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Physiological and Evolutionary Implications of the Pattern of Expression of Oxygen-Binding Hemoproteins in Antarctic Notothenioid Fishes

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PHYSIOLOGICAL AND EVOLUTIONARY IMPLICATIONS OF THE PATTERN OF EXPRESSION OF OXYGEN-BINDING HEMOPROTEINS IN ANTARCTIC NOTOTHENIOID FISHES

By

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A DISSERTATION

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By Kimberly Borley

Thesis Advisor: Dr. Bruce Sidell

An Abstract of the Dissertation Presented
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Antarctic icefish do not express hemoglobin (Hb). Icefishes possess cardiovascular modifications including increased densities of blood vessels, larger ventricles and increased blood volume compared to red-blooded relatives. In addition to delivering oxygen to tissues, Hb degrades nitric oxide (NO), a small signaling molecule. To investigate the mechanism driving development of icefish cardiovascular characteristics, I present and test the hypothesis that loss of Hb results in increased steady-state levels of NO, triggering downstream signaling pathways such as angiogenesis. I measured NO breakdown products, as a proxy for NO, and found that icefish have higher steady-state levels of NO metabolites in their plasma compared to red-blooded notothenioids. Expression of angiogenesis genes (HIF-1α, PHD2, and VEGF) did not differ significantly between red- and white-blooded notothenioids indicating that, while NO levels are higher in adult icefish, angiogenesis is not active. To

investigate whether loss of Hb directly can increase NO and stimulate angiogenesis, hematocrit of red-blooded *N. coriiceps* was severely reduced using the hemolytic agent, phenylhydrazine HCl. Anemic fish exhibited a significantly higher concentration of NO metabolites in the plasma than did control fish, indicating an increase in NO. Expression of HIF-1α, PHD2, and VEGF mRNA 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* NO signaling.

In addition to lacking Hb, several species of icefish have lost ability to express myoglobin (Mb), an oxygen-binding protein expressed in the ventricle of most notothenioids. Previous studies have indicated Mb expression was lost on four separate occasions during evolution of the icefish family. Sequencing of the Mb gene from *D. hunteri* identified a duplicated TATA box previously proposed as the mechanism responsible for loss of Mb in *C. aceratus.* Sequencing of Mb from all 16 species of icefish identified the duplicated TATA box is present in all but two icefish species. The presence of the duplicated TATA box in Mb-expressing icefish suggests that the loss of Mb in *C. aceratus* and *D. hunteri* may occur by a mechanism independent of the duplicated TATA box.

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Chapter 1

INTRODUCTION

August Krogh stated that "for such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (1929). Although it could easily be argued that Antarctic notothenioids are anything but convenient to study, these animals have proved to be remarkable models for addressing evolution-based questions. This suborder of fish originated from a benthic ancestor living in temperate waters of the Gondwana continental shelf in the region destined to become Antarctica. Arising from this benthic temperate ancestor, the modern notothenioid suborder contains eight families, five of which are endemic to the Southern Ocean. The Antarctic notothenioids dominate the fish fauna of the continental shelf where the water hovers around 0ºC year round (Eastman, 1993). While some species are confined to benthic habitats, others have evolved a pelagic lifestyle on multiple occasions within the suborder (Montgomery and Clements, 2000). Even more remarkable, one family of notothenioids, the channichthyids, has lost the ability to express hemoglobin (Hb), an oxygen binding protein that previously was presumed to be absolutely necessary for adult vertebrates (Ruud, 1965). In this dissertation, I will examine some of the evolutionary questions surrounding the Antarctic notothenioids. In particular, I will focus on the Channichthyid family. In chapters 2 and 3, I will focus on the question of how icefish were able to survive the loss of hemoglobin. In chapter 4, I will switch gears to examine the phylogenetic relationships of the icefish species using the myoglobin gene. In order to ask these evolution-based questions, I must first examine the challenges

and obstacles the Antarctic notothenioids have faced that have brought about the modern fish fauna of the Southern Ocean.

The Southern Ocean

The Southern Ocean was originally recognized by the International Hydrographic Organization in the second edition of the *Limits of Oceans and Seas* in 1937, but it was retracted in the third edition due to controversy surrounding the definition of an ocean (IHO, 1953). A working 4th draft, currently lists the Southern Ocean as extending from the continent to 60ºS. Mariners and scientists have long recognized the unique nature of the Southern Ocean, also known historically as the Antarctic Ocean.

> "… if a body of water may be known by its characteristics, there are few who have been to the Antarctic who would quibble at giving a specific name to the ferocious sea which is unique on our earth…" ~ Russell Owen, *The Antarctic Ocean* (1941)

Even those who recognize the existence of the Southern Ocean have a hard time agreeing on the northern boundary. While the IHO prefers 60ºS, the northern boundary of the Southern Ocean has also been described as either the subtropical convergence or the Antarctic Polar Front (APF; also known as the Antarctic convergence or the Polar Frontal Zone) (Gon and Heemstra, 1990). For the purposes of this paper, when referring to the Southern Ocean, I am referring to the waters south of the APF. The APF, located between 50 and 60ºS, is the point where Antarctic surface water sinks below the warmer subantarctic surface water resulting in a significant (3-4ºC) change in the temperature of the surface water over a short distance. This change in surface water temperature forms a zoogeographic barrier for epipelagic animals (Eastman, 1993).

