Effects of Hyperoxia on Thermal Tolerance and Indicators of Hypoxic Stress in Antarctic Fishes That Differ in Expression of Oxygen-Binding Proteins

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Devin Patrick Devor

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This thesis titled
Effects of Hyperoxia on Thermal Tolerance and Indicators of Hypoxic Stress in Antarctic
Fishes That Differ in Expression of Oxygen-Binding Proteins

by

DEVIN PATRICK DEVOR

has been approved for
the Department of Biological Sciences
and the College of Arts and Sciences by

Elizabeth L. Crockett
Associate Professor of Physiology

Robert Frank
Dean, College of Arts and Sciences
ABSTRACT

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Effects of Hyperoxia on Thermal Tolerance and Indicators of Hypoxic Stress in Antarctic Fishes That Differ in Expression of Oxygen-Binding Proteins

Director of Thesis: Elizabeth L. Crockett

Antarctic icefishes (Family Channichthyidae) are unique in lacking the oxygen-carrier hemoglobin (Hb) in their blood, leaving them with only 10% of the oxygen-carrying capacity observed in closely related red-blooded fishes. Previous studies have shown that icefishes have lower critical thermal maxima (CTMAX) than red-blooded notothenioids and that there is a positive correlation between CTMAX and hematocrit, indicating that oxygen-carrying capacity may contribute to thermal tolerance. I tested the hypothesis that the lower CTMAX of icefishes is associated with their reduced oxygen-carrying capacity. I also hypothesized that, as temperature rises, correlates of oxygen limitation (hypoxia inducible factor-1α and lactate) would increase to a greater extent in icefishes compared to red-blooded fishes and that this increase would be minimized under hyperoxic conditions. I sampled *Chaenocephalus aceratus* (Hb-) and *Notothenia coriiceps* (Hb+) at three temperatures (ambient, 8°C, and CTMAX) during an experimental heat ramp (4°C * hr⁻¹) under normoxia and environmental hyperoxia. I have determined that a 3- to 4-fold elevation in environmental oxygen does not extend CTMAX in either species despite a 1.7-fold reduction in plasma lactate accumulation and a 2- to 3-fold increase in oxygen utilization as indicated by the differences in arterial and venous oxygen (PaO₂-PvO₂). The absence of an effect of hyperoxia on thermal tolerance may be
explained by the lack of increased cardiac (venous) oxygen supply. I have determined that cardiac lactate increases with temperature in the icefish, *C. aceratus* but not in *N. coriiceps* and that HIF-1α mRNA levels in the heart are insensitive to increases in both temperature and oxygen. My results indicate that the hearts of icefishes may be especially hypoxia-sensitive and that the inability to maintain cardiac metabolism may contribute to the relatively low thermal tolerances of channichthyid fishes.
PREFACE

Work contained in this thesis will be submitted for publication to the Journal of Experimental Biology. The formatting of this document has been modified for consistency with the guidelines of that journal.
This thesis is dedicated to my incredibly supportive family and my loving girlfriend, Emily, for their words of encouragement and inspiration throughout my academic career.
ACKNOWLEDGEMENTS

This document is a testament to the caring support of my thesis advisor, Dr. Elizabeth Crockett. Without her patient guidance I would not be where I am today nor would I have the opportunity to go where I may in the future. She has nurtured my growth as a scientist as well as a person to an extent beyond all expectation. I will forever count myself lucky to have had the opportunity to work with her, for both the knowledge and experiences she has shared with me.

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Support for this work was provided by the National Science Foundation (ANT-0741301, ANT-0739637).

I am, as always, thankful for the love of my family and friends. Their patient endurance is a constant reminder of their companionship through all of the obstacles life presents me with. It is their positivity and humor that keeps me determined in the face of any difficulty.
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<tr>
<td>ACC</td>
<td>Antarctic Circumpolar Current</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APF</td>
<td>Antarctic Polar Front</td>
</tr>
<tr>
<td>CaO₂</td>
<td>arterial oxygen concentration</td>
</tr>
<tr>
<td>CvO₂</td>
<td>venous oxygen concentration</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CT&lt;sub&gt;MAX&lt;/sub&gt;</td>
<td>critical thermal maximum</td>
</tr>
<tr>
<td>EF-1α</td>
<td>elongation factor – 1α</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor-1α</td>
</tr>
<tr>
<td>LRR</td>
<td>loss of righting response</td>
</tr>
<tr>
<td>Mb</td>
<td>myoglobin</td>
</tr>
<tr>
<td>MS-222</td>
<td>ethyl m-aminobenzoate methanesulfonate</td>
</tr>
<tr>
<td>OLTT</td>
<td>oxygen limited thermal tolerance</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>WAP</td>
<td>Western Antarctic Peninsula</td>
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INTRODUCTION

The fish fauna of the Southern Ocean has existed in isolation for more than 20 million years due to geographical separation and several oceanographic features, including deep ocean basins, the Antarctic Circumpolar Current (ACC) and the Antarctic Polar Front (APF) (reviewed in Eastman, 2005). Though the point at which the ACC was formed is not clear as a result of uncertainties in geological data, it is likely that Antarctic marine taxa have been restricted to the waters surrounding Antarctica since sometime near the Eocene-Oligocene boundary (Livermore et al., 2005; Barker et al., 2007). Changes in circulatory patterns in the Southern Ocean along with the establishment of steep gradients in temperature and salinity associated with the APF were followed by a collapse in marine biodiversity occurring approximately 30 million years ago. Since then, the fish fauna of the continental shelf and upper continental slope has been dominated by the perciform suborder, the Notothenioidei (Eastman, 1993).

Since their isolation, Antarctic nototheniid fishes have thrived in the constantly frigid Southern Ocean. Even in the region of the Western Antarctic Peninsula (WAP), one of the warmer regions of the Southern Ocean, waters do not typically deviate from the range of -1.8 to +1.5°C year-round (Dewitt, 1971). Notothenioids possess some exceptional traits, which have allowed them to exist in the freezing temperatures associated with the Southern Ocean. These traits include antifreeze glycoproteins, which prevent the propagation of ice crystals in their tissues (DeVries, 1971; DeVries and Cheng, 2005), as well as cold-stable microtubules, which facilitate cellular function at low environmental temperatures (Detrich, 1997). Notothenioids have also been shown to lack a heat shock response, most probably due to the likelihood that they never experience an acute exposure to heat stress in their thermally stable environment (Hoffmann et al. 2000; Buckley et al., 2004; Hoffmann et al., 2005).

Notothenioids benefit from the high dissolved oxygen content in their chronically cold native waters, an effect of the inverse relationship of water temperature and oxygen solubility (Littlepage, 1965). Additionally, though some are pelagic, most notothenioids
are relatively inactive, benthic species with markedly reduced metabolic rates compared to temperate species (Johnston et al., 1991, White et al., 2011). High oxygen solubility and low metabolic rates as well as depressed competition resulting from diminished species diversity (Eastman, 1993) have allowed the persistence of a unique nototheniid family, the Channichthyidae, whose species lack oxygen-binding proteins. Members of the family Channichthyidae are exceptional because all species of this clade lack erythrocytes and expression of hemoglobin (Hb) (Ruud, 1954; Hureau et al., 1977), a trait unique to them among all adult vertebrates. Members of this group are known as “icefishes” due, in part, to the translucent appearance of their blood caused by the absence of heme pigment (Ruud, 1954). The lack of Hb is the result of a deletion of the β-globin gene and much of the α-globin gene, which is likely to have occurred in a species ancestral to the icefish clade (di Prisco et al., 2002; Near et al., 2006). This condition is an example of a “disaptation” (Montgomery and Clements, 2000) given the reduction of oxygen-carrying capacity by more than 90% compared to closely related red-blooded notothenioids (Holeton, 1970). All oxygen that diffuses across the gill epithelium and the scale-less skin of icefishes is transported dissolved in the plasma. In addition to the loss of hemoglobin, six of the 16 known species of channichthyids do not express myoglobin (Mb) in their hearts (Sidell et al., 1997; Moylan and Sidell, 2000; Grove et al., 2004). Unlike the case of Hb, the loss of cardiac Mb in this subset of icefishes is thought to have occurred via four separate mutational events during the diversification of the icefish clade (Small et al., 1998; Small et al., 2003; Grove et al., 2004). However, like the hemoglobinless condition (-Hb), the further lack of cardiac Mb (-Mb) confers a decrease in the efficiency of oxygen delivery, specifically via reduced facilitation of oxygen storage and intracellular transport. Indeed, icefishes lacking myoglobin show a more rapidly diminished cardiac output in the face of increasing afterload pressures compared with species of icefish that express the protein in their hearts (Acierno et al., 1997).

It is widely accepted that lack of these oxygen-binding proteins could only have persisted in fishes living in perpetually cold, oxygen-rich waters such as those found in the Southern Ocean (Montgomery and Clements, 2000; Sidell and O’Brien, 2006; Cheng
and Detrich, 2007). Icefishes have been successful in the absence of Hb by possessing several cardiovascular traits that facilitate oxygen delivery to tissues and intracellular diffusion. Such traits include blood volumes that are 2- to 4-fold higher than in red-blooded species (Hemmingsen and Douglas, 1970, Twelves, 1972). Channichthyids also have approximately 4-fold greater relative ventricular mass (Holeton, 1970; Tota et al., 1991) and increased vascular densities compared to red-blooded nototheniid species (Fitch et al., 1984; Wujcik et al. 2007) in addition to capillary diameters that are 2- to 3-fold larger than in other teleosts (Fitch et al., 1984, Hemmingsen and Douglas, 1977). Members of this family also have 2-fold higher mitochondrial membrane densities in their cardiomyocytes relative to red-blooded notothenioids (O’Brien and Sidell, 2000; Urschel and O’Brien, 2008). This is likely of direct benefit to icefishes because the increased amount of intracellular lipid provides a pathway of less resistance for oxygen diffusion (O’Brien and Sidell, 2000) since oxygen is more soluble in these nonpolar lipid membranes than it is in water (Battino et al., 1968).

The Western Antarctic Peninsula, home to many icefish species, is experiencing the most rapidly rising temperatures in the southern hemisphere and, in fact, is among the most rapidly warming regions in the world (Meredith and King, 2005; Vaughan et al., 2003). This is of particular relevance for notothenioids, since their upper thermal limits are relatively low compared to temperate and even arctic species (Somero and DeVries, 1967; Podrabsky and Somero, 2006; Bilyk and DeVries, 2011, Beers and Sidell, 2011). Additionally, two studies have shown that warm-acclimated Antarctic notothenioids have lower thermal limits than temperate species cold-acclimated to similar temperatures (Somero and DeVries, 1967; Podrabsky and Somero, 2006). This further highlights the stenothermal nature of the nototheniid suborder (Somero and DeVries, 1967).

