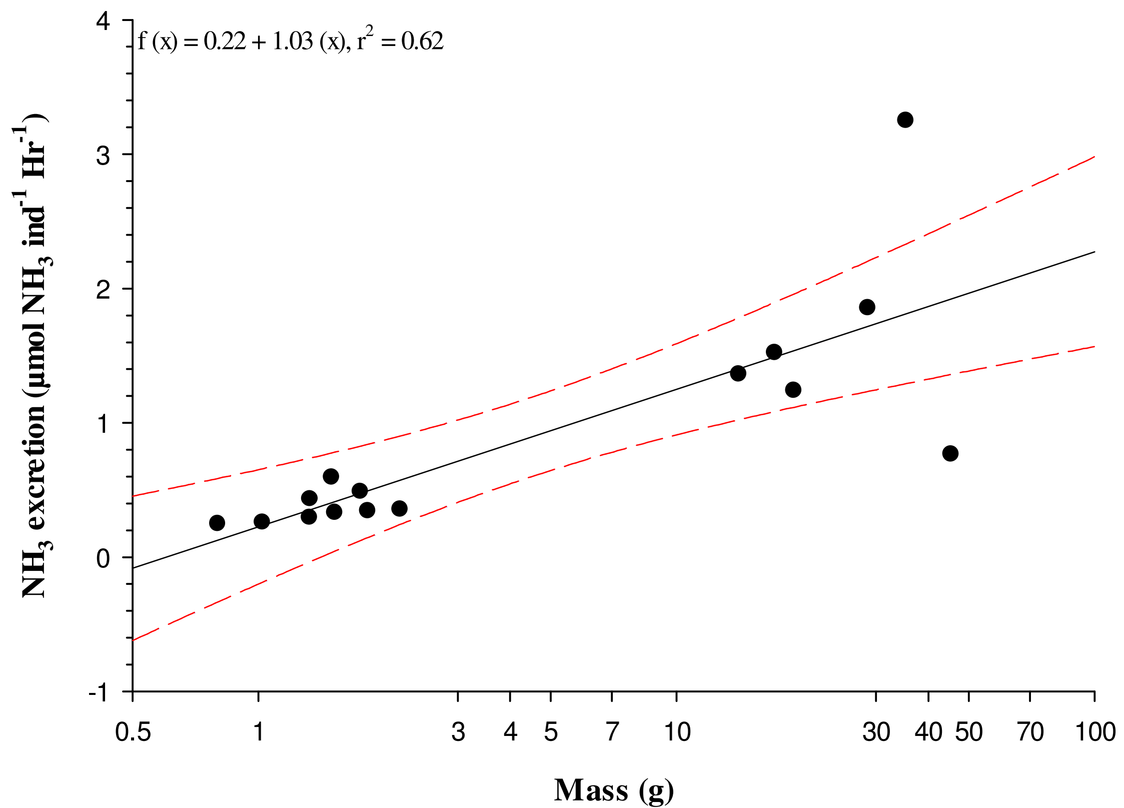

Figure 3.3: Ammonia excretion rates from *Pleuragramma antarcticum* specimens. A linear regression line is show (solid line), along with the 95% confidence interval of the regression.

Mass specific ammonia excretion decrease with increasing specimen mass (Figure 3.4). Specimens of the year class 1 and 2 showed ammonia excretion rates ranging from 0.40 to 0.16 µmol NH_3 g WM^{-1} Hr^{-1}. Ammonia excretion rates decreased sharply with increasing size, from 0.04 µmol NH_3 $ind^{-1}Hr^{-1}$ in smaller specimens to 0.02 µmol NH_3 $ind^{-1}Hr^{-1}$ in the largest specimen analyzed.

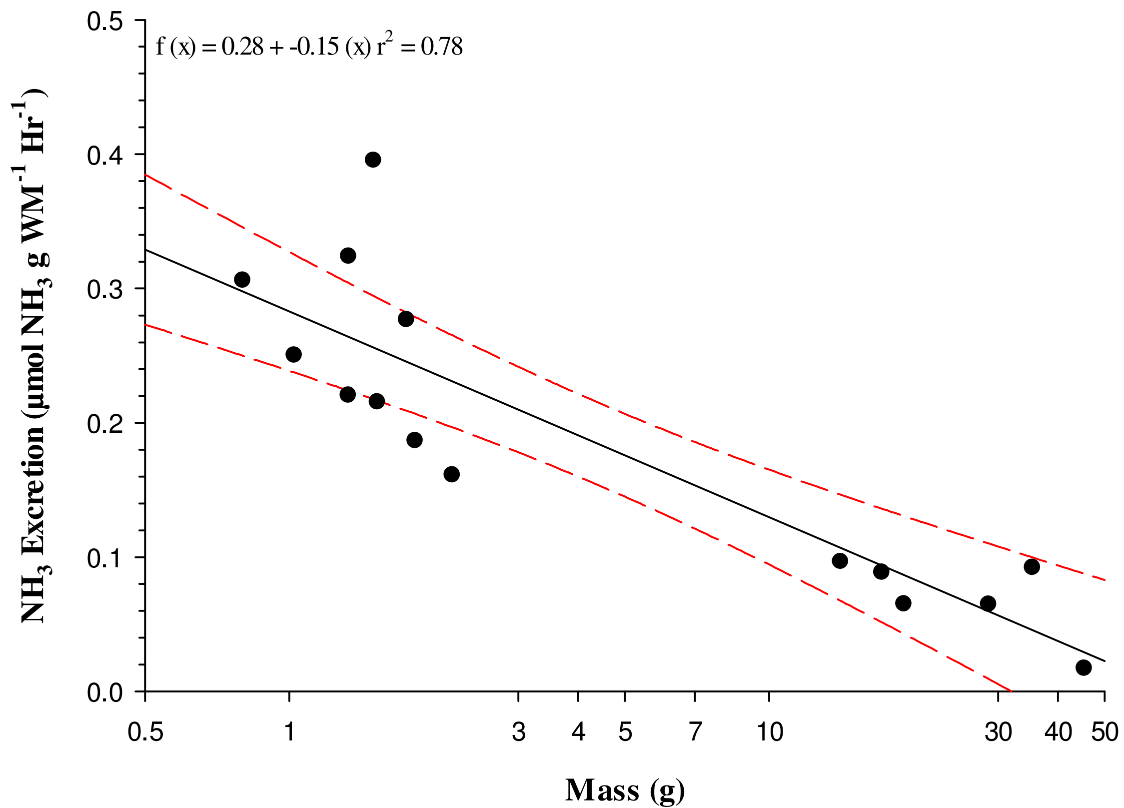

Figure 3.4: Mass specific ammonia excretion rates of *Pleuragramma antarcticum*. Linear regression is shown in solid line, along with the 95% confidence interval of the regression.

Oxygen: NH_3 ratios of *Pleuragramma antarcticum*. The relative proportion of oxygen consumed to the ammonia excreted of *P. antarcticum* is illustrated in Figures 3.5 and 3.6. Overall, O: NH_3 ratios ranged from 53.7 to 17.66 through the investigated size range (Figure 3.5). The majority of the specimens exhibited ratios in between 17.66 and 30.9 (n = 9), with three adult individuals displaying ratios of 36.8, 43.20 and 53.74, respectively.

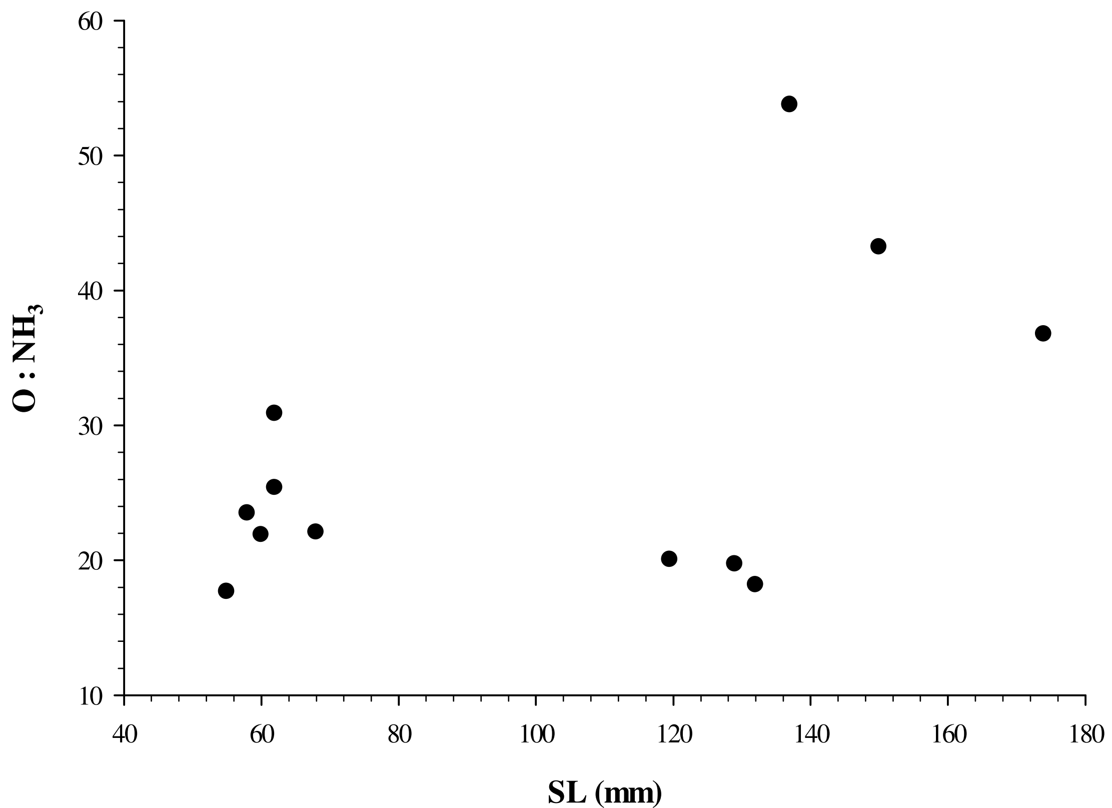

Figure 3.5: O : NH_3 ratios obtained for *Pleuragramma antarcticum*.

Although no discernible relationship between standard length and O : NH_3 ratio was found, ammonia excretion was positively correlated with oxygen consumption (Figure 3.6). In other words, ammonia excretion increased with increasing respiration rate.

Enzyme activity. Enzymatic activity was measure in two enzymes associated with aerobic pathways of ATP production (CS and MDH), as well as two enzymes associated to anaerobic pathways (LDH and ADH). No significant mass specific correlations were found. Summarized finding are presented in Table 3.1.