The APF is located within the Antarctic circumpolar current (ACC). Running clockwise around the continent, the ACC is the most voluminous current in the world. The ACC acts as a thermal barrier insulating Antarctica from the warmer waters of the Indian, Atlantic and Pacific Oceans (Eastman, 1993). The water south of the APF is thermally stable varying little with depth and season. The temperature ranges from 4ºC at the APF in the summer to approximately -1.86º year round in McMurdo Sound (Somero and Devries, 1967; Clarke and Johnston, 1996). The Southern Ocean is also characterized by high oxygen solubility. Oxygen solubility in McMurdo Sound is 6-8 mg/L and varies little with depth (Littlepage, 1965). While nutrients are readily available, primary productivity is limited by the photoperiod which varies drastically with season (Eastman, 1993).

The modern fish fauna of the Antarctic continental shelf is dominated by the suborder Notothenioidei. This suborder alone accounts for approximately 35% of the species of fish and approximately 90% of the fish biomass in the Southern ocean. While notothenioids are known to have evolved from a benthic fish lacking a swimbladder, modern notothenioids are found living throughout the entire water column (Eastman, 1993). One extreme example is *Pagothenia borchgrevinki*. This species has adapted to live at the undersurface of the sea ice (Eastman and DeVries, 1985). Habitat use by modern notothenioids has evolved in response to changes in their environment as Antarctica detached from South America and Australia resulting in the formation of the Southern Ocean.

Geological History of the Southern Ocean

In the early Tertiary period, Antarctica was connected to South America and Australia as a part of Gondwana (Eastman, 1993). Current data suggests the Drake Passage formed between South America and Antarctica ~41 Ma (Scher and Martin, 2006). Complete separation of Antarctica did not occur until ~35.5 Ma when then Tasmanian Gateway formed between Antarctica and Australia (Stickley *et al.*, 2004). As these passageways enlarged and deepened, the Antarctic circumpolar current formed. The timing of the formation of the ACC is a matter of great interest since it would provide information on the cooling of the Southern Ocean and glaciation of the continent. However, the exact timing of formation of the ACC is still a matter of controversy (Barker *et al.*, 2007).

Fossil records from the middle to late Eocene (~40 Ma) indicate the fish fauna was similar to fauna currently inhabiting the continental shelves of New Zealand, Tasmania and southern South America. Approximately 40 Ma, at least 29 taxa inhabited the continental shelf in the peninsula region. From the fossils, 21 chondrichthyans and 8 actinopterygians have been identified. Approximately 25 Ma shelf fauna was subjected to harsh conditions as temperatures dropped and the ice sheet expanded, resulting in the loss of much of the coastal and intercoastal habitat. These conditions resulted in a massive species extinction that allowed for the subsequent radiation of fish fauna that had found areas of refugia (Eastman, 2005).

The Notothenioid Radiation: Survival of the Fittest and Luckiest?

In the struggle for survival, the fittest win out at the expense of their rivals because they succeed in adapting themselves best to their environment – Charles Darwin

One reason notothenioids have been able to survive and thrive in the Southern Ocean is because they adapted to the cooling environment. Examples of cold-adapted proteins in the Antarctic notothenioids include, α- and ß-tubulin and lactate dehydrogenase A4 (Fields and Somero, 1998; Detrich *et al.*, 2000). Of course maintaining protein function at cold temperatures would be useless if the animals often came in contact with temperatures below the freezing point of their plasma. The freezing point of a typical temperate marine teleost plasma is around -0.6ºC (DeVries, 1988). The osmolarity of plasma from Antarctic notothenioids in McMurdo Sound is higher than temperate marine teleosts. Based on the osmolarity, the freezing point of Antarctic notothenioid plasma is predicted to be approximately -1ºC. However, the freezing point of plasma from notothenioids captured in McMurdo Sound is -2.2 ºC. The lower than predicted freezing point is due to the presence of the antifreeze glycoproteins (AFGPs). Given that Antarctic notothenioids can encounter temperatures as low as -1.86ºC, the evolution of the AFGP was pivotal in the survival of this group of animals.

Antifreeze glycoproteins bind to the surface of developing ice crystals inhibiting the growth of the crystal depressing the freezing point of biological fluids (DeVries, 1971). The AFGP gene evolved from a pancreatic gene encoding a pancreatic trypsinogen-like protease (Chen *et al.*, 1997a; Cheng *et al.*, 2006). Arctic cod express AFGPs that are similar to the notothenioid AFGP in structure and function, but arose from an unrelated gene (Chen *et al.*, 1997b). The independent evolution of two unique

proteins from different genes demonstrates a remarkable case of convergent evolution that has allowed both Antarctic and Arctic fishes access to habitats where the water temperature would otherwise be lethal.

While it is clear that the notothenioids have adapted to their environment, their survival definitely appears to have been partially due to chance or luck. Eastman (2000) points out "there was also probably an element of chance in the emergence of the notothenioid stock- being in the right place at the right time." The ancestral notothenioids evolved prior to separation of Antarctica from South America and Australia. In support of this hypothesis, the Pseudaphritidae and Eleginopidae families are basal notothenioid families restricted to Australia and South America, respectively. The notothenioids that inhabited the Antarctic continental shelf after the geographic isolation of the continent were in 'the right place at the right time' during the expansion of the ice sheet ~25 Ma. By surviving in areas of refugia, and developing AFGPs, the Antarctic notothenioids were poised to dominate once the ice sheet retracted, exposing the continental shelf (Eastman, 2000).