Given their reduced oxygen-carrying capacity, icefishes may be more likely than red-blooded notothenioids to experience hypoxic stress associated with warming. Indeed, it was recently reported that icefishes are not as tolerant of acute increases in temperature as their red-blooded relatives (Beers and Sidell, 2011). Using the critical thermal maximum (CT\textsubscript{MAX}) as an indicator of upper thermal tolerance, Beers and Sidell examined the upper thermal tolerances of five species of notothenioids that differ in hematocrit and
expression of oxygen-binding proteins. CT_{MAX} is quantified by subjecting an individual to a steady heating regime at a pre-determined rate of increase until a behavioral indicator of thermal limit is reached (Lutterschmidt and Hutchison, 1997). In their 2011 study, Beers and Sidell used the loss of a righting response (LRR), or the point at which an animal was no longer able to correct itself when equilibrium was lost, as the behavioral indicator. The three red-blooded species studied by Beers and Sidell, *Notothenia coriiceps* (Richardson, 1844), *Gobionotothen gibberifrons* (Lönnberg, 1905), and *Lepidonotothen squamifrons* (Günther, 1880), had CT_{MAX} ranging from 14.2 ± 0.4 to 17.1 ± 0.2°C (mean ± s.e.m.). The two icefishes in their study, *Chionodraco rastrospinosus* (DeWitt and Hureau, 1979) and *Chaenocephalus aceratus* (Lönnberg, 1906), had CT_{MAX} of 13.3 ± 0.2 and 13.9 ± 0.4°C, respectively. Beers and Sidell concluded that a positive correlation exists between CT_{MAX} and hematocrit in notothenioid fishes (Beers and Sidell, 2011). Considering the narrow thermal windows of icefishes as well as their reliance on chronically cold and oxygen-rich waters, many researchers have expressed concern regarding the ability of channichthyids to respond to projected increases in environmental temperature (Somero, 2010, Tota et al., 2012).

Further evidence that thermal tolerance is set by oxygen limitation in teleosts comes from studies utilizing respirometry and nuclear magnetic resonance (Mark et al., 2002; reviewed in Pörtner et al., 2004). Rates of oxygen consumption in the Antarctic eelpout, *Pachycara brachycephalum*, at elevated temperatures (12-15°C) are reduced nearly 2-fold with environmental hyperoxia, and the rise in blood flow through the dorsal aorta that accompanies elevated temperatures under normoxic conditions is not observed with hyperoxia (Mark et al., 2002). Mark et al. suggest that, under normoxic conditions, the hearts of Antarctic fishes may be unable to obtain sufficient oxygen at elevated temperatures to supply a higher systemic oxygen demand (2002). These findings, together with the correlation of CT_{MAX} with hematocrit (Beers and Sidell, 2011), may indicate that oxygen-carrying capacity contributes to thermal tolerance limits of Antarctic notothenioid fishes.

My aim, in the present study, was to determine whether oxygen limitation is a contributing factor underlying thermal tolerances in Antarctic notothenioid fishes. I
expected the positive correlation between CT_{MAX} and hematocrit (Beers and Sidell, 2011) to stem from the reduced oxygen supply and delivery inherent in fishes with lower levels of Hb (icefishes represent the extreme case, having no hemoglobin). I hypothesized that fish exposed to elevated oxygen concentrations (environmental hyperoxia) experience an extension of their thermal limits (i.e., elevated CT_{MAX}) relative to animals held at normoxia. I also hypothesized that the effect of hyperoxia on thermal tolerance is more pronounced in an icefish than in a red-blooded fish. In addition, I hypothesized that the levels of correlates of oxygen limitation are greater in the icefish compared to the red-blooded species, and that levels of these indicators of oxygen limitation would be mitigated by hyperoxia.

I chose two correlates of oxygen limitation, L-lactate and hypoxia inducible factor (HIF-1\(\alpha\)). Lactate production increases in tissues when ATP demand exceeds the rate at which ATP can be supplied by aerobic pathways of energy metabolism. I tested the hypothesis that, as body temperature rises, lactate levels increase in icefish tissues to a greater extent than in the tissues of a red-blooded notothenioid, and that elevated environmental oxygen concentration (hyperoxia) minimizes lactate accumulation more so in the icefish than in the red-blooded species. The basis for this hypothesis is the expectation that lactate will increase to a greater extent in animals whose oxygen transport and delivery are more limited (i.e., the icefish relative to the red-blooded species and individuals under normoxia compared to those under hyperoxia). Lactate was measured in the plasma, oxidative and glycolytic skeletal muscles and ventricle.

HIF functions as a master regulator of the overall hypoxic response by activating the transcription of numerous hypoxia-induced genes (reviewed in Wenger et al., 2005). HIF-1\(\alpha\), the variable subunit of the HIF heterodimer, is degraded under normoxia and is stabilized immediately when hypoxia inactivates regulatory proteins acting on HIF (Jewell et al., 2001). This allows HIF-1\(\alpha\) to bind hypoxia response elements in promoter regions of the genome. As such, the HIF-1\(\alpha\) subunit is a widely used indicator of the molecular response to hypoxia (Semenza, 2000; Wenger et al., 2005; Ke and Costa, 2006). The heart ventricle was selected for quantification of HIF-1\(\alpha\) mRNA levels because previous work demonstrated an increase in HIF-1\(\alpha\) mRNA only in this tissue at
elevated temperature ($CT_{MAX}$) compared to ambient temperature (Beers and Sidell, 2011). I hypothesized that transcript levels of HIF-1α are higher in an icefish relative to a red-blooded notothenioid. Additionally, I expected that the HIF response would be greater under environmental oxygen limitation (normoxia) compared to conditions of greater oxygen availability (hyperoxia). I based the hypotheses that HIF-1α mRNA levels will be higher in icefish and in normoxic conditions (relative to red-blooded fishes and in hyperoxic conditions, respectively) on the expectation that greater hypoxic insult occurs under normoxic conditions and in animals with reduced oxygen-carrying capacity (i.e., icefishes).

To further examine the extent to which oxygen limitation contributes to the thermal tolerances of icefishes (-Hb) and red-blooded (+Hb) notothenioids, this study builds upon the thermal tolerance work of Beers and Sidell (2011). Here I compare a red-blooded notothenioid and an icefish lacking expression of both Hb and cardiac Mb. This allows for a clearer description of the effects of oxygen-binding proteins on indicators of hypoxia during thermal stress relative to studies using Mb-expressing icefishes. This study marks the first use of hyperoxic treatments to investigate the physiological underpinnings of the upper thermal limits of Antarctic notothenioids.
MATERIALS AND METHODS

Animal Collection

Animals were collected from the ARSV Laurence M. Gould in April-May 2011 off the southwest shore of Low Island (63°30’S, 62°42’W) and off Brabant Island in Dallmann Bay near Astrolabe Needle (64°08’S, 62°40’W). Specimens of Chaenocephalus aceratus (Lönnberg, 1906) were collected from otter trawls, while Notothenia coriiceps (Richardson, 1844) were collected from both trawls and baited, benthic traps. Animals were held in flow-through seawater tanks aboard the ship during transport to the US Antarctic research station, Palmer Station. Upon arrival, animals were immediately transferred to covered, flow-through seawater tanks on-station where they were maintained at ambient temperature for a minimum recovery period of 36 hours prior to experiments.

Thermal Tolerance Experiments

Following recovery, three animals were transferred to each of two insulated, 700L experimental tanks (polyethylene tanks containing polyurethane foam insulation, Xactics International, Inc., Cornwall, Ontario, Canada). Animals were then held in filtered seawater at ambient temperature (0.3 ± 0.04°C) for a minimum of 12 hours to allow animals to overcome handling stress prior to temperature ramping. Both tanks were supplied with flowing, filtered seawater and aerated using air pumps (Silent Giant, Aquarium Pump Supply, Prescott, AZ, USA) connected to air stones (1”x1”x9”). Upon transfer of animals to experimental tanks, the tank designated for hyperoxic treatment was switched from a supply of air to a supply of oxygen using an oxygen concentrator (DeVilbiss Healthcare, Somerset, PA, USA) at a maximal rate of 5 L O₂ * min⁻¹. A data logger recorded environmental PO₂ of the water in the hyperoxic tank overnight and during experiments. Readings of environmental PO₂ in the normoxic tank were taken every 20 minutes from a handheld dissolved oxygen and temperature meter (Yellow Springs Inst., Yellow Springs, OH, USA). Levels of oxygen in the hyperoxic tank were consistently between 3- to 4-fold higher than levels in the normoxic tank at similar
temperatures, ranging from 28.7 to 42.3 mg * L\(^{-1}\) (hyperoxia) and 7 to 11.2 mg * L\(^{-1}\) (normoxia). Light intensity in both tanks was kept to a minimum using tank lids and black plastic curtains. Each tank was also fitted with a water temperature logger (HOBO, Onset Computer, Pocasset, MA, USA) to record the temperature (1x per min).

One animal from each tank was sampled at ambient temperature (0.3 ± 0.04°C) just prior to temperature ramping, at which point the incoming flow was turned off and a heater (900 EVO, Electro Engineering, Stevenage Hertfordshire SG1 2BH, UK), and the in-series pump supplying it, were turned on. Heaters were set to 22.2°C ensuring the set point would not be reached prior to completion of the experiment (CT\(_{\text{MAX}}\) of the final animal in the tank). These settings produced a rate of increase of 4°C * hour\(^{-1}\). This rate of temperature ramping was chosen in order to avoid thermal acclimation (which could occur with a slower heating regime) while at the same time, preventing a lag time prior to equilibration of water and body temperatures which might have occurred with a more rapid rate of warming in these relatively large-bodied fishes. A second animal was sampled from each tank at 8°C (approximately intermediate to ambient and CT\(_{\text{MAX}}\)) and a third animal at was sampled at CT\(_{\text{MAX}}\).

Animals were removed from the experimental tanks and anesthetized in a 20 L solution (1:7500 g * ml\(^{-1}\)) of ethyl m-aminobenzoate methanesulphonate (MS-222; Finquel, Argent Chemical Laboratories, Inc., Redmond, WA, USA) in an insulated container. All seawater used to prepare solutions of anesthesia was collected from the experimental tanks in order to match the animals’ body temperatures. All solutions of anesthesia were aerated using a pump fitted with an air stone as in the normoxic experimental tank. Fish no longer demonstrating signs of opercular pumping (after 8-12 minutes in anesthesia), were transferred to a bin of ice.