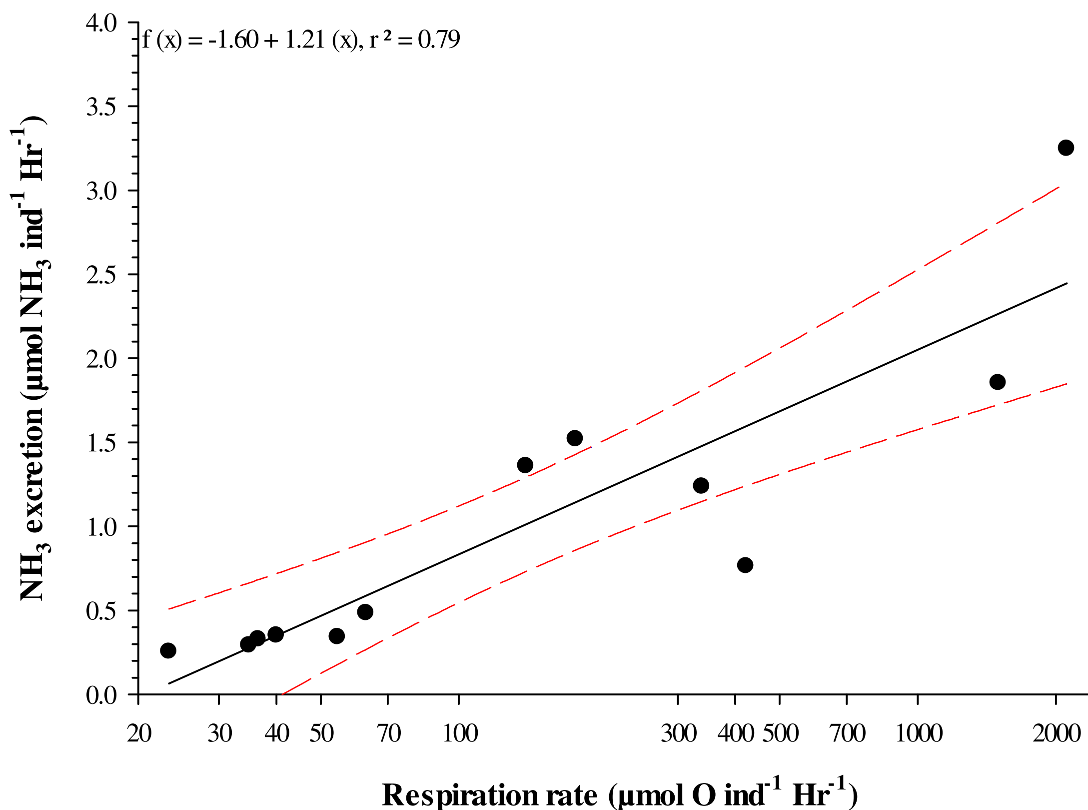

Figure 3.6: Ammonia excretion as a function of respiration in *Pleuragramma antarcticum*.

Table 3.1: Standard length, wet mass, enzyme activities at 10°C, and total protein content for specimens analyzed. All values are mean ± 95% confidence interval, along with minimum and maximum values in parenthesis. Enzyme activities are expressed as µmoles of substrate converted per gram of wet mass. Protein content is expressed in milligrams of protein per milliliter of homogenate.

Species (n)	Length (cm)	Mass (g)	LDH	CS	MDH	ADH	Total Protein
Pleuragramma	12.95±2.18	21.98±13.42	10.71±5.14	1.16±0.21	17.43±4.43	7.5±3.81	84.8±21.6
antarcticum (8)	(9.03-18.4)	(3.86-58.74)	(5.0-23.8)	(0.8-1.6)	(11.3-27.3)	(2.4-14.8)	(36.4-123.6)

Discussion

Respiration measurements. In this study, the routine metabolism of *P. antarcticum* was determined, to provide a comparative analysis of those respiration rates obtained in benthic notothenioids (Wohlschlag 1960; Wohlschlag 1963b; Holeton 1974; Forster et al. 1987), as well as routine metabolic rates found in non-notothenioid mesopelagic fishes of the Southern Ocean (Torres and Somero 1988a).

Mass specific metabolic rates obtained throughout the mass range scaled with size, with the exception of two individuals (Figure 3.2). These individuals showed variable mass specific metabolic rates, where lower values found were up to six times higher than would have been expected for their size. It has been shown that variability of metabolic rates observed may be attributed to the specific dynamic action, where a post-prandial increase in oxygen consumption in fishes immediately after feeding have been observed (Beamish 1974; Jobling 1981). This increase in oxygen consumption has been shown to be proportional to the quantity of food, and inversely proportional to temperature (Jobling and Davies 1980; Jobling 1981). Thus, it is plausible that the adult specimens displaying the highest respiration rates were responding to digestive processes taking place after recent feeding activity.

Slope values (b-value) of VO_2 as a function of weight found for *P. antarcticum* (0.94) fall well within those values found for Antarctic fishes (Wohlschlag 1960; Wohlschlag 1963a; Wohlschlag 1963b; Holeton 1974; Torres and Somero 1988a). Furthermore, the proximity of the slope to 1.0 indicates a close scaling with mass rather than surface area, which was also found for other Antarctic mesopelagic teleosts (Torres

and Somero 1988b). Overall, mass specific VO_2 for *P. antarcticum* was found to be in the lower spectrum of VO_2 rates found by Wohlschlag (1960; 1963b), but gross weight specific comparisons among notothenioids without considering mass/age relationships and life habits should be made with caution (Zimmermann and Hubold 1998). Although there is a reduced number of pelagic notothenioid species in the Southern Ocean, metabolic rates obtained for cryopelagic species could provide, to a certain extent, an 'apples to apples' comparison of their corresponding energetic requirements.

With the exception of the two individuals previously described as to be potentially displaying SDA, mass specific respiration rates obtained from *P. antarcticum*, these results are in close agreement with those rates found in the cryopelagic notothenioid *Pagothenia borchgrerevinki* (Forster et al. 1987). In this study, a 50g individual has an estimated VO_2 of 39.6 uL O_2 g WM Hr^{-1}, which is comparable with an estimated VO_2 of 48.74 uL O_2 Hr^{-1} for a 50g *P. antarcticum* specimen, derived from the slope of the respiration equation found in this study (Figure 3.1).

Nitrogen excretion. Nitrogenous waste excretion, in the form of ammonia, scaled inversely with specimen mass. Although this feature could be indicative of a high protein diet in earlier age classes, evidence points towards changes in metabolic substrate utilization, from nitrogen-based metabolic substrates to carbohydrate or fatty acid oxidation. Stomach contents of *P. antarcticum* did not show significant quantities of carbohydrates, in comparison with lipids and protein (Reisenbichler 1993).Previous studies have shown that proximate composition of distinctive populations of *P. antarcticum* have similar protein content values, as well as no discernible changes in the protein content of their stomachs (Reisenbichler 1993). Stomach contents of *P.*

antarcticum did not show significant quantities of carbohydrates, in comparison with lipids and proteins. A proximate composition analysis of *P. antarcticum* prey shows that *E. superba*, often a primary food item for adult *P. antarcticum*, has slightly higher protein content than smaller prey items (e.g. amphipods) consumed by earlier year classes (Torres et al. 1994). Considering the large quantities of adipocytes in the muscle tissue of *P. antarcticum* known to be used a metabolic reservoir (Eastman and DeVries 1989) and the high lipase activity observe in notothenioids (Sidell and Hazel 2002), these results point to an ontogenic metabolic reorganization favoring the oxidation of lipids in the slow growing *P. antarcticum*.

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Chapter Four:

A comparative study on the thermal resilience of the mitochondrial energy balance in subtropical fishes

Introduction

Estuaries are considered one of the most fascinating coastal ecosystems, particularly interesting since these ecosystems are in a perpetual state of change. Estuaries such as the Tampa bay are subject to tidal cycles, which in conjunction with precipitation cycles, influence salinity, temperature and nutrient profiles. For example, average water temperature could vary as much as 15°C in less than a month (Badylak et al. 2007). Thus, the organisms inhabiting such a challenging environment must be able to cope at various levels of biological organization; from the organismal to the sub cellular levels.

Most coastal fishes depend in one way or another on estuarine systems (Blaber et al. 1989; Able 2005). For the most part, juvenile stages are dominant in estuaries, where structured habitats are abundant, thus potentially decreasing mortality associated with predation. Most of these structured habitats are located in shallow (< 5 m) waters, where seagrass beds (e.g. Thalassia, Halodule) and mangrove (Rhizophora) covered coastlines predominate. Although protective in nature, these shallow water habitats constitute a set

of unique challenges for fishes, as the water column can drastically change in its thermal characteristic and oxygen content in a matter of hours.

Body temperature, analog to environmental temperature in most ectotherms, has profound implication on physiological performance. As a result, physiological restraints limit the habitat selection and range distribution, where most of the time the individual resides close to its thermal optimum (Pörtner 2001; Pörtner 2002; Somero 2005). In this optimal thermal range, biochemical processes, especially enzyme mediated processes, often exhibit a higher performance. However, fishes inhabiting estuaries often encounter a highly variable thermal regime, which may alter the energy balance, overall homeostasis and performance of the individual.

The production of mitochondrial ATP is a temperature sensitive process; mostly due to the high number of enzyme-catalyzed reactions that take place in the process, including complex I and II of the electron transport system (ETS), along with cytochrome *c* oxidase and the F_0F_1-ATPase. Complexes I and II provide the reducing power of the ETS through oxidation of the reduced electron carriers NADH and $FADH_2$ produced in the citric acid cycle (Nicholls and Ferguson 2002).

The goal of this study was **to evaluate the thermal sensitivity of the oxidative phosphorylation (OXPHOS) system of liver mitochondria from estuarine teleosts inhabiting the waters of Tampa Bay.** Further, **mitochondrial function of subtropical fishes will be discussed and compared to values obtained from Antarctic teleosts**. To achieve this, mitochondrial function was assayed at various temperatures, and the thermal sensitivity of the mitochondrial complex I (NADH oxidoreductase) and complex II

(succinate dehydrogenase) was determined in the pelagic carangid *Caranx crysos* and the more demersal *Lagodon rhomboides*. In addition, the capacity of the OXPHOS system was evaluated in liver mitochondria from *Opisthonema oglinum*, *Fundulus similis*, *Mugil cephalus* and *Mugil curema* to investigate the thermal sensitivity of complexes I and II throughout the naturally occurring thermal range.