The Icefish: The "Bloodless" Fish

Within the notothenioid suborder, the Antarctic icefish (Family: Channichthyidae) are arguably the most remarkable group. These fish are the only adult vertebrates known not to express the oxygen-binding blood protein, hemoglobin (Hb) (Ruud, 1954). Expression of hemoglobin was lost due to a gene mutation event that resulted in loss of the entire ß-globin gene and part of the α-globin gene (Cocca *et al.*, 1995; Near *et al.*, 2006). The 3' end of the α-globin gene is the only remnant of the

globin genes present in the genome of icefishes except for *Neopagetopsis ionah,* which maintains a nonfunctional αβ-globin complex (Near *et al.*, 2006). The loss of Hb was originally thought to be beneficial. The hypothesis was that the loss of Hb and red blood cells resulted in a decrease in blood viscosity resulting in an overall energetic advantage. Since viscosity is inversely related to temperature, a less viscous blood would cost less energy to pump through the cardiovascular system of an Antarctic fish. In support of this hypothesis, many scientists have found that the hematocrit of animals is inversely correlated with the temperature of their environment, and fish in temperate climates have lower hematocrits in the winter than in the summer (Powers, 1974). However, this hypothesis does not take into account the fact that icefish have a larger blood volume than their red-blooded relatives. For the same sized animal, icefish have approximately a 2-3X larger blood volume than closely related red-blooded notothenioids (Hemmingsen and Douglas, 1970). Although the blood is less viscous, energetic cost to maintain cardiac output of these animals is approximately twice that required in red-blooded Antarctic notothenioids. The energy used to maintain this cardiac output far exceeds the energy that is saved by having less viscous blood. These data indicate that, instead of being advantageous, loss of Hb is the result of a sublethal disadvantageous mutation (Wells, 1990; Sidell and O'Brien, 2006). Sublethal disadvantageous mutations are typically removed via natural selection, but this has not occurred in the icefish. Today the family has a circumpolar distribution with 15 species of icefish endemic to the Southern Ocean and one species located along the southern tip of South America (Gon and Heemstra, 1990).

If the loss of Hb has led to compensatory responses that are energetically disadvantageous, how did these fish survive? Natural selection dictates that disadvantageous traits will be removed from the population. However, natural selection requires competition (Sidell and O'Brien, 2006). The mutation resulting in the loss of Hb occurred 8.5 Ma, well after the crash in species diversity that relaxed competition in the Southern Ocean (Near, 2004). In addition to evolving in a period of relaxed competition, the loss of Hb also occurred after cooling of the Southern Ocean. In the absence of Hb, oxygen is carried in the blood only in physical solution in the plasma. This results in an oxygen-carrying capacity that is less than 10% that of the red-blooded Antarctic notothenioids (Ruud, 1954). The higher oxygen solubility due to the cold temperature was likely invaluable to survival of the ancestral icefish. But, additional differences in physiologies between modern icefish and red-blooded Antarctic notothenioids provide insight into how the animals have compensated for the loss of Hb.

Icefishes have evolved unique cardiovascular systems that assists in the delivery of oxygen. As mentioned previously, icefishes have a larger blood volume than red-blooded species. Using the capillary bed of the retina as a 2-D model, Wujcik *et al.* (2007) recently demonstrated that the blood vessels of icefish are larger and more dense than their red-blooded relatives. The larger bore blood vessels and greater vascularization help to contain the large blood volume and decrease the diffusion distance of oxygen from the blood vessels to each individual cell. In addition, icefish have larger ventricles that are much spongier than red-blooded notothenioids (O'Brien *et al.*, 2000). The spongy nature of the tissue enhances diffusion of oxygen into the cardiac cells from blood in the lumen of the heart. Ultrastructure of the ventricle is also altered. Cell

volume of the ventricle occupied by mitochondria is greater in the icefish than in red-blooded fish (O'Brien and Sidell, 2000). While at first glance it might seem that more mitochondria would result in increased ATP production, the activity of cytochrome c oxidase in the electron-transport chain is the same per mass of tissue in red- and white-blooded notothenioids. Instead it appears that the increased mitochondrial density is present to assist in the diffusion of oxygen through the cell via a "lipid highway" formed by the mitochondrial membranes.

How Did the Icefish Cardiovascular System Evolve?

While the cardiovascular characteristics present in these fish have been described in detail, we do not understand the mechanisms that resulted in the cardiovascular system present in icefish today. This question is examined in more detail in chapters 2 and 3. These chapters represent two submitted publications that are the result of a collaborative project with Jody Beers. In chapter two, Jody presents the hypothesis that the loss of Hb resulted in an increase in nitric oxide (NO) which in turn stimulated downstream pathways such as angiogenesis. To test this hypothesis, I measured the concentration of NO metabolites in the plasma of red- and white-blooded adult fish to determine if NO is, in fact, higher in fish that do not express Hb. Next I looked at the expression of genes involved in angiogenesis in the retinae of red- and white-blooded notothenioids and Jody measured nitric oxide synthase activity in different tissues of red- and white-blooded notothenioids. In chapter 3, I present the results of experiments in which we manipulated red-blooded notothenioids to make an Antarctic icefish 'model' in which to further test

our original hypothesis. In these fish, we greatly reduced the amount of Hb in circulation to see if removing Hb could directly influence NO levels and thus stimulate angiogenesis.

Who is *C. aceratus's* Sister?

In the study of the physiology and biochemistry of the icefish, it is important that we place the information we have learned within the context of evolution. In order to understand how these animals have adapted to the loss of Hb, we must know how the animals we study are related. As with many other organisms, the icefish phylogeny has been revised as different phylogenetic techniques have become available. The original phylogenies were based on morphological indices (Iwami, 1985; Chen *et al.*, 1998; Balushkin, 2000; Voskoboinikova, 2000). Current phylogenies were developed from a mix of morphological and molecular data (Near *et al.*, 2003). Ongoing research is aiming to add nuclear gene sequencing to the data already known for mitochondrial genes. In chapter 4, I describe sequencing the myoglobin (Mb) gene from all species of icefish and a few species of red-blooded notothenioids to investigate the evolution of the icefish family.

