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Collaborative Research: Linkages among Mitochondrial Form, Function and Thermal Tolerance of Antarctic Notothenioid Fishes

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Collaborative Research: Linkages among Mitochondrial Form, Function and Thermal Tolerance of Antarctic Notothenioid Fishes

Project Participants

Senior Personnel

Name: Sidell, Bruce
Worked for more than 160 Hours: Yes
Contribution to Project:

Post-doc

Graduate Student

Name: Beers, Jody
Worked for more than 160 Hours: Yes
Contribution to Project:
Jody Beers is conducting Ph.D. research supported by our project. She has been executing laboratory analyses of tissue samples from Antarctic fishes in our CONUS laboratory throughout the 08-09 academic year and also was a field team member of our project during the April - June 2009 period. During our field work, Ms. Beers worked closely with the Co-PI (Sidell) on thermal tolerance experiments with Antarctic fishes.

Name: Borley, Kimberly
Worked for more than 160 Hours: Yes
Contribution to Project:
Ms. Borley is conducting her Ph.D. research on adaptive changes in gene expression associated with the presence or absence of hemoglobin in Antarctic notothenioid fishes. The work's inception was with our previous award (ANT 0437887), which is directly antecedent and to our present award.

Undergraduate Student

Technician, Programmer

Other Participant

Research Experience for Undergraduates

Organizational Partners

University of Alaska Fairbanks Campus
Our Collaborative Research project is being conducted in collaboration with Dr. Kristin M. O'Brien of the University of Alaska and her students.

Ohio University
Our Collaborative Research project is also being conducted in collaboration with Dr. Elizabeth L. Crockett of Ohio University and her students.

University of Rhode Island

The University of Rhode Island's ARMADA Program facilitated the participation of an high school teacher from Fairhope, AL (Ms. Megan O'Neill) during our April - June 2009 Antarctic field season. Ms. O'Neill participated in our research cruise, posted a journal on the Web and broadcast televideo conferences to her classes in Alabama.

Other Collaborators or Contacts

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

Findings: (See PDF version submitted by PI at the end of the report)

Training and Development:

Ph.D. thesis research of graduate students Kimberly A. Borley and Jody M. Beers at the University of Maine has been supported by this award.

Borley successfully defended her Ph.D. in Molecular Biology (Department of Biochemistry, Microbiology and Molecular Biology) in April 2010 and her doctoral degree was conferred in May 2010.

Beers is a Ph.D. candidate in Marine Biology (School of Marine Sciences). Beers is scheduled to defend her Ph.D. thesis on 13 July 2010 with conferral of degree expected in August 2010.

Outreach Activities:

Graduate student Kimberly Borley gave a presentation to third grade classes at Holden (ME) Elementary School in February 2010.

Journal Publications

Beers. JM, Borley, KA, Sidell, BD, "Relationship among circulating hemoglobin, nitric oxide synthase activities and angiogenic poise in red- and white-blooded Antarctic notothenioid fishes.", Comparative Biochemistry and Physiology, Part A, p. 422, vol. 156, (2010). Published, 10.1016/j.cbpa.2010.03.027

Borley, KA, Beers, JM, Sidell, BD, "Phenylhydrazine-induced anemia causes nitric oxide-mediated upregulation of the angiogenic pathway in Notothenia coriiceps.", Journal of Experimental Biology, p. , vol. , (2010). Accepted,

Beers, JM, Sidell, BD, "Blood oxygen-carrying capacity affects thermal tolerance in Antarctic notothenoid fishes.", American Journal of Physiology, p. , vol. , (2011). In preparation,

Borley, KA; Sidell, B.D., "Evolution of the myoglobin gene in antarctic icefishes (Family Channichthyidae).", Polar Biology, p. , vol. , (2011). In preparation,

Books or Other One-time Publications

Web/Internet Site

Other Specific Products

Contributions

Contributions within Discipline:

Our experiments of thermal tolerance of Antarctic notothenioid fish species will contribute insight into the sensitivity of these animals to potential elevation of habitat temperature due to global climate change. Results of experiments described in this report support our hypothesis that hemoglobinless Antarctic icefishes succumb early to elevations in water temperature, suggesting that this group may be particularly imperiled by climate change.

Results of these experiments will be communicated in a paper authored by J.M. Beers & B.D. Sidell at the 2010 American Physiological Society Intersociety Meeting entitled 'Global Change & Global Science: Comparative Physiology in a Changing World'. This meeting will be held 3-7 August in Westminster, CO.

Contributions to Other Disciplines:

Contributions to Human Resource Development:

Two graduate students (Beers and Borley) have benefited from training opportunities under this award.

Contributions to Resources for Research and Education:

Contributions Beyond Science and Engineering:

Conference Proceedings

Special Requirements

Special reporting requirements: None

Change in Objectives or Scope: None

Animal, Human Subjects, Biohazards: None

Categories for which nothing is reported:

Any Book

Any Web/Internet Site

Any Product

Contributions: To Any Other Disciplines

Contributions: To Any Resources for Research and Education

Contributions: To Any Beyond Science and Engineering

Any Conference

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Findings

Each enumerated entry below is followed by a bulleted list of essential findings. Immediately after that list, I have included the full text of manuscripts or draft manuscripts for each paper that has not yet been published.

1. **Comparison of nitric oxide synthase activities and expression levels of key genes in pathways of hypoxia sensitivity and angiogenesis between red- and white-blooded notothenioid fishes.** See Beers *et al.* (2010). *Comparative Physiology and Biochemistry*. Part A. **156**: 422-429. [.pdf of final publication sent to Program Officer]
    - Chemical indices of the corporeal content of nitric oxide (NO) are greater in hemoglobinless icefishes than red-blooded relatives and proportional to circulating hemoglobin content of blood in the latter group.
    - Inverse relationship between blood Hb concentration and NO concentration is driven predominantly by rate of Hb-mediated NO degradation and not by differences in capacity for synthesis of NO by nitric oxide synthase.
    - Expression of hypoxia-sensitive and angiogenic genes is similar in adults of both red- and white-blooded notothenioids, indicating well oxygenated tissues in the fully developed animals.

2. **Effect of chemically-induced anemia upon expression of hypoxia-sensitive and angiogenic genes in the red-blooded notothenioid, *Notothenia coriiceps.***
    - Chemically-induced anemia (phenylhydrazine) in a red-blooded notothenioid results in elevation of corporeal NO but does not affect the activity of nitric oxide synthase.
    - Anemic fishes show elevation of expression of genes from pathways of hypoxia sensitivity and leading to angiogenesis, documenting the presence of an hypoxia-inducible mechanism for promoting proliferation of vasculature in notothenioid fishes that appears to be mediated by NO.

The following manuscript is in press in *Journal of Experimental Biology:*

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PHENYLHYDRAZINE-INDUCED ANEMIA CAUSES NITRIC OXIDE-MEDIATED UPREGULATION OF THE ANGIOGENIC PATHWAY IN *NOTOTHENIA CORIICEPS.*

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Keywords: Antarctic fish, icefish, hemoglobin, phenylhydrazine, angiogenesis, nitric oxide

Short Title: Nitric Oxide and Cardiovascular Remodeling

Symbols and abbreviations

Hb:	Hemoglobin
Hb-:	Hemoglobinless channichthyid icefishes
Hb+:	Red-blooded notothenioid fishes

Hct:	Hematocrit
Mb:	Myoglobin
NO:	Nitric oxide
NO_3^-:	Nitrate
NO_2^-:	Nitrite
NO_x:	Nitrate + nitrite
NOS:	Nitric oxide synthase
eNOS:	Endothelial nitric oxide synthase
iNOS:	Inducible nitric oxide synthase
nNOS:	Neuronal nitric oxide synthase
PHZ:	Phenylhydrazine HCl
L-NAME:	N-ω-nitro-L-arginine methyl ester hydrochloride
QPCR:	Quantitative real-time PCR
dpi:	days post injection
HIF-1:	Hypoxia-inducible factor 1
HRE:	Hypoxia-response element
PHD2:	Prolyl hydroxylase domain containing protein 2
VEGF:	Vascular endothelial growth factor

Summary

Antarctic icefishes possess several cardiovascular characteristics that enable them to deliver oxygen adequately in the absence of hemoglobin (Hb). To investigate the mechanism driving development of these cardiovascular characteristics of icefish, we chemically induced severe anemia in a red-blooded notothenioid, *N. coriiceps*. After ten days of treatment with phenylhydrazine HCl, the hematocrit and Hb concentration of *N. coriiceps* decreased by >90% and >70%, respectively. Anemic fish exhibited a significantly higher concentration of nitric oxide (NO) metabolites in their plasma compared to control animals, indicating that corporeal levels of NO are higher in anemic animals than control fish. Activity of nitric oxide synthase (NOS) was measured in brain, retina, pectoral muscle, and ventricle of control and anemic animals. With the exception of retina, no significant differences in NOS activities were observed, indicating that the increase in plasma NO metabolites is due to loss of Hb and not due to an overall increase in NO production. To determine if loss of Hb can stimulate remodeling of the cardiovascular system, we measured expression of HIF-1α, PHD2, and VEGF mRNA in retinae of control and anemic fish. Expression of all three genes was higher in anemic animals compared to control *N. coriiceps*, suggesting a causative relationship between loss of Hb and induction of angiogenesis that likely is mediated *via* nitric oxide signaling.

Introduction

It was once believed that hemoglobin (Hb) expression was a distinguishing characteristic of vertebrates. Antarctic icefishes (Suborder: Notothenioidei, Family: Channichthyidae) are the exception to this rule (Ruud, 1954). Channichthyids lost the ability to express Hb through a gene deletion event that occurred when they diverged from red-blooded Antarctic notothenioids approximately 8.5 Ma (Cocca et al., 1995; Near, 2004). In the absence of Hb, icefish blood carries oxygen in physical solution in plasma, resulting in an oxygen-carrying capacity that is less than 10% that of red-blooded notothenioids (Holeton, 1970). Loss of Hb expression would be a lethal mutation in most environments. However, in the Southern Ocean, low temperature results in high oxygen solubility that undoubtedly contributed to the survival of icefish. While it has been hypothesized that decreased blood viscosity due to lack of red blood cells (RBCs) is

energetically favorable, icefishes have a higher cardiac output than red-blooded fish, resulting in an overall greater energetic cost of circulation (Wells, 1990; Sidell and O'Brien, 2006). Thus, loss of Hb is an energetically disadvantageous trait. Like all notothenioids, icefishes benefited from very low competition due to a crash in species diversity that occurred sometime after the mid-tertiary (Eastman, 1993). With greatly relaxed competition, negative selection will not operate on sublethal disadvantageous traits within the population. The combination of a cold, well oxygenated environment and low competition allowed early icefish to survive and persist even though they possessed a mutation that impaired their physiology (Sidell and O'Brien, 2006).

Today, 16 species of icefishes inhabit the Southern Ocean. Modern icefishes are genetically very closely related to their red-blooded relatives; however, red- and white-blooded fish have notably different cardiovascular systems. During the course of evolution, icefishes developed a unique cardiovascular system that appears to compensate for the loss of Hb. Channichthyids are characterized by increased blood volume, larger bore blood vessels, greater ventricular mass, higher cardiac output, denser vascularization, and increased ventricular mitochondrial densities compared to red-blooded Antarctic notothenioids (Eastman, 1993; O'Brien and Sidell, 2000; Wujcik et al., 2007). Together, these cardiovascular characteristics facilitate delivery of oxygen throughout the body. Although these characteristics have been well described, the underlying mechanisms responsible for driving them has not been elucidated. In this paper, we describe how nitric oxide-mediated signaling pathways can be triggered by the loss of Hb to stimulate angiogenesis in a red-blooded Antarctic notothenioid. Our observations provide insight into the evolutionary path that may have led to the cardiovascular characteristics of modern icefishes.

Nitric oxide (NO) is a pervasive signaling molecule produced from arginine and oxygen by nitric oxide synthases (NOS) (Alderton et al., 2001). In most vertebrates, the half-life of this potent molecule is very short *in vivo* because it is broken down rapidly through reactions with oxygenated Hb or myoglobin (Mb), resulting in the formation of nitrate (Gow et al., 1999; Flogel et al., 2001). NO degradation also occurs at a much slower rate through reactions with oxygen free radicals and thiols resulting in the formation of nitrate and nitrite (Kelm, 1999).

In the absence of Hb, we predicted that the steady-state levels of NO would be higher in icefish than red-blooded notothenioids due to loss of the primary breakdown pathway for NO

(Sidell and O'Brien, 2006). In previous work, we measured the concentration of nitrate plus nitrite (NO_x), in plasma of several species of notothenioids (Beers et al., submitted). Due to technical difficulties surrounding accurately measuring NO directly, the aggregate concentration of NO_x is often measured as a proxy for NO (Sun et al., 2003; Tsikas, 2005). Consistent with our hypothesis, we found that icefish species generally had higher concentrations of NO_x in their plasma than did red-blooded species (Beers et al., submitted). When results are adjusted for the larger blood volume of fish lacking Hb expression, it is clear that corporeal levels of NO are higher in icefishes than red-blooded notothenioids.

NO stimulates angiogenesis and mitochondrial biogenesis in mammals. Expression of genes in both signaling pathways increases in response to NO (Ziche and Morbidelli, 2000; Nisoli et al., 2003). Tissues of hemoglobinless icefishes display both dramatically greater mitochondrial densities (O'Brien and Sidell, 2000) and vascular densities (Wujcik et al, 2006) than their red-blooded relatives. Yet, despite the higher level of NO in icefish, there is no significant difference in expression of mitochondrial biogenesis genes in ventricles and angiogenesis genes in retinae between red- and white-blooded adult notothenioids (Beers et al., submitted; Urschel and O'Brien, 2008). Feedback inhibition could be responsible for lack of upregulation in the genes, once stable well-oxygenated phenotypes are attained. Indeed, NOS activity is lower in icefish than red-blooded species, indicating that feedback inhibition may account for a decrease in the rate of NO production in adult icefish (Beers et al., submitted).

Although not evident in adults, where the anatomy has stabilized, NO may play a role in remodeling the cardiovascular system early in the development of icefishes. However, due to inability to capture or manipulate early life history stages of icefish, we cannot measure NO levels or gene expression in developing icefish. To test our hypothesis, we produced an icefish model by treating adults of the red-blooded notothenioid, *N. coriiceps,* for 10 days with phenylhydrazine HCl (PHZ). Phenylhydrazine is a hemolytic agent that lyses RBCs leading to the degradation and clearance of Hb and drastically reducing Hct and Hb concentration of fish injected with the compound (Smith et al., 1971; Gilmour and Perry, 1996; McClelland et al., 2005). We reasoned that treatment of a red-blooded notothenioid with PHZ would induce severe anemia that could provide insight into what happens when the primary breakdown pathway for NO is removed.

Materials and methods

Animals

Notothenia coriiceps Richardson 1844 were collected from Dallmann Bay in the Antarctic Peninsula region (64°08′S, 62°40′W) during the austral autumn of 2007 and 2009. Fish were caught from approximately 150 m depth using Otter trawls and baited traps deployed from the ARSV *Laurence M. Gould*. Animals were held in flowing seawater tanks during transit to Palmer Station on Anvers Island. Fish were transferred to the Palmer Station aquarium and held in covered flowing seawater tanks at 0±0.5°C.

Experimentally induced anemia

Specimens of *N. coriiceps*, a red-blooded notothenиid with a normal hematocrit of 35-40%, were made anemic by treatment with phenylhydrazine HCl (PHZ), a hemolytic agent. PHZ was administered by an initial intraperitoneal injection, followed by continuous delivery of the drug by a surgically implanted osmotic pump as described below. Moderately sized animals (39-43 cm total length; 1000-1400 g wet weight) were used for experimental treatment.