Methodology

Specimen collection. Benthic and pelagic specimens were collected in the southern portion of Tampa Bay, Florida. The species *Opisthonema oglinum*, *Mugil cephalus*, *Mugil curema* and *Fundulus similis* were caught by using a 3m wide cast net, and *Lagodon rhomboides* and *Caranx crysos* specimens were caught using hook and line. After collection, all specimens were transported in aerated 19 L bucket to the aquarium facility of the University of South Florida, College of Marine Science. Specimens were transferred to acclimation tanks equipped with a flow-through bay water system for at least two weeks prior to analysis, and feed a combination of commercial fish pellets and pathogen-free frozen mysid shrimps every 48 hours. Acclimation tanks consisted of three 570 L fiberglass rectangular tanks, and specimens were held at low densities (less than 10 individuals per tank) at any given time. Temperature was controlled (28 ± 2.0°C), along with biweekly nutrient analysis.

Isolation of liver mitochondria. Fresh livers were excised and processed according to Weinstein and Somero (1998), with minor modifications. Briefly, liver tissue from one or more individuals (~1.0 g of liver tissue) were minced in an ice-cold petri dish, then homogenized in 8 mL of a sucrose-based isolation medium (250 mM

Sucrose, 1 mM EGTA, 10 mM K_2PO_4, 1 % BSA, pH = 7.4, 20 °C) using an ice-cold Dounce homogenizer (Kontes, Vineland, NJ) and 5 passes with a loose fitting pestle followed by 2 passes with a tight fitting pestle. Homogenate was distributed among 1.5 mL centrifuge tubes and centrifuged at 650 *g* for 10 min at 4°C to remove cellular debris and undisrupted tissue. The supernatant was collected and again centrifuged at 9,600 *g* for 15 min at 4°C to sediment the mitochondrial fraction. Pellets were washed with isolation medium, resuspended and recollected by centrifugation at 9,600 *g* for 15 min at 4 °C two consecutive times. The final pellet was suspended in 300 – 500 µL of isolation medium and stored in ice until assayed.

Thermal sensitivity of ETS complexes I and II in liver mitochondria. To assess the thermal sensitivity of mitochondrial complexes I and II, which feed electrons to the ETS from the citric acid cycle products NADH and $FADH_2$, a high-resolution respirometry system was employed. This system comprise a 2.0 mL water-jacketed respirometric chamber (DW-1, Hansatech Instruments, Norfolk, England) attached to a Clark-type polarographic electrode (C-1, Hansatech Instruments, Norfolk, England). Respirometric chambers were calibrated at the assay temperature in the presence of 500 µL of respiration medium prepared following Weinstein and Somero (1998), with minor modifications (100 mM KCl, 1% w/v BSA, 2 mM $MgCl_2$, 1mM EGTA, 25 mM K_2PO_4, 10 mM Tris-HCl, pH = 7.5, 20°C). At each temperature, background signal was recorded prior to mitochondrial injection. For each run, 10 – 50 µL of purified mitochondria was injected into the respirometer chamber containing 500 µL of respiration medium. Bennett and coworkers (1992) found a critical thermal minimum (CTMin) for *L. rhomboides* to be about 11.7°C on specimens acclimated to 22°C. With this in mind, oxygen

consumption was monitored at assay temperatures ranging from 10 - 40 °C. Substrate stock preparation were carefully prepared according to Lemieux and Gnaiger (2010). To assess the temperature sensitivity of OXPHOS substrates, ADP, and inhibitors were added in the following order:

1. Mitochondria → Establish state I, or respiration without any added substrates
2. Pyruvate (5mM), malate (2mM), glutamate (10mM) → State II with complex I
3. ADP (2mM)→ State III with complex I
4. Succinate (10mM)→State III with complex I and II
5. Rotenone (0.5µM)→Inhibits complex I, establishing state III with complex II
6. Oligomycin (5mM) →Inhibits F_1F_0-ATPase, establishing state II with complex II

Respiratory states were determined as described in Nicholls and Ferguson (2002). Briefly, substrate induced (state II, Leak) respiration rate was recorded for approximately 10 minutes, followed by the addition of 2 mM ADP (10 µL from a 100 mM neutralized ADP stock). ADP stimulated respiration (state III, OXPHOS) for each complex was recorded for approximately 10 minutes. The complex I inhibitor rotenone was added to the chamber in order to determine state III respiration of complex II. Respiration rates via $FADH_2$ from complex II in state II was determined after the addition of oligomycin, a F_0F_1 ATPase inhibitor. The ratio of ADP-induced respiration over leak respiration serves as indicator for the coupling efficiency of the oxidation system (ETS) with the phosphorylation system and is termed respiratory control ratio (RCR). RCR values were calculated from average respiration rates at each temperature dividing state III respiration rates by state II rates.

Mitochondrial protein quantification. Total protein in sample was quantified according to Bradford (1976), using the commercially available Better Bradford Commassie Stain Assay (Thermo Scientific, Rockford, IL). Samples were diluted 20:1 in deionized water and absorption values were determined after 10 min incubation with a Cary 1 spectrophotometer at 20 °C and $\lambda = 595$ nm. Protein values in the isolation buffer were measured, and samples were corrected for the concentration of BSA present in the isolation buffer.

Overall thermal sensitivity of OXPHOS in estuarine teleosts. Livers samples of all specimens were processed as described above. The respirometer was calibrated at 10°C with 500µL of respiration medium and 10 – 50 µL of purified mitochondria was added to each chamber. Each isolation was assayed at substrate combinations that feeds electrons through complex I and II (P+M+G+S) simultaneously, at final concentrations reported previously. Respiratory states II and III with the activation of complexes I and II was recorded, which were utilized to calculate the respiratory control ratio (RCR) at each temperature.

Results

The activation of the OXPHOS system and its thermal window of tolerance was evaluated in various subtropical estuarine species. Overall, there was a significant amount of variability in the observed thermal tolerance of the OXPHOS system among species. However, respiratory control ratios were significantly reduced beyond temperatures warmer than habitat temperatures for most species.

Thermal sensitivity of ETS complexes I and II in liver mitochondria. Complex specific thermal sensitivity was evaluated for the species *L. rhomboides* and *C.*

crysos. As illustrated in Figure 1.1a, complexes I and II of *L. rhomboides* showed significant differences in thermal sensitivity. At 10°C, both complexes I and II of *L. rhomboides* showed respiration rates of 15.34 ± 1.9 and 12.55 ± 1.4 nmol O_2 min^{-1} mg $protein^{-1}$, respectively. As assay temperature increased, so did their individual complex activities. However, *L. rhomboides* complex I exhibited a higher Q_{10} than complex II at each temperature The highest Q_{10}'s were found between 10° and 20°C with 2.99 for complex I and 1.98 for complex II. Moreover, significant differences were found among state III rates obtained when only complex II was activated and those rates obtained with both complexes activated simultaneously. Throughout the thermal range assayed, a trend characterized by high state III was observed when both complexes were simultaneously activated.

Complex specific state III rates in *C. crysos* liver mitochondria were highly variable at each temperature assayed (Figure 4.1b). As shown, no significant differences were found among state III obtained from individual and simultaneous activation of complexes I and II (two way ANOVA with temperature and complexes as factors, $P > 0.05$, $n = 3\text{-}8$, ± SE). However, there was a significant difference between state III rates obtained at 30°C and those values obtained at 10°C and 40°C (two way ANOVA with temperature and complexes as factors, $P < 0.05$, $n = 3\text{-}8$, ± SE). Simultaneous activation of complexes elicited a Q_{10} value of 2.77 between 10°C and 30°C.

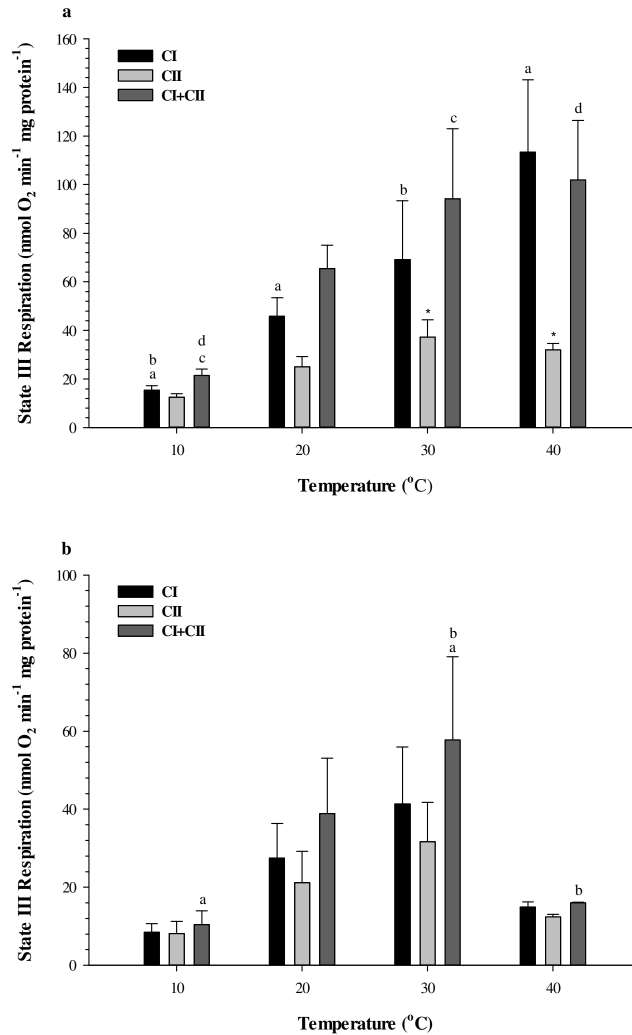

Figure 4.1: Temperature dependent contributions of complexes I and II to the oxidative phosphorylation (OXPHOS) system of liver mitochondria from *Lagodon rhomboides* (**a**) and *Caranx crysos* (**b**). Differences in state III respiration between simultaneous and individual activation of complexes are shown with an asterisk (two-way ANOVA on complexes and temperature as factors, $P < 0.00$, $n = 3\text{-}8$, ± SE). Complex specific differences in state III rates at different temperatures are identified with letters (a-d).

Respiratory control ratios for *L. rhomboides* and *C. crysos* are shown in Figure 4.2. Coupling of substrate oxidation with the phosphorylation system was not significantly different between NADH or $FADH_2$ generating complexes in both species. RCR values were above four indicating high coupling of OXPHOS at assay temperatures close to the acclimation temperature of 28°C. RCR values differed between both species

only at 10°C, where control ratios for both complexes I and II were lowest in *C. crysos*. At an assay temperatures of 40°C, both species exhibited a sharp decrease in the RCR (two way ANOVA with temperature and complex as factors, $P < 0.05$, n = 3-8, ± SE).