Myoglobin is an intracellular oxygen-binding protein typically expressed in the oxidative muscle of teleosts. Antarctic notothenioids lack the ability to express myoglobin in the pectoral muscle. Myoglobin is typically expressed in the ventricle of Antarctic notothenioids (Sidell *et al.*, 1997). However, a few species of Antarctic icefish have completely lost the ability to express Mb protein. Within the icefish family, Mb expression has been lost at least four times resulting in the lack of Mb expression in 6 out of 16 species (Vayda *et al.*, 1997; Small *et al.*, 1998; Moylan and Sidell, 2000; Small *et*

al., 2003; Grove *et al.*, 2004; Sidell and O'Brien, 2006). In chapter 4, I present the genomic DNA sequence for Mb from *Dacodraco hunteri,* one of the icefish species known to lack Mb expression. The *D. hunter* Mb promoter contains the same 15 bp insertion/deletion (INDEL) previously shown to responsible for the loss of Mb expression in *Chaenocephalus aceratus*. This was of particular interest since these species are not sister taxa in the current icefish phylogenies. I sequenced the Mb gene from all 16 species of icefish and 5 red-blooded Antarctic notothenioids with the hypothesis that a Mb-based phylogeny of the family would conclude that *C. aceratus* and *D. hunteri* are sister-species. Chapter 4 presents the Mb sequence data obtained from the 21 species sequenced.

Chapter 2

RELATIONSHIP AMONG CIRCULATING HEMOGLOBIN CONCENTRATION, NITRIC OXIDE SYNTHASE ACTIVITIES AND ANGIOGENIC POISE IN RED- AND WHITE-BLOODED ANTARCTIC NOTOTHENIOID FISHES [1]

Abstract

Nitric oxide (NO)-mediated angiogenesis may play a role in establishing dense retinal vasculatures of Antarctic hemoglobinless icefishes (suborder: Notothenioidei). We hypothesized that loss of hemoglobin (Hb) leads to elevation in [NO] due to decreased degradation of the compound when the NO-scavenger Hb is absent, thereby inducing vascular growth. We found that plasma levels of NO metabolites, nitrite plus nitrate (NO_x), generally are greater in icefishes than in red-blooded notothenioids [*e.g. C. aceratus* (Hb-), 22.7±2.9 μM; *N. coriiceps* (Hb+), 14.7±1.7 μM], suggesting a higher NO load in hemoglobinless animals. High NO levels do not appear to be a result of greater NO synthesis; we consistently measured lower activities of the enzyme catalyzing NO production, nitric oxide synthase, in tissues of icefishes than in Hb-expressing notothenioids [*e.g.* 96±10 and 216±39 pmol(min • g wet wt.)$^{-1}$ in brain tissue of *C. aceratus* (Hb-) and *G. gibberifrons* (Hb+), respectively]. Levels of mRNA for hypoxia-induced (HIF-1α and PHD2) and angiogenic genes (VEGF) were similar in red- and

[1] This chapter contains the text of the following article:
Beers, Jody M., Kimberly A. Borley and Bruce D. Sidell (201X). "Relationship among circulating hemoglobin concentration, nitric oxide synthase activities and angiogenic poise in red- and white-blooded Antarctic notothenioid fishes." Accepted by Comparative Biochemistry and Physiology
Jody took the lead drafting the article. She is also responsible for collecting the hemoglobin concentration data and measuring NOS activity.

white-blooded species, indicating that vascular maintenance in adult animals does not require differences in angiogenic tone. This does not preclude a cause-and-effect relationship between absence of Hb and NO-mediated angiogenesis during earlier ontogenetic stages of icefishes.

Introduction

Antarctic icefishes (Family Channichthyidae) are the only known vertebrate animals that completely lack the oxygen transport protein hemoglobin (Hb) in their blood as adults (Ruud, 1954). Loss of this important respiratory pigment has resulted in a characteristic pale white appearance of channichthyid blood, hence common names of 'white-blooded fishes' and 'icefishes.' This extraordinary group of animals is one of eight families belonging to the perciform suborder Notothenioidei. Notothenioids comprise approximately 35% of fish species and 90% of fish biomass south of the Antarctic Polar Front, therefore dominating waters surrounding Antarctica in what is the coldest, most thermally stable marine habitat on the planet (Eastman, 1993).

Absence of Hb has caused icefishes to transport oxygen strictly in physical solution of the blood, resulting in an oxygen-carrying capacity that is less than 10% of that seen in red-blooded notothenioids (Holeton, 1970). On the surface, one might think that this deficiency presents an insurmountable limitation for delivering adequate oxygen to tissues. However, these animals have compensated for the low oxygen-carrying capacity of their blood by developing dramatic cardiovascular modifications. Icefishes have larger diameter capillaries, 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). These features contribute to weight-specific cardiac outputs in white-blooded fishes that are many times greater than those of red-blooded species (Holeton, 1970; Hemmingsen and Douglas, 1972). Decreased systemic flow resistance creates a high throughput conduit that allows channichthyids to circulate large blood volumes at relatively high flow rates while requiring only modest vascular pressures. Ultimately, the combination of high throughput circulatory systems, low absolute metabolic rates and exceptionally well-oxygenated waters of the Southern Ocean allows this group of animals to oxygenate their tissues sufficiently and survive without Hb (Hemmingsen, 1991)

We recently have documented yet another remarkable circulatory feature in icefishes that further highlights differences in the cardiovascular traits between white- and red-blooded species. Blood vasculature supplying oxygen to retinal tissue in the eyes of icefishes is unusually dense compared to that of red-blooded notothenioids and, most notably, these observed densities are inversely correlated to the amount of Hb in circulation (Wujcik *et al.*, 2007). This indicates that icefishes may compensate for oxygen deficiency by increasing vascular proliferation and, thus, oxygen delivery to the highly aerobic retina. The benefit of an enhanced vasculature to aid in oxygen transport in hemoglobinless fishes is apparent, but the underlying mechanisms driving development of these elaborate vascular patterns are presently unknown.