Fish were anaesthetized prior to surgery using MS-222 (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) at a dosage of 1:7500 w/v. Once unresponsive, animals were transferred to an inclined surgical table where their gills were irrigated continuously with chilled seawater containing anaesthetic at 1:12000 w/v throughout the surgical procedure. A section of ventral abdominal surface, just anterior to the vent and lateral to the midline, was swabbed thoroughly with antiseptic (0.02% chlorhexidine) prior to making a 2 cm long incision through the abdominal wall. An Alzet® 2ML1 osmotic pump (DURECT Corporation, Cupertino, CA, USA), containing 440 mM PHZ in notothenioid Ringer solution, was inserted into the peritoneal cavity through the incision. Notothenioid Ringer solution was composed of: 260 mM NaCl, 2.5 mM $MgCl_2$, 5.0 mM KCl, 2.5 mM $NaHCO_3$, 5.0 mM NaH_2PO_4, pH 8.0 at 1 °C. According to the algorithm provided by the manufacturer, this pump should deliver PHZ solution at a constant rate of ~4.5 $\mu L \cdot hr^{-1}$ for the duration of the experiment under these conditions. After implantation of the pump, the incision was closed by suturing with 4/0 polypropylene monofilament. Animals then were injected intraperitoneally with PHZ at 10

$mg \cdot kg^{-1}$ prior to initial transfer to a shallow holding tank for recovery. During the recovery period, a hose delivering flowing seawater at ambient temperature was held in the animal's buccal cavity to ensure irrigation of the gills until the fish was able to resume autonomous ventilation. Animals then were transferred to circular 4000 L flowing seawater tanks where they were held for 10 days.

Tissue preparation

All animals were anesthetized with MS-222 (1:7500 w/v). Blood was drawn from the caudal vein and a small volume was drawn immediately into heparinized glass capillary tubes for hematocrit determination. The remainder of the whole blood was mixed with a 3.2% sodium citrate solution (9 parts blood to 1 part sodium citrate) to prevent clotting. Whole blood samples were stored for hemoglobin determination. Plasma samples were obtained by centrifuging the blood at 5300 *x g* for 10 min at 4ºC; plasma was drawn off and frozen at -80ºC for later NO_x determination. After drawing blood, anesthetized animals were killed by severing of the spinal cord followed by rapid excision of the brain. All tissues collected for gene expression and enzyme activity measurements were flash-frozen with liquid nitrogen and stored at -80˚C.

Hemoglobin determination

Hemoglobin concentration was determined using the cyanmethemoglobin method (Stadie, 1920). Briefly, 20 µl of whole blood containing sodium citrate was mixed with 5 ml of Drabkin's Reagent (Sigma Aldrich, St. Louis, MO, USA) and then incubated for 30 min at room temperature before spectrophotometric measurement at 540 nm. Total Hb concentration was calculated using a bovine Hb (Sigma Aldrich) standard curve. All samples were performed in triplicate and mean values were computed for each individual.

Plasma nitrate + nitrite (NO_x) determination

Plasma was deproteinated using an acetonitrile/chloroform treatment based on a protocol from Romitelli *et al.* (2007). Plasma was mixed 1:1 with acetonitrile, vortexed for 60 s and centrifuged at 21000 *x g* for 10 min at 4ºC. The supernatant was transferred to a new tube, mixed with two volumes of chloroform and centrifuged at 12000 *x g* for 15 min at 4ºC. The

aqueous phase, containing the deproteinated plasma, was transferred to a new tube and frozen at -80ºC.

Differences in NO concentration between species were inferred by measuring combined break-down products, nitrate (NO_3^-) plus nitrite (NO_2^-), according to the Griess method, as described by Grisham *et al.* (1995). First, NO_3^- was converted to NO_2^- by incubating 100 µl of deproteinated plasma with 0.2 units/mL of nitrate reductase in 50 mM HEPES, 5 µM FAD and 0.1 mM NADPH, pH 7.4 (final volume 500 µl) at 37ºC for 30 min. To oxidize any remaining unreacted NADPH, 7.5 units of LDH and 50 µl of 100 mM pyruvic acid were added next and incubated for an additional 10 min at 37 ºC. Finally, 1 mL of Griess reagent (0.1% napthyleneethylenediamine, 1% sulfanilamide and 2.5% phosphoric acid) was added to each sample and then incubated at 25ºC for 10 min. NO_2^- concentration was determined by spectrophotometric measurement of diazonium salt formation at 543 nm. Samples were run in duplicate and compared to a NO_2^- standard curve to determine total concentration of NO_x in the samples. Corrections for plasma volumes were performed to account for the addition of sodium citrate to the blood. Absorbance of PHZ-treated *N. coriiceps* plasma samples at 543 nm was measured before the addition of the Griess reagent. This baseline reading then was subtracted from the final reading to correct for the presence of trace PHZ.

Measurement of NOS activity

NOS activity was measured in brain, retina, ventricle and pectoral muscle of control *N. coriiceps,* and PHZ-treated *N. coriiceps* by quantifying conversion of [^{14}C]arginine to [^{14}C]citrulline. Crude extracts were prepared by homogenizing tissue in a 5% w/v ice-cold buffer solution (25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA; pH 7.4) with a ground-glass homogenizer. Homogenates were centrifuged at 12000 x *g* for 5 min at 4°C to remove cellular debris. Supernatants were drawn off, transferred to fresh tubes and kept on ice.

Tissue extracts (10 µl per reaction) were incubated for 3 hr at 5°C in reaction medium containing 25 mM Tris-HCl, 3 µM tetrahydrobiopterin, 1 µM FAD, 1 µM FMN, 10 mM NADPH, 6 mM $CaCl_2$ and [^{14}C]arginine (0.05 µCi per reaction) (40 µl final volume; pH 7.4). Reactions were terminated by adding to each tube 400 µl of 50 mM HEPES stop buffer (pH 5.5) containing 5 mM EDTA. [^{14}C]citrulline was separated from unreacted [^{14}C]arginine using batch

ion-exchange chromatography. Briefly, 100 µl of Dowex® 50WX8 resin (Na^+ form) was added to each reaction and mixed thoroughly. Samples then were transferred to 0.45 µM cellulose acetate Spin-X® columns (Costar®, Corning Life Sciences, Lowell, MA, USA) and centrifuged at 16000 *x g* for 30 s. Finally, 450 µl of filtrate, containing the [^{14}C]citrulline, was transferred to a vial with 3 ml of scintillation cocktail and quantified by liquid scintillation spectrometry. All reactions were run in triplicate and parallel controls were carried out by adding 5 µl of the competitive NOS inhibitor, L-NAME (N-ω-nitro-L-arginine methyl ester hydrochloride; 1 µM), to the reaction mix. Enzyme activity is reported strictly as L-NAME-inhibitable activity.

Measurement of VEGF, HIF-1α and PHD2 mRNA expression in the retina

Total RNA was extracted from notothenioid retinae using an AllPrep™ DNA/RNA/Protein mini kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instructions. RNA concentration and purity were analyzed by spectral analysis with a Beckman DU540 spectrophotometer. The RNA then was analyzed using an Agilent 2100 Bioanalyzer to ensure the samples were not degraded. DNA contamination was removed from RNA samples using Turbo DNA-free™ (Applied Biosystems/Ambion, Austin, TX, USA). First-strand cDNA was synthesized from total RNA using Superscript® III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and oligo(dt) primer. DNAse-treated RNA was added to each reaction with a final concentration of 45 ng/µl.

Sequencing of Antarctic notothenioid VEGF, HIF-1α, and PHD2 is described in Beers *et a.* (submitted). QPCR primers (Table 1) were designed in regions of the genes conserved among Antarctic notothenioids using Primer3 (Rozen and Skaletsky, 2000). All genes were amplified using Invitrogen's SYBR® GreenER™ (1 cycle of 5 min at 50ºC, 1 cycle of 10 min at 95ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC). QPCR reactions had a final volume of 30 µl with 2 µl of cDNA (diluted 1:10) and primer concentrations ranging from 20 to 40 nM, depending on the gene. Each sample was run in triplicate. QPCR products were subjected to a melt-curve analysis and sequenced to ensure primer specificity.

Due to the complex nature of retinal tissue and differences in tissue composition, as demonstrated by the increased vascular endothelial tissue in icefish compared to red-blooded species (Wujcik et al., 2007), samples were normalized to total RNA. This was completed by

several rounds of careful quantification and dilution until all samples had the same RNA concentration. cDNA synthesis of all samples was done simultaneously with the same master mix to ensure the same efficiency of the reverse transcription reaction between samples. Finally, the same amount of cDNA was added to each QPCR reaction and all samples were run on the same plate for each primer set. A standard curve of linearized plasmid containing the gene of interest spanning five logs was run on each qPCR plate.

Statistical analyses

Comparisons among species for differences in NOS activity were performed in SigmaStat (Version 3.1; Systat Software, Inc., Chicago, IL) using a Kruskal-Wallis One-Way ANOVA on Ranks for all tissue types assayed ($p \leq 0.05$). A one-way ANOVA followed by a *post-hoc* Tukey's Honestly Significantly Different test was used to determine significant differences in gene expression and plasma NO_x ($p \leq 0.05$). Hb concentration and Hct of treated and untreated *N. coriiceps* were analyzed using a two-sample T test. Hct readings of <1% were conservatively considered 1% for statistical purposes. With the exception of NOS activity data, all statistics were performed in SYSTAT (Version 12; Systat Software, Inc., Chicago, IL).

Results

Phenylhydrazine treatment significantly reduces hematocrit and hemoglobin concentration

Blood samples from *N. coriiceps* treated for ten days with PHZ were analyzed for Hct and Hb concentration. Compared to control animals, the Hct and Hb concentration measurements were significantly lower in PHZ-treated animals ($p<0.001$; Fig. 1). Hcts of PHZ-treated fish ranged from 4.8% to less than 1% while the control fish had an average Hct of 36.1 ± 1.5%. These numbers represent a greater than 90% decrease in Hct in the PHZ-treated *N. coriiceps*. The Hb concentration also was reduced dramatically in the PHZ-treated fish. Compared to the controls, Hb content of PHZ-treated animals was reduced by >70%. Residual Hb in plasma of PHZ-treated animals is responsible for the less dramatic change seen in the concentration of Hb compared to the reduction in Hct (Fig. 2). These results demonstrate that we have successfully induced severe anemia in a red-blooded Antarctic notothenioid.

Phenylhydrazine treatment significantly increases concentration of nitrate + nitrite in the plasma of N. coriiceps

The aggregate concentration of plasma NO_x was measured in control and anemic (PHZ-treated) *N. coriiceps* as a proxy for NO (Fig. 3). Plasma concentration of NO_x in *N. coriiceps* increased by 33% when animals were treated with PHZ ($p \leq 0.01$). While significantly different from untreated *N. coriiceps,* the concentration of plasma NO_x in anemic *N. coriiceps* is not significantly different from that of *C. aceratus* (Hb-) reported in Beers *et al.* (submitted). Thus, PHZ treatment results in a significant elevation in NO metabolites compared to control animals.

NOS activity in N. coriiceps treated with phenylhydrazine

Nitric oxide synthase (NOS) activity was determined in four different tissues of control and PHZ-treated *N. coriiceps* using a radiochemical method to measure the conversion of [^{14}C]arginine to [^{14}C]citrulline (Fig 4). There is no significant difference in NOS activity in the brain, ventricle, and pectoral muscle between control and PHZ-treated animals. However, PHZ-treatment results in approximately a 2.5-fold increase in NOS activity in the retina compared to control animals ($p<0.05$). For both treated- and untreated *N. coriiceps*, the highest NOS activity was observed in brain tissue [approximately 160 and 170 pmol (min·g wet wt.)$^{-1}$, respectively]. Brain NOS activity was more than 50-fold higher than activities measured in ventricle, retina and pectoral muscle of control and treated *N. coriiceps*. Brain, retina, and ventricle NOS activity of treated and untreated *N. coriiceps* is consistently higher than observed for *C. aceratus* (Hb-) (Beers et al., submitted).

PHZ-treatment increases mRNA expression of VEGF, PHD2 and HIF-1α

Treatment of *N. coriiceps* with PHZ for 10 days resulted in an increase in retinal mRNA levels of PHD2, HIF-1α, and VEGF (Fig. 5). Messenger RNA levels of PHD2, an oxygen-dependent regulator of HIF-1α, were approximately 8-fold higher in animals treated with PHZ than control animals ($p<0.001$). Expression of mRNA encoding HIF-1α, part of the HIF-1 transcription factor, was approximately 4-fold higher in anemic than in control animals ($p<0.01$). VEGF, a growth factor regulated by HIF-1 that stimulates blood vessel growth, showed the largest increase in mRNA levels with PHZ treatment. VEGF mRNA was approximately 30-fold

higher in PHZ-treated animals than in control *N. coriiceps* ($p<0.001$). Increases in VEGF expression are an indication of ongoing angiogenesis. Beers *et al.* (submitted) found the steady-state mRNA expression of all three genes to be statistically the same in *N. coriiceps* (Hb+) and *C. aceratus* (Hb-). Retinae of PHZ-treated fish have mRNA levels of PHD2, HIF-1α and PHD2 that are significantly higher than levels measured in both control *N. coriiceps* and *C. aceratus* (Hb-) (Fig. 5). These data indicate PHZ-treatment stimulates angiogenesis in the retina of *N. coriiceps*.

Discussion

Our experiments were designed to provide insight into how icefishes have developed drastically different cardiovascular characteristics than red-blooded Antarctic notothenioids. We induced severe anemia in a red-blooded notothenioid to model the conditions the icefish might experience early in development. Using this experimental model, we investigated a possible mechanism that could be responsible for some of the alterations to the cardiovascular system of the Antarctic icefish. We show that treatment with PHZ results in increased levels of NO and stimulates angiogenesis in red-blooded *N. coriiceps*.

Phenylhydrazine treatment results in increased nitric oxide levels in a red-blooded Antarctic notothenioid

Adult icefish have higher levels of NO metabolites than red-blooded Antarctic notothenioids (Beers et al., submitted). To confirm that loss of Hb can induce an increase in NO, red-blooded *N. coriiceps* were treated with PHZ for 10 days. In previous studies that have utilized PHZ to induce anemia in salmonids, one intraperitoneal injection with PHZ (10 or 12.5 $\mu g \cdot g^{-1}$) was sufficient to dramatically and rapidly decrease the Hct (Smith et al., 1971; Gilmour and Perry, 1996; McClelland et al., 2005). By 1 day post injection (dpi), the Hct was significantly lower than control animals and continued to decrease for several days before starting to recover 8-10 dpi. Full recovery took ≥ 5 weeks depending on the species. To ensure the Hct would not recover during the ten day treatment, we initially injected the animals with 10 μg PHZ per gram body mass and also surgically implanted an Alzet osmotic pump containing PHZ into the animal. Osmotic pumps are preferable to repeated injections because the animal

receives a continual low dose of the chemical without the stress of repeated injections (Theeuwes and Yum, 1976). The fish responded remarkably well to the PHZ-treatment. All animals survived the surgery, injection and ten day treatment. Despite the fact that some animals had a Hct of <1% at the end of the treatment, there was no noticeable change in the health or behavior of the animals. The survival rate of PHZ-treated animals documents that, in a cold and well oxygenated environment, the loss of Hb is nonlethal.

PHZ-treated *N. coriiceps* have higher levels of NO_x than control *N. coriiceps*. In fact, the elevated concentration of NO_x in the plasma of anemic *N. coriiceps* is not significantly different from icefish (Hb-). Because the concentration of NO_x is often measured as a proxy for NO, we can infer that the PHZ-treatment results in an increase in NO concentration in red-blooded notothenioids. While our results strongly suggest that the increase in NO is due to the loss of Hb, they do not rule out a possible contribution of increased NO production. To more closely examine whether the loss of Hb is solely responsible for the increase in NO, we measured catalytic capacity for NO production in several different tissues from control and PHZ-treated animals.