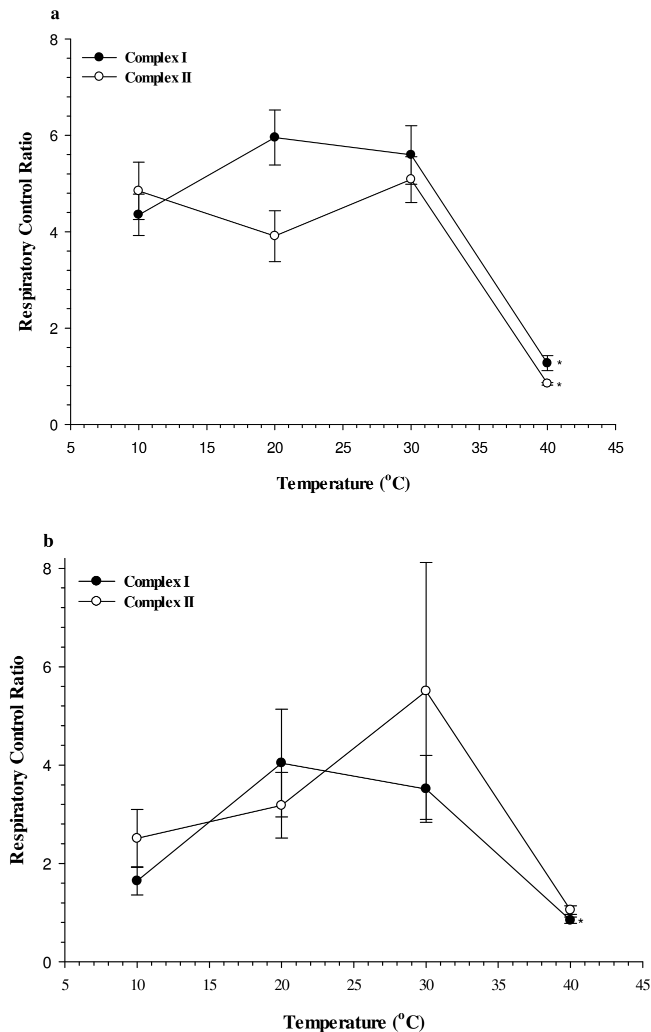

Figure 4.2: Complex-specific respiratory control ratio (RCR) as a function of temperature of liver mitochondria from *Lagodon rhomboides* (**a**) and *Caranx crysos* (**b**).Significant differences are highlighted with an asterisk (*) (one one way ANOVA, $P < 0.05$, n = 38 ± SE). RCR values obtained from the activation of complex II in *C. crysos* did not differ significantly among temperatures.

The response to temperature differed between respiration rates of individual complexes, as shown in Figure 4.3. Significant differences were found for *L. rhomboides*

at 40˚C. Complex I-to-complex II ratios (CI:CII) ranged from 1.33 ±0.22 at 10°C to 3.35 ±0.62 at 40°C in *L. rhomboides* (*n* = 5-8, ± SE). However, complex specific responses of *C. crysos* did not differ with assay temperature.

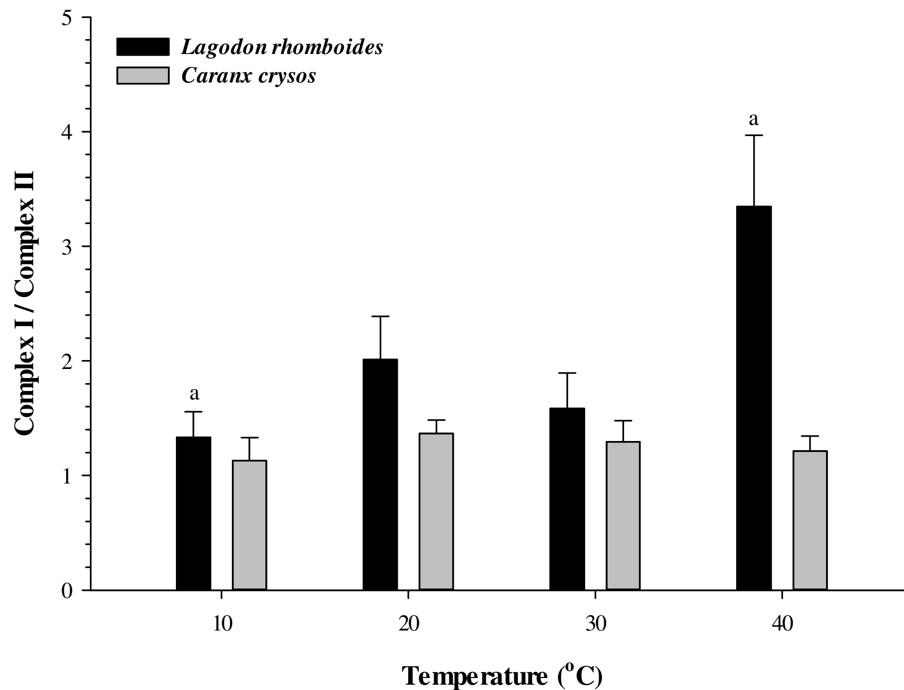

Figure 4.3: Complex I / Complex II ratios of mitochondrial state III respiration for *Lagodon rhomboides* and *Caranx crysos*. Vertical bars represent means, and standard error is shown (*n* = 3-8). Differences in calculated ratios was found only in *L. rhomboides*, represented with a letter (a) (one way ANOVA on ranks, P = 0.032)

Overall thermal sensitivity of OXPHOS in estuarine teleosts. The relationship between leak respiration (state II) and the respiration rate after the addition of saturating levels of ADP (OXPHOS) was evaluated for the estuarine species *O. oglinum*, *F. similis*, *M. cephalus* and *M. curema*. Respiration rates associated with OXPHOS was variable, where even congeners (*M. cephalus* and *M. curema*) showed significant differences in their maximal RCR. Despite this variability, changes in RCR values with increasing temperature have shown a similar pattern amongst the species range. Optimal RCR

values were found at 20°C for each species, and all species showed a sharp decrease in RCR from 30°C to 40°C. Within species, excluding *F. similis* due to low sample size, significant differences in RCR values were found within species at all temperatures (one way ANOVA on individual species RCR, P < 0.001, *n* = 4 – 8)

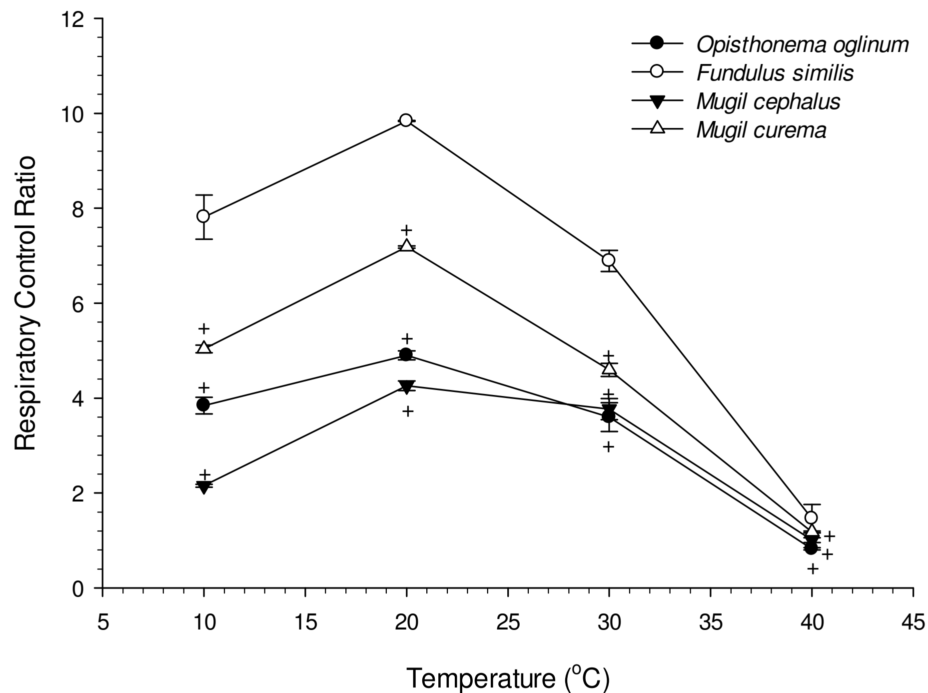

Figure 4.4: Respiratory control ratios (RCR) of liver mitochondria of subtropical teleosts. Values represent the mean and standard error (*n* = 4 - 8), with the exception of *Fundulus similis* (*n* = 2) where error bars represent the median range. RCR values at any given temperature were significant different among species, except for *F. similis*. In addition, significant differences were found in RCR values obtained within species at each temperature (two way ANOVA, P < 0.001, *n* = 4-8, ± SE)

Discussion

Thermal sensitivity of ETS complexes I and II in liver mitochondria.

Energetic processes in the cell are highly reliant on the functionality of the OXPHOS

system. In subtropical, estuarine systems like Tampa Bay, thermal fluctuations could exert a challenge to fishes that inhabit it. In this study, the thermal tolerance of the OXPHOS system utilizing NADH (complexes I) and or $FADH_2$ (complex II) generating substrates was evaluated. Results obtained from this study suggest that mitochondrial ATP production in subtropical teleosts could be adversely affected by temperatures close to those found for the summer months in the Tampa bay. This may indicate that species in subtropical regions are already operating close to their upper thermal tolerance and will be particularly impacted by the warming trend projected for marine ecosystems.

As shown in Figure 4.1, in *L. rhomboides* state III activity of complex I increased with increasing temperature. Despite this continuous increase in respiration, a sharp decrease in RCR values was observed by raising the temperature from 30°C to 40°C. This decrease in RCR could be associated with an increase in fluidity of the inner mitochondrial membrane at higher temperatures (Hazel 1995), which in turns increases the rate of uncoupled ('LEAK') respiration. Increasing uncoupled respiration thus reduces the efficiency of the OXPHOS system, potentially reducing the amount of ATP produced per oxygen consumed (P/O ratio).