Loss of Hb in icefishes has another important, but often overlooked, implication; Hb is the primary reactant in degradation of nitric oxide (NO) to nitrate in vertebrate animals (Gow *et al.*, 1999; Gardner, 2005). NO is a small, highly diffusible, gaseous signaling molecule that mediates a broad array of physiological functions (Kerwin *et al.*,

1995). NO and citrulline are oxidation products of L-arginine and oxygen in a reaction enzymatically catalyzed by nitric oxide synthase (NOS). There are three distinct forms of NOS: neuronal NOS (nNOS, NOS-I), inducible NOS (iNOS, NOS-II), and endothelial NOS (eNOS, NOS-III) (Andrew and Mayer, 1999; Alderton *et al.*, 2001). eNOS and nNOS are constitutively expressed in mammals, whereas iNOS is induced by factors such as cytokines and hypoxia (Kerwin *et al.*, 1995). Each of these isoforms has been reported in teleosts, although presence of eNOS has not been definitively resolved due to lack of molecular evidence to corroborate apparent detection of the isoform by heterologous antibodies (Fritsche *et al.*, 2000; Oyan *et al.*, 2000; Ebbesson *et al.*, 2005; Tota *et al.*, 2005; Hyndman *et al.*, 2006).

Nitric oxide is a molecular signal in a number of physiological processes, including neurotransmission, cardiovascular regulation, immunological reactions, and inflammatory responses (Kerwin *et al.*, 1995; Moncada, 1997). Although perhaps best known as a vasodilator (Palmer *et al.*, 1987), NO also is implicated in angiogenesis, the growth and proliferation of new blood vessels from preexisting vasculature (Conway *et al.*, 2001). While there are many factors involved in the angiogenic pathway, the key stimulator of blood vessel proliferation is vascular endothelial growth factor (VEGF). VEGF contains a hypoxia-response element (HRE) within its promoter region that enables hypoxia-inducible factor 1 (HIF-1) to stimulate transcription via its alpha subunit (HIF-1α) in response to either hypoxic insult or NO (Kimura *et al.*, 2000; Wenger *et al.*, 2005).

HIF-1α combines with HIF-1β to form HIF-1, a transcription factor known to regulate expression of many genes when oxygen levels decline, including VEGF

(Wenger *et al.*, 2005). While HIF-1β is expressed constitutively, the expression of HIF-1α is highly regulated. When oxygen is present, prolyl hydroxylase domain protein 2 (PHD2) hydroxylates proline residues on HIF-1α targeting the protein for proteosomal degradation. PHD2 (also known as EGLN1: egg laying abnormal nine homolog 1 or HPH2: HIF prolyl hydroxylase 2) is inactive in the absence of oxygen, thus blocking degradation of HIF-1α and allowing its accumulation, which stimulates transcription of downstream genes (Berra *et al.*, 2003). As mentioned already, NO induces VEGF transcription through HIF-1α. NO also blocks PHD2 from binding oxygen, inhibiting degradation of HIF-1α and resulting in its accumulation regardless of oxygen availability. These responses combine to produce an NO-mediated induction of angiogenesis (Kimura *et al.*, 2000; Kimura *et al.*, 2001; Metzen *et al.*, 2003).

Antarctic notothenioids have been exploited by scientists for decades now as 'Krogh-type' models with which to study cold-adapted cardiovascular traits. We propose that the naturally-occurring expression pattern of Hb in these animals provides us with an opportunity to pursue yet another intriguing line of research— nitric oxide-mediated angiogenesis. Indeed, we believe that the exceptional vascular patterns seen in the eyes of icefishes may have developed via this mechanism (Sidell and O'Brien, 2006). We hypothesize that loss of Hb in these animals has led to an elevation in steady-state concentration of NO due to a decreased rate of degradation of the compound, thereby inducing vascular proliferation. In this study, we have begun to investigate this possibility by utilizing several approaches: 1) we have exploited the differential expression pattern of Hb among notothenioids to determine if absence of Hb leads to an elevation of the ubiquitous signaling molecule, NO, 2) given that endogenous [NO] is a

result of both rate of breakdown and rate of production, we have evaluated the contribution of synthesis to the overall NO pool by examining the catalytic capacity of NOS, and 3) we have quantified mRNA expression of genes that encode factors involved in NO-mediated angiogenesis to determine if differences observed in circulating NO levels are correlated with angiogenic poise of the tissues.

Materials and Methods

Animal Collection

Six species of Antarctic notothenioid fishes were collected during the austral autumn (April-May) of 2005 and 2007 from waters of the Antarctic Peninsula region. *Chaenocephalus aceratus, Champsocephalus gunnari, Chionodraco rastrospinosus*, *Pseudochaenichthys georgianus*, *Notothenia coriiceps* and *Gobionotothen gibberifrons* were caught primarily by otter trawls deployed from the ARSV *Laurence M. Gould* at water depths of 75-150 m in the vicinity of Astrolabe Needle in Dallmann Bay (64°08′S, 62°40′W). *N. coriiceps, G. gibberifrons* and *Lepidonotothen kempi* also were caught in baited traps set at 200-500 m depth in either Dallmann Bay or 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 the US Antarctic research base, Palmer Station, on Anvers Island, where they were maintained in covered aquaria with flowing seawater at ambient water temperatures of 0±0.5°C. Additionally, a small number of specimens generously were collected during 2008 by H. William Detrich, III and coworkers of Northeastern University, using the same collection methods and sites described above.