Does loss of hemoglobin affect the rate of nitric oxide production?

Nitric oxide is produced by NOS isoforms. At least one isoform of NOS is present across the phylogenetic spectrum of animals from insects to mammals. Mammals express three NOS isoforms. Endothelial (eNOS), inducible (iNOS) and neuronal NOS (nNOS) differ in how they are regulated and tissues in which they are expressed (Alderton et al., 2001). Less is known about NOS in lower vertebrates. While mammals have three isoforms of NOS, the number of NOS isoforms in lower vertebrates is unresolved. Genomes of zebrafish and pufferfish contain a gene for nNOS, and iNOS is present in the zebrafish genome (www.ensembl.org). To date, eNOS has not been identified in a fish genome. However, several labs have reported the presence of eNOS in different fish species based on cross-reactivity with mammalian antibodies (Fritsche et al., 2000; Ebbesson et al., 2005; Garofalo et al., 2009).

We measured NOS activity in untreated and PHZ-treated *N. coriiceps* by measuring conversion of radioactively labeled arginine to citrulline. This method does not discriminate among different NOS isoforms. PHZ treatment resulted in an increase in NOS activity in retina,

but not in brain, pectoral muscle or kidney. Measureable activity indicates that at least one isoform is expressed in each of the tissues analyzed for both control and anemic animals. Lack of difference in NOS activity between PHZ-treated and control animals in three out of four tissues measured indicates NO production is generally not upregulated in anemic fish. These results suggest that anemia-induced increase in NO is primarily due to loss of Hb and not due to a change in the rate of NO production. Unlike adult icefish, capacity for NO production apparently is not downregulated in the PHZ-treated *N. coriiceps*. Perhaps longer exposure to PHZ would decrease NOS activity to the level seen in icefishes. PHZ-treated animals experience a low Hb, high NO environment for only 10 days whereas icefish are exposed to high NO levels for their entire life history.

Phenylhydrazine-induced anemia triggers a hypoxic response

We have focused on the role of Hb in NO metabolism; however, the primary role of fish Hb is to carry oxygen from the gills and deliver it throughout the body. Loss of Hb in PHZ-treated animals drastically reduces the oxygen-carrying capacity of blood. Decreases in oxygen availability at the cellular level trigger hypoxic-signaling pathways regulated by transcription factor, HIF-1 (Ke and Costa, 2006). Decreased oxygen results in a build up of HIF-1α protein, which binds to constitutively expressed HIF-1ß to form HIF-1. This transcription factor then stimulates expression of genes containing a hypoxia-response element, HRE, in their promoters. Via the HRE, HIF-1 regulates expression of genes involved in erythropoiesis, angiogenesis, vascular tone, glucose metabolism, and cell survival.

We know that HIF acts as a global regulator of hypoxia-responsive genes in all vertebrates. While it is believed that regulation of HIF-1α by PHD2 is conserved throughout vertebrates, it has not been confirmed (Nikinmaa and Rees, 2005). PHD2 (also known as HPH2, HIF prolyl hydroxylase 2, or EGLN1, egg laying abnormal nine homolog 1) regulates HIF-1α protein expression in mammals. In the presence of oxygen, PHD2 hydroxylates HIF-1α on two proline residues in the oxygen-dependent degradation domain, targeting the protein for proteosomal degradation (Berra et al., 2003; Berra et al., 2006). Increased expression of PHD2 mRNA during hypoxia primes the system so that when oxygen becomes available, PHD2 degrades HIF-1α rapidly (Metzen et al., 2005). Genes encoding PHD2 and other prolyl

hydroxylases have been located in the genomes of multiple fish species, and the HIF-1α oxygen dependent degradation domain is conserved in fish and mammals (Soitamo et al., 2001). However, other than our own recent results (Beers et al., submitted), we are unaware of any studies that have examined the expression of PHD2 in fish species.

Expression of PHD2 mRNA in retina is not significantly different between adult red- and white-blooded Antarctic notothenioids (Beers et al., submitted). This suggests either that icefish are not hypoxic, or that the transcription of PHD2 is not responsive to hypoxia in fish. Increased PHD2 mRNA expression in anemic *N. coriiceps* establishes that PHD2 is hypoxia-responsive in Antarctic notothenioids. Demonstration that PHD2 is hypoxia-responsive in notothenioids, but not upregulated in adult icefish, indicates that the cardiovascular characteristics of adult icefishes ensure normoxia of tissues despite the absence of Hb. These experiments also support that regulation of HIF-1α by PHD2 is conserved in vertebrates.

In addition to PHD2, HIF-1α mRNA expression also was higher in retinae from PHZ-treated animals compared to untreated *N. coriiceps*. Expression of HIF-1α mRNA is often thought to be unaffected by hypoxia and solely regulated at the protein level by PHD2. However, changes in HIF-1α mRNA expression have been observed in hypoxic fish. HIF-1α mRNA expression in grass carp changes in response to length of hypoxia exposure and the tissue type and HIF-1α mRNA expression increases in zebrafish embryos exposed to hypoxic conditions (Ton et al., 2003; Law et al., 2006). In the present paper, we have presented another example of hypoxia-induced HIF-1α mRNA expression. Many studies have examined only expression of HIF-1α protein. It is possible that regulation of HIF-1α expression at the mRNA level is more widespread than is currently appreciated.

Hypoxia may induce angiogenesis via a nitric oxide-mediated pathway

We have shown that PHZ-treatment of red-blooded *N. coriiceps* results in both low oxygen levels and high NO levels. These conditions may mimic those experienced by developing icefishes. We hypothesize that this unique set of circumstances stimulates remodeling of the icefish cardiovascular system early in development by stimulating angiogenesis. To test whether loss of Hb stimulates angiogenesis, we measured mRNA expression of the angiogenic growth factor VEGF, vascular endothelial growth factor.

Angiogenesis, the growth of new blood vessels from preexisting blood vessels, is stimulated by hypoxia and/or nitric oxide (Ziche and Morbidelli, 2000; Pugh and Ratcliffe, 2003). During hypoxia, HIF-1α and HIF-1ß, binds in concert to an HRE in the promoter of VEGF, stimulating transcription. NO also is known to stimulate transcription of VEGF via an increase in HIF-1α protein expression. NO can block ability of PHD2 to bind oxygen, inhibiting the enzyme from hydroxylating HIF-1α. This inhibition of PHD2 by NO results in accumulation of HIF-1α protein (Kimura et al., 2000; Kimura et al., 2001; Metzen et al., 2003).

What happens to HIF-1α protein levels, and thus the expression of genes downstream of HIF-1α in the angiogenic pathway (*e.g.* VEGF), when oxygen is low and NO is high is a matter of debate. Conflicting evidence for the effect of NO upon PHD2 activity during hypoxia also has been reported. Several studies have indicated that NO increases activity of PHD2 during hypoxia and that treatment of cultured cells with NO donors inhibits accumulation of HIF-1α (Brune and Zhou, 2003). Hagen *et al.* (2003) suggested that NO binds to cytochromes in the electron transport chain at low oxygen levels, making oxygen available to other oxygen-binding proteins, such as PHD2. Such a mechanism would enable PHD2 to hydroxylate HIF-1α, thus targeting the protein for degradation, even when oxygen is in short supply. This effect of NO upon PHD2 activity may, however, be dose-dependent with respect to NO. Hypoxic HIF-1α protein expression in human liver HepG2 cells is diminished by low levels of NO, but higher levels of NO stabilized hypoxic HIF-1α protein (Callapina et al., 2005). High levels of NO during hypoxia inhibit PHD2 activity by blocking the ability of the protein to bind oxygen. This results in the accumulation of HIF-1α stimulating the transcription of genes such as VEGF, downstream in the angiogenic pathway. We believe this to be the situation observed during PHZ-induced anemia of *N. coriiceps.*

Retinae of PHZ-treated *N. coriiceps* experience both high levels of NO and low levels of oxygen. Our data indicate that PHZ-induced anemia in these animals results in elevation of NO metabolites, presumably reflecting increased NO levels. Concomitant with these effects, we observe an increase in HIF-1α gene expression and an approximately 30-fold increase in expression of VEGF mRNA in PHZ-treated animals compared to untreated fish. In the retina, we conclude that inhibition of PHD2 by the presence of NO and absence of oxygen results in accumulation of HIF -1α protein, stimulating angiogenesis. This demonstrates a possible

mechanism that could be activated early in the development of icefish, resulting in the higher density of vasculature present in retinae of adult animals.

Loss of Hb triggers endogenous signaling pathways

Since loss of Hb by their progenitor, the Family Channichthyidae has radiated to contain 16 species of fish that have exploited different niches in the Southern Ocean. While there certainly have been changes in the genome of icefishes as the family has evolved, we present a mechanism that may account for inception of their cardiovascular adaptations and may still contribute to ontogenetic development of these traits in modern species. In this paper, we have demonstrated that removal of Hb stimulates the angiogenic pathway in an adult red-blooded Antarctic notothenioid. Severe anemia in the adult fish resulted in high levels of NO and presumed hypoxia. Presence of this homeostatic system would have helped early icefishes to compensate immediately for the lower oxygen-carrying capacity of blood due to loss of Hb expression.

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Figure Legends

Fig. 1. Effect of phenylhydrazine treatment upon hematocrit (A) and hemoglobin (B) concentration in *N. coriiceps*. *N. coriiceps* were treated for ten days with the hemolytic agent, phenylhydrazine (PHZ; see methods). Asterisk denotes significance between control and PHZ-treated *N. coriiceps* ($p \leq 0.05$). Values are means ± s.e.m; $N=8$ for both control and treatment groups.

Fig. 2. Reduction in hematocrit of *N. coriiceps* by treatment with phenylhydrazine. Photographs of gills and Hct capillary tubes from control *N. coriiceps* (A) and *N. coriiceps* treated with phenylhydrazine (PHZ) for 10 days (B). Plasma of PHZ-treated *N. coriiceps* is red-tinted due to the release of Hb when red blood cells are lysed by PHZ.

Fig. 3. Plasma concentration of nitrate plus nitrite (NO_x) is increased in *N. coriiceps* treated with phenylhydrazine. *N. coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb) while *C. aceratus* is a species that lacks Hb. *N. coriiceps* treated with phenylhydrazine, a hemolytic agent, are labeled 'PHZ.' Values are means ± s.e.m.; $N=8$ for each species. Letters (a, b) signify the samples are significantly different from one another at $p \leq 0.05$. *C. aceratus* data, as reported in Beers *et al.* (submitted), are illustrated for comparison.

Fig. 4. Nitric oxide synthase (NOS) activity in tissues of control and PHZ-treated *N. coriiceps*. *N. coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb) while *C. aceratus* is a species that lacks Hb (Hb-). *N. coriiceps* treated with phenylhydrazine, a hemolytic agent, are labeled 'PHZ.' Values are means ± s.e.m. ($N=4$ per group). Letters (a, b) signify the samples are significantly different from one another at $p \leq 0.05$ within a tissue type. *C. aceratus* data, as reported in Beers *et al.* (submitted), are illustrated for comparison. NOS activity was not detected in *C. aceratus* retina.

Fig. 5. PHZ-treatment results in an increase in expression of genes associated with angiogenesis. *N. coriiceps* (Hb+) expresses hemoglobin (Hb) while *C. aceratus* lacks Hb (Hb-). *N. coriiceps* treated with phenylhydrazine, a hemolytic agent, are labeled 'PHZ.' Expression was normalized

to total RNA and is expressed as relative to *N. coriiceps*. Values are expressed as means ± s.e.m. (*N= 4* per group). Letters (a, b) signify the samples are significantly different from one another at $p \leq 0.05$ within a gene. *C. aceratus* data, as reported in Beers *et al.* (submitted), are illustrated for comparison.

Table 1: QPCR Primers

Gene	Forward Primer	Reverse Primer
VEGF	5' CAAGGGAGCGGAGAAGAGTA 3'	5' CAAGGGAGCGGAGAAGAGTA 3'
HIF-1α	5' TCTCTACAACGATGTAATGCTTCC 3'	5' AATCTGATTTCATCTCCGAGTCC 3'
PHD2	5' AAACGGGCAAGTTCACAGAC 3'	5' TCCCAATTTGCCGTTACAAT 3'

from Beers *et al.* (submitted)

Figure 1

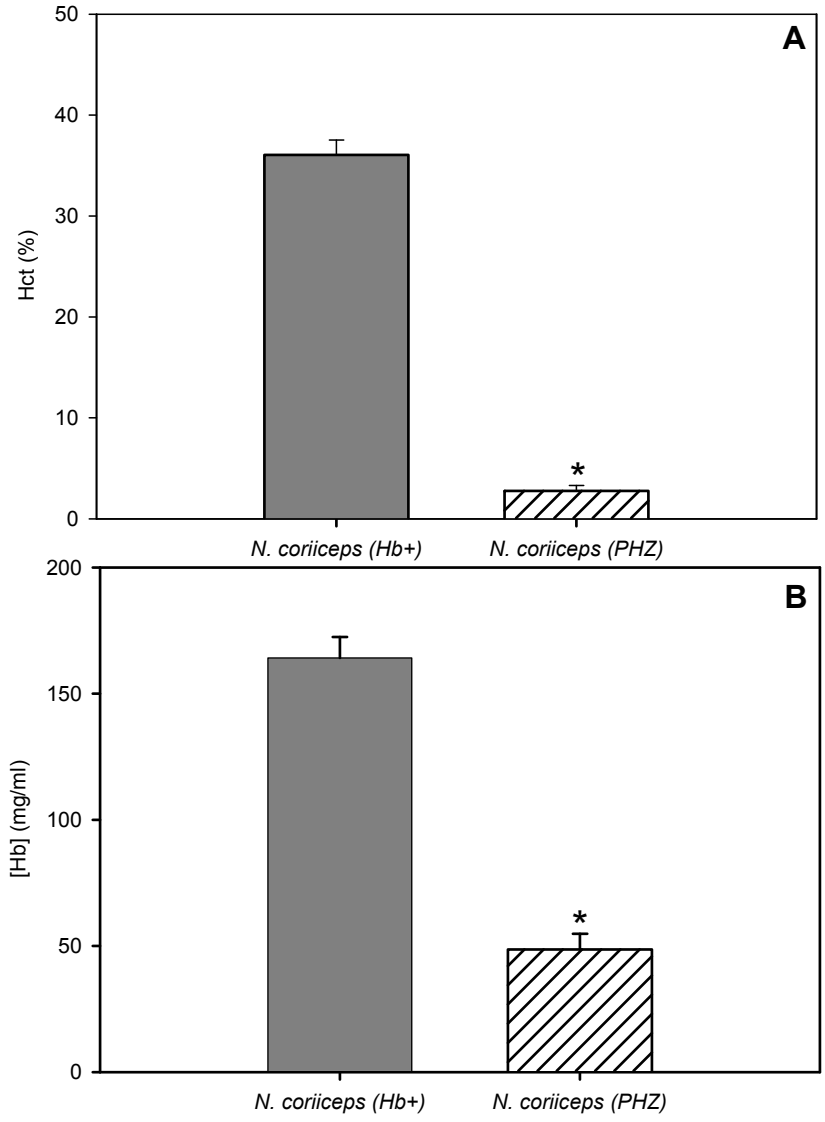

A
Hct (%)
50
40
30
20
10
0
*
N. coriiceps (Hb+)
N. coriiceps (PHZ)
B
[Hb] (mg/ml)
200
150
100
50
0
*
N. coriiceps (Hb+)
N. coriiceps (PHZ)