While on ice, immediately following anesthetization, arterial and venous blood was sampled from the dorsal aorta and the heart, respectively. Blood PO\(_2\) was measured for each in a MC100 Microcell chamber (Strathkelvin Instruments, North Lanarkshire, Scotland) thermostated with a circulating water bath. The oxygen electrode in this chamber was connected to a 782 single/dual channel oxygen meter (Strathkelvin Instruments). Venous blood was used to measure hematocrit (for *N. coriiceps*) and
prepare plasma samples following collection via sterile syringes and 1.5 inch needles. One cc syringes with 20 gauge needles were used for red-blooded fish and 3 cc syringes with 18 gauge needles were used for icefish. All syringes and needles used for blood collection were chilled on ice prior to use to prevent blood from warming. For hematocrit measurements, heparinized capillary tubes were filled with venous blood and centrifuged for 5 minutes in a hematocrit centrifuge to separate red cells. Following this, digital calipers were used to calculate the percentage of the capillary tube occupied by red cells. Venous blood was used for preparation of plasma. Venous blood was treated with ice-cold 3.2% sodium citrate to prevent clotting (4:1 (vol/vol) blood/sodium citrate for icefish; 9:1 (vol/vol) blood/sodium citrate for red-blooded fish). Citrated blood was centrifuged (5415C Microfuge, Eppendorf, Hamburg, Germany) at 5300 x g (8000 rpm) for 10 minutes at 3°C. Plasma isolates were frozen in liquid nitrogen and stored at -80°C for use in lactate analyses.

Following blood draws, fish morphometrics (mass and total length) were recorded and the spine was transected posterior to the skull. After excising the heart and rinsing it in ice-cold notothenioid Ringer solution (240 mM NaCl, 2.5 mM MgCl₂, 5.0 mM KCl, 2.5 mM NaHCO₃, 5.0 mM NaH₂PO₄, pH 8.0) the ventricle was separated, blotted dry, weighed, and bisected longitudinally. Half of the ventricle was freeze-clamped for quantification of tissue lactate. Portions of the oxidative and glycolytic skeletal muscles (pectoral adductor and axial muscles, respectively) were excised, cleaned of connective tissue, and similarly freeze-clamped. Freeze-clamping was employed due to findings that this method produces significantly lower tissue lactate levels compared to freezing by submersion in liquid nitrogen (Adcock and Dando, 1983). All samples for lactate analyses were processed on an ice-cold stage and transferred to storage at -80°C. Animals sampled at 8°C were processed for the collection of tissues in the same way as ambient animals.

Approximately one hour before tank temperatures approached average CTMAX values (from Beers and Sidell, 2011), behavior of the third and final animal in each tank was carefully monitored. Observation was performed in the dark using red-filtered headlamps to minimize stress experienced by the fish. During this time, anecdotal
observations of animal behavior and ventilatory movements were made. For this study, as in Beers and Sidell (2011), CT_MAX was determined by the loss of a righting response (LRR). Upon reaching CT_MAX animals were anesthetized and their tissues collected as before. All procedures involving fishes were approved by the University of Alaska, Fairbanks Institutional Animal Care Committee (134774-2).

Loss of righting response is an often-used indicator of upper thermal limits in notothenioid fishes, as it is more easily observed in this group compared to an alternative CT_MAX endpoint, the onset of muscular spasms (Beers and Sidell, 2011; Bilyk and DeVries, 2011). In fact, Lutterschmidt and Hutchison, who recommend onset of spasms as a more precise CT_MAX endpoint, admit that it is often difficult to observe in fishes (Lutterschmidt and Hutchison, 1997). Regardless of any potential differences in CT_MAX obtained using either the methods recommended by Lutterschmidt and Hutchison (1997) or LRR, the present study does allow for a valid relative comparison of thermal limits among species or treatments.

**Lactate quantification**

Lactate levels were determined in heart ventricle, oxidative and glycolytic skeletal muscles, and blood plasma of experimental animals (collected as described above) from each treatment combination. A commercial lactate kit (Eton Bioscience, San Diego, CA, USA) was used to measure lactate colorimetrically at 490nm with some modification to the kit protocol. Solid tissues were homogenized by hand in Ten Broeck ground-glass homogenizers. For quantification of solid tissue lactate, the kit recommends extraction of homogenates in 80% ethanol, however, the supplied standards are dissolved in H_2O and ethanol was found to interfere with the color-generating reaction. Therefore, I substituted the ethanol extraction with a vigorous centrifugation step in which homogenates were centrifuged for 30 minutes at 21000 x g (model 5424 centrifuge with HL004 aerosol-tight rotor, Eppendorf, Hamburg, Germany) and supernatants were removed and centrifuged for an additional 20 minutes at 21000 x g. The final supernatant was diluted and used directly used in the assay. Using this modified protocol, the kit yielded repeatable results for cardiac and skeletal muscle samples and these results were comparable to those
obtained using other assays. Plasma was diluted and measured directly in the assay without extraction. Absorbances were quantified in a SpectraMax M2e plate reader (Molecular Devices, Sunnyvale, CA, USA) using the software SoftMax Pro v.5.4.1 (Molecular Devices).

In addition to expressing lactate levels as mM concentrations (plasma) and µmol * g tissue\(^{-1}\) (ventricle), calculations were made to assess the total lactate load in plasma and cardiac muscle. This was important in order to account for the significantly higher blood volume (Twelves, 1972) and cardiac mass (Hemmingsen and Douglas, 1977) in icefishes compared to red-blooded species. For these calculations, plasma lactate was normalized to gram body mass using an estimate of blood volume per gram body mass (Twelves, 1972) and cardiac lactate was calculated as total lactate load in the ventricle for each individual using recorded ventricle masses. When presenting data for plasma and cardiac lactate levels, accounting for differences in blood volume and heart size offers more meaningful comparisons of organismal metabolic stress than concentrations (expressed as mM or per g tissue).

**HIF-1α quantification**

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for ventricles from each individual in order to quantify changes in the expression of HIF-1α with increases in temperature or environmental oxygen (HIF-1α GenBank accession numbers: GU362089 and GU362090 for *N. coriiceps* and *C. aceratus*, respectively). Cardiac muscle was selected for HIF-1α quantification because Beers and Sidell (2011) demonstrated an increase in HIF-1α mRNA levels at elevated temperature in this tissue only. Elongation factor-1α (EF-1α) was used as a housekeeping gene since it showed no response to temperature or oxygen treatments in either species and was equivalent between species. This was determined using BestKeeper v1 (Pfaffl et al., 2004) as described by Orczewska et al. (2010).

RNA was extracted from heart homogenates using a commercial kit (RNeasy™ fibrous tissue kit, Quiagen, Valencia, CA, USA). I used a slightly modified protocol for extraction wherein DNase I treatment of homogenates was performed at room
temperature for 25 minutes followed by an additional DNase I treatment of 20 minutes. Purity of RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA extracts having an absorbance ratio >1.6 (260 nm/230 nm) and > 1.8 (260 nm/280 nm) were considered to be sufficiently pure; samples having lower ratios were re-extracted. RNA integrity was determined via gel electrophoresis on a 2% agarose gel stained with ethidium bromide. Reverse transcription of complementary DNA (cDNA) was carried out using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). For each sample, an additional aliquot of RNA was not reverse-transcribed in order to act as a control, ensuring PCR did not amplify genomic DNA. Genetic material for all samples was then amplified in a thermocycler (iCycler, BioRad, Hercules, CA) and amplified cDNA was diluted to 1 ng * µL⁻¹ for use in PCR.

Primers for hypoxia-inducible factor were designed using Primer Express software (Perkin-Elmer, Foster City, CA). These were designed from partial gene sequences obtained in a previous study (Beers et al., 2010). Primers were selected at sequences that are shared by both species. The forward primer for HIF-1α was designed over a splice site to avoid amplification of genomic DNA. Primers for elongation factor-1α (EF-1α) were obtained from Invitrogen (Carlsbad, CA). See Table 1 for primer sequences.

Using the fluorescent probe SYBR Green, qRT-PCR was carried out in triplicate in 384-well plates in an ABI-Prism 7900HT system (Applied Biosystems, Foster City, CA). Water blanks were used for both HIF-1α and EF-1α. A dissociation curve was run after PCR for each 384-well plate to confirm that only the gene of interest was amplified (HIF-1α or EF-1α). All qRT-PCR was performed in the laboratory of Dr. Kristin O’Brien at the University of Alaska, Fairbanks.

**Calculation of arterial and venous oxygen concentrations**

In order to further describe changes in blood oxygen levels, I have calculated the concentration of oxygen dissolved in both arterial (CaO₂) and venous blood (CvO₂). As explained by Henry’s Law, the partial pressure exerted by a gas is a function of both the
amount of oxygen present and the solubility of the gas, the latter of which is affected by
temperature of the system. Therefore, the same concentration of oxygen will exert more
pressure in a liquid as temperature rises. Using the temperature dependence term for
oxygen solubility (1700K: Loomis, 1928; Kavanaugh and Trussell, 1980; Dean, 1992)
with Henry’s solubility coefficient under standard conditions ($k_H^o$) and the standard
temperature ($T^\theta$), the Van’t Hoff equation (1) yields Henry’s coefficient for each
experimental temperature ($T$). This temperature-specific solubility coefficient ($k_H(T)$) was
used in the Henry’s Law equation (2) to calculate the concentration of oxygen (mol * L$^{-1}$)
for each experimentally determined value of PO$_2$.

$$k_H(T) = k_H^o \exp \left[1700K \times \left(\frac{1}{T} + \frac{1}{T^\theta}\right)\right]$$  \hspace{1cm} (1)

plasma $O_2$ concentration = $k_H(T) \times PO_2$  \hspace{1cm} (2)

It is important to note that calculated values of blood oxygen concentration (CaO$_2$ and
CvO$_2$) do not account for oxygen bound to Hb and therefore do not reflect the total
amount of oxygen available to the red-blooded species as they do in the icefish.