Tissue Preparation

Animals were anesthetized in MS-222 (Finquel®) in seawater (1:7500 w/v) for a 10-15 min period prior to blood collection and tissue harvest. Plasma samples were prepared for determination of nitrite and nitrate concentration by 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 (*G. gibberifrons, L. kempi* and *N. coriiceps*) were mixed at a 9:1 blood to sodium citrate ratio, while white-blooded samples (*C. gunnari, C. rastrospinosus* and *C. aceratus*) 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 processing. Animals then were killed by severing the spinal cord, followed by rapid excision of the brain. Tissues collected for measurement of enzyme activity and gene expression were excised quickly, frozen in liquid nitrogen and stored at -80°C.

Hemoglobin and Hematocrit Determination

Blood was collected from *G. gibberifrons, L. kempi* and *N. coriiceps* as described above. 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) and then incubated for approximately 30 min at room temperature before spectrophotometric measurement of absorbance at 540 nm. Total Hb concentration was calculated from a calibration curve with bovine Hb (Sigma) as the standard. For hematocrit (Hct) calculation, whole blood was drawn into heparinized capillary tubes (sodium citrate was not added to these samples), centrifuged for five min in a hematocrit microcentrifuge and then red blood cell content was measured using

digital calipers. All samples were measured in triplicate and mean values were computed for each individual for both Hb and Hct determinations.

Measurement of Nitric Oxide Metabolites in Plasma

Plasma samples were deproteinated using an acetonitrile/chloroform treatment based on a protocol from Romitelli et al. (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 determined indirectly by measuring break-down products, nitrite plus nitrate (NO_x), according to the Griess method as described by Grisham et al. (Grisham *et al.*, 1995). First, nitrate was converted to nitrite 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 500µl) at 37ºC for 30 min. To oxidize any remaining unreacted NADPH, 7.5 units of LDH and 50 µl of 100mM 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. Nitrite concentration was determined by spectrophotometric measurement of diazonium salt formation at 543 nm and absorbance values were compared to a nitrite standard curve to determine total concentration of (NO_x) in the samples. Samples were run in duplicate and corrections for

plasma volumes were performed to account for the addition of sodium citrate to the blood.

Measurement of Nitric Oxide Synthase Activity

NOS activity was determined by measuring the conversion of [^{14}C]-arginine to [^{14}C]-citrulline in four different tissues: brain, heart, retina, and pectoral muscle. 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 vortexed thoroughly. Samples then were transferred to 0.45 µm cellulose acetate Spin-X® columns (Costar®, Corning Life Sciences, Lowell, MA, USA) and centrifuged in a microcentrifuge at 16000 *x g* for 30 s. Finally, 450 µl of filtrate, containing the [^{14}C]-citrulline, was transferred to a vial containing 3 ml of scintillation cocktail fluid 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-omega-nitro-L-arginine methyl ester hydrochloride;1 μM), to the reaction mix. Enzyme activity is reported strictly as L-NAME-inhibitable activity.

Cloning and sequencing of VEGF, HIF-1α, and PHD2

Total RNA was extracted from notothenioid retinae using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA concentration and purity were determined by spectral analysis using a Beckman DU540 spectrophotometer. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer. DNA contamination was removed from RNA samples using Turbo DNA-free™ (Applied Biosystems/Ambion, Foster City, CA, USA). First-strand cDNA was synthesized from total RNA using Superscript® III Reverse Transcriptase (Invitrogen Life Technologies) and oligo(dt) primer. DNAse-treated RNA was added to each reaction with a final concentration of 45 ng/μl.

Degenerate primers were designed from vertebrate protein sequences available on GenBank (Benson *et al.*, 2009) and Ensembl (Hubbard *et al.*, 2006) using CODEHOP(Rose *et al.*, 2003). *P. georgianus*, a white-blooded notothenioid, was used for initial cloning and sequencing of all genes. Degenerate primer sequences for VEGF, PHD2, and HIF-1α are listed in Table 2.1. VEGF and HIF-1α were amplified with degenerate primers and Taq polymerase (Invitrogen Life Technologies) with the following thermocycler conditions: 1 cycle of 2 min at 94ºC, 35 cycles of 30 s at 94ºC, 45 s at 55ºC, 2.5 min at 72ºC, and 1 cycle of 10 min at 72ºC. PHD2 was amplified with Phusion® DNA polymerase (Finnzymes) and the following thermocycler conditions:

1 cycle of 30 s at 98ºC, 30 cycles of 10 s at 98ºC, 45 s at 60ºC, 30 sec at 72ºC, and 1 cycle of 5 min at 72ºC. PCR products were cloned for sequencing using Invitrogen's PCR4 TOPO TA Cloning® Kit. Samples were sequenced at the University of Maine DNA Sequencing Facility (Orono, ME) and submitted to tblastn (http://blast.ncbi.nlm.nih.gov) to confirm gene identity.

Table 2.1. Degenerate primers used to clone genes from the angiogenic pathway of white-blooded Antarctic notothenioid, *P. georgianus*.

Gene	Primers	
VEGF	Forward	5' CCCTGGTGGACATCTTCCARGARTAYCC 3'
	Reverse	5' GAGCACTTGCAGGTCTGAGGRTCYTGNAC 3'
HIF-1α	Forward	5' GGGMGCACTGTCAAYGTMAAATC 3'
	Reverse	5' CTGKGGYTGGGAGTTCTT 3'
PHD2	Forward	5' CCAAGGACATCCGAGGAGAYMARATHAC 3'
	Reverse	5' CGTCTGCGTCGAAGTACCANACNGT 3'

Degenerate Nucleotides: K= G or T; H= A, C, or T; M=A+C; Y= C or T; N= A, C, G, or T; R=A+GK=G/T, H=A/C/T, M=A/C, Y=C/T, N=A/C/G/T, R=A/G

We obtained additional coding sequence for each gene using the Marathon® cDNA Amplification Kit (BD Biosciences) and gene specific RACE (Rapid Amplification of cDNA Ends) primers (Table 2.2) designed with Primer3 (Rozen and Skaletsky, 2000). Two or three nested PCR reactions were completed using either high fidelity Phusion™ DNA polymerase (Finnzymes) (1 cycle of 30 s at 98ºC, 30 cycles of 10 s at 98ºC, 2 min at 72ºC, and 1 cycle of 10 min at 72ºC) or high fidelity Platinum® Taq (Invitrogen Life Technologies) (1 cycle of 2 min at 94ºC, 30 cycles of 30 s at 94ºC, 30 s at 65-75ºC and 1 cycle of 3 min at 68ºC, and 10 min at 68ºC). All sequence data was submitted to GenBank (Table 2.3).