Figure 2

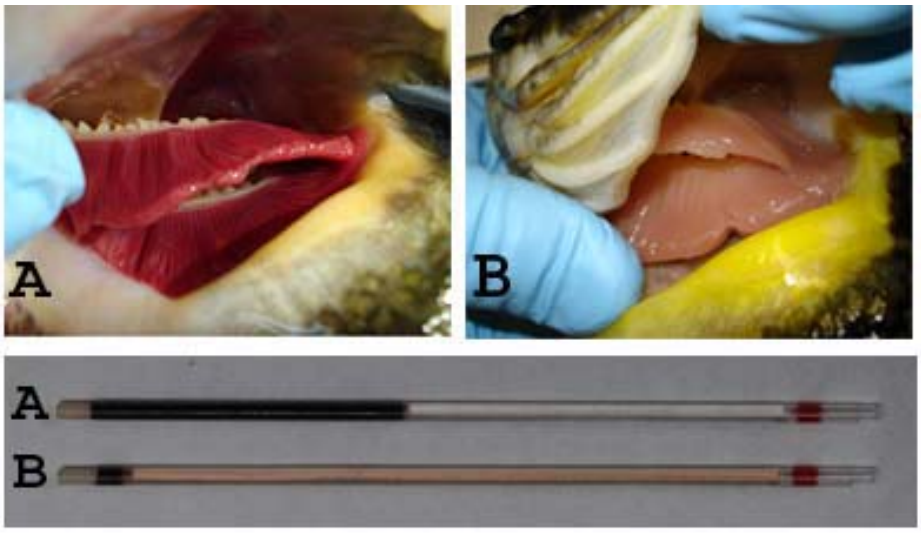


Figure 3

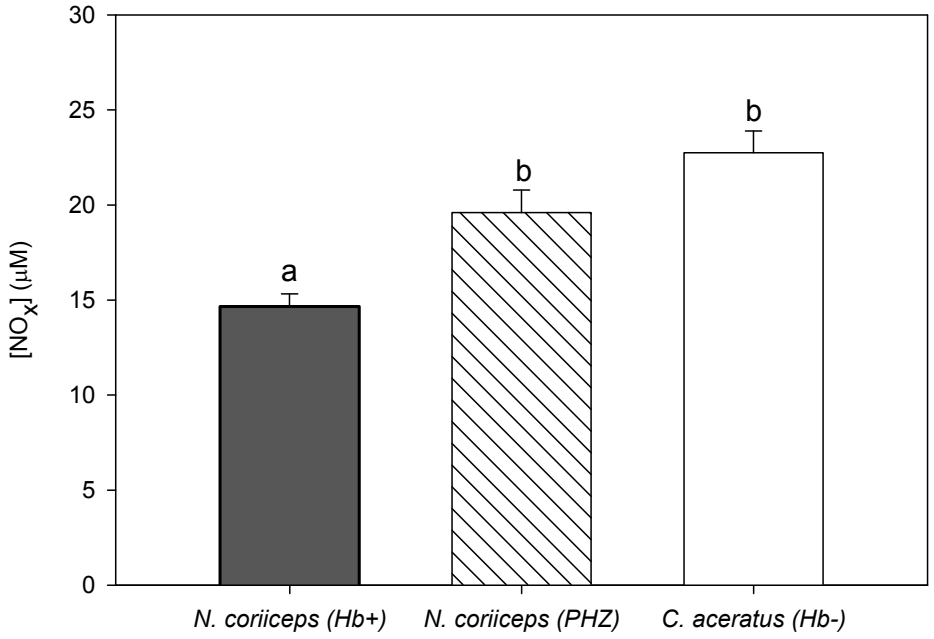


Figure 4

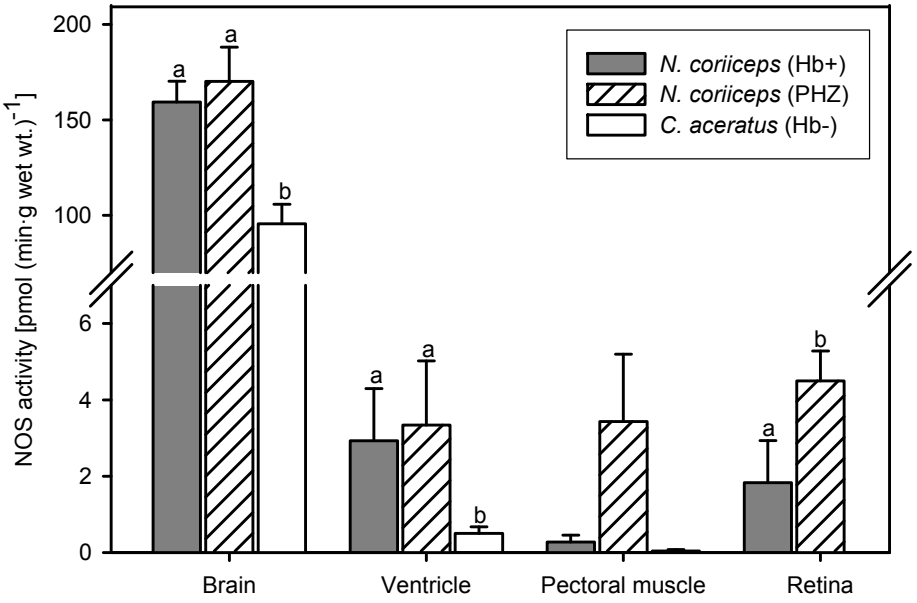
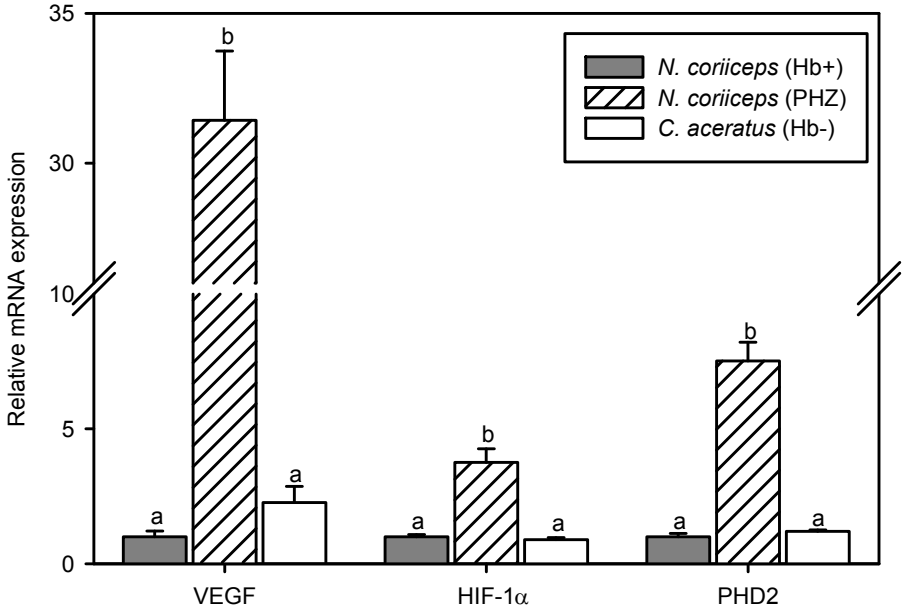


Figure 5



3. **Thermal tolerance and effect of thermal insult upon hypoxia-inducible genes in notothenioid fishes.**

- Hemoglobinless icefishes are more susceptible to rapidly warming temperature than are closely related red-blooded notothenioid fishes.
- Among hemoglobin-expressing notothenioid species examined, thermal tolerance is directly positively correlated with blood hemoglobin concentration. *i.e.* The higher the normal blood hemoglobin concentration, the greater the tolerance of the species to temperature elevation. This strongly suggests that an oxygen limitation dictates thermal tolerance of these fish species.
- Within the very short duration (5-6 hr) of our modified Critical Thermal Maximum (CT_{max}) experimental design, there are few signs of elevation in expression of hypoxia-sensitive genes in any tissues other than heart ventricle. More pronounced elevations in gene expression may be encountered over longer intervals of exposure and cardiac function may be particularly sensitive to thermal insult leading to insufficient supply of oxygen to the tissue.

The following is a draft of a manuscript (Authorship: Beers, J.M. and B.D. Sidell) to be submitted to a peer-reviewed journal (tentatively *American Journal of Physiology*) for publication by August 2010:

INTRODUCTION

The Antarctic Peninsula is recognized widely as one of the planet's "hot spots" of rapid elevation in climatic temperature (Steig et al., 2009). Effects on the terrestrial ecosystem have been extensive—collapse of ice shelves, glacier shrinkage and exposure of new habitat (Clarke et al., 2007; Schofield et al., 2010). The bordering oceanic environment has been impacted as well. Surface waters near the West Antarctic Peninsula (WAP) have risen by ~1°C over the past 50 years and are predicted to rise by another 2°C in the coming century (Meredith and King, 2005; Murphy and Mitchell, 2005). Significant changes have been observed in sea ice coverage adjacent to the WAP (Jacobs and Comiso, 1993; King and Harangozo, 1998) and warming of the Upper Circumpolar Deep Water (UCDW) of the Antarctic Circumpolar Current has been documented (Levitus et al., 2000; Levitus et al., 2005). Temperature elevation of the UCDW

may have a significant influence on marine fauna because it penetrates into WAP continental shelf waters, where a majority of marine life resides (Schofield et al., 2010).

Cascading effects from rapid regional warming of the WAP already have become evident in the surrounding marine ecosystem. Changes in species abundance and distribution have been documented for many species, consequences of which have been shifts in community structure and alterations in food web dynamics (McClintock et al., 2008; Aronson et al., 2009; Schofield et al., 2010). Much focus has been aimed at broad scale ecological impacts of climate change, but there also has been considerable interest about the physiological effects of temperature elevation on individual species. Evaluation of thermal tolerance limits is a key component of understanding species-specific responses to temperature change. Delineation of an organism's thermal 'window' (entire temperature range that permits survival) yields insight into physiological plasticity to adapt to alternate temperature regimes.

Antarctic marine ectotherms live in the coldest, most thermally stable waters of any marine environment. Consequently, many of these species have become highly stenothermal, meaning that they are specialized in their low and narrow temperature window (Somero and DeVries, 1967; Pörtner et al., 2007). Some invertebrate species, such as the bivalve, *Limopsis marionensis*, and the scallop, *Adamussium colbecki*, have incredibly narrow thermal windows and are affected by thermal increases as small as 1-2°C above habitat temperature (Pörtner et al., 2007). Other species, like the limpet, *Nacella concinna*, and the predatory nemertean, *Parborlasia corrugatus*, can tolerate temperatures up to 10°C (Pörtner et al., 2007). Thermal tolerance limits of Antarctic notothenioid fishes also have been investigated, although thermal sensitivities of the suborder's most distinctive family member, white-blooded icefishes, have not

been assessed (Somero and DeVries, 1967; Hofmann et al., 2000; Mark et al., 2002; Podrabsky and Somero, 2006; Franklin et al., 2007).

Antarctic icefishes (Family Channichthyidae) are one of eight families of the perciform suborder, Notothenioidei, which dominates the fish fauna surrounding Antarctica (Eastman, 1993). Icefishes are unique in being the only known vertebrate animals that completely lack the circulating oxygen-binding protein, hemoglobin (Hb), in their blood as adults (Ruud, 1954). Many species within the family also do not express the intracellular oxygen-binding protein, myoglobin (Mb) (Moylan and Sidell, 2000). A suite of cardiovascular modifications appears to have compensated for loss of these important respiratory hemoproteins. Icefishes have larger and more extensive vasculatures, greater blood volumes, larger hearts, and more numerous cardiac mitochondria compared to similar-sized red-blooded notothenioids (Hemmingsen and Douglas, 1970; Fitch et al., 1984; O'Brien and Sidell, 2000; Wujcik et al., 2007). Combination of high throughput circulatory systems, low absolute metabolic rates and exceptionally well-oxygenated waters of the Southern Ocean ultimately permit this group of animals to supply enough oxygen to their tissues and survive without Hb and/or Mb (Hemmingsen, 1991).

Lack of expression of oxygen-binding proteins is not without costs. Loss of Hb has led to greater energetic expense to the circulatory system and loss of Mb has resulted in decreased cardiac performance (Sidell and O'Brien, 2006). Additionally, oxygen-carrying capacity of the blood in icefishes is reduced to less than 10% of that in red-blooded fishes (Holeton, 1970). Current environmental conditions of the Southern Ocean (*i.e.*, cold, stable, well-oxygenated waters) have allowed icefishes to compensate for reduced cardiac efficiency and oxygen-carrying capacities of the blood. However, future climatic warming may exacerbate icefishes' already difficult situation of maintaining oxygen homeostasis. As warming occurs, oxygen

availability will decline due to inverse proportionality of oxygen solubility and temperature. Thus, we anticipate that icefishes might be more susceptible to thermal warming than red-blooded fishes because of inability to supply adequate levels of oxygen to tissues.

We evaluated thermal sensitivities of Antarctic notothenioid fishes, including two icefish species, using a modified CT_{max} (Critical Thermal Maximum) experimental design (Cowles and Bogert, 1944). This design has benefits over other methods because of its ease of use, requirement for fewer animals and shorter experimental exposure times (Lutterschmidt and Hutchison, 1997). The magnitude of thermal insult experienced by an organism is an integral of both exposure temperature and duration of exposure. Consequently, the very short experimental duration of CT_{max} design inherently yields upper thermal limits that are much warmer than the physiological habitat range for an organism. Likewise, use of criteria such as loss of righting response (LRR) to define CT_{max} produces a thermal limit higher than temperatures at which many physiological systems are impacted. CT_{max}, nonetheless, provides a useful means of comparing relative thermal tolerances of organisms and reliably rank-ordering thermal sensitivities among species; it has been widely used for this purpose (Lutterschmidt and Hutchison, 1997). Species that have a CT_{max} close to their normal habitat temperature, *i.e.* narrow thermal window, undoubtedly will be more vulnerable to rise in temperature than animals with a broader thermal window. Objectives of our study were three-fold: 1) test the hypothesis that white-blooded icefishes are more susceptible to temperature elevation than red-blooded notothenioids, 2) evaluate the role of oxygen in setting thermal tolerance limits, and 3) assess the capacity of notothenioids to adjust for rise in temperature.

MATERIALS AND METHODS

Animal collection

We collected five species of Antarctic notothenioid fishes from waters of the Antarctic Peninsula region during austral autumns (April-May) of 2007 and 2009. *Chaenocephalus aceratus* (Lönnberg, 1906), *Chionodraco rastrospinosus* DeWitt and Hureau, 1979, *Notothenia coriiceps* Richardson, 1844 and *Gobionotothen gibberifrons* (Lönnberg, 1905) were caught with otter trawls deployed from the ARSV *Laurence M. Gould* at water depths of 75-150 m in Dallmann Bay (64°08′S, 62°40′W). Bottom-time of each trawl was restricted to 20-30 minutes to minimize capture stress. *N. coriiceps, G. gibberifrons* and *Lepidonotothen kempi* (Norman, 1937) also were caught in baited traps set at 200-500 m depth in both Dallmann Bay and Palmer Basin (64°50′S, 64°04′W). Upon capture, animals were held aboard the vessel in flowing seawater tanks until transfer to the aquarium facility at US Antarctic research base, Palmer Station, where they were maintained in covered, flowing-seawater aquaria at ambient water temperatures of 0±0.5°C. Animals were maintained in aquaria for several days prior to experiments to enable recovery from capture-related stress. Only fish that were in healthy condition were chosen for our study.