Statistics

A factorial analysis of variance (ANOVA) was used to detect the effects of
species, temperature and oxygen treatments. The levels of these three factors were,
species: *N. coriiceps* (Hb+/Mb+) and *C. aceratus* (Hb-/Mb-), oxygen availability:
normoxic and hyperoxic, temperature: ambient, 8$^\circ$C, and CT$_{MAX}$. When ANOVA’s
reported significant effects of one or more factors, multiple pairwise comparisons, using a
Bonferroni correction, were performed to identify differences among treatment
combinations. Normality and homogeneity of variance were tested using a Shapiro-Wilk
test and a Modified Levene test, respectively. Where the assumptions of normality and/or
homogeneity of variance were unmet for a dataset ($p > 0.05$), transformations
(logarithmic, exponential, or root) were carried out. Imputation using the arithmetic mean
was used for CT$_{MAX}$ and hematocrit data to achieve equal sample sizes (this was
determined to yield similar statistical results as did random deletion of single data points to achieve equal sample sizes). Blood PO$_2$ data were analyzed using a “type III” three-way ANOVA to account for more substantial inequality of sample sizes. For most data sets, samples sizes were seven or eight. For PO$_2$ data, samples size ranged from four to ten, reflecting the greater difficulty in obtaining arterial blood from the hemoglobinless, C. aceratus. All statistical analyses were performed in R 2.12.2 (The R Foundation for Statistical Computing) using a significance level of 0.05.
RESULTS

Critical thermal maxima

Critical thermal maxima (CTMAX) were unchanged by the enhancement of environmental oxygen (i.e., hyperoxia) in both species (p > 0.05; Fig. 1). CTMAX for Chaenocephalus aceratus was significantly lower than CTMAX for Notothenia coriiceps (two-way factorial ANOVA; F_{1, 32} = 92.60; p < 0.001). Critical thermal maxima were 13.1 ± 0.4°C and 16.8 ± 0.2°C for C. aceratus and N. coriiceps, respectively (mean ± s.e.m.; Fig. 1).

Blood oxygen and hematocrit

Arterial PO2 (PaO2) was up to 2.5-fold higher in C. aceratus under hyperoxia compared to normoxia (F_{1, 89} = 7.944; p < 0.01, Fig. 2A). Venous PO2 (PvO2), on the other hand, did not differ between normoxic and hyperoxic treatments in either species (Fig. 2B). Both PaO2 and PvO2 were significantly higher in the icefish, C. aceratus, than in the red-blooded N. coriiceps by approximately 4-fold (‘Type III’, three-way factorial ANOVA; for PaO2: F_{1, 89} = 45.64, p < 0.001; for PvO2: F_{1, 74} = 63.02, p < 0.001; Fig. 2A,B). For both arterial and venous blood, there was no significant change in blood oxygen pressures at CTMAX relative to ambient temperature (Fig. 2A,B).

Similar to the results for blood oxygen tensions, the concentrations of dissolved oxygen in blood (mol * L^{-1}) were between 7- and 8-fold greater in the icefish than in the red-blooded species (‘Type III’, three-way factorial ANOVA; for CaO2: F_{1, 89} = 45.64, p < 0.001; for CvO2: F_{1, 74} = 39.94, p < 0.001; Fig. 3A,B). In contrast to oxygen tensions, calculated arterial and venous oxygen concentrations decreased significantly with elevated temperature (‘Type III’, three-way factorial ANOVA; for CaO2: F_{2, 89} = 27.49, p < 0.001; for CvO2: F_{2, 74} = 58.36, p < 0.001). There was, on average, a 7-fold reduction in dissolved oxygen concentrations in arterial blood and a 16-fold reduction in venous blood when comparing ambient and CTMAX temperatures (Fig. 3). As was the case for PO2, arterial oxygen concentrations were significantly elevated in the icefish, C. aceratus, in
response to hyperoxia ($F_{1, 89} = 7.944; p < 0.01; \text{Fig. 3A}$) but venous oxygen concentrations were not similarly affected.

Hematocrit, measured in the red-blooded N. coriiceps, increased with temperature (two-way factorial ANOVA; $F_{2, 54} = 7.82; p = 0.001; \text{Fig. 4}$) showing significantly elevated levels at $C_{T_{\text{MAX}}}$ compared to ambient temperature (1.2-fold on average between oxygen treatments). There was no significant difference in hematocrit between animals held under normoxia and hyperoxia. Furthermore, hematocrit did not respond differently to increased temperature in the two oxygen treatments (Fig. 4).

**Lactate**

Concentrations of lactate in the plasma (mM) were 2.4-fold greater in Notothenia coriiceps than in Chaenocephalus aceratus (three-way factorial ANOVA; $F_{1,72} = 66.0; p < 0.001; \text{Fig. 5}$). Plasma lactate levels were highly sensitive to temperature in both species, experiencing an increase of 9- to 15-fold at $C_{T_{\text{MAX}}}$ compared to levels at ambient temperature (three-way factorial ANOVA; $F_{2, 72} = 222.0; p < 0.001; \text{Fig. 5}$). Further, plasma lactate concentrations at 8°C were intermediate to levels measured at ambient and $C_{T_{\text{MAX}}}$ temperatures ($p < 0.001; \text{Fig. 5}$). Hyperoxic treatments were accompanied by a 1.7-fold reduction in plasma lactate in both species ($F_{1, 72} = 23.79; p < 0.001; \text{Fig. 5}$).

When taking into account differences in blood volume per gram body mass between the two species, levels of plasma lactate expressed per gram body mass were statistically similar between C. aceratus and N. coriiceps (Fig. 6). The fold changes in plasma lactate in response to temperature and oxygen when normalized to blood volume are the same as those for molar lactate concentrations (Fig. 6).

At all levels of temperature and oxygen, the amount of lactate in the heart ventricle (expressed per gram tissue) was, on average, 1.7-fold higher in the red-blooded N. coriiceps than in the icefish, C. aceratus (three-way factorial ANOVA; $F_{1, 72} = 99; p < 0.001; \text{Fig. 7}$). Cardiac lactate was the only tissue in which lactate responded differently to temperature between the two species. Cardiac lactate was elevated in C. aceratus at $C_{T_{\text{MAX}}}$ relative to both ambient temperature and 8°C ($p < 0.01$), while there was no change in N. coriiceps (Fig. 7). The responses of cardiac lactate to elevated temperature
in each species were similar under hyperoxia compared to the responses observed in the same species under normoxia.

In order to obtain the most precise measure of lactate in the hearts of the two notothenioid species, interspecific differences in heart mass were considered. *C. aceratus* had a 2.4-fold higher overall lactate load in their hearts (mol * ventricle-1) than the red-blooded, *N. coriiceps* due to the larger heart size of the former species (*three-way factorial ANOVA; *F*$_{1,72}$ = 88.73; *p* < 0.001; Fig. 8). As in non-normalized data, species responded differently to thermal treatments; *C. aceratus* had an elevated total cardiac lactate load at CT$_{\text{MAX}}$ compared to lower temperatures but *N. coriiceps* showed no effect of temperature (Fig. 8). Similar to data for cardiac lactate concentrations (per gram tissue), there was no effect of oxygen treatment on the total lactate load in the heart.

Lactate concentrations in the oxidative pectoral muscle were significantly higher in the icefish than in the red-blooded fish (*three-way factorial ANOVA; *F*$_{1,72}$ = 20.86; *p* < 0.001; Fig. 9A). The glycolytic axial muscle, however, was the only tissue type among those tested in which lactate concentrations were equivalent between the two species (Fig. 9B). There was a significant effect of temperature on lactate levels in both oxidative (*F*$_{2,72}$ = 13.93; *p* < 0.001) and glycolytic skeletal muscle (*F*$_{2,72}$ = 10.43; *p* < 0.001) in both species. However, the magnitude of this increase (1.3-fold and 1.5-fold in oxidative and glycolytic muscle, respectively) was much less than that seen in the plasma (9- to 15-fold, described above). Lactate levels in both skeletal muscle types were unchanged in response to hyperoxic treatment in both *C. aceratus* and *N. coriiceps* (Fig. 9A,B).

**Hypoxia Inducible Factor-1a**

Levels of ventricular HIF-1a mRNA were insensitive to oxygen treatment and elevated temperature (Fig. 10). There was a significant species difference in ventricular mRNA levels of the HIF-1a compared to the constitutively expressed, elongation factor-1a (EF-1a). The red-blooded *N. coriiceps* has relative cardiac transcript levels of HIF-1a that are 6.6-fold higher than those observed in the icefish, *C. aceratus*, when combining data from all levels of oxygen and temperature (*three-way factorial ANOVA; *F*$_{1,84}$ = 822.0; *p* < 0.001; Fig. 10).
DISCUSSION

This study marks the first use of hyperoxic treatments to test directly the hypothesis that oxygen availability limits thermal tolerance in hemoglobinless icefishes to a greater extent than in red-blooded fishes. I have determined that increasing environmental oxygen during upward temperature ramping does not extend thermal tolerance (indicated by critical thermal maxima; CTMAX) in either the red-blooded Antarctic notothenioid, *Notothenia coriiceps*, or the icefish, *Chaenocephalus aceratus*, despite elevated arterial PO2 in the latter species. Additionally, while lactate levels increase with temperature in both species, indicating increased reliance on anaerobic metabolism, plasma lactate levels are mitigated by hyperoxic treatments. Despite lower levels of the anaerobic end product under hyperoxia, thermal tolerance was not extended. The accumulation of lactate in the hearts of *C. aceratus* at elevated temperatures (while levels of cardiac lactate in *N. coriiceps* remain stable over the range of temperatures tested) indicates that this tissue in icefishes may be particularly sensitive to hypoxia. Furthermore, the finding that HIF-1α transcript levels do not vary with oxygen availability demonstrates that, in notothenioid fishes, cardiac ventricle may be unable to respond to hypoxic stress through the typical hypoxia-inducible pathway. It is most likely that icefishes are less tolerant of elevated temperatures than red-blooded notothenioids due to cardiac hypoxia which occurs during warming (see Table 2 for a summary of results).

The use of a heating rate of 4°C * hr⁻¹ in the present study yields similar critical thermal maxima (16.8 ± 0.2°C and 13.1 ± 0.4°C for *N. coriiceps* and *C. aceratus*, respectively) to those reported previously in studies using loss of righting response under different heating regimes. A more rapid heating rate of 0.3°C * min⁻¹ (equivalent to 18°C * hr⁻¹) yielded average CTMAX of 16.2 ± 0.3°C for *N. coriiceps* (Bilyk and DeVries, 2011) and 12.4°C for *C. aceratus* (K. T. Bilyk, personal communication). Additionally, using a somewhat slower heating rate of 3.6°C * hr⁻¹, others have measured CTMAX of 17.1 ± 0.2°C and 13.9 ± 0.4°C for *N. coriiceps* and *C. aceratus*, respectively (Beers and Sidell, 2011). It has been argued that a heating rate of 4°C * hr⁻¹ is relatively slow and may result
in partial thermal acclimation, therefore producing elevated CTMAX as an artifact (Lutterschmidt and Hutchison, 1997). However, the similarities between CTMAX in this study and the results of Bilyk and DeVries, using a more rapid heat ramp (mentioned above), indicate that any acclimation that took place during the heat ramping process was insignificant.