ETS complexes I and II have been shown to reduce their activity with increasing temperature. In triplefin blennies, state III respiration rates obtained with the activation of complex I decrease with increasing temperature, from 50% of the state III rates obtained with complex II activated at 15°C, to 30% of complex II rates at 30°C (Hilton et al. 2010). In the study presented here, state III respiration rates obtained by activating complex I were comparably higher that those values obtained by activating complex II, with a CI:CII higher than one for both *L. rhomboides* and *C.crysos* at all assay

temperatures. In both *L. rhomboides* and *C.crysos*, complex I elicited more than 100% of the state III respiration obtained by activation complex II alone (Figure 4.3), which are found to be higher than those found by Hilton and Colleagues (2010). Moreover, CI:CII increases with temperature in *L. rhomboides*, which suggest that there is a wider thermal tolerance of complex I than those species assayed by Hilton et. al (2010). In *C. crysos*, CI:CII found to be consistent throughout the temperature range, suggesting no comparative differences in thermal sensitivity of both complexes I and II. Results found for *C. crysos* agrees with a similar study in Antarctic notothenioids, where it was found that complex I was as thermally stable as complex II (Mark et al. 2012). The reported differences in thermal stability of *L. rhomboides* between complexes I and II in this study have not been described in the past, and may reflect the implications of the differences in biochemical properties (e.g. size, function) between complexes I and II. Considering the differences in size alone, complex I is comprised of 43 polypeptides, compared to 4 polypeptides in complex II (Nicholls and Ferguson 2002). Furthermore, state III respiration rates observed when both complexes are activated are not simple additions of the complex specific rates (non-additive), which also agrees with previous studies in Antarctic teleosts (Mark et al. 2012).

Functional capacity of the OXPHOS system, measured as RCR, remained stable over most of the thermal range. Fluctuation in habitat temperatures could alter the efficiency of the OXPHOS system, as shown between intertidal and subtidal fishes (Hilton et al. 2010). Field observations of changes in fish community structure along seagrass beds of Tampa bay during summer low tides suggest that *L. rhomboides* are highly tolerant to temperatures close to 40°C over short tidal periods (Martinez, pers.

obs.). This constitutes a higher than average temperature values found for the warmest months in Tampa Bay, where average water column temperatures can be around 30°C (Badylak et al. 2007). Critical thermal maximum values obtained for other estuarine species suggests that water temperatures above 30°C may reduce the physiological performance of estuarine species (Sylvester 1975; Menasveta 1981). In addition, critical thermal values found in tropical reef fish species are within a similar range (35-41°C) of those found for estuarine species (Mora and Ospina 2001), indicating that estuarine teleosts may experience thermal stress over warm summer periods. Further, this high tolerance of *L. rhomboides* towards warm temperatures, combined with the data gathered in this study, points towards a moderate tradeoff between reduced coupling of the OXPHOS system with respiration at water temperatures above 30°C, and the advantage of predator avoidance (Jordan et al. 1997) and prey availability (Main 1987) by remaining in shallow (less than 1 meter) seagrass beds. Further work on oxidative stress in *L. rhomboides* as well as other species exposed to warm temperatures will be necessary to confirm this hypothesis.

As described in Figures 4.1 and 4.2, thermal tolerance was observed to be higher in *L. rhomboides* than in the blue runner *C. crysos*. State III respiration rates in *C. crysos* below 20°C and above 30°C in presence of either NADH or $FADH_2$-generating substrates are a clear reflection of the low thermal tolerance, which translates to the low RCR values observed for the species. The evidenced high leak respiration could be beneficial to reduce the production of reactive oxygen species in a highly-active pelagic fish (Skulachev 1998); however it will only benefit the organisms as long as enough ATP is produced to comply with the metabolic demands of the individual. When both *C.*

crysos and *L. rhomboides* average state III respiration rates are compared throughout the thermal range, it shows that thermal window of tolerance are species specific. Further investigation will be necessary to establish whether the nature of complex specific variability resides on quantitative or qualitative strategies.

Overall thermal sensitivity of the OXPHOS system in estuarine teleosts. Subtropical estuarine environments such as the Tampa Bay are particularly dynamic with respect to variations in salinity and temperature, among other potential environmental stressors. Average water temperatures have been observed to drop by 15°C in a matter of weeks (Badylak et al. 2007), and greater fluctuations could be observed in shallower regions, where most of the species studied spend a large portion of their life. Despite the challenges that arise from such large variation in water temperatures, estuarine fishes thrive in these waters, and the physiological underpinnings describing how they cope with thermal insults at the biochemical levels are yet to be clearly understood.

Intraspecific differences in mitochondrial thermal tolerance along a latitudinal gradient have been documented in estuarine fishes (Fangue et al. 2009). At the same acclimation temperature, subspecies from higher latitudes (colder water temperatures) exhibited higher organismal metabolic rates, in addition to higher state III at each acclimation temperature (Fangue et al. 2009). From the overall RCR analysis of various estuarine species, the thermal sensitivity of the OXPHOS system of estuarine teleosts exhibited a wide functional range (Figures 4.4 and 4.2). Furthermore, this pattern conforms to the theoretical performance of a thermal generalist (Angilletta Jr 2009). If we compare RCR values found of a low-temperature specialist such as *Pleuragramma antarcticum* (See Ch. 2) with RCR of subtropical species, it becomes clear that

mitochondrial function of subtropical species exhibit a lower performance but a wider thermal tolerance window. This feature is evidenced by a low RCR across the investigated thermal range of subtropical species, which may serve to reduce constant biochemical compensation in a thermally variable environment.

The effects of thermal heterogeneity upon teleost thermal tolerance have been evaluated in the past. In most fish studies, the thermal window of tolerance was somewhat dependent on acclimation temperature (Beitinger et al. 2000; Eme and Bennett 2009). Furthermore, thermal tolerance polygons of both reef and estuary associated fishes are considerably different (Eme and Bennett 2009). Thus, the ability of organisms to tolerate a wider range of temperature could be attributed in part to fluctuations in environmental temperature. Along these lines, further work on the thermal plasticity of mitochondria of estuarine fishes acclimated to a fluctuating thermal regime versus non-fluctuating regime could prove useful to establish whether the acclimation capacity could be reduced by reducing environmental thermal heterogeneity.

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Chapter Five:

Thermal thresholds of mitochondrial respiration from estuarine tropical teleosts

Introduction

Fluctuations of the thermal regime in coastal tropical regions are relatively small, in comparison to those found for subtropical regions. However, it is in the tropical regions where temperatures in the warm end of the spectrum (25-30°C) are observed year round. Thus, tropical organisms with small thermal tolerance polygon (Brett 1952) like fishes associated with coral reefs and estuaries (Eme and Bennett 2009) could seem often challenged in their thermal regimes.

Critical thermal tolerance and sensitivity have been utilized in fishes to determine potential effects of climate change in tropical marine teleosts (Mora and Ospina 2001; Eme et al. 2011). From these studies, authors have suggested that tropical estuarine species may have what it takes to survive long term warming trends associated with climate change (Eme et al. 2011). In additional studies elucidating the thermal tolerance polygons for estuarine and coral-reef associated fishes, estuarine species sustained a larger tolerance window when compare to coral reef-associated fishes (Eme and Bennett 2009). Although the tolerance window of estuarine fish species seems beyond any of those values found in the wild, there may be other implications of gradual change in

temperature, like those affecting the energy budget of fishes. Currently, there is little information about sub-lethal effects of changes in the thermal regime of tropical fishes. More specifically, there is a need for mechanistic explanations to describe how the energetic machinery could adapt to thermal change, and to what extent the thermal polygons will also represent reproductive growth performance.

To further understand the biochemical aspects of thermal stress in tropical fishes, it is relevant to describe how the mitochondrial energy metabolism could be affected. Our present understanding of the thermal window of tolerance provides a relevant piece of information in the determination of a specie's thermal window of tolerance, but fails in describing sub-lethal effects that could be addressed by describing how mitochondrial function is affected by temperature.

The goal of this study was **to describe the functional thermal window of the oxidative phosphorylation (OXPHOS) system of liver mitochondria from the tropical teleosts *Eugerres plumieri*, *Micropogonias furnieri* and *Trachinotus goodei*.** By analysing the respiratory states of the mitochondria at a series of temperatures, we were able to elcudiate how temperature could affect one of the most important energetic process; ATP production by the mitochondria. Moreover, we were able to determine interspecific differences in the OXPHOS system, thus pointing towards species particularly sensible to thermal stress.

Methodology

Specimen collection. Tropical specimens were collected in neighboring waters of Punta Santiago beach area in Humacao, Puerto Rico (18° 09' 47.36" N; 65° 44' 36.85"

W). Collection and experimental procedures were performed from December 1–15 of 2012. Specimens were collected using a 20-meter long beach seine net, and later transported in aerated 19 L buckets to a 190 L holding tank at the University of Puerto Rico, Humacao Campus. No acclimation period was performed; specimens were held for less than 3 days before the experiment in artificial seawater prepared in the laboratory at habitat salinity and aquarium room temperature (25.0 ± 2.0°C)(Instant Ocean - Spectrum Brands, Madison, WI).

Isolation of liver mitochondria. Fresh livers were excised and processed according to (Weinstein and Somero 1998), with minor modifications. Briefly, liver tissue from one or more individuals (~1.0 g of liver tissue) were minced in an ice-cold petri dish, then homogenized in 8 mL of a sucrose-based isolation medium (250 mM Sucrose, 1 mM EGTA, 10 mM K_2PO_4, 1 % BSA, pH = 7.4, 20 °C) using an ice-cold Dounce homogenizer (Kontes, Vineland, NJ) and 5 passes with a loose fitting pestle followed by 2 passes with a tight fitting pestle. Homogenate was distributed among 1.5 mL centrifuge tubes and centrifuged at 650 *g* for 10 min at 4°C (Minispin®, Eppendorf, Hauppauge, NY) to remove cellular debris and undisrupted tissue. The supernatant was collected and again centrifuged at 9,600 *g* for 15 min at 4°C to sediment the mitochondrial fraction. Pellets were washed with isolation medium, resuspended and recollected by centrifugation at 9,600 *g* for 15 min at 4 °C two consecutive times. The final pellet was suspended in 300–500 µL of isolation medium and stored in ice until assayed.

Thermal sensitivity of liver mitochondria. To assess the thermal sensitivity of liver mitochondria, a high-resolution respirometry system was employed. This system

comprise a 2.0 mL water-jacketed respirometric chamber (DW-1, Hansatech Instruments, Norfolk, England) attached to a Clark-type polarographic electrode (C-1, Hansatech Instruments, Norfolk, England). Each respirometric chamber (2 in total) was calibrated at the desired assay temperature in the presence of 500 µL of respiration medium. At each temperature, background signal was recorded prior to mitochondrial injection. For each run, 10 – 50 µL of purified mitochondria was injected into the respirometer chamber containing 500 µL of a potassium-based respiration medium prepared following (Weinstein and Somero 1998), with minor modifications (100 mM KCl, 1% w/v BSA, 2 mM $MgCl_2$, 1mM EGTA, 25 mM K_2PO_4, 10 mM Tris-HCl, pH = 7.5 at 20°C). Oxygen consumption was monitored at assay temperatures ranging from 10-40 °C. Substrate stock preparation were carefully prepared according to (Lemieux and Gnaiger 2010). To assess the temperature sensitivity of the OXPHOS system, substrates were injected to the chamber to give final concentrations of 5 mM pyruvate [P], 2 mM malate [M], 10 mM glutamate [G] and 10mM succinate [S]. Respiratory states were determined as described in Nicholls and Ferguson (2002). Substrate induced state II respiration rate was recorded for approximately 10 minutes, followed by the addition of 2 mM ADP (10 µL from a 100 mM neutralized ADP stock). ADP stimulated respiration (state III) was recorded until rates stabilized or the chamber became anoxic. To determine whether state III respiration rate was only reflecting F_0F_1 ATPase activity, respiration rate was recorded after the addition of Oligomycin (1 µL; 5.0 mM).