Thermal tolerance experiments

We assessed thermal tolerance of Antarctic notothenioid species using a modified CT_{max} (critical thermal maximum) experimental design (Lutterschmidt and Hutchison, 1997). Animals were transferred in groups of 2-3 to an 180 gallon insulated experimental tank at least 2 hr prior to the start of each experiment (Figure 1). The aquarium room was kept dark during the time

course of each experiment to minimize stress to these relatively dark-adapted fishes; only red lights were used for monitoring animal behavior. Temperature was elevated acutely from ambient at a constant rate of +3.6°C per hour and CT_{max} was defined as the temperature where animals lost righting response. Seawater circulation in the experimental tank was maintained using a saltwater compatible pump that drew water from the tank and directed the flow through a 3 kV titanium inline heater (AquaLogic, Inc., San Diego, CA). Heated water then reentered the tank and mixed with incoming ambient seawater as it went through an air lift centered on the bottom of the tank. Incoming ambient seawater flow was controlled by a flow meter mounted on the wall next to the tank; during experimental runs, this flow was stopped and only heated seawater was permitted to recirculate through the tank. We were able to maintain constant rates of temperature change using this technique. Time and temperature data were recorded throughout each experiment using a HOBO® Water Temp Pro V2 temperature logger (Figure 2). Raw data were imported into Microsoft Excel and linear regression analysis was used to compute CT_{max} endpoints and verify a constant rate of temperature change (Figure 3). Upon reaching their CT_{max}, each animal was removed from the tank and then processed as described below.

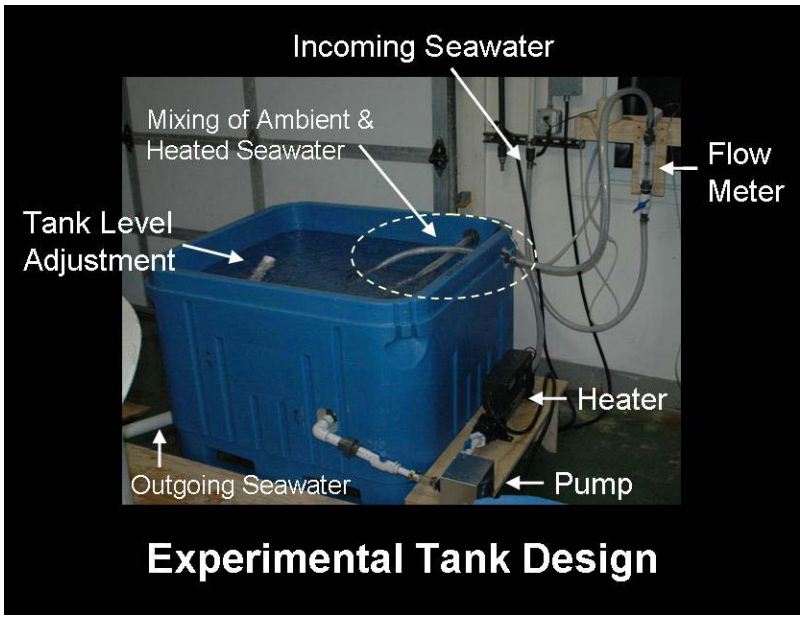


Fig. 1. Experimental tank design for assessing thermal tolerance of Antarctic notothenioid fishes. Animals were put into 180 gallon insulated tanks and subjected to continuous elevation of temperature following a modified CT_{max} method. See text for description of seawater flow and adjustment.

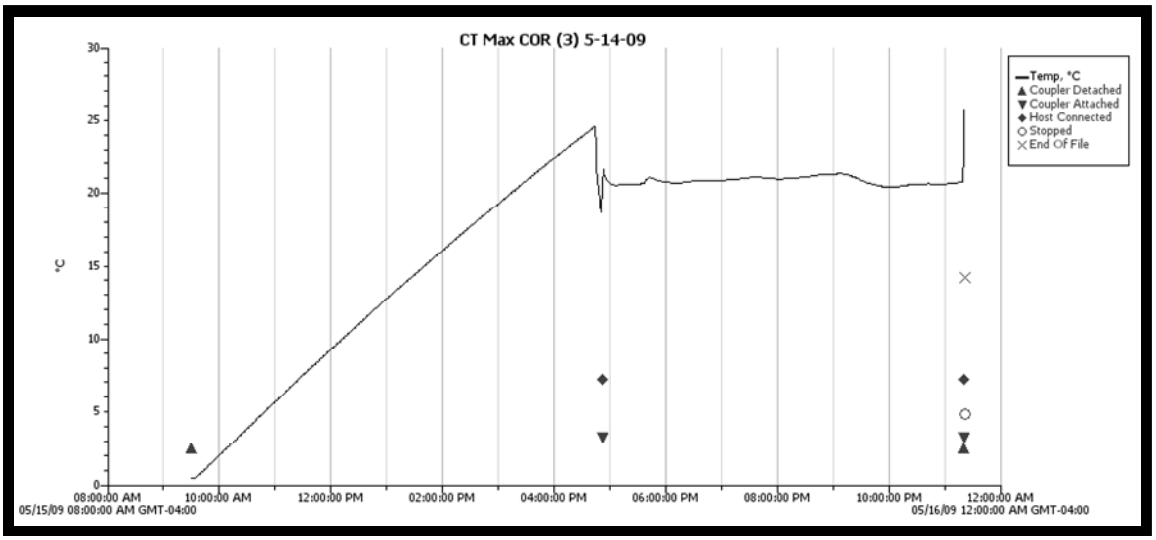


Fig. 2. Representative temperature log from HOBO® Pro V2 temperature logger. Raw data from temperature traces were imported into Microsoft Excel and linear regression analysis was performed to calculate CT_{max} values.

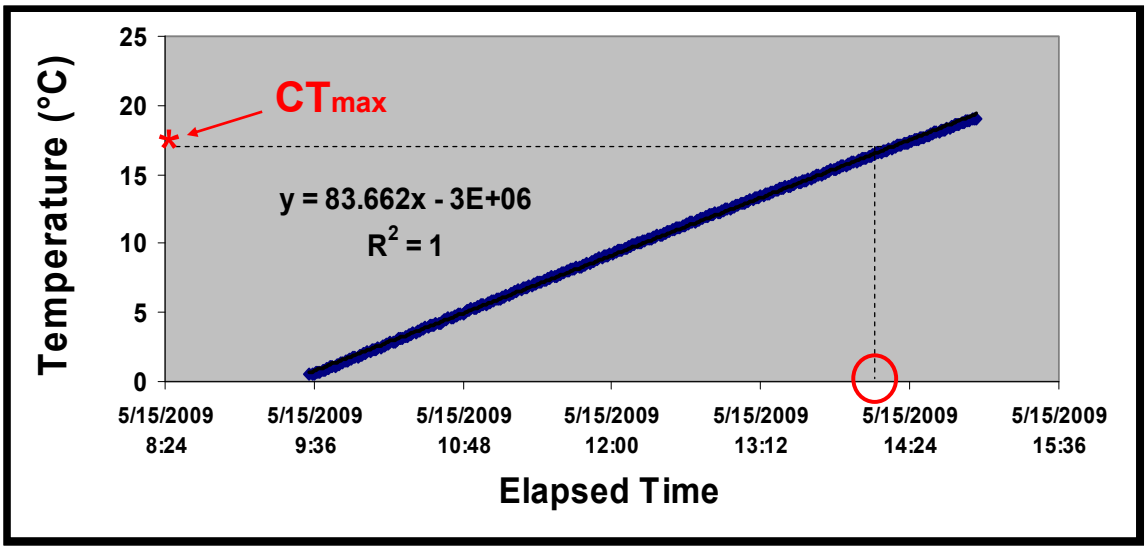


Fig. 3. Linear regression analysis using raw data from HOBO® Pro V2 temperature logger. Red circle denotes the time that animal lost its righting response. CT_{max} temperature was computed by inserting 'time' into the linear regression equation. A correlation coefficient (R^2) value of one indicates a constant rate of temperature change.

Tissue preparation

Animals were anesthetized in MS-222 (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) in seawater (1:7500 w/v) prior to blood collection and tissue harvest. Specimens were killed by severing the spinal cord, followed by rapid excision of the brain. Tissues collected for measurement of gene expression were excised quickly, frozen in liquid nitrogen and stored at -80°C.

Measurement of plasma lactate concentration and hematocrit

Samples were prepared for determination of plasma lactate concentration by first drawing blood from the caudal vein of anesthetized fish and mixing it with a 3.2% sodium citrate solution

to prevent clotting. Red-blooded samples (*N. coriiceps*) were mixed at a 9:1 blood to sodium citrate ratio (volume:volume), while white-blooded samples (*C. rastrospinosus*) were prepared at 4:1. Whole blood samples were centrifuged at 5300 *x g* for 10 min at 4ºC; plasma was drawn off and frozen at -80ºC until later analyses.

Plasma lactate was measured using a commercial lactate kit according to manufacturer's instructions (BioVision Research Products, Mountain View, CA, USA). Briefly, diluted samples (controls 1:10 and thermally-treated 1:200) were analyzed in a VERSAmax™ microplate reader at 450nm and then compared to a lactate standard curve. All samples were measured in duplicate and mean values were computed for each individual. For hematocrit (Hct) measurement, blood was drawn from anesthetized red-blooded fishes (*G. gibberifrons, L. kempi* and *N. coriiceps*) into heparinized capillary tubes and then centrifuged for 5 min in a hematocrit centrifuge. The percentage of red blood cells in whole blood was calculated using digital calipers. Hct measurements were performed in triplicate and mean values were calculated for each individual.

Isolation of RNA

Total RNA was extracted from brain, heart and pectoral muscle using an RNeasy® Fibrous Tissue Mini Kit according to manufacturer instructions (Qiagen, Valencia, CA, USA). RNA concentration and quality were assessed by spectral analysis using a Beckman DU®640 spectrophotometer. Only samples with an A_{260}:A_{280} ratio of 1.8-2.1 and an A_{260}:A_{230} ratio of 1.6-2.0 were used for further analyses. Samples were visualized on 1% agarose gels stained with ethidium bromide to evaluate RNA integrity. RNA was stored at -80°C until later processing.

Quantitative real-time PCR of hypoxia-inducible genes

Levels of mRNA abundance for two hypoxia-inducible genes, hypoxia-inducible factor-1 alpha (HIF-1α) and prolyl hydroxylase domain containing protein 2 (PHD2), were measured using quantitative real-time PCR (qRT-PCR). Gene-specific primers (Table 1) were prepared previously and designed from conserved regions of cDNA sequence among notothenioid species of interest (Beers et al., 2010). Quantitative RT-PCR was performed using qScript™ One-Step SYBR® Green qRT-PCR Kit, Low ROX™ (Quanta BioSciences, Gaithersburg, MD, USA) with a Stratagene MX4000™ set at the following cycling parameters: 1 cycle of 10 min at 50ºC, 1 cycle of 5 min at 95ºC, 40 cycles of 10 s at 95ºC and 30 s at 60ºC. Final reaction volumes for HIF-1α and PHD2 contained 2.5 $ng \cdot \mu l^{-1}$ of total RNA. Only 0.01 $ng \cdot \mu l^{-1}$ of total RNA was used to measure expression level of the 'housekeeping' gene, 18S, to adjust for its greater cellular abundance compared to target genes. Primer concentrations ranged from 100 nM for HIF-1α and 18S to 200 nM for PHD2. Reactions were run in triplicate and negative controls were included on each plate to ensure lack of amplification of genomic or contaminating DNA. A melt-curve analysis was performed at the completion of every run to verify amplification of only a single product in each reaction.

Standard curves of linearized plasmid were run on each experimental plate for both target and reference genes and copy number was computed for each sample. We then normalized expression of each target gene to that of 18S rRNA. Evaluation of 18S as a suitable housekeeping gene in tissues of notothenioids has been reported previously (Urschel and O'Brien, 2008). LinRegPCR (Version 11.0) was used to evaluate PCR efficiencies for all reactions (Ramakers et al., 2003).

Table 1: Primers used for qRT-PCR of hypoxia-inducible and housekeeping genes

Gene	Forward Primer 5′→3′	Reverse Primer 5′→3′
HIF-1α	TCTCTACAACGATGTAATGCTTCC	AATCTGATTTCATCTCCGAGTCC
PHD2	AAACGGGCAAGTTCACAGAC	TCCCAATTTGCCGTTACAAT
18S	ACCACATCCAAGGAAGGCAG	CCGAGTCGGGAGTGGGTAAT

* Primers from Beers et al., 2010

Statistical analyses

Comparisons among species for differences in thermal tolerance (*i.e.*, CT_{max}), lactate concentration and Hct level were performed in SigmaStat (Version 3.1; Systat Software, Inc.) using a one-way ANOVA with a *post-hoc* Student-Newman-Keuls test ($P \leq 0.05$). A student's t-test was used to determine significant differences in gene expression ($P \leq 0.05$).

RESULTS

Oxygen-carrying capacity of blood influences thermal tolerance

Fishes that lack completely or have low levels of the oxygen-binding protein, hemoglobin (Hb), are sensitive to acute elevations in temperature. Hemoglobinless icefishes, *C. rastrospinosus* and *C. aceratus*, displayed greatest thermal sensitivity with the lowest recorded CT_{max} values of 13.3±0.2°C and 13.9±0.4°C, respectively (Figure 1). Hb-expressing *L. kempi*, a species with relatively low Hct compared to other red-blooded notothenioids, had a CT_{max} temperature (14.2±0.4°C) similar to those of icefishes. However, this is where similarities end. Thermal tolerance data for red-blooded species, *G. gibberifrons* and *N. coriiceps*, showed significantly higher CT_{max} values, approximately 1.5-3.0°C higher than icefishes and *L. kempi*. Most impressively, the data illustrate a strong positive correlation between CT_{max} and oxygen-

carrying capacity of the blood, *i.e.*, Hct (Figure 2). *N. coriiceps*, a species with a normal Hct of approximately 35%, had the greatest thermal tolerance to acute rise of water temperature (17.1±0.2°C).

To test whether Antarctic notothenioids have capacity to compensate for elevations in temperature, we subjected a group of *N. coriiceps* to one week of acclimation at 4°C prior to measurement of CT_{max}. There was no change in CT_{max} (17.0±0.1°C) of *N. coriiceps* under these experimental conditions, indicating inability of previous thermal history to influence acute thermal tolerance.

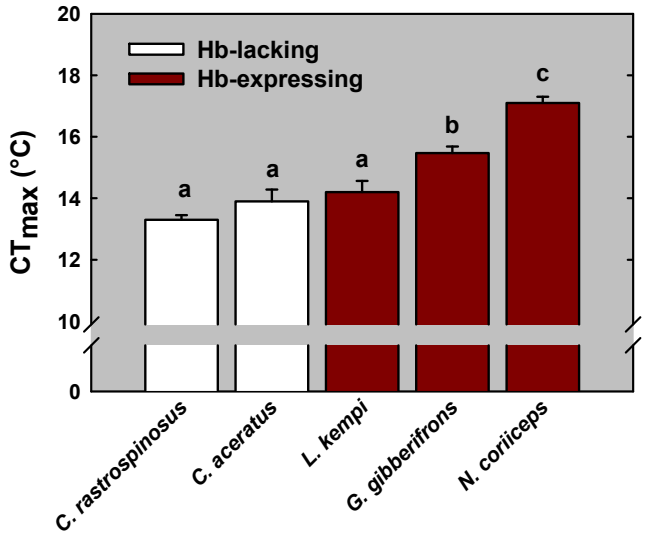


Fig. 1. Thermal tolerance of Antarctic notothenioid fishes. Critical thermal maximum (CT_{max}) differs among fishes lacking (white bars) and expressing (red bars) the oxygen-binding protein, hemoglobin (Hb). Data are presented as means ± s.e.m.; N=8 for each species. Different lower case letters denote statistically significant differences between species ($P \leq 0.001$).