Hyperoxic Treatment Enhances Oxygen Utilization but Does not Affect Thermal Tolerance

The results of this study demonstrate that a 3- to 4-fold elevation in environmental oxygen concentration (6.5 – 12 mg * L⁻¹ and 25 – 47 mg * L⁻¹ under normoxia and hyperoxia, respectively; Table 3) does not extend upper thermal limits in either the icefish or the red-blooded notothenioid. Hyperoxic conditions were similarly shown to have no effect on the CTMAX of several species of temperate teleosts (Rutledge and Beitinger, 1989; Healy and Schulte, 2012).

Significant elevations in PaO₂ (more than 2-fold) were found with hyperoxic treatment in the icefish, C. aceratus, but not in the red-blooded N. coriiceps. This increase in PaO₂ in C. aceratus indicates a passive rise in oxygen levels during hyperoxia. Furthermore, the difference in levels of dissolved oxygen between arterial and venous blood (e.g., PaO₂ – PvO₂) is greatest in hyperoxic treatments indicating 3.2-fold enhanced oxygen utilization under hyperoxia compared to normoxia. The apparent lack of an effect of hyperoxia on PO₂ and oxygen concentration in the red-blooded N. coriiceps is likely due to the presence of Hb, which, in arterial blood, is typically near oxygen saturation (Wells et al 1982). It is possible that levels of oxygen bound to Hb in N. coriiceps are affected by hyperoxic conditions, although this was not determined in the present study. Lower PO₂ in the blood of N. coriiceps compared to C. aceratus may also reflect the presence of Hb in N. coriiceps which is absent in icefishes.

The inverse relationship between oxygen content and temperature, resulting in reduced blood oxygen concentrations as temperature approaches CTMAX, is likely to be particularly problematic for icefishes since their aerobic energy supply is entirely reliant on maintaining adequate levels of dissolved oxygen. The threat of limited oxygen may be
greatest in the highly aerobic heart. In contrast to arterial oxygen levels, venous oxygen (PvO$_2$) did not increase in either species under hyperoxia. These findings indicate that much of the additional oxygen supplied in the hyperoxic treatment was removed from systemic circulation prior to reaching the heart, from which venous blood is drawn.

It appears as if elevated oxygen levels in this study were insufficient to provide the heart with increased oxygen relative to normoxic treatments. The absence of extended thermal tolerance in the hyperoxic treatment, coupled with the accumulation of cardiac lactate (in the icefish) and the lack of a change in PvO$_2$, indicates that hyperoxia did not provide the heart with an oxygen supply sufficient to meet oxygen demands, which would be expected to rise with temperature (see Table 3 for a summary of oxygen levels in each treatment).

One study in the eurythermal killifish, *Fundulus heteroclitus*, provides evidence that thermal tolerance in fishes depends on levels of blood oxygen rather than environmental oxygen (Haakons, 2010). Haakons (2010) determined that CT$_{MAX}$ is not correlated with critical environmental oxygen tension in the killifish. Critical environmental oxygen tension refers to the oxygen tension below which fish metabolism conforms to external oxygen availability (Speers-Roesch et al., 2012a). This observation in killifish supports my finding that venous oxygen supply, rather than environmental oxygen, is central to setting thermal limits.

It has been proposed that increased reliance on anaerobic metabolism may contribute to setting the upper critical temperature for ectotherms (Pörtner et al., 1998; Pörtner, 2001). Indeed, I have determined that levels of the anaerobic metabolite lactate increase in plasma by 9- to 15-fold at CT$_{MAX}$ over levels at ambient temperature. This increase is similar in magnitude to that seen in lactic acid (approximately 10-fold) in the plasma of *C. aceratus* held at 10$^\circ$C for 4 hours (Hemmingsen and Douglas, 1972). Lactate also accumulates in the ventricle (icefish only) and skeletal muscles (elevations in all muscle types range from 1.3- to 1.6-fold). Accumulation of lactate indicates an increased reliance on anaerobic metabolism in both species as temperatures rise, and suggests that oxygen is limiting at elevated temperature. My results, however, show that a 1.7-fold reduction in plasma lactate with hyperoxic treatment (on average between
species) is not accompanied by an extension of $C_{T_{\text{MAX}}}$. In combination, these data suggest that the amount of plasma lactate is not responsible for limiting the thermal tolerance of Antarctic notothenioids. Instead, it is more likely that tissue-specific difference in lactate accumulation (e.g., in cardiac muscle) is a contributing factor for setting thermal tolerance in these fishes.

**Oxygen Limitation of Thermal Tolerance and Cardiac Hypoxia**

The theory of “oxygen-limited thermal tolerance” (OLTT) (Pörtner, 2002) addresses the physiological underpinnings of thermal tolerance in ectotherms and predicts that, at elevated temperatures, animals lose the ability to match increased oxygen demand through ventilatory and circulatory modifications in oxygen supply (Pörtner, 2001; Pörtner, 2002). Applying the theory of OLTT to my research question, one would expect that the lower $C_{T_{\text{MAX}}}$ of icefishes, which lack a heme-based oxygen delivery system, could be explained by organismal hypoxia resulting from oxygen imbalance. A recent study comparing hypoxia-tolerant and hypoxia-sensitive elasmobranchs supports this (Speers-Roesch et al., 2012a). In this study the authors determined that hypoxia tolerance is associated with the efficiency of oxygen transport in the blood (i.e., Hb-oxygen binding affinity).

Several studies have suggested that the heart may be among the first tissues to experience hypoxic stress during warming in a variety of ectotherms (Frederich and Pörtner, 2000; Pörtner 2002; Sartoris et al., 2003; Farrell, 2002). In fact, this highly aerobic organ is thought to play a central role in the ability of some fishes to tolerate hypoxia (Speers-Roesch et al., 2012b). In their elasmobranch studies, Speers-Roesch and colleagues found that the critical environmental PO$_2$ at which cardiac output and heart rate decline is equal to the whole animal critical PO$_2$ at which the fish becomes an oxyconformer (Speers-Roesch et al., 2012b). This finding indicates that the ability to maintain cardiac function plays an important role in setting the tolerance levels of fishes to low oxygen availability. It may not be surprising then, that notothenioid hearts (perhaps especially those of the hemoglobinless icefishes) are also sensitive to hypoxia,
given the aerobic nature of this tissue and the lack of a coronary blood flow in these fishes, allowing only oxygen-poor venous blood to reach the heart.

The pattern of cardiac lactate accumulation in the present study also serves as evidence of hypoxic stress in the hearts of icefishes because, while cardiac lactate levels are stable in the red-blooded *N. coriiceps* when temperature is elevated, there is an accumulation of the anaerobic end-product in the ventricle of *C. aceratus*. This is reminiscent of studies in hypoxia-sensitive elasmobranchs, which experience an accumulation of cardiac lactate while hypoxia-tolerant elasmobranchs do not (Speers-Roesch et al., 2012b). The apparent sensitivity of channichthyd cardiac tissue to hypoxia is likely to reflect an inability to maintain flux through oxidative pathways due to decreased oxygen delivery during warming. The high lactate dehydrogenase activity of the icefish heart, relative to red-blooded species (Feller and Gerday, 1987; O’Brien and Sidell, 2000), indicates that icefish hearts possess a greater potential for the generation of ATP by anaerobic means or, alternatively, for oxidizing lactate. However, the accumulation of lactate in the hearts of icefish indicates that lactate oxidation is unable to keep up with increasing amounts of lactate, whether generated endogenously or deposited from the plasma during thermal treatments. Given the constancy of cardiac lactate in the red-blooded *N. coriiceps*, it is possible that thermal tolerance in this Hb-expressing fish is not limited by cardiac hypoxia as is likely the case for icefishes. Given the greater oxygen-carrying capacity of Hb-expressing species, it is not surprising that these species are restricted by different physiological factors than fishes lacking Hb. We cannot, however, exclude the possibility that, despite the lack of increased cardiac lactate in *N. coriiceps*, this group does experience cardiac hypoxic stress.

Further evidence that oxygen limitation plays a crucial role in limiting thermal tolerance in teleosts is seen in temperate fishes (Alabaster and Welcomme, 1962; Weatherly, 1970; Healy and Schulte, 2012). Studies with the eurythermal killifish have found that, while CT$_{MAX}$ is not affected by hyperoxic treatments (as in my own study), there is a significant reduction in heat tolerance associated with reduced oxygen availability (i.e., hypoxia). The authors suggest that, while environmental oxygen does not directly influence thermal limits under normoxic and hyperoxic conditions, it may
play a role when levels are significantly reduced prior to heating (Healy and Schulte, 2012). While the effects of reduced environmental oxygen on $CT_{MAX}$ have not been tested in notothenioid fishes, such studies may not be physiologically relevant for Antarctic fauna, as they are unlikely to experience such severe hypoxic stress even under climate change scenarios.

Hypoxia-related oxidative stress in cardiac muscle during warming has been suggested to play a role in setting the thermal limits of notothenioids (Mueller et al., 2011; Mueller et al., 2012). Recent studies have found that levels of oxidized macromolecules (protein carbonyls and peroxidized lipids) increase in the hearts of icefishes but not in the hearts of red-blooded species upon exposure to $CT_{MAX}$ (Mueller et al., 2012). In addition, reactive oxygen species, which can lead to oxidative damage to macromolecules, are produced more rapidly in the icefish, *C. aceratus*, than in the red-blooded *N. coriiceps* when the electron transport chain is chemically inhibited by antimycin A (which is similar in effect to hypoxia) (Mueller et al., 2011). These studies also determined that the overall antioxidant defense of icefishes is lower than that of red-blooded species, and neither icefishes nor red-blooded species were able to increase the transcript levels or activity of antioxidant enzymes in response to increasing oxidative stress (Mueller et al., 2011; Mueller et al. 2012). An increased level of hypoxia-induced oxidative stress, apparent in the hearts of icefishes, is likely to be a contributing factor to the low thermal tolerance of icefishes relative to Hb-expressing species (Mueller et al., 2012). The role of cardiac hypoxia in limiting thermal tolerance is also supported by the present finding that the hearts of *C. aceratus* experience a greater accumulation of the anaerobic end-product lactate than the hearts of *N. coriiceps*.

Constitutive levels of HIF-1α mRNA are greater in the red-blooded, *N. coriiceps* relative to *C. aceratus*, which may be attributed, at least in part, to the lower levels of dissolved oxygen reaching the hearts of the former species under ambient conditions (5-fold lower $PvO_2$ compared with *C. aceratus*) as well as elevated activity levels relative to icefishes. The species difference in HIF-1α transcript levels, together with the consistently higher levels of oxidized protein in the hearts of *N. coriiceps* relative to icefishes (Mueller et al., 2012), suggests that cardiac muscle in *N. coriiceps* may
normally experience greater hypoxia-related oxidative stress than in *C. aceratus*. This is also supported by the higher concentration of lactate in the cardiac muscle (µmol * g ventricle⁻¹) of *N. coriiceps* compared to *C. aceratus* (Fig. 7). The more robust antioxidant defense observed in red-blooded species relative to icefishes (Mueller et al., 2011; Mueller et al., 2012) may help mitigate the greater vulnerability to oxidative stress (at ambient temperature) in the red-blooded species.