The ratio of ADP-induced respiration over leak respiration serves as indicator for the coupling efficiency of the oxidation system (ETS) with the phosphorylation system and is termed respiratory control ratio (RCR). RCR’s were calculated from average

respiration rates at each temperature by dividing state III respiration rates by state II rates To understand the effects of temperature on the OXPHOS system, breakpoints in state II and state III respiration rates was calculated using a 2-parameter piecewise regression using average respiration rates, where least squares were calculated through 200 iterations (Sigma plot 12.3, Systat Software).

Oxidative capacities and complex-specific contributions to the ETS. To investigate the contribution of specific mitochondrial complexes to overall mitochondrial function, a variety of metabolic substrate combinations were tested following the rationale of Gnaiger (2010). Livers samples of all specimens were processed as described above. The respirometer was calibrated at 10 °C with 500 µL of respiration medium, and 10–50 µL of purified mitochondria was added to each chamber. Each liver sample was assayed at substrate combinations that feeds electrons through complex I; P+M, M+G, P+M+G, at final concentrations reported previously. Succinate dehydrogenase (complex II) activity was measured after the addition of 10 mM succinate in the presence of 0.5 µM rotenone. To investigate whether respiration was limited by the ETS of the phosphorylation system, stepwise titrations of the uncoupler FCCP (0.5 µM increments) were performed.

Mitochondrial protein quantification. Total protein in sample was quantified according to Bradford (1976), using the commercially available Better Bradford Commassie Stain Assay (Thermo Scientific, Rockford, IL). Samples were diluted 20:1 in deionized water and absorption values were determined after 10 min incubation with a Cary 1 spectrophotometer at 20°C and λ = 595 nm. Protein values were corrected for the concentration of BSA present in the isolation buffer.

Results

Thermal sensitivity of liver mitochondria. Mitochondrial respiration rates obtained at various temperatures in the presence of metabolic substrates and saturating ADP were obtained, and representative raw values are illustrated in Figure 5.1. Substrate induced respiration (state II) rates increase with increasing assay temperature. However, ADP induced respiration (state III) rates displayed variable responses to rising assay temperatures. Overall, coupling of respiration with OXPHOS was obtained at temperatures ranging from 10°C to 30°C for all three species. Interestingly, only in *E. plumieri* (Figure 5.1a) elicited any ATP-coupled respiration response at 40°C. Also, it is a worth noting feature that the overall low respiration rates observed in mitochondria from the pelagic *T. goodei* (Figure 5.1c) were considerably lower than respiration rates found for *E. plumieri* and *M. furnieri*. The highest state III respiration rate at low temperature was found in the benthic *M. furnieri,* characterized by high coupling of respiration with the OXPHOS system.

Individual respiration states II and III were evaluated for *E. plumieri*, *M. furnieri* and *T. goodei* (Figure 5.2). Further evaluation, employing a two-piece regression, showed temperature related changes in state II and III respiration rates with increasing temperature. State II respiration rates observed in *E. plumieri* was significantly altered from 12.57 ± 0.53 at 10°C to 149.49 ± 12.49 nmol O_2 min^{-1} mg $protein^{-1}$ at 40°C (one-way ANOVA on ranks, $P < 0.001$, $n = 5$, ± SE). Significant differences were also found for state II rates of *M. furnieri* and *T. goodei*, where values averaged 9.93 ± 0.47 and 9.235 ± 1.39 at 10°C and increased to 78.07 ± 8.84 and 36.07 ± 1.92 nmol O_2 min^{-1} mg $protein^{-1}$ at 40°C, respectively.

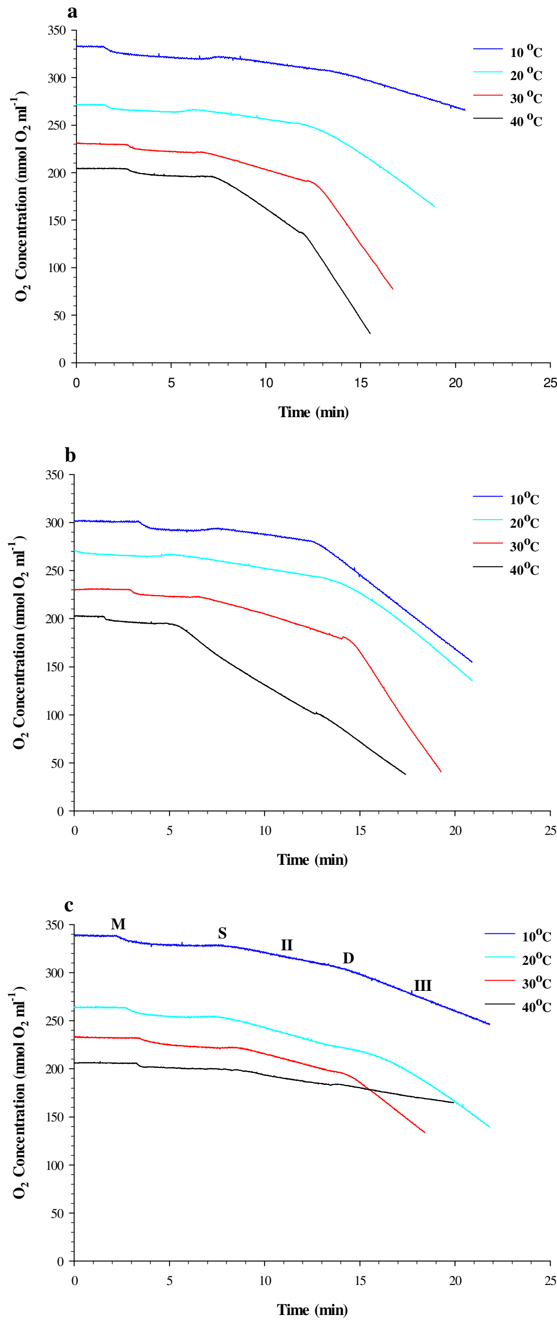

Figure 5.1: Representative oxygen consumption rates of liver mitochondria from *Eugerres plumieri* (**a**), *Micropogonias furnieri* (**b**) and *Trachinotus goodei* (**c**), at various respiratory states. Upper case letters in Figure 1c represent the addition of mitochondria (M), further activation of complexes I and II with the addition of pyruvate, malate, glutamate and succinate (S), followed by the addition of saturating levels of ADP (D). Substrate induced respiration rate (II) and ADP induced respiration rate (III) are also identified.

Comparative analysis of state III respiration showed significant differences of respiration rates associated with OXPHOS at different temperatures (Figure 5.2). State III respiration of *E. plumieri* liver mitochondria increased with assay temperature, where respiration rates increased from 40.03 ± 5.64 at 10°C to 311.25 ± 30.96 nmol O_2 min^{-1} mg $protein^{-1}$ at 40°C (one-way ANOVA on ranks, $P < 0.001$, $n = 5$, ± SE). Interestingly, state III respiration showed a reduced tolerance towards temperatures warmer than 30°C in both *M. furnieri* and *T. goodei*. State III respiration rates obtained for *M. furnieri* and *T. goodei* plummeted from 176.04 ± 26.99 and 109.30 ± 10.30 at 30° C, to a low 100.21 ± 15.32 and 35.11 ± 1.69 nmol O_2 min^{-1} mg $protein^{-1}$ at 40°C, respectively.

Respiratory control ratios (RCR) are shown for *E. plumieri*, *M. furnieri* and *T. goodei* in Figure 5.3. Significant differences in RCR values across the thermal range were found for *E. plumieri*, where significant differences in OXPHOS coupling efficiency were observed throughout the investigated thermal range (Figure 5.3a) (one-way ANOVA, $P < 0.001$, $n = 5$, ± SE). Similarly for *M. furnieri*, average RCR values obtained from 10°C to 30°C were around 4, and significantly dropped to around 1.25 at 40°C (Figure 5.3b). In the more thermally sensible *T. goodei*, RCR values found at all temperatures differ from each other, with the exception of RCR values obtained at 20°C and 30°C (Figure 5.3c). In summary, coupling efficiency measured in all three species varied with assay temperature, where *E. plumieri* exhibited the highest coupling efficiency at warmer temperatures, *M. furnieri* coupling efficiency extended to the lowest temperature assayed and *T. goodei* liver mitochondria was remarkably sensible to the investigated thermal range.