Table 2.2. Primers used in RACE PCR reactions of genes in the angiogenic pathway of white-blooded Antarctic notothenioid, *P. georgianus*.

Gene	Primers	
VEGF	Forward 1 [a]	5' AGAACACACGTACATTCCCTCCTGCGTGGT 3'
	Forward 2 [b]	5' TAGCGACGAAGCATTGGAGTGTGTTCCTAC 3'
	Reverse 1 [a]	5' CTTCCTTCTCTCTGAGCAAGGCGCACAGT 3'
	Reverse 2 [b]	5' TCTTCACTGGAAGATCTGGCTTTTGTCTGC 3'
HIF-1α	Forward 1 [a]	5' GTGTATGACGGCTGCACTGAGGAGACT 3'
	Forward 2 [b]	5' TAACATCGAGGTCCCTCTGGACACCAAGAC 3'
	Reverse 1 [a]	5' GGTTCAACAGGTCCTCCGGGTCATAA 3'
	Reverse 2 [b]	5' TGTCCATTGTGTGGCGGCTGA 3'
	Reverse 3 [c]	5' TGGTGTCCAGAGGGACCTCGAT 3'
PHD2	Forward 1 [a]	5' GAGAAGATCCGCTTTCTGATGAGTCGTGTG3'
	Forward 2 [b]	5' GGTCAGACATTGTAACGGCAAATTGGGAAA 3'
	Forward 3 [c]	5' CGACGGACGCTGTGTCACATGCATATATTA 3'
	Reverse 1 [a]	5' ACCAGAACAGGAGCAGGCGGTCAAATCT 3'
	Reverse 2 [b]	5' GTCAAATCTGGGCTCGATATCGGCAAACT 3'
	Reverse 3 [c]	5' CCGTGTTCCTTGGCAGTCCAATCTTTGTTA 3'

[a]Primary PCR Reaction, [b]Secondary PCR reaction, [c]Tertiary PCR reaction

Table 2.3. GenBank accession numbers for genes in the angiogenic pathway of red- and white-blooded Antarctic notothenioid fishes.

Gene	***N. coriiceps***	***G. gibberifrons***	***C. gunnari***	***C. aceratus***	***P. georgianus***
	(Hb+)	**(Hb+)**	**(Hb-)**	**(Hb-)**	**(Hb-)**
VEGF	GU362084	GU362087	GU362086	GU362085	GU362083
HIF-1α	GU362089	GU362091	GU362088	GU362090	GU362092
PHD2	GU214992	GU214995	GU214993	GU214994	GU214991

Quantification of VEGF, HIF-1α, and PHD2 mRNA Expression by Quantitative Real-Time PCR (QPCR)

Samples from two individuals of each species were sequenced with gene specific primers (Table 2.4) designed from the complete cDNA sequence to identify regions of 100% homology between species. QPCR primers (Table 2.5) were designed in conserved regions using Primer3 (Rozen and Skaletsky, 2000). All genes were amplified using Invitrogen's SYBR® GreenER™ with a Stragene MX4000™ (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.

Table 2.4. Primers used to amplify genes in the angiogenic pathway of red- and white-blooded Antarctic notothenioid fishes.

Gene	Primers	
VEGF	Forward	5' GCGGTTCTTCACCTATCCACAGTAA 3'
	Reverse	5' TCTTCACTGGAAGATCTGGCTTTTG 3'
HIF-1α	Forward	5' GAGCTCGGACCGGAGGAA 3'
	Reverse	5' AGCTGGAAGTCATCGTCCATG 3'
PHD2	Forward	5' CGGGCAGGTCTTTTTAGGAGCAGAG 3'
	Reverse	5' CCTTTCTCTCCTGCACCCGTTAGGT 3'

Table 2.5. Primers used to measure mRNA expression of genes in the angiogenic pathway of red- and white-blooded Antarctic notothenioid fishes using quantitative PCR.

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

Due to the complex nature of retinal tissue and differences in tissue composition, as demonstrated by the increased vascular endothelial tissue in icefish than in 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 simultaneous

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. No reverse-transcriptase controls were included to ensure the samples were not contaminated with gDNA.

Statistical Analyses

Comparisons among species for differences in NOS activity were performed in SigmaStat (Version 3.1; Systat Software, Inc.) using a Kruskal-Wallis one-way ANOVA on Ranks for all tissue types assayed with the exception of brain tissue, in which case a one-way ANOVA with a *post-hoc* Student-Newman-Keuls (SNK) test was performed ($P \leq 0.05$). A one-way ANOVA with a *post-hoc* SNK test also was used to examine differences in Hb and Hct levels among species. A one-way ANOVA followed by a *post-hoc* Tukey's Honestly Significantly Different (HSD) test was used to determine significant differences in gene expression and plasma NO_x ($P \leq 0.05$).