The absence of changes in HIF-1α expression in response to elevated temperature is perhaps surprising considering that some HIF target genes are important in maintaining cardiac function when the heart becomes hypoxic (Huang et al., 2004, reviewed in Giordano, 2005). My results stand in contrast to the only previous study to quantify notothenioid HIF-1α transcript levels in response to elevated temperature. In the previous study, the authors report a 1.6-fold increase in HIF-1α expression in the hearts of *N. coriiceps* (but not in the icefish, *Chionodraco rastrosinosus*) upon exposure to CT_MAX (Beers and Sidell, 2011). Although I do not have a definitive explanation for this discrepancy it is possible that the difference in results reflects the use of different housekeeping genes or standards between our two studies. Beers and Sidell used 18S ribosomal RNA as a housekeeping gene and linearized plasmid in their standard curve while I have used EF-1α as a housekeeping gene and genomic DNA for my standards. It is unlikely that the lack of an effect in the present study is due to thermal treatments that were too rapid to allow changes in expression, since the rates of heating in this study (4°C * hr⁻¹) and the previous study (3.6°C * hr⁻¹) were similar. Additionally, expression levels of 262 notothenioid genes have been shown to respond to temperature after a 4 hour heat stress at 4°C (Buckley and Somero, 2009), although variable HIF-1α itself was not detected. My results indicate that notothenioid fishes lack the ability to generate a hypoxic response through the up-regulation of HIF-1α. This is perhaps similar to the characteristic inability of notothenioids to mount a heat shock response (Hoffmann et al. 2000; Buckley et al., 2004; Hoffmann et al., 2005) and, possibly, an antioxidant response (Mueller et al., 2011; Mueller et al., 2012). Future studies are required to determine whether notothenioids are able to generate a HIF response through post-translational
modifications or whether HIF-3α plays a role in the hypoxic response of these fishes as has been suggested in mammals (Heidbreder et al., 2003; Li et al., 2006).

Conclusions

My results indicate that the limited oxygen-carrying capacity of channichthyids and the resultant cardiac hypoxia appear to be factors contributing to the reduced thermal tolerance in the icefish *Chaenocephalus aceratus* compared with the red-blooded notothenioid, *Notothenia coriiceps*. The role of cardiac hypoxia in limiting thermal tolerance is supported by previous findings in fishes (Pörtner, 2002; Farrell, 2002; Mueller et al., 2012) and other aquatic ectotherms (Frederich and Pörtner, 2000; Braby and Somero, 2006). My data demonstrate that, with hyperoxic treatment, there is a reduction in plasma lactate and a rise in oxygen utilization (as reflected in the difference between PaO₂ and PvO₂), indicating that both species make use of additional oxygen under hyperoxia. Despite enhanced oxygen utilization, however, hyperoxia did not expand the thermal tolerance of either species. This may be explained, at least partially, by the lack of increased levels of dissolved oxygen in venous blood available to the heart during hyperoxic treatment. In my study, hyperoxic treatment was insufficient to provide this highly aerobic tissue with an enhanced oxygen supply. The increase in cardiac lactate observed in the heart of the icefish, but not in that of the red-blooded species, implicates the heart of the icefish as an especially hypoxia-sensitive tissue.

Projected warming of atmospheric and ocean surface temperatures in the Western Antarctic Peninsula (WAP), due to global climate change (Meredith and King, 2005; Vaughan et al., 2003), could pose a significant threat to the stenothermal notothenioid fishes endemic to this area. Icefishes, in particular, may be at a greater risk in the face of rising temperatures compared to closely related, red-blooded notothenioids given the lower thermal tolerances of the former group. Although elevations in temperature resulting from climate change will continue to be more gradual than the heating rate used in the current study, my research provides insight into the relative responses of icefishes and red-blooded notothenioids to thermal stress. The present data support previous work (Beers and Sidell, 2011) which shows icefishes to be a particularly oxygen-limited group.
Additionally, my study elucidates the physiological underpinnings associated with the reduced thermal tolerance of icefishes relative to red-blooded species.

Future research should address the acclimatory capacities of icefishes and red-blooded notothenioids to warmer temperatures and whether warm acclimation affects thermal tolerance. Though some studies have tested the capacity for enhanced heat tolerance after warm acclimation in notothenioids, these studies have been limited to red-blooded species and have yielded mixed results (Seebacher et al., 2005; Podrabsky and Somero, 2006; Franklin et al., 2007; Beers and Sidell, 2011). Further acclimation studies might yield valuable information regarding potential responses to more progressive thermal stress.

While cardiac hypoxia appears to play a central role in limiting thermal tolerance in icefishes, it is unclear whether a similar hypoxic insult occurs in *N. coriiceps*, given the constancy of cardiac lactate levels in this species. It is possible that different physiological factors limit thermal tolerance in red-blooded notothenioids and further investigations into the physiology of Antarctic fishes could determine what these factors may be. Regardless of potential differences in the underlying mechanisms contributing to thermal limits in icefishes and red-blooded species, the relatively narrow thermal ranges of channichthiid fishes will likely lead to the establishment of the biologically unique icefishes as an indicator taxon for global climate change.
Figure 1. Critical thermal maxima (CT_{MAX}) was not affected by hyperoxic treatment in either *Notothenia coriiceps* or *Chaenocephalus aceratus*. Imputation using arithmetic mean provided equal samples. Different letters indicate significant differences between species (*p* < 0.001). Minimum *n* = 8. Data are presented as mean ± s.e.m.
Figure 2. Both arterial (A) and venous (B) oxygen tension were higher in the icefish Chaenocephalus aceratus (ACE) compared to Notothenia coriiceps (COR) (different letters indicate significant species difference, $p < 0.001$). Blood oxygen tensions were not significantly different at $CT_{\text{MAX}}$ compared to ambient temperature. $PaO_2$ was significantly elevated in hyperoxic treatments (HO) compared to normoxia (Norm) ($p < 0.01$), but this effect is only seen in the icefish $C. aceratus$. Analysis performed on Log-transformed data. Type III ANOVA used to account for unequal sample sizes ($n$ between 4-10). Data are presented as mean ± s.e.m.
Figure 3. Both arterial (A) and venous (B) oxygen concentration (mol * L\(^{-1}\)) were higher in the icefish *Chaenocephalus aceratus* (ACE) compared to *Notothenia coriiceps* (COR) (different letters indicate significant species difference, \(p < 0.001\)). Oxygen concentrations were significantly reduced in all treatments as temperature increased (\(p < 0.001\)). CaO\(_2\) was significantly elevated in hyperoxic treatments (HO) compared to normoxia (Norm) (\(p < 0.01\)), but this effect is only seen in the icefish *C. aceratus*. Analysis performed on Log-transformed data. Type III ANOVA used to account for unequal sample sizes (\(n\) between 4-10). Data are presented as mean ± s.e.m.
Figure 4. Hematocrit in the red-blooded Notothenia coriiceps is significantly higher at $CT_{MAX}$ than at ambient temperature. Hematocrit at 8°C is not significantly different from hematocrit at either higher or lower temperatures. This effect of temperature does not differ significantly between normoxic and hyperoxic treatments. Imputation using arithmetic mean provided equal samples. Minimum $n = 9$. Data are presented as mean ± s.e.m.
Figure 5. Plasma lactate (mM) increased sharply with temperature (A,D) \((p < 0.001)\). Lactate accumulation was reduced under hyperoxia (HO) relative to normoxia (Norm) in both species (A,C) \((p < 0.001)\). Plasma lactate (mM) was significantly lower in *C. aceratus* than in *N. coriiceps* (A,B) \((p < 0.001)\). Panel A shows all factors while panels B, C, and D show main factor effects. \(n = 7\). Analysis performed on Log-transformed data. Data are presented as mean ± s.e.m.
Figure 6. When accounting for species differences in plasma volume (mL plasma * g body mass (M_b)^{-1}), plasma lactate levels were similar between species (A,B). Plasma lactate (µmol * g M_b^{-1}) increased sharply with temperature (A,D) \((p < 0.001)\). This lactate accumulation was reduced under hyperoxia (HO) relative to normoxia (Norm) in both species (A,C) \((p < 0.001)\). Plasma lactate (µmol * g M_b^{-1}) was significantly lower in *C. aceratus* than in *N. coriceps* (A) \((p < 0.001)\). Panel A shows all factors while panels B, C, and D show main factor effects. Analysis performed on Log-transformed data. \(n = 7\). Data are presented as mean ± s.e.m.
Figure 7. Cardiac lactate concentration (µmol * g ventricle⁻¹) was significantly higher in *N. coriiceps* than in *C. aceratus* (indicated by *) (*p < 0.001*). Cardiac lactate in *C. aceratus* was higher at CT_{MAX} than at either lower temperature (indicated by different letters) (*p < 0.05*) while lactate was stable in the hearts of *N. coriiceps*. Neither species exhibited an effect of hyperoxic treatments. Analysis performed on Log-transformed data. *n = 7*. Data are presented as mean ± s.e.m.
Figure 8. Accounting for species differences in heart size, total cardiac lactate load (µmol ventricle⁻¹) was significantly higher in C. aceratus than in N. coriiceps (indicated by *) (p < 0.001). Cardiac lactate in C. aceratus was higher at CT_{MAX} than at either lower temperature (indicated by different letters) (p < 0.05) while lactate was stable in the hearts of N. coriiceps. Neither species exhibited an effect of hyperoxic treatments. Analysis performed on Log-transformed data. n = 7. Data are presented as mean ± s.e.m.
Figure 9. Lactate in oxidative pectoral muscle (A) was significantly higher in *C. aceratus* (ACE) than in *N. coriiceps* (COR) (indicated by different letters; $p < 0.001$) while lactate levels in glycolytic axial muscle (B) did not differ between species. Both muscle types experienced elevated lactate at CTMAX compared to ambient temperatures ($p < 0.001$). Neither species exhibited an effect of hyperoxic treatments (HO) compared to normoxia (Norm). Analysis performed on Log-transformed data. $n = 7$. Data are presented as mean ± s.e.m.
Figure 10. Cardiac transcript levels of HIF-1α relative to EF-1α were significantly higher in *N. coriiceps* (ACE) than in *C. aceratus* (COR) (indicated by different letters; \( p < 0.001 \)). Neither temperature nor hyperoxia had a significant effect on HIF-1α mRNA levels. HO = Hyperoxia, Norm = Normoxia. Analysis performed on 4th root-transformed data. \( n = 8 \). Data are presented as mean ± s.e.m.
Table 1: Primers for qRT-PCR of hypoxia-inducible factor-1α (HIF-1α) and housekeeping gene elongation factor-1α (EF-1α)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’ to 3’</th>
<th>Reverse Primer 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>ACTTCAGCTGCCCTGATTCAAG</td>
<td>CACTGGTGAGGGAAGCATT</td>
</tr>
<tr>
<td>EF-1α</td>
<td>CTGGAAGCCAGTGAAGAGATGAC</td>
<td>ACGCTCAACCTCCATCCC</td>
</tr>
</tbody>
</table>
Table 2. Summary of factor effects