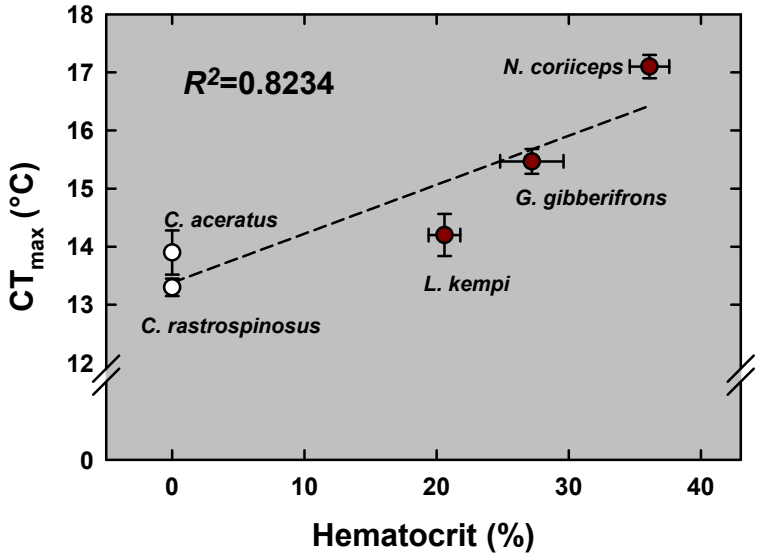


Fig. 2. Thermal tolerance correlates to oxygen-carrying capacity in Antarctic notothenioids fishes. There is a strong positive relationship (R^2=0.8234) between CT_{max} temperature and hematocrit of both white- and red-blooded species (white- and red-filled circles, respectively), indicating that fishes with a relatively high oxygen-carrying capacity are less sensitive to elevation in temperature than species having a low capability to transport oxygen. Values are presented as means ± s.e.m.; *N*=8 for each species.

Effect of acute temperature elevation on hematocrit and plasma lactate levels

Red-blooded notothenioids subjected to an acute elevation in temperature responded by increasing the number of red blood cells (*i.e.*, Hct) in their circulation (Figure 3), a direct determinant of oxygen-carrying capacity. *G. gibberifrons* and *N. coriiceps* showed the most significant increases in Hct upon reaching CT_{max} (approximately 24% and 16%, respectively), whereas *L. kempi* had only a modest 7% rise in Hct.

We analyzed plasma lactate concentrations of the two species with minimum and maximum CT_{max} temperatures recorded in our study. There were significant increases in plasma lactate concentrations in both *C. rastrospinosus* and *N. coriiceps* over those of control animals (Figure 4). The approximate 10-fold treatment effect of *C. rastrospinosus* was double that of the

5-fold change seen in *N. coriiceps*; however, absolute lactate concentrations were greater in the red-blooded species. Taken as a whole, this blood parameter indicates a stress response to elevation in temperature and an increase in anaerobic metabolism to compensate for an apparent shortfall in oxygen supply.

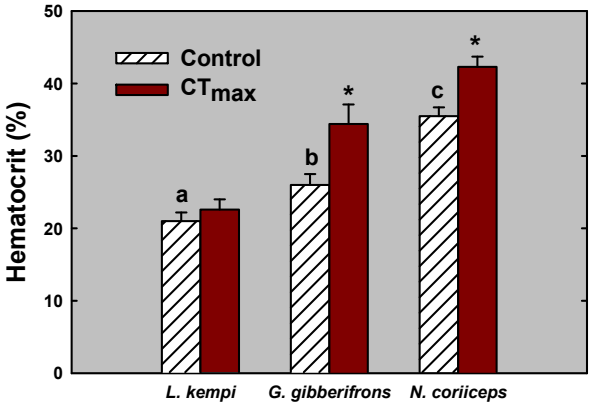


Fig. 3. Hematocrits of three red-blooded Antarctic notothenioid fishes. Exposure to an acute rise in ambient temperature (CT_{max}) causes an increase in the number of red blood cells in circulation. Data are presented as means ± s.e.m.; $N \geq 6$. Different lower case letters denote statistically significant differences among control species ($P \leq 0.001$). Asterisk denotes a significant difference between control and treated (CT_{max}) groups within each species ($P \leq 0.05$).

Effect of acute temperature elevation on mRNA levels of hypoxia-inducible genes

We measured mRNA levels of two genes associated with an hypoxia-inducible response, HIF-1α and PHD2, in tissues of animals exposed to an acute elevation in temperature. As with lactate measurements, we only performed experiments on two species having the lowest and highest recorded CT_{max} temperatures. Although the duration of exposure to elevated temperature was relatively short for changes in mRNA expression to occur, we were able to detect some differences in levels between control and treated animals. The most notable result was the increase in mRNA abundance of HIF-1α in cardiac tissue of *N. coriiceps* (Figure 5). This

increase represented an approximate 1.6-fold change in relative mRNA copy number. We did not detect a treatment effect on HIF-1α levels in either brain or pectoral muscle of *N. coriiceps*. The expression pattern of PHD2 in tissues of *N. coriiceps* was similar to that of HIF-1α; the increase of mRNA abundance in heart, however, fell just short of statistical significance (P=0.06).

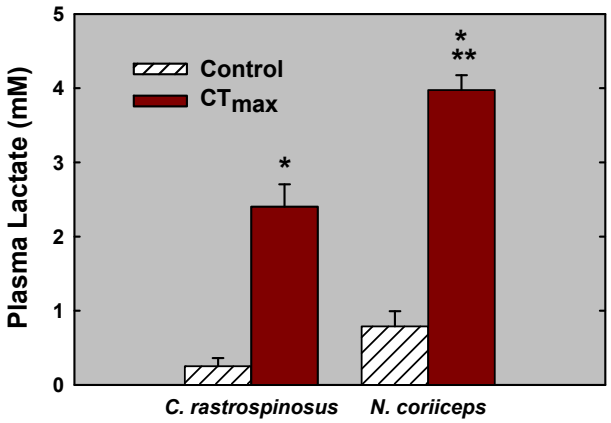


Fig. 4. Lactate concentrations in blood plasma of a white- and red-blooded Antarctic notothenioid fish. Plasma lactate levels are elevated in fishes subjected to an acute rise in temperature (CT_{max}). Values are presented as means ± s.e.m.; N=6 for each group. Single asterisk denotes statistical significance between control and treated groups of Hb-lacking *C. rastrospinous* and Hb-expressing *N. coriiceps* ($P \leq 0.001$). Double asterisk denotes a significant difference in lactate concentration between treated *C. rastrospinosus* and treated *N. coriiceps* ($P \leq 0.001$).

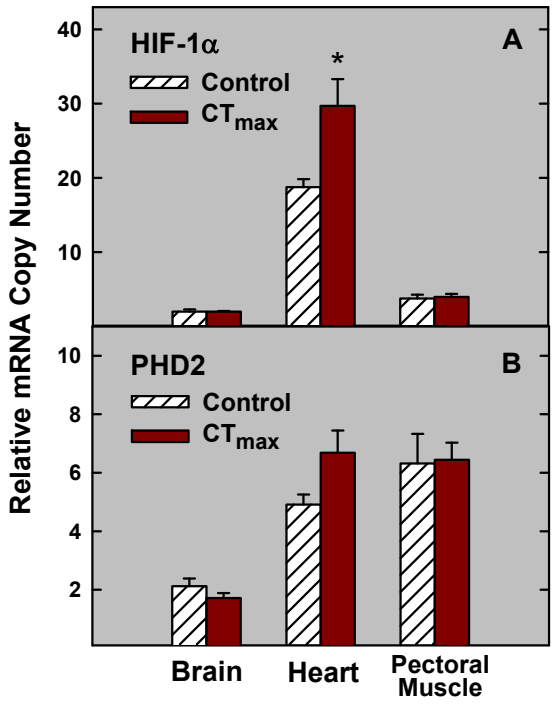


Fig. 5. Messenger RNA abundance of hypoxia-inducible genes in a red-blooded Antarctic notothenioid, *N. coriiceps*. Relative mRNA copy numbers were computed for HIF-1α (A) and PHD2 (B) using quantitative real-time PCR (qRT-PCR). Expression of each target gene was normalized to 18S rRNA. Values are presented as means ± s.e.m.; $N \geq 5$ for each group. Asterisk denotes treatment effect within that tissue ($P \leq 0.05$).

Examination of expression levels of the same genes in tissues of *C. rastrospinosus* revealed both parallels and differences to results from *N. coriiceps* (Figure 6). Similar to *N. coriiceps*, there was an increase in mRNA abundance of HIF-1α in cardiac tissue of treated *C. rastrospinosus*; however, these data also fell short of statistical significance from one another ($P \leq 0.423$) due to high variation in the data set. An exception to results seen for *N. coriiceps* was a small, but significant ($P = 0.05$), difference in levels of HIF-1α mRNA in pectoral muscle between control and CT_{max} animals of *C. rastrospinosus*. This represented the only significant decrease in HIF-1α mRNA level in response to an acute temperature elevation. Overall, the

combination of results for both HIF-1 α and PHD2 suggest a hypoxia-inducible response in hearts of thermally-treated *N. coriiceps* and a potential treatment effect in hearts of *C. rastrospinosus*; additional analyses are required for the latter group.

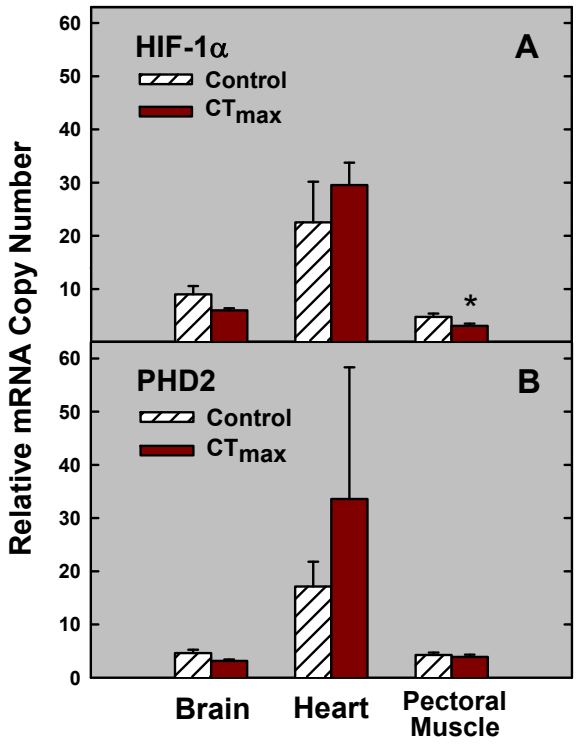


Fig. 6. Messenger RNA abundance of hypoxia-inducible genes in a white-blooded Antarctic notothenioid, *C. rastrospinosus*. Relative mRNA copy numbers were computed for HIF-1α (A) and PHD2 (B) using quantitative real-time PCR (qRT-PCR). Expression of each target gene was normalized to 18S rRNA. Values are presented as means ± s.e.m.; $N \geq 4$ for each group. Asterisk denotes treatment effect within that tissue ($P=0.05$).

DISCUSSION

Waters surrounding the West Antarctic Peninsula are warming at a more rapid rate than other regions of the world's oceans (Vaughan et al., 2003; Meredith and King, 2005). Much research has focused on the influence of temperature elevation upon different species within the Southern Ocean ecosystem, including a handful of studies that have given attention to notothenioid fishes inhabiting these waters (Somero and DeVries, 1967; Hofmann et al., 2000; Mark et al., 2002; Podrabsky and Somero, 2006; Franklin et al., 2007). None of these investigations, however, have assessed the temperature sensitivities of white-blooded notothenioids. We believe this study to be the first to give insights into the thermal tolerance window of icefishes.

Thermal tolerance correlates to oxygen-carrying capacity of notothenioids

We report a clear pattern of temperature sensitivities among notothenioid fishes that vary in their expression of the oxygen-binding protein, hemoglobin. Results indicate that notothenioids that lack completely or have low levels of Hb have narrower thermal windows than species with comparatively high Hb levels. These findings suggest that animals with low oxygen-carrying capacities, particularly icefishes, may be highly susceptible to effects of global warming. The narrower thermal window of icefishes compared to closely-related red-blooded relatives is not a surprise, but the absolute CT_{max} temperatures of all species are higher than we might have anticipated. Previous studies report lower thermal tolerance limits for notothenioid fishes than the findings we present here. However, not all of these studies are directly comparable due to vastly different experimental designs. The classic determination of Upper

Incipient Lethal Temperature (UILT) requires exposure of experimental animals to elevated temperatures for one week (Fry, 1971). CT_{max} designs, on the other hand, expose fish to a continuously greater thermal shock over a period of only minutes or hours (Lutterschmidt and Hutchinson, 1997). Because the magnitude of thermal insult is undoubtedly a product of time and temperature, for any given species, CT_{max} temperatures are invariably higher than UILT temperatures. Comparisons of absolute thermal tolerance limits must be considered with this in mind.

The seminal study on temperature sensitivities of Antarctic fishes by Somero and DeVries documented an UILT of approximately 6°C for three red-blooded notothenioids of the genus *Trematomus* (Somero and DeVries, 1967). Other reports since have used modified CT_{max} designs to show survival temperatures ranging from 9-13°C for the Antarctic eelpout, *Pachycara brachycephalum*, and 9-10°C for the red-blooded notothenioid, *Lepidonotothen nudifrons* (Hardewig et al., 1999; van Dijk et al., 1999; Mark et al., 2002). In light of these results, the relatively high CT_{max} temperatures that we found for icefishes are somewhat surprising. There are at least two possible explanations. First, prior thermal history strongly influences CT_{max} and UILT (see section below); many of the animals from the above mentioned studies had different acclimation temperatures. Second, as mentioned already, it is difficult to obtain data that compare exactly when using thermal tolerance protocols that deviate even slightly (Lutterschmidt and Hutchison, 1997). What is clear, though, is that there are species-specific responses to temperature change and there is no 'one size fits all' model for Antarctic notothenioids. To assess how climate change will affect the Southern Ocean ecosystem as a whole, it will be necessary to collect data on the effects of temperature upon many member species.

Oxygen limitation of thermal tolerance?

An inadequate supply of oxygen to tissues as a result of increasing temperature is thought to be an early cause of functional limitation in marine ectotherms (Pörtner and Knust, 2007; Pörtner et al., 2007; Pörtner, 2010). The concept of oxygen-limited thermal tolerance implies a hypoxic milieu within tissues. Although results of our CT_{max} experiments point toward an oxygen deficiency setting thermal tolerance limits, we did not directly measure either ambient or intracellular oxygen concentrations and can only speculate as to what these levels may be within tissues. Calculations of oxygen solubility in seawater indicate that animals should have been exposed to a 26-33% reduction in ambient oxygen concentration at their CT_{max}. Oxygen demand by tissues simultaneously will increase as body temperature increases. These two factors would translate to a much greater decrease in available oxygen at the tissue level. Just as there are species-specific differences in thermal tolerance, the same is true for tolerance of hypoxia. Cold-adapted species typically require high oxygen levels, while warmer water species, *e.g.* cyprinids, can survive from full anoxia to hyperoxia (Lushchak and Bagnyukova, 2006). A 26-33% reduction in oxygen concentration most likely causes a significant shortfall in oxygen supply at the tissue level in cold-adapted notothenioids. Furthermore, this reduction in oxygen at the tissues will be particularly pronounced for icefishes that have blood with less than 10% the oxygen-carrying capacity of red-blooded-fishes (Holeton, 1970).

Even without direct measurements to confirm an oxygen deficiency, our results provide convincing evidence for oxygen-limited thermal tolerance in notothenioid fishes. High correlation between CT_{max} and normal Hct, in combination with elevation of Hct and blood lactate concentration in CT_{max} animals, support that thermally-treated fish have experienced an

hypoxic insult. Increased Hct often is a first response to low oxygen conditions as a means to elevate oxygen-carrying capacity. While Hct in white-blooded animals is zero due to lack of red blood cells, we found that all experimental species of red-blooded notothenioids increased their Hcts in response to acutely elevated temperature. Elevated Hct in CT_{max} animals not only verifies a stress response to experimental treatment, but also suggests a compensatory adjustment by red-blooded species to facilitate greater oxygen-delivery to their tissues. Greater plasma lactate concentrations in thermally-treated animals compared to control animals indicate a shift from aerobic to anaerobic metabolism, which occurs during hypoxic conditions.