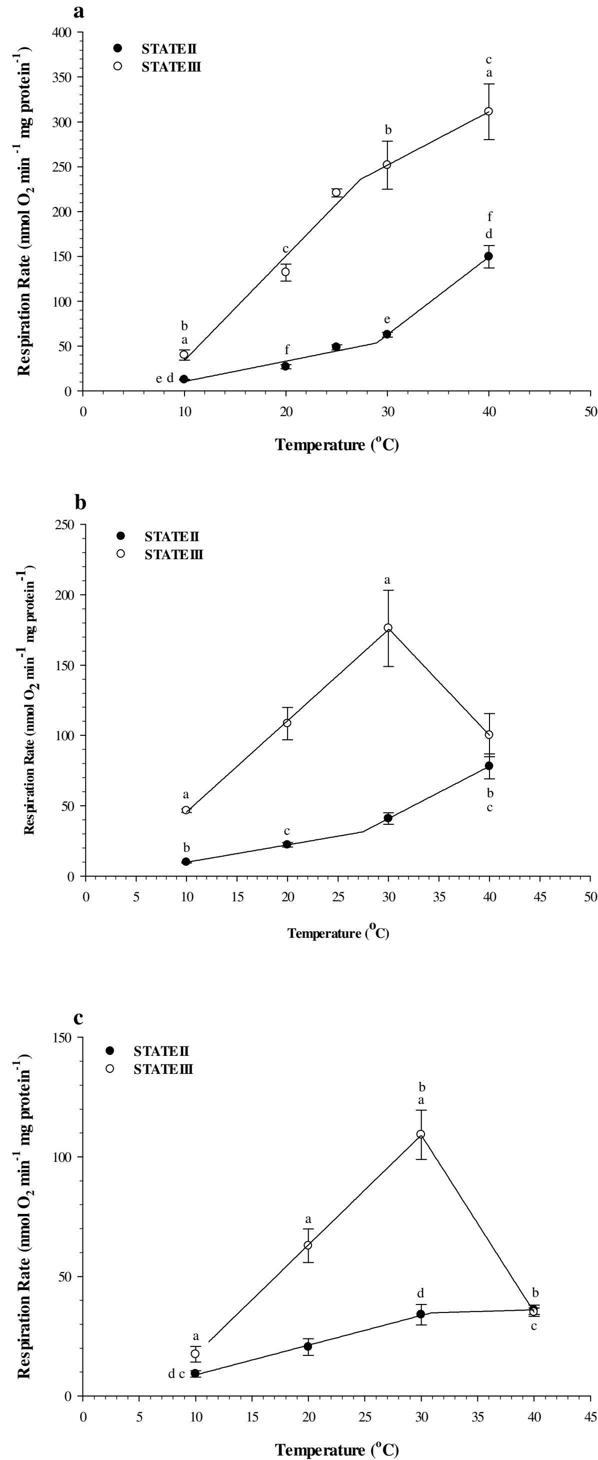

Figure 5.2: Thermal sensitivity of state II and state III respiration of liver mitochondria from *Eugerres plumieri* (**a**), *Micropogonias furnieri* (**b**) and *Trachinotus goodei* (**c**). Average respiration rates obtained with the addition of pyruvate, malate, glutamate and succinate (state II) are shown, along with their corresponding standard error (n = 5). State

III respiration rates under saturating ADP are shown for each species; *E. plumieri* exhibited the lowest thermal sensitivity, where no breakpoint in state III respiration was found throughout the thermal regime. Average values with the same letters (a - f) are significantly different from each other (one way ANOVA on ranks, $P < 0.05$, $n = 5$, ± SE).

Oxidative capacities and complex specific contributions to the ETS. Complex specific contribution to the ETS was evaluated in *E. plumieri*, *M. furnieri* and *T. goodei* at 30°C (Figure 5.4). Overall, state III respiration rates was highest in *E. plumieri*, where average state III respiration rates for both complexes I and II were 196.01 ± 11.24 and 243.67 ± 44.88 nmol O_2 min^{-1} mg $protein^{-1}$, respectively. Although the simultaneous activation of both complexes yielded consistent high state III respiration rates, no significant differences were found between individual and simultaneous complex activation (two way ANOVA, $P = 0.073$, $n = 5$ ± SE). Pairwise comparison of complex specific state III among species described significant differences among groups. State III respiration rates with individually activated complexes I and II in *E. plumieri* were different from complex specific state II rates obtained with *T. goodei*. Similar differences were observed in state III values obtained from the simultaneous activation of both complexes, where interspecific differences were also found for *E. plumieri* and *T. goodei*.

Comparative evaluation of the state III respiration obtained with the individual activation of complex I and complex II at 30°C is summarized in Table 5.1. Within each species, complex I consistently elicited about 80% of the state III respiration rate observed with complex II activated. No significant differences were found amongst average ratios (one way ANOVA, $P = 0.54$, $n = 5$, ± SE)

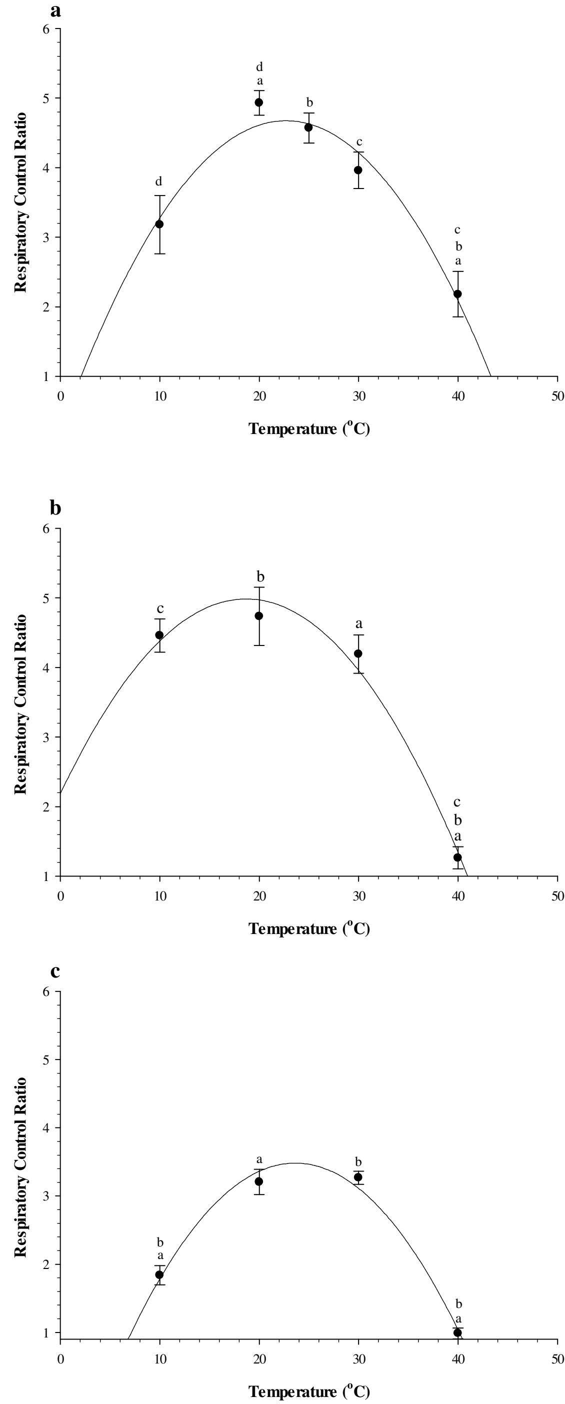

Figure 5.3: Respiratory control ratios (RCR) of liver mitochondria from *Eugerres plumieri* (**a**), *Micropogonias furnieri* (**b**) and *Trachinotus goodei* (**c**). Values identified with the same letters are significantly different (one way ANOVA, P <0.05, *n* = 5 ±SE).

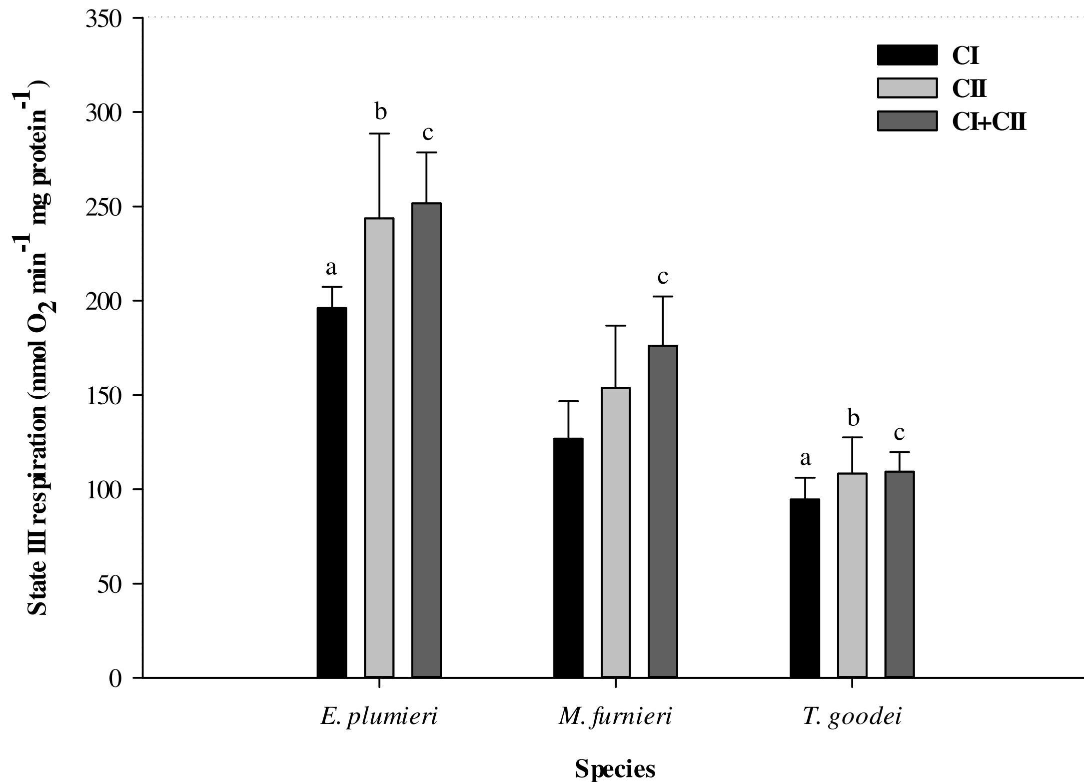

Figure 5.4: Complex specific contributions to OXPHOS respiration in liver mitochondria of tropical estuarine fishes at 30°C. Activation of the ETS was achieved by the addition of milimolar levels of pyruvate, malate, glutamate and succinate. State III respiration rates were obtained after the addition of saturating levels of ADP. Letters over columns (a-c) illustrate significant differences within ETS complexes (two way ANOVA, $P < 0.05$, $n = 5$, ± SE). Columns with the same letters display significantly different values.

Table 5.1: Complex specific state III respiration and their relative contribution to the ETS in tropical fishes. Oxygen consumption rates are expressed in nmol O_2 min^{-1} mg $protein^{-1}$; standard error is shown.

Species	Complex I state III	Complex II state III	C I / C II
Eugerres plumieri **(*n* = 5)**	196.01 ±11.24	243.67 ±44.88	0.85 ± 0.09
Micropogonias furnieri **(*n* = 5)**	126.71 ±19.94	153.88 ±32.79	0.80 ± 0.01
Trachinotus goodei **(*n* = 5)**	94.45 ±11.58	108.34 ±19.16	0.89 ± 0.07

Discussion

Thermal sensitivity of the OXPHOS system in tropical teleosts. Thermal thresholds in the bioenergetic machinery of cells influence the overall performance of ectothermic fauna (Angilletta Jr et al. 2002; Angilletta Jr 2009). Within a given tolerance range, physiological performance is primarily dependent on how much energetic currency, in the form of ATP, is available for metabolic and anabolic processes. In this study, mitochondrial function, primarily the OXPHOS system function, of tropical fishes was evaluated at various temperatures, with the objective of better understand how alterations in the thermal regime could affect mitochondrial ATP production. Results obtained for all three coastal species analyzed indicate that increasing temperatures such as the warming trend projected in tropical regions could reduce the efficiency of the

OXPHOS system, potentially reducing long-term growth performance and reproductive output of estuarine fishes.