<table>
<thead>
<tr>
<th>Species</th>
<th>Elevated temperature</th>
<th>Hyperoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CT\textsubscript{MAX}</strong></td>
<td><em>N. coriiceps</em></td>
<td>--</td>
</tr>
<tr>
<td><strong>Hematocrit</strong></td>
<td>--</td>
<td>↑</td>
</tr>
<tr>
<td><strong>PaO\textsubscript{2}</strong></td>
<td><em>C. aceratus</em></td>
<td>NS</td>
</tr>
<tr>
<td><strong>PvO\textsubscript{2}</strong></td>
<td><em>C. aceratus</em></td>
<td>NS</td>
</tr>
<tr>
<td><strong>CaO\textsubscript{2}</strong></td>
<td><em>C. aceratus</em></td>
<td>↓</td>
</tr>
<tr>
<td><strong>CvO\textsubscript{2}</strong></td>
<td><em>C. aceratus</em></td>
<td>↓</td>
</tr>
<tr>
<td><strong>HIF-1α</strong></td>
<td><em>N. coriiceps</em></td>
<td>NS</td>
</tr>
<tr>
<td><strong>Plasma lactate</strong> (µmol/g Mb) ‡</td>
<td>NS</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Cardiac lactate</strong> (µmol/ventricle)</td>
<td><em>C. aceratus</em></td>
<td>↑†</td>
</tr>
<tr>
<td><strong>Oxidative muscle lactate</strong></td>
<td><em>C. aceratus</em></td>
<td>↑</td>
</tr>
<tr>
<td><strong>Glycolytic muscle lactate</strong></td>
<td>NS</td>
<td>↑</td>
</tr>
</tbody>
</table>

Arrows show direction of change in response to factor. NS = no significant effect of factor.

* Species having significantly higher levels displayed for each independent variable.
† Increase seen in *C. aceratus* only.
‡ From data incorporating plasma volume/g body mass (Twelves, 1972).
Note: Hyperoxic environmental oxygen concentration did not decline with temperature due to the continued pumping of oxygen when inflowing sea water was shut off (Ambient time point)

<table>
<thead>
<tr>
<th></th>
<th>$[O_2]$ (mg/L)</th>
<th>PaO$_2$</th>
<th>PvO$_2$</th>
<th>PaO$_2$</th>
<th>PvO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. aceratus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>11.2</td>
<td>39.3</td>
<td>16.8</td>
<td>11.1</td>
<td>4.5</td>
</tr>
<tr>
<td>8°C</td>
<td>9.1</td>
<td>34.5</td>
<td>23.2</td>
<td>5.0</td>
<td>2.7</td>
</tr>
<tr>
<td>CT$_{MAX}$</td>
<td>7.6</td>
<td>28.2</td>
<td>15.2</td>
<td>28.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Hyperoxic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>31.1</td>
<td>73.8*</td>
<td>21.7</td>
<td>11.1</td>
<td>4.7</td>
</tr>
<tr>
<td>8°C</td>
<td>40.1</td>
<td>86.1*</td>
<td>29.2</td>
<td>13.1</td>
<td>7.9</td>
</tr>
<tr>
<td>CT$_{MAX}$</td>
<td>31.4</td>
<td>60.0*</td>
<td>19.8</td>
<td>17.3</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* = Significant effect of hyperoxic treatment

Note: Hyperoxic environmental oxygen concentration did not decline with temperature due to the continued pumping of oxygen when inflowing sea water was shut off (Ambient time point)
REFERENCE LIST


APPENDIX A: THERMAL TOLERANCE PROTOCOL

CT\textsubscript{MAX} Experimental Protocol
(Treatment apparatus designed by B. D. Sidell. Modified from Beers and Sidell, 2011)

Night before each CT\textsubscript{MAX} run complete the following checklist:
\begin{itemize}
  \item CryoVials and foil for freeze-clamped samples are labeled
  \item Experimental tanks are clean prior to transfer of fishes
  \item Temperature and oxygen conditions are at ambient levels (Heaters are off)
  \item Seawater inflow reduced to 3 gal/min
  \item Air stones are bubbling air in each (O\textsubscript{2} generator is OFF)
  \item Check battery level in oxygen meter
  \item Transfer 3 healthy fishes of same species/similar size to each tank
  \item Replace tank lids. Note time, temperature, oxygen levels
  \item Turn ON O\textsubscript{2} Generator and turn off air stone in Hyperoxic tank
  \item Allow animals to acclimate overnight
\end{itemize}

\textbf{CT}\textsubscript{MAX} Experiments

1. Put temperature logger in Normoxic tank.

2. Allow \sim 1-1.5 hrs for temperature logger to reach tank temperature.

3. Remove “Ambient” animal from Normoxic tank into aerated insulated anesthesia solution. Note time, temperature, and oxygen level of tank.

4. Turn ON pump for heater and heater. Turn OFF seawater inflow.

5. Place anesthetized fish into ice bin ventral side up for blood draws.

6. Measure PaO\textsubscript{2} and PvO\textsubscript{2} in thermostated oxygen electrode.

7. Aliquot some venous blood for plasma isolation and hematocrit quantification.

8. Weigh and measure total length of fish.


Appendix A: Thermal Tolerance Protocol Continued…

11. On ice-cold metal stage, isolate ventricle, bisect longitudinally.

12. Freeze-clamp one portion of ventricle for lactate assay.

13. Collect pectoral muscle and white muscle. On ice-cold stage, clean off connective tissue. Divide each sample.

14. Freeze-clamp one portion of each muscle for lactate assay.

15. Remaining portions of tissues frozen in liquid nitrogen.

(Left and Right anterior gill arch as well as brain also frozen in liquid nitrogen)

Hematocrit quantification (Complete Steps 16-18 During Steps 7-13)

16. Fill 2 capillary tubes with venous blood and seal end.

17. Centrifuge capillary tubes in hematocrit centrifuge.

18. Measure hematocrit using digital caliper.

Plasma preparation (Complete Steps 19-21 During Steps 7-13)

19. Treat venous blood ice-cold 3.2% sodium citrate (4:1 (vol/vol) blood/sodium citrate for *C. aceratus*; 9:1 (vol/vol) blood/sodium citrate for *N. coriiceps*).

20. Centrifuge at 8000 rpm for 10 minutes at 3°C.

21. Remove plasma (supernatant) and freeze in liquid nitrogen.

22. Remove “Ambient” animal from Hyperoxic tank into anesthesia. Note time, temperature, and oxygen level of tank. DO NOT start heater or change tank conditions.

23. Repeat Steps 5-21 for this animal.

24. When Normoxic tank reaches 8°C, remove a second animal into anesthesia solution. Note time, temperature, and oxygen level of tank.
25. Repeat Steps 5-21 for this animal.

26. Repeat Step 4 for Hyperoxic tank.

27. When Normoxic tank reaches 10°C (C. aceratus) or 14°C (N. coriiceps), observe “CT_MAX” animal under red light.

28. When animal loses righting response, remove to anesthesia. Note time, temperature, and oxygen level of tank.

29. Repeat Steps 5-21 for this animal.

30. When no animals remain in Normoxic tank, turn ON seawater inflow. Turn off heater and heater pump.

31. When Hyperoxic tank reaches 8°C, remove a second animal into anesthesia solution. Note time, temperature, and oxygen level of tank.

32. Repeat Steps 5-21 for this animal.

33. When Normoxic tank reaches 10°C (C. aceratus) or 14°C (N. coriiceps), observe “CT_MAX” animal under red light.

34. When animal loses righting response, remove to anesthesia. Note time, temperature, and oxygen level of tank.

35. Repeat Steps 5-21 for this animal.

36. When no animals remain in Hyperoxic tank, turn ON seawater inflow. Turn off heater and heater pump. Turn OFF O₂ concentrator.
APPENDIX B: LACTATE QUANTIFICATION PROTOCOL

Lactate Assay
(Modified from Eton Bioscience, San Diego, CA, USA)

-- Keep samples, homogenates and supernatants on ice during entire protocol.

1. While frozen, weigh 1-2g of tissue.
   For Plasma thaw on ice, dilute as in Table 1 below, continue to “Plating and Analyzing Samples”.

2. Thaw (on ice) enough Lactate Assay Solution (provided, aliquoted) for the current assay run.
   Thaw (on ice) Lactate Standards (provided).

3. Add tissue to ice cold Ten-Broeck ground glass homogenizer.
   Add ice-chilled ddH₂O (10mL per 1g tissue).


5. Centrifuge homogenates 30min at 21,000xg.
   Using Pasteur pipet, transfer supernatant to new microfuge tube.

6. Repeat Step 4, centrifuging for 20min.

7. Dilute Final Supernatant as described in the following table:

| Dilutions of final supernatant (1 part supernatant/x parts total diluted volume) |
|-----------------|-------|-------|-----------|-----|
|                  | Oxidative | Glycolytic | Ventricle | Plasma |
|                  | Muscle    | Muscle    |           |       |
| N. coriceps      |           |           |           |       |
| Normoxic         |           |           |           |       |
| 8°C              | 4        | 10        | 8         | 1    |
| CTMAX            | 8        | 8         | 8         | 15   |
| Hyperoxic        |           |           |           |       |
| 8°C              | 8        | 8         | 8         | 1    |
| CTMAX            | 8        | 8         | 10        | 8    |
| C. aceratus      |           |           |           |       |
| Normoxic         |           |           |           |       |
| 8°C              | 4        | 10        | 6         | 2    |
| CTMAX            | 8        | 8         | 8         | 18   |
| Hyperoxic        |           |           |           |       |
| 8°C              | 8        | 8         | 8         | 1    |
| CTMAX            | 8        | 8         | 6         | 8    |
Appendix B: Lactate Quantification Protocol Continued…

--Dilution Factors in table above determined by dilution tests for each treatment combination in each tissue type.

Plating and Analyzing Samples
-- Use multichannel pipettor when able.
-- Samples run in quadruplicate, Standards run in duplicate.