As a final means of probing an oxygen-limitation hypothesis, we analyzed mRNA expression levels of two genes that have key roles during hypoxic response, HIF-1α and PHD2. HIF-1α is one of two subunits that comprise the heterodimeric transcription factor, HIF-1. HIF-1 often is called the 'master regulator' for its role in inducing expression of many genes during hypoxia, including those involved in angiogenesis, glycolysis, and erythropoiesis (D'Angelo et al., 2003; Wenger et al., 2005). Expression of HIF-1α is highly regulated, while HIF-1β, the other subunit of HIF-1, is expressed constitutively. During normoxia, PHD2 hydroxylates proline residues on HIF-1α targeting the protein for proteosomal degradation. PHD2 is inactive without oxygen, thus blocking degradation of HIF-1α and allowing its accumulation, which stimulates transcription of downstream genes (Berra et al., 2003). Upregulation of PHD2 during hypoxia acts as a feedback mechanism to prevent the continuation of a hypoxia-inducible response upon reoxygenation (D'Angelo et al., 2003).

The relatively short duration (~5 hours) of our modified CT_{max} design may not be a sufficiently long treatment period to observe major changes in gene expression. It is not particularly remarkable that our results did not demonstrate a robust response in all tissues

assayed. Despite this limitation, however, we did see a change in mRNA abundance of hypoxia-inducible genes in heart of *N. coriiceps*. An hypoxic response in cardiac tissue is not surprising given the circulatory anatomy of fish; because coronary vessels are absent, the heart is reliant upon lumenal venous blood from the systemic circulation and would be exposed to lower levels of oxygen than the other tissues that we sampled. Cardiac function has been shown to decrease precipitously for a number of animals upon reaching acute thermal limits, thereby implicating it as "an obvious candidate for a cause of acute thermal death" (Somero, 2010). Reports that CT_{max} of cardiac function is the same as the UILT of porcelain crabs provide strong support for a mechanistic connection between whole-animal thermal tolerance and collapse of a specific organ (Stillman and Somero, 1996; Stillman, 2002; Stillman, 2003). However, intriguing as the results by Stillman and Somero are, we must consider that the mechanistic underpinnings of thermal tolerance in a vertebrate fish (myogenic heart) might be very unlike that in an invertebrate crab (neurogenic heart) due to differences in cardiovascular anatomy and regulation.

In light of the limitations described above, the lack of response observed in hearts of *C. rastrospinosus* is not entirely unanticipated. It is possible that the duration of the treatment exposure for these animals was not long enough to induce changes in expression of mRNA or that HIF-1α and PHD2 were regulated by posttranslational modifications and not via transcription. An additional explanation stems from the fact that changes in mRNA expression due to heat stress and/or hypoxia can be highly transient and tissue-specific (Buckley et al., 2006; Law et al., 2006; Rissanen et al., 2006) Tissue-specificity also would explain the small, but significant, change in level of HIF-1α in pectoral muscle of *C. rastrospinosus*. Although not clear-cut, these data support the concept of oxygen-limited thermal tolerance.

Capacity of Antarctic fishes to modify thermal tolerance limits

The degree to which animals are able to adjust their thermal sensitivity is an important consideration when assessing overall impact of climate change on species, populations, and ecosystems (Stillman, 2003; Pörtner et al., 2007; Somero, 2010). Thermal history (*i.e.*, acclimation or acclimatization) plays a key role in determining how an organism will respond to future environmental temperature change (Stillman, 2003). Thus, the very cold, stable temperatures of Antarctic waters have resulted in a high degree of thermal specialization and extreme stenothermy of endemic species (Pörtner et al., 2007; Somero, 2010).

There has been debate about the potential for highly stenothermal Antarctic species to adapt to rising temperature. Thermal specialization has been thought to be accompanied by trade-offs that compromise ability to respond to changes in temperature (Pörtner et al., 2007; Somero, 2010). The few investigations into this claim have yielded mixed results. Studies by Franklin, Seebacher and colleagues have shown that a red-blooded notothenioid, *Pagothenia borchgrevinki*, has capacity to acclimate cardiac function to elevated temperatures (Seebacher et al., 2005; Franklin et al., 2007). Podrabsky and Somero saw increased heat tolerance in two species of *Trematomus* (*T. pennellii* and *T. bernacchii*), but they did not see an increase in tolerance of *P. borchgrevinki* or in a deep-water zoarcid species, *Lycodichthys dearborni* (Podrabsky and Somero, 2006). Their finding that *P. borchgrevinki* did not increase heat tolerance, thus, is in direct contrast to results of Franklin, Seebacher and colleagues.

Our data indicate that the red-blooded notothenioid, *N. coriiceps*, is unable to increase its heat tolerance after acclimation to 4°C for one week. Although, one week may not seem like a particularly long acclimation period, increases in thermal tolerance have been shown to develop within 1-2 days of acclimation to 4°C in *T. bernacchii* (Podrabsky and Somero, 2006). Due to a

shortage of animals and time constraints in the field, we were unable to run the same acclimation experiment with an icefish species. Based upon available information, we cannot assume that icefishes will respond in the same manner as *N. coriiceps* due to species-specific responses in capacity to compensate for elevated temperatures.

Summary and conclusions

Results from our study show that temperature sensitivity is highly dependent upon the oxygen-carrying capacity of the blood. Thus, Antarctic notothenioids that completely lack or have low levels of the oxygen-binding protein, Hb, may be more susceptible to elevated temperatures that accompany climate change than species with comparatively high levels of Hb. Although we do not have a detailed understanding of the mechanism(s) setting thermal tolerance limits, our data give strong support to the concept of oxygen-limited thermal tolerance. Additional research to uncover the mechanistic underpinnings of thermal sensitivity at the cellular and molecular levels is needed. The degree to which animals can adjust thermal tolerance also is an important consideration when assessing the impact of future climate change. Acclimation to 4°C for one week revealed that red-blooded, *N. coriiceps*, does not alter its thermal tolerance upon acute elevation of temperature. Taken as a whole, our results suggest that Antarctic notothenioids, particularly icefishes, may be vulnerable to effects of global warming, which may have profound consequences in physiological performance, geographic distribution and species survival.

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4. **Molecular evolution of the myoglobin gene in Channichthyid Icefishes.**
    - We sequenced the myoglobin gene, including substantial sequence upstream of the core promoter, from genomic DNA of all 16 known species of Channichthyid icefishes.
    - We unexpectedly found that a 15 bp insertion upstream of the core promoter, which includes duplication of the muscle-specific TATAAAA promoter element, was present in 14 species, including several that produce myoglobin protein. This sequence previously had been attributed as being responsible for lack of Mb expression in *Chenocephalus aceratus.*
    - Although the duplicated TATAAAA sequence does appear to be involved in preventing Mb expression in *C. aceratus*, the underlying mechanism is unresolved and appears to be more complex than previously believed, probably involving interaction with other sequences at locations remote from the Mb gene proper.

The following is a draft of a manuscript (authorship: Borley, K.A. and B.D. Sidell) that will be submitted to a peer-reviewed journal (*Polar Biology*) for publication during Summer 2010:

EVOLUTION OF THE MYOGLOBIN GENE IN

ANTARCTIC ICEFISHES (FAMILY: CHANNICHTHYIDAE)

Abstract (150-250 words)

Antarctic icefishes (Family: Channichthyidae) are the only adult vertebrate animals that do not express the circulatory oxygen-binding protein, hemoglobin. Six species of icefishes also do not express myoglobin (Mb), an oxygen-binding protein found in ventricular tissue of Antarctic notothenioids. Sequence of the Mb gene from the icefish, *Dacodraco hunteri,* contains a duplicated TATA box in the promoter that is absent from the promoter of *Chionodraco rastrospinosus*, an icefish species known to express Mb. The 15 base pair insertion in *D. hunteri* is identical to the duplicated TATA box previously identified in *C. aceratus*, another icefish species that lacks Mb expression, and is thought to be directly responsible for

inhibiting transcription of the Mb gene. Sequencing of the Mb promoter from the remaining channichthyid species revealed the duplicated TATA box in 14 of 16 icefish species, including 8 species known to express Mb. Based on the presence of the duplicated TATA box in promoters of both Mb-expressing and Mb-lacking species, loss of Mb expression in *D. hunteri* and *C. aceratus* is either independent of the duplicated TATA box, or the duplicated TATA box requires presence or absence of a distant regulatory element to inhibit transcription.

Keywords: Antarctic Icefish, Myoglobin, Promoter Elements, Notothenioids

Introduction

Antarctic icefishes are the only known adult vertebrates that do not express the circulating oxygen-binding protein, hemoglobin (Hb) (Ruud, 1954). Lack of Hb expression in icefish has been traced to a gene deletion event that occurred approximately 8.5 Ma when icefishes diverged from red-blooded bathydraconids (Near, 2004).

In an interesting twist in the story of icefish evolution, several channichthyid species also do not express myoglobin (Mb). Myoglobin is an oxygen-binding protein that assists in the storage and delivery of oxygen in cardiac myocytes and oxidative skeletal muscle fibers (Ordway and Garry, 2004). In red-blooded Antarctic notothenioids and Mb-expressing icefishes, Mb expression is restricted to heart ventricle (Sidell *et al.*, 1997). Six icefish species, in four genera, do not express Mb. The location within the channichthyid phylogeny (Near *et al.*, 2003)

of six species lacking Mb suggests Mb expression has been lost on four distinct occasions during the evolution of the family (Sidell *et al.*, 1997; Moylan and Sidell, 2000; Grove *et al.*, 2004).

Several distinctly different mutations have led to loss of Mb expression within the icefish family. In two genera that do not express Mb protein, *Champsocephalus* and *Pagetopsis*, the Mb gene is transcribed but not translated (Vayda *et al.*, 1997; Grove *et al.*, 2004). Myoglobin expression was lost by a common ancestor to the congeners *Champsocephalus esox* and *C. gunnari* due to a 5-base pair (bp) frameshift duplication in exon 2 resulting in a premature stop codon (Grove *et al.*, 2004). The mechanism leading to loss of Mb protein expression in *Pageotopsis* species is distinct from that of the *Champsocephalus* species. *P. macropterus* and *P. macultus* Mb gene sequences contain an aberrant polyadenylation sequence. The aberrant poly-A signal may inhibit addition of a poly-A tail to the 3' end of the mRNA, resulting in early degradation of the transcript (Vayda *et al.*, 1997). Transcription of the Mb gene followed by rapid degradation explains why low levels of transcript are produced in *P. macropterus*, while no protein has been detected in the heart ventricle.

Two species of icefish, *Chaenocephalus aceratus* and *Dacodraco hunteri*, do not express myoglobin mRNA or protein (Moylan and Sidell, 2000). In *C. aceratus*, a 15 bp insertion results in duplication of the TATA box sequence (TATAAAA), a promoter element typically located 30 bp upstream of the transcription start site. Binding of TATA binding protein, a subunit of transcription factor IID (TFIID), to the TATA box stimulates the formation of the RNA Polymerase II preinitiation complex (Burley and Roeder, 1996). The duplicate TATA box in *C. aceratus* is located approximately 600 bp upstream of a correctly positioned TATA box. A putative E-box, a promoter element that binds basic helix-loop-helix transcription factors, is located 10 bp upstream of the duplicated TATA box. Close proximity of the duplicated TATA

box and putative E-box suggests that binding of RNA Polymerase II to the duplicated TATA box could block access to the E-box, inhibiting transcription of Mb (Small *et al.*, 2003). Although *D. hunteri* does not express Mb mRNA or protein, the mechanism resulting in the loss Mb transcription has yet to be elucidated.

In this paper, the genomic DNA (gDNA) sequence of *D. hunteri* is reported to include a 15 bp insertion containing a duplicated TATA box that is identical in sequence and position to the insertion previously described in *C. aceratus.* Although the consensus phylogeny of the icefish family does not group *C. aceratus* and *D. hunteri* together, the most parsimonious explanation for both species containing this 15 bp insertion is that the duplicated TATA box mutation occurred once in the family, suggesting that *C. aceratus* and *D. hunteri* may be sister species. To test this hypothesis, we sequenced Mb genes from all 16 species of icefishes. Analyses of promoter sequences from these species revealed that the duplicated TATA box is not restricted to *D. hunteri* and *C. aceratus*. In fact, the duplicated TATA box is present in promoters of species known to express Mb. Based on these new data, we reject our initial hypothesis that *C. aceratus* and *D. hunteri* are sister species and consider possible roles of the duplicated TATA box in regulating myoglobin transcription.

Methods

Preparation and Isolation of Myogobin Genomic DNA

D. hunteri ventricular tissue was collected by William Detrich on the 2003 AMLAR cruise (need more collection info). DNA was extracted using the Qiagen DNEasy kit as described in the kit protocol. gDNA samples for all other species were generously provided by Dr. Thomas Near of *Yale University*.

Amplification and Sequencing of Myoglobin

The myoglobin gene was amplified from gDNA samples using primers shown in Table 1, Phusion® DNA polymerase (Finnzymes) and the following thermocycler conditions: 1 cycle of 30 s at 98ºC, 35 cycles of 10 s at 98ºC, 30 s at 60ºC, 2 min at 72ºC, and 1 cycle of 5 min at 72ºC. PCR products were cloned for sequencing using either Invitrogen's PCR4 vector and Top10 cells or Promega's pGEM-Teasy and Invtrogen's DH5-α cells. Colonies were screened by PCR amplification, sequenced by the University of Maine DNA Sequencing Facility (Orono, ME), and then submitted to tblastn (http://blast.ncbi.nlm.nih.gov) to confirm gene identity.

Table 1. Nucleotide sequence of primers used to amplify the myoglobin gene. Position refers to the location of the primer in the *Chionodraco rastrospinosus* myoglobin gene where zero is the putative transcription start site for the gene.

Primer Name	Nucleotide Sequence	Position
Mb Upstream	5'-ACCATAGCGTGTACAGTTGTTC-3'	-1265 → -1244
Mb TATA1[a]	5'-CGATTTGAAGACGCTATTGGA-3'	-580 → -557
Mb TATA2[a]	5'-CCTGCAGAGTAGTAAAATGTCCTG-3'	+36 ← +59
Mb Mid F	5'-AGCTGAGGTGATGAAGACGCCTCTTTC-3'	+312 → +339
Mb Mid R	5'-CAGCACTTCAGCACCATGTCAAAGTC-3'	+526 ← +551
Mb Rev[a]	5'-TAATTAGGCTTACAGAAAATCAGACC-3'	+1,649 ← +1,674
Mb Coding Rev	5'- GGAGGACACAAAAAGTTGGAGGAAAGATC-3'	+1,367 ← +1,395

[a] Primer sequences from Grove *et al.* (2004)

Sequence Analysis

Sequence chromatographs were analyzed using Chromas (Technelysium Pty Ltd) and aligned using Clustal W2 (Larkin *et al.*, 2007). Alignment was done with the following settings: Gap open 25, Gap Extension 0.5, Gap distance 1, and then manually checked. Alignment (Supplementary Figure 1) is labeled with transcription start sites, introns, exons, and putative promoter elements that were identified in *C. rastrospinosus* and *C. aceratus* by Small *et al.*

(1998). MEGA 4 (Tamura *et al.*, 2007) was used to calculate percent similarity between different species.