In the tropical species investigated here, it was found that the thermal tolerance of the OXPHOS system was variable among species. These results agrees with the variability found in previous studies of the OXPHOS system sensitivity to temperature in subtropical and polar teleosts (Weinstein and Somero 1998; Hardewig et al. 1999; Urschel and O'Brien 2009; Hilton et al. 2010; Mark et al. 2012). Despite the variability observed, results from the piecewise regression establish a breakpoint in state III respiration at 30°C for all species investigated, where ADP induced state III respiration rates at temperatures warmer than 30°C are reduced or completely impaired.

Coupling of respiration with the OXPHOS was highly sensitive to assay temperature in all three species. It was not surprising that higher RCR values reported for all three species were between 20 and 30°C, as this range represents the natural range of annual water temperature of the area (25.5–28.7°C) (Colón-Rivera, Unpublished data). However, coupling efficiency is severely reduced after 30°C, suggesting that the OXPHOS system of the species assayed in this study could be adversely affected with increasing water temperature. Although previous studies evaluating the thermal tolerance polygons on estuarine species have concluded that some teleost species may not be compromised by gradual increase in habitat temperatures (Eme, Dabruzzi et al. 2011), the energetic repercussion at sublethal levels must be evaluated to assess their performance. Additional studies evaluating the capacity for acclimation of mitochondria to water temperatures above 30°C will be instructive, to confirm that whether the observed reduction in coupling efficiency could not be improved through acclimation periods.

Oxidative capacities and complex-specific contributions to the ETS. Complex specific state III respiration was evaluated for tropical coastal fishes at 30°C. Among all species investigated, state III respiration elicited with NADH (complex I) substrates yielded 80-89% of those state III rates obtained with $FADH_2$ (complex II) substrates. These values are higher than 40-50% found on triplefin blennies acclimated to 15°C (Hilton et al. 2010). These differences may be attributed to the type of tissue utilized (liver in this study, heart ventricular fibers in Hilton et. al 2010), which could yield variable respiratory capacities due to the *in vitro* effect of the mitochondrial isolation protocol utilized in this study. However, state III respiration rates obtained with NADH generating substrates have yield 50-150% of state III rates with $FADH_2$ substrates in ventricular fibers of Antarctic notothenioids (Brand 1990). These results suggest that the contribution to mitochondrial energy metabolism could vary from 30-100% of complex II contribution. Furthermore, Complex specific state III respiration rates of subtropical species (See Ch. 4) were in close agreement to the rates obtained for tropical fishes, where activation of complex I yielded state III rates similar or higher than those found when complex II is activated.

In conclusion, this study provides evidence suggesting that the OXPHOS system, responsible for mitochondrial ATP formation, reduce its efficiency at temperatures above 30°C in wild specimens. In addition, state III respiration with NADH generating substrates could be up to 90% of the contribution estimated for complex II, Suggesting that complex I contribute to a higher extent to the mitochondrial energy metabolism in warm adapted fishes. In comparison with a similar study performed in subtropical species (See Ch. 4), mitochondrial function of tropical species did not exhibited higher tolerance

towards warmer temperature, although average annual water temperature is higher than the subtropical estuary examined in Chapter 4. This evidence points towards two scenarios, 1) mitochondria from tropical specimens are close to their thermal tolerance window or 2) that mitochondrial function must uncouple respiration from ATP production to reduce the oxidative stress caused by warmer temperatures. Further work in the acclimation capacity of the investigated species to temperatures warmer than 30°C will be instrumental a more in depth analysis.

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Chapter Six:

Concluding Remarks

From the studies here provided regarding the comparative physiology of teleost inhabiting Antarctic waters, it is concluded that:

- In the Antarctic notothenioid *Pleuragramma antarcticum*, liver mitochondrial function is not significantly altered at temperatures lethal to Antarctic notothenioids. This might not be the same scenario in brain synaptosomal mitochondria, which was suggested to be investigated in the future.
- In comparison with other notothenioids studied, *P. antarcticum* exhibited the highest ATP-coupled respiration. It is suggested herein that the increasing dependence of lipid metabolism observed on *P. antartcticum* in previous studies, which in turns increase the oxidative capacity of the citric acid cycle, could be the causative for the rates obtained.
- Further work determining routine metabolic rates of *P. antarcticum* showed that the metabolic rates strongly correlated with specimen wet mass. Intriguingly, two out of the 11 specimens analyzed exhibited unusually high respiration rates, to which it was suggested that those specimens might be exhibiting specific dynamic action. Besides these two cases, mass specific metabolic rates decreased with specimen wet mass. Overall, routine metabolism of *P. antarcticum* was found to

be within the lower end of previous studies, which confirms that *P. antarcticum* pelagic activity is low.

From the series of comparative studies on mitochondrial function across thermal gradients, it is concluded that:

- Mitochondrial function exhibited performance curves analogous to those expected for both stenothermal and eurythermal fishes (Figure 6.1). In other words, the scale of mitochondrial performance (measured as the respiratory control ratio), is tightly related to the level of thermal specialization of the species.
- In tropical and subtropical teleosts species studied, it was found that the oxidative phosphorylation (OXPHOS) system is highly sensitive to assay temperatures of 40°C. Therefore it is suggested that further work regarding the thermal tolerance of estuarine tropical and subtropical species should take into account the OXPHOS thermal sensitivity of the species, and that classical thermal tolerance polygons might not reflect reduction in individual growth (somatic and reproductive) performance.

Of outmost importance, the energetic repercussions of the projected warming trends in tropical and subtropical regions could directly impact long-lived teleosts species (e.g. Centropomidae, Megalopidae), along with their associated fisheries. Often, these species rely on a reproductive stock dependent of multiple year classes. If no further acclimation capacity could be observed in these species, reproductive or somatic growth of early year classes may be compromised. Likewise, fast-growing species (e.g. Clupeidae) growing in a warmer-than-optimal temperature range may be detrimental to reproductive success. Often, these fast growing species are short-lived, and a reduction in the reproductive

performance may impact both the quality (i.e. amount of lipids) and the quantity (density per individual) of eggs produced. Therefore, it is imperative to establish study models that will provide mechanistic answers regarding how a reduced mitochondrial performance may impact the reproductive capacities and overall population dynamics of fishes in the face of a changing climate.

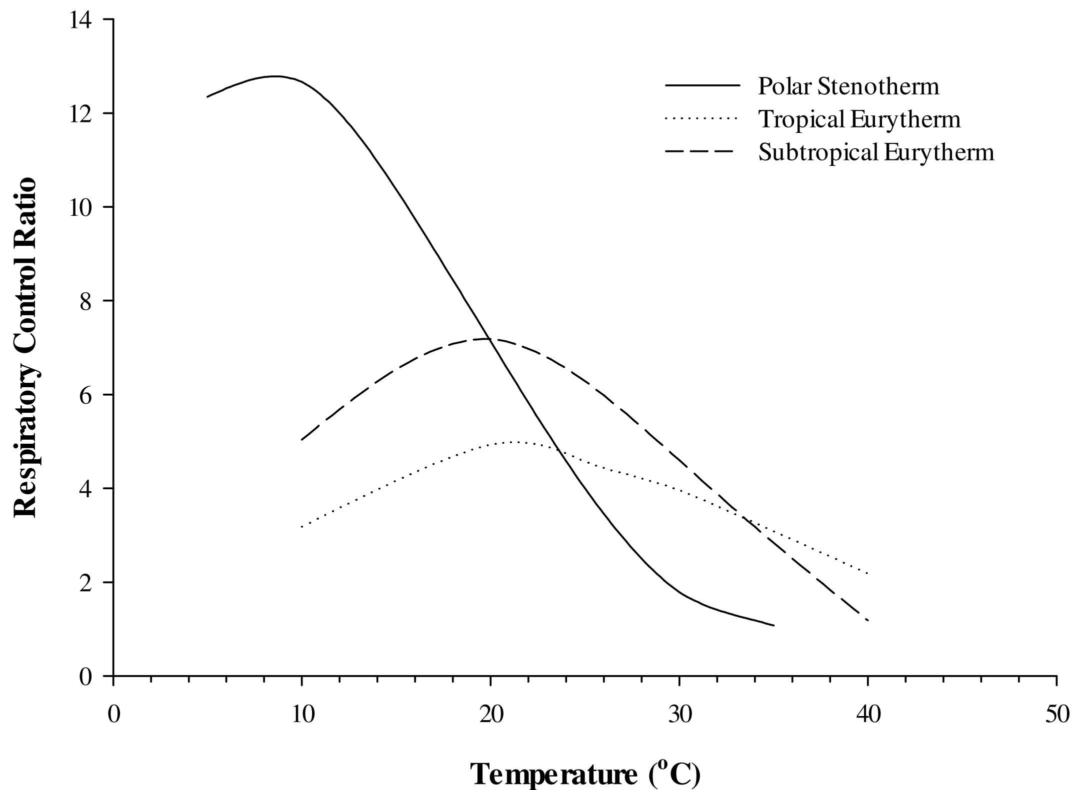

Figure 6.1: thermal sensitivity of the coupling of oxidative phosphorylation system with mitochondrial oxygen consumption (quantified as the RCR) in fishes from various thermal regimes. Antarctic stenotherms are represented by *Pleuragramma antarcticum*, subtropical values are represented by *Mugil curema* and the tropical values are shown for *Eugerres plumieri*.

About the Author

Eloy Martinez was born and raised in the city of Bayamón, Puerto Rico. He graduated *Cum* Laude in the year of 2006 with a B.S. in Coastal Marine Biology, from the University of Puerto Rico at Humacao. During his bachelors, Eloy gained valuable research experience in the fields of larval ecology and physiology during research performed in Shannon Point Marine Center (Anacortes, WA) and the Chesapeake Biological Laboratory (Solomon Island, MD), respectively. In the year 2007, Eloy was admitted to embark in graduate studies, particularly in the field of fish physiology under the mentorship of avid physiologist Dr. Joseph J. Torres. Since then, his biochemical studies have been funded by the USF College of Marine science, as well as NSF-funded multicultural programs. His research described in this dissertation and other collaborative efforts have taken him into the treacherous waters of the Southern Ocean, as well as in oceanographic research cruises in the Gulf of Mexico and the Caribbean Sea. Among various recognitions, Eloy was awarded a US-Congress medal in recognition for time served performing oceanographic research in Antarctica during the year of 2010, in addition to other meritorious recognitions for multiple scholastic achievements.