1. In dim light, add ddH2O (as described in table below) to designated sample wells in 96-well clear bottom plate.

<table>
<thead>
<tr>
<th>µL Water added to plate</th>
<th>µL Sample added to plate</th>
<th>Final Sample Vol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>30</td>
<td>20*</td>
<td>50</td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>

* Glycolytic muscle Hyperoxic CT\textsubscript{MAX} samples were an exception
-- Dilution Factors in table above determined by dilution tests for each treatment combination in each tissue type.

2. Plate Standards 50µL each replicate.

3. Add samples to wells already containing water.

4. Add Lactate Assay Solution to all wells (multichannel). Pipetting to mix as it is added to wells.

5. Cover Plate. Incubate 30min at 37°C.

6. Stop reaction by addition of 0.5M Acetic acid (multichannel). Pipetting to mix as it is added to wells.

7. Using clean syringe needle, pop bubbles in wells.
Appendix B: Lactate Quantification Protocol Continued…

8. Read plate at 490nm.
   Using pre-loaded template file.

APPENDIX C: HIF-1α QUANTIFICATION PROTOCOL
(From K. M. O’Brien Lab)

--During all PCR preparation steps keep bench, gloves and instruments free of RNase using RNase Away (Molecular BioProducts).
--Use filtered pipette tips only.

RNA Extraction
(RNeasy™ fibrous tissue kit, Quiagen, Valencia, CA, USA)

1. Thaw samples on ice.
   Set water bath to 55°C.
   Mix β-Mercaptoethanol 10µL into every 1mL RLT buffer needed.
   Prepare DNase I Incubation Mix containing the following for each sample:
   10µL DNase I stock solution
   70µL RDD Buffer
   Mix “DNase Mix” by inverting tube!

2. Homogenize ≤ 30mg sample in ground glass homogenizer containing 300µL RLT Buffer. Transfer homogenate to microfuge tubes.

3. Add 590µL RNase-Free water and 10µL proteinase K to each sample.
   Incubate sample at 55°C for 10min.
   Centrifuge at 10,000xg for 3min.
   Pipet supernatant (900uL) to new 1.5mL microfuge tube.
   Add 450µL of 96-100% EtOH. Pipet to mix.

4. Transfer 700µL of sample to RNeasy Mini spin column.
   Centrifuge at 8,000xg for 15sec. Discard filtrate.
   Repeat filtration for remaining sample.
   Add 350µL RW1 Buffer to column.
   Centrifuge at 8,000xg for 15sec. Discard filtrate.

5. Add 80µL DNase I Incubation Mix directly to spin column membrane.
   Let sit at room temperature 25min.
   Add 350µL RW1 Buffer.
   Centrifuge at 8,000xg for 15sec. Discard filtrate.
   REPEAT Step 5 letting sit for 20min at room temperature.
Appendix C: HIF-1α Quantification Protocol Continued…

6. Add 500µL RPE Buffer to column.
   Centrifuge at 8,000xg for 15sec. Discard filtrate.
Add 500µL RPE Buffer to column.
Centrifuge at 8,000xg for 2min. Discard filtrate.
Place column in NEW 2mL collection tube.
Centrifuge at 8,000xg for 1min. Discard filtrate.
Place column in NEW 1.5mL collection tube.
Add 30µL Milli-Q purified water (pH 8.0).
Let sit at room temperature for 5min.
Centrifuge at 8,000xg for 1min.
Filter filtrate again through same column.

--KEEP RNA Extracts ON ICE. Freeze at -80°C ASAP

Nanodrop Test for RNA Purity
1. Start program for “Nucleic acids”. Wipe loading area clean.

2. Load 1.5µL pure H2O. Press Okay.


4. Load 1.5µL sample. Press Measure. Wipe loading area clean (top and bottom).

   260/280 ratio > 1.8 = no protein contamination
   260/230 ratio > 1.6 = no organic solvent contamination

Agarose Gel Test for RNA Integrity
1. Mix gel:
   60mL RNase-free TXE Buffer 1x
   1.2g agarose
   Microwave 2min swirling at 3sec.
   Add 4µL Ethidium Br in hood, nitrile gloves.

2. Pour into gel chamber with comb. When hardened, Fill gel chamber with Buffer.
Appendix C: HIF-1α Quantification Protocol Continued…

3. Add to wells:
   8µL pH8.0 H2O
   1µL RNA loading dye
   1µL Sample (or DNA Ladder - exACTGene low range Plus)
   PIPET to mix

4. Run Gel at 100V for 45-60min. Turn off power. Remove gel and wrap in plastic wrap.

5. Photograph on light box.
   2 distinct bands indicate high RNA integrity.

**cDNA Synthesis and Dilution**
(TaqMan reverse transcription reagents, Applied Biosystems, Foster City, CA, USA)

1. Prepare Reverse Transcription Master Mix as follows:
   **Master Mix**
   1µL 10x RT Buffer
   2.2µL MgCl2 25 mM
   2.0µL dNTP 10 mM
   0.5µL random hexamers
   0.2µL RNase inhibitor

2. Add 5.9µL Master Mix to each PCR tube

3. Add 200 ng RNA to each.

4. Add Milli-Q H2O to each (3.6µL for +RT tubes, 4.1µL for –RT tubes)

5. To **+RT tubes ONLY**, add 0.5µL RT (Kept on ice). This is added immediately prior to thermocycling.

6. Vortex for 3sec at low speed to bring all fluid to bottom of tubes.

7. Place in thermocycler for the following protocol:
   10min at 25°C, 30min at 48°C, 5min at 95°C
Appendix C: HIF-1α Quantification Protocol Continued…

8. Store DNA at -80°C. Unless proceeding immediately to dilution in the following steps.

9. Mix 1µL cDNA solution (starting concentration of 20ng/uL) with 19µL to obtain
1ng/µL cDNA to be used in PCR.

Making Standard Curve
1. Pool 2µL of each +RT cDNA Sample for standard stock.

2. Add 3µL Milli-Q H2O for each µL pooled sample. Vortex to Mix.

3. Dilute 1:4 (stock:H2O) in new tube – Standard #1

4. Dilute 1:2 (Standard 1:H2O) in new tube – Standard #2

5. Dilute Std#2 as in Step 4 – Standard#3

6. Continue as above for a total of 7 Standards.

Testing HIF-1α Primers
1. Thaw 1 sample of each species (both +RT and –RT)

2. Make 300nM Master Mix (multiplied by number of wells needed):
   10µL 2x SYBR Green
   1.2µL Forward Primer 5 µM
   1.2µL Reverse Primer 5 µM
   2.6µL pure H2O

3. Make 600nM Master Mix (multiplied by number of wells needed):
   10µL 2x SYBR Green
   2.4µL Forward Primer 5 µM
   24µL Reverse Primer 5 µM
   2.6µL pure H2O

4. Add 15µL Master mixes to plate.
Appendix C: HIF-1α Quantification Protocol Continued…

5. Add 5µL +RT Sample (duplicate), -RT sample (1 well), Standard (duplicate), or water (1 well) 
   Plate this many replicates for each primer set and each Master Mix concentration.

6. Continue analysis as in “Plating and qRT-PCR” Steps below.

7. Calculate Primer efficiency as in “qRT-PCR Result Analysis” Steps below. 
   Primer with best correlation and efficiency is used for PCR of samples. 
   Check that both Master Mix concentrations yield same results. If so use 300nM Mix in PCR of samples.

Test of Housekeeping Genes 
(as in Orczewska et al., 2010)

1. Thaw on ice 4 samples from each species and treatment.

2. Prepare 300nM Master Mix as follows (multiplied by number of wells needed): 
   10µL 2x SYBR Green 
   1.2µL Forward Primer 5 µM 
   1.2µL Reverse Primer 5 µM 
   2.6µL pure H2O 

   -- Separate Master Mix must be made for each potential Housekeeping Gene (EF-1α and 18S rRNA).
   -- For each housekeeping gene, Run each sample in triplicate with one well devoted to RNA of the same sample (-RT). One well devoted to a negative (water) control for each housekeeping gene.

3. Follow “Plating and qRT-PCR” Steps below

4. In BestKeeper v1 (Pfaffl et al., 2004) input all C_i averages.

5. For housekeeping gene to be viable check that: 
   Standard deviation < 1. 
   Pearson correlation coefficient p-value is significant 
   Gene vs. BestKeeper p-value is significant 
   NO significant differences among all treatment combinations (ANOVA).
Appendix C: HIF-1α Quantification Protocol Continued…

**Plating and qRT-PCR**

1. Add 15µL 300nM Master Mix to each well used in 384-well plate. 
   Add 5µL Sample in triplicate to appropriate wells and pipet to mix. 
   Add 5µL Standards in duplicate to appropriate wells pipet to mix. (Not included in housekeeping gene plate).

2. Seal plate with optical plate cover, eliminating bubbles. Spin 10sec in plate centrifuge.

3. Set up SDS 2.2.2 PCR program as follows: 
   New File. 
   Select “Relative Quantification (ΔΔCt)” Press Okay. 
   Select “Import” File. 
   Import prepared PCR template text file. 
   Check temperature profile. 
   Save plate. Press OPEN. Insert Plate. CLOSE. **START Program.**

4. When program is finished: 
   Select “Relative Quantification (ΔΔCt) Study”. Press Okay. 
   Add plate from File. Press green “Analyze” button. 
   Change Detector to “all” 
   In Results tab highlight all. Copy Ct values into Excel document. 
   **Check that –RT Ct values are at least 4 cycles lower than +RT Ct values.**

5. Run Dissociation Curve: 
   New File 
   Select “Import” File. 
   Import same prepared PCR template text file. 
   Instrument Tab, select “60°C, 10min” portion of profile. 
   Select “Add dissociation curve” 
   Delete all steps before the dissociation curve. 
   Save Plate. Press Start.

6. When Dissociation Curve is finished: 
   Press green “Analyze” button. 
   For Detector, select “all” under Dissociation Curve tab.
Appendix C: HIF-1α Quantification Protocol Continued…

Check Dissociation Curve for each sample and each gene.
(Single peak indicates amplification of only the target gene).

7. Repeat for as many plates as needed to complete all samples.

qRT-PCR Result Analysis

1. Standard curve and Primer analysis:
   Calculate average C<sub>t</sub> values for each standard curve point.
   Plot average C<sub>t</sub> values of standards against the log of the concentration of each standard relative to undiluted standard.
   Calculate efficiency of primer = \[10^{(-1/slope\ of\ std\ crv\ trend\ line)-1}\]

2. Sample analysis:
   Calculate average C<sub>t</sub> values for each.
   Calculate log(input) from standard curve equation.
   Calculate input from log(input).
   Calculate relative expression of HIF-1α (HIF input/EF-1α input).