Results

Dacodraco hunteri Myoglobin Sequence

D. hunteri Mb gDNA sequence was aligned with genomic sequences from *C. aceratus* (Genbank Accession Number: U71153) and *C. rastrospinosus* (Genbank Accession Number: U71059) previously published in Small *et al.* (1998). Sequence of *D. hunteri* contains a duplicated TATA Box 722 bp upstream of the putative transcription start site. The duplicated TATA box is identical in both sequence and position to that previously identified by Small *et al.* (1998) in *C. aceratus* (Figure 1). The putative E-box located upstream of the duplicated TATA Box is intact in *D. hunteri* Mb; however, an INDEL is present immediately upstream of the E-box (Figure 1).

```
                                                  -650
                                                   ↓
C. rastro.    (Mb +)  TACCACCGCTAAACAGCTGATGTAAAAC----------------AAATCTTAAAAC
C. aceratus   (Mb -)  TACCACCGCTAAACAGCTGATGTAAAACCTATAAAACAAAAACAAATCTTAAAAC
D. hunteri    (Mb -)  TACC--------ACAGCTGATGTAAAACCTATAAAACAAAAACAAATCTTAAAAC
                                  Putative       Duplicated
                                   E Box          TATA Box
```

Figure 1. The promoter of *D. hunteri* contains a duplicated TATA box previously identified in the promoter of *C. aceratus*. Species are labeled Mb+ or Mb- indicating if myoglobin protein is expressed. *C. rastrospinosus* and *C. aceratus* sequences were reported in Small *et al.* (1998); Accession numbers: U71153 and U71059, respectively. Numbers above the sequence correspond to the position in the sequence in reference to the putative transcription start site for the reference *C. rastrospinosus* sequence.

Sequences of Myoglobin Genes from All Icefish Species

Supplementary Figure 1 contains alignment of the Mb gene for all 16 species of channichthyid icefishes. With the exception of *P. maculatus*, each species' sequence stretches from -1067 to +1366 compared to the reference *C. rastrospinosus* sequence. These sequence data cover approximately 1067 bp of the promoter region upstream of the transcription start site through the first two exons and ending 54 bp into the third intron. The *P. maculatus* sequence ends shortly after the start codon (*C. rastrospinosus* +550) due to inability to amplify the 3' end with primers listed in Table 1. For 15 icefish species that have been sequenced into the third intron, sequence length ranged from 2262 bp in *C. esox* to 4789 bp in *P. macropterus* due to presence of insertions and deletions.

Compared to the 2425 nucleotides of the *C. rastrospinosus* reference sequence, 2343 of the nucleotides are conserved among species sequenced (with the exception of *P. maculatus* due to the incomplete sequence for this species). Percentage similarity between species ranged from 99.76% (*Chionodraco rhinoceratus* vs. *Cryodraco antarcticus*) to 97.62% (*Champsocephalus esox* vs. *Chionodraco hamatus*).

P. macropterus (Mb-) contains a 2,293 nucleotide insertion located at +665 in the *C. rastrospinosus* reference sequence placing the insertion within the second intron. Nucleotide and translated amino acid sequences were run against NCBI's nucleotide and protein databases using blastn and blastx, respectively. In both cases, the most significant hits were LIM domain containing sequences; however, when compared to the two databases, the nucleotide database produced the most significant hits. The most significant blastn result (E value: 8e-26) was to a

D. rerio mRNA sequence predicted to be LIM domains containing protein 1 (GenBank Accession Number: XM686167.3).

15 bp INDEL Containing a Duplication of the TATAAAA Sequence is Not Unique to *C. aceratus* and *D. hunteri*

The 15 bp INDEL that encodes the duplicated 'TATAAAA' sequence of interest, that was thought to be responsible for loss of Mb expression in *C. aceratus* (Small *et al.*, 2003) and presumed to be responsible for loss of Mb expression in *D. hunteri,* is located at -651 bp relative to the *C. rastrospinosus* sequence. Interestingly, the 15 bp INDEL was found in 14 out of the 16 icefish species sequenced (Figure 2). Of the 14 species possessing the duplicated TATA box, 8 species express Mb in ventricle. The only two icefish species that do not contain the duplicated TATA box are *C. rastrospinosus* and *C. hamatus*, both of which express Mb. *C. gunnari* (Mb-) contains an additional 5 bp 'aatac' in the middle of the 15 bp INDEL, that does not interrupt the 'TATAAAA' sequence.

Figure 2. The Duplicated TATA Box is Present in the Myoglobin Sequence of Most Icefish Species. Species are labeled Mb+ or Mb- indicating if myoglobin is expressed. Numbers above the sequence correspond to the position in the sequence in reference to the putative transcription start site for the reference *C. rastrospinosus* sequence.

Conservation of Putative Promoter Elements

Putative promoter elements identified by Small *et al.* (2003) are marked in the alignment in Supplemental Figure 1 and summarized in Table 2. The TATA box (TATAAAA) is conserved in all 16 species at -25. The TATAAAA element in mammalian Mb interacts synergistically with YTAAAATATAR elements. The YTAAAATATAR element in human Mb binds transcription factors MEF-2 (myocytes enhancing factor-2) which is integral to myocyte development and the unknown factor ATF35 (A/T-binding factor 35 kDa) (Grayson *et al.*, 1995). Small *et al.* (2003) identified two possible YTAAAATATAR sites (-457 and -347). The first site is located 457 bp upstream of the *C. rastrospinosus* transcription start site. At this site, none of the sequences match the consensus sequence perfectly. *C. hamatus* (Mb+) and *C. rastrospinosus* (Mb+) have TTAAAATAA while all the other species have TTAAATAA. At

347 bp upstream, most of the species encode TTAAAATAA at while *C. gunnari* (Mb-), *C. esox* (Mb-), and *C. dewitti* (Mb+) are missing the sequence. Potential E-boxes, CACGTG at -575 and CAGCTG at -663, are 100% conserved in all 16 species, as are the two potential binding sites for a zinc-finger transcription factor GATA-4, which is involved in embryogenesis and function and differentiation of myocardial cells (-261 and -478).

Other landmarks previously identified within the *C. rastrospinosus* sequence are also conserved, including the transcription start site, Kozak consensus sequence, translation start codon, and intron/exon boundaries.

Table 2. Myoglobin Putative Promoter Elements. Sequences from putative promoter elements originally identified in *C. rastrospinosus* by Small *et al.* (1998). The distance from the promoter is relative to the *C. rastrospinosus* sequence. Myoglobin mRNA and protein expression is indicated with a +, -, or ? for myoglobin expressing, not expression, or unknown expression respectively.

	Myoglobin Expression		Putative Promoter and Regulatory Elements							
	mRNA	Protein	CANNTG[1] (-663)	Duplicated TATA Box[2] (-651)	CANNTG[1] (-575)	WGATAMS[3] (-478)	YTAAAATATAR[4] (-457)	YTAAAATATAR[4] (-347)	WGATAMS[3] (-261)	TATA BOX[2] (-25)
C.rastrospinosus	+	+	cagctg	-------	cacgtg	agataac	**Ttaaaaataa**	ttaaaataa	agataag	tataaaa
P.maculatus	?	-	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
P.macropterus	+	-	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
D.hunteri	-	-	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.antarcticus	+	+	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.gunnari	+	-	cagctg	**tataaaa**	cacgtg	agataac	Tttaaataa	---------	agataag	tataaaa
C.esox	?	-	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	---------	agataag	tataaaa
C.dewitti	+	+	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	---------	agataag	tataaaa
P.georgianus	+	+	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.aceratus	-	-	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.wilsoni	+	+	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.rhinoceratus	+	+	cagctg	**tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.hamatus	+	+	cagctg	-------	cacgtg	agataac	**Ttaaaaataa**	ttaaaataa	agataag	tataaaa
C.myersi	+	+	cagctg	**Tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
N.ionah	+	+	cagctg	**Tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa
C.atkinsoni	+	+	cagctg	**Tataaaa**	cacgtg	agataac	Ttaaaataa	ttaaaataa	agataag	tataaaa

[1] Putative E-box, binding sites for basic helix-loop-helix transcription factors
[2] Putative binding sites for TATA-Binding Protein
[3] Putative binding sites for transcription factor GATA-4
[4] Putative binding sites for transcription factors MEF-2 (Myocyte Enhancing Factor 2) and ATF35 (A/T-binding factor 35 kDa)

Discussion

The Mb gene sequence of *D. hunteri* contains a 15 bp INDEL in the promoter region (Figure 1). This 15 bp INDEL is identical to an INDEL containing a duplication of the TATA box (TATAAAA) that was found in *C. aceratus* (Small *et al.*, 1998; Small *et al.*, 2003). Small *et al.* (2003) demonstrated that a 41 bp oligonucleotide containing this duplicated TATA box was capable of binding HeLa transcription factor IID (TFIID) or binding factors present in extracts from the nuclei of *C. aceratus*. Using a transient transcription assay, they demonstrated that the promoter region of *C. rastrospinosus* is sufficient to stimulate transcription of a reporter gene, while the promoter region of *C. aceratus* is not, even though these promoters are 99% identical. They proposed that the duplicated TATA box may result in loss of Mb transcription by binding TFIID, which could inhibit ability of basic helix-loop-helix (bHLH) transcription factors to bind at the putative E-box 10 bp upstream. Presence of the same 15bp INDEL in *D. hunteri* suggested this mechanism might also be responsible for lack of Mb mRNA expression in *D. hunteri*. Shared sequence and position of the 15 bp INDEL led to the hypothesis that these species may be sister species, although they are not grouped together in the most current phylogeny of the icefish family.

Sequences of the Mb promoter from the remaining 15 icefish species, however, clearly show that presence of the duplicated TATA box alone cannot determine whether the Mb gene will be transcribed. The 15 bp INDEL originally identified in *C. aceratus* is present in 14 of the 16 species sequenced (Figure 2). Of the 14 species containing the duplicated TATA box, 8 species are icefishes known to express Mb. These new data led us to two possible conclusions about loss of Mb expression in *C. aceratus* and *D. hunteri*.

Either loss of Mb expression in *C. aceratus* and *D. hunteri* is independent of TATA box duplication or the duplicated TATA box requires the appropriate context to inhibit transcription.

To investigate the possibility that the loss of Mb expression in *C. aceratus* and *D. hunteri* may be due to a mechanism independent of the duplicated TATA box, Mb sequences of these species were compared to the sequences of species known to express Mb. Promoter elements and the transcription start sites identified by Small *et al.* (1998) do not differ between species that do express Mb and those that do not. The E-box, an element present in human Mb that binds bHLH transcription factors, is conserved in *D. hunteri.* However, *D. hunteri* lacks eight nucleotides immediately upstream of the putative E-box, which may impede transcription factors from binding to the E-box, resulting in loss of transcription of the Mb gene. However, the importance of the E-box in regulating transcription of human Mb is uncertain. Mutations to the two E-boxes in human Mb only had a minimal effect on transcription of the gene in skeletal and cardiac muscle (Bassel-Duby *et al.*, 1992; Bassel-Duby *et al.*, 1993). *C. aceratus* and *D. hunteri* sequences also were checked for an early RNA cleavage site or an early translation stop codon, but neither was identified. No definitive reason for lack of transcription of Mb in *C. aceratus* and *D. hunteri* can be identified based on sequence data.

An unidentified promoter element or one or more distant regulatory element(s) could be responsible for lack of transcription of the Mb gene in *D. hunteri* and *C. aceratus*. Enhancers, regions of DNA that activate transcription of a gene, can be located throughout a genome. While enhancers can be located close to or within a gene, some enhancers are located many kb upstream or downstream of a gene (Kleinjan and van

Heyningen, 2005). Chromatin looping brings distant regions of DNA into close proximity allowing distant regulatory elements to regulate transcription of a gene (Sexton *et al.*, 2009). Changes in a remote area of the *C. aceratus* and *D. hunteri* genomes potentially could inhibit transcription of the Mb gene.

Despite the fact that promoters of *C. aceratus* and *C. rastrospinosus* are 99% conserved, they differ significantly in their ability to stimulate transcription. Transcription of the *C. aceratus* myoglobin reporter gene construct is approximately 7x lower than the *C. rastrospinosus* reporter gene construct. These data indicate that a region of the *C. aceratus* sequence is sufficiently different from *C. rastrospinosus* to inhibit transcription of the reporter gene. When compared to the Mb gene sequence of *C. rastrospinosus*, that of *C. aceratus* also contains 4 point mutations, 2 single nucleotide deletions and, the 'ATCT' repeat is longer by 9 units (Small *et al.*, 1998). Because our current information indicates that the duplicated TATA box may not be responsible for inhibiting transcription, loss of Mb may be due to a point mutation located in a yet unidentified promoter element.

A second possibility is that the duplicated TATA box inhibits transcription of Mb, but only when the promoter is able to interact with other regions of the genome. Reporter gene constructs allow us to study the transcriptional activity of a promoter with all of the necessary binding factors present. However, this method simplifies transcription and does not take into consideration how distant regulatory elements can regulate the activity of a promoter. Technologies such as the chromosome conformation capture (3C) technique have demonstrated how chromatin looping can bring genes into close

proximity of each other, allowing distant regions of the DNA to control transcription of unrelated genes (Sexton *et al.*, 2009). There are two possible mechanisms by which the duplicated TATA box may cause lack of Mb transcription in *C. aceratus* and *D. hunteri*. Genomes of *C. aceratus* and *D. hunteri* could contain a distant regulatory element that is absent from the genomes of other icefish species, enabling TFIID to bind to the duplicated TATA box, resulting in loss of transcription. An alternative explanation is that the genomes of *C. aceratus* and *D. hunteri* may lack a distant regulatory element that, in other icefish species, blocks binding of TFIID to the duplicated TATA box and maintains transcription of Mb.

Presence of the identical 15 bp INDEL in both *C. aceratus* and *D. hunteri* led to an initial hypothesis that these species may be sister-species in the channichthyid family. Myoglobin gene sequences from other channichthyid species reveal that this INDEL is not unique to *C. aceratus* and *D. hunteri*. Based on these observations, we conclude that placement of the species in the current phylogeny (Near *et al.*, 2003) is accurate. At this time, we are unable definitively to describe the mechanism(s) responsible for loss of Mb transcription in *C. aceratus* and *D. hunteri.*

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Annual Progress Report
1 September 2009 – 31 August 2010

Activities

During the past year, we:

1. Completed analysis of data comparing nitric oxide synthase activities and expression levels of key genes in pathways of hypoxia sensitivity and angiogenesis between red- and white-blooded notothenioids. (See Beers *et al., Comparative Physiology and Biochemistry,* Part A. **156**:422-429, 2010).
2. Completed analysis of data from organismal thermal tolerance experiments conducted during our 2009 Field Season.
3. Completed experiments designed to evaluate possible upregulation of hypoxia-inducible genes in tissues of thermally exposed animals from #1 above.

   (Results from #s 2,3 above form the basis for a manuscript currently in draft form that will be submitted for journal publication by August 2010 – see Beers and Sidell, 2011 in Publications section of the report.)

4. Completed analyses of data from experiments designed to evaluate the effect of chemically induced anemia in the red-blooded notothenioid, *Notothenia coriiceps*, upon expression of key genes in hypoxia-sensitive and angiogenic pathways. (Results are presented in Borley *et al.*, 2010 – in press in *Journal of Expermental Biology*).
5. Completed sequencing of the myoglobin gene from genomic DNA of all sixteen known channichthyid icefish species. Findings are currently in draft manuscript form and will be submitted for journal publication later this summer (see Borley and Sidell, 2011 – in Publications section of the report).
6. Designed the system and specified components for the experimental system that will be used during the 2011 field season for examining the effect of hyperbaric oxygen concentration upon thermal tolerance of Antarctic fishes.

In addition:

7. K. A. Borley successfully defended her Ph.D. thesis Molecular Biology in April 2010 and her doctoral degree was conferred in June 2010. This thesis research was supported by the current award.
8. J.M. Beers is scheduled to defend her Ph.D. thesis in Marine Biology on 13 July 2010. Assuming successful defense of thesis, her doctoral degree will be conferred in August 2010.