Results

Blood Characteristics of Red-Blooded Fishes

Both Hct and concentrations of Hb varied significantly among three red-blooded notothenioids. Values for both of these blood parameters were lowest in *L. kempi*, intermediate in *G. gibberifrons* and highest in *N. coriiceps* (Table 2.6). Additionally, our results showed a direct correlation between Hb and Hct levels in the three fishes sampled

(linear regression coefficient $R^2 = 0.91$, data not shown). These data indicate that there are differences in oxygen-carrying capacities among red-blooded species.

Table 2.6. Blood characteristics of red-blooded Antarctic notothenioid fishes.

	L. kempi	***G. gibberifrons***	***N. coriiceps***
Hematocrit (%)	20.56 ± 1.24^a	27.19 ± 2.37^b	36.05 ± 1.48^c
Hemoglobin (mg/ml)	74.51 ± 2.63^a	87.34 ± 2.70^b	164.14 ± 8.35^c

Values are means ± s.e.m.; *N*=8 for all species.
Different superscript letters denote significant differences between species ($P \leq 0.05$).

Nitric Oxide Metabolites in Plasma

Nitrite and nitrate (NO_x) are the primary breakdown products of NO. We measured NO_x in blood plasma, which should reflect differences in NO levels among red- and white-blooded Antarctic notothenioids. In general, species lacking Hb had higher plasma [NO_x] than fish expressing Hb (Figure 2.1). NO_x levels were greater in plasma of two of the three hemoglobinless species; *C. aceratus* 22.7 ± 2.9 μM and *C. rastrospinosus* 22.4 ± 2.1 μM. These values were significantly greater than those of *G. gibberifrons*, *N. coriiceps* and *L. kempi* (18.1 ± 2.0, 14.7 ± 1.7 and 14.4 ± 2.0 μM, respectively). Generally higher levels of plasma NO_x in white-blooded notothenioids than in Hb-expressing species suggest that icefishes have greater levels of circulating NO than their red-blooded relatives.

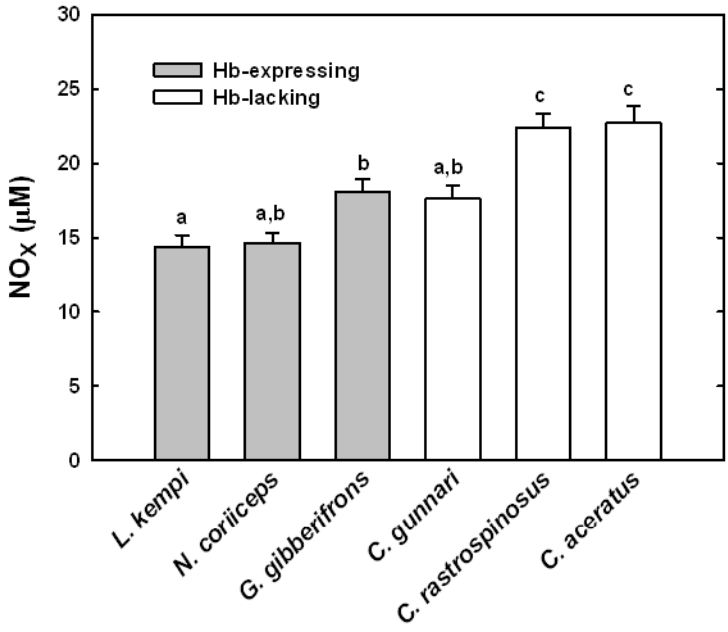

Figure 2.1. Plasma NO metabolites, nitrite plus nitrate (NO_x), of red- and white-blooded Antarctic notothenioid fishes. Concentrations of NO_x were measured using the Griess Assay. Solid color bars indicate that the species expresses hemoglobin (Hb), while clear bars denote lack of Hb. Values are presented as means ± s.e.m.; *N*=8 for *L. kempi* and *C. rastrospinosus*, *N*=7 for *G. gibberifrons* and *C. gunnari*, *N*=6 for *N. coriiceps* and *C. aceratus*. Statistical significance between species is denoted by different lowercase letters ($P \leq 0.05$).

Catalytic Activity of Nitric Oxide Synthase

Nitric oxide synthase (NOS) activity was determined in red- and white-blooded notothenioids using a radiochemical method to measure conversion of [^{14}C]-arginine to [^{14}C]-citrulline. Determinations were done in four different tissues: brain, heart, retina and pectoral muscle. Results showed that NOS activity was highest in brain and only minimal or not detectable in the other three tissues assayed (Table 2.7). This pattern was consistent among all species in the study. *G. gibberifrons*, an Hb-expressing species, displayed approximately 2-fold greater NOS activity in brain than hemoglobinless icefishes, *C. aceratus* and *C. gunnari*. NOS activity in *G. gibberifrons* retina also was

significantly higher than in white-blooded species and, surprisingly, also higher than that seen in retina of red-blooded *N. coriiceps*. We did not detect any interspecific differences in NOS activity in either heart or pectoral muscle. Taken as a whole, our data indicate that NOS activity is greater in red-blooded notothenioids than in white-blooded species, thus suggesting a higher level of NO synthesis in the former group.

Table 2.7. Nitric oxide synthase activities in tissues of red- and white-blooded Antarctic notothenioid fishes.

Tissues	Enzyme activity [pmol (min • g wet wt.)$^{-1}$]			
	N. coriiceps **(Hb+)**	***G. gibberifrons*** **(Hb+)**	***C. gunnari*** **(Hb-)**	***C. aceratus*** **(Hb-)**
Brain	$159.3 \pm 10.9^{a,b}$	216.2 ± 39.3^{b}	126.6 ± 9.8^{a}	95.7 ± 10.1^{a}
Heart	2.9 ± 1.4	1.1 ± 0.6	0.6 ± 0.3	0.5 ± 0.2
Retina	1.8 ± 1.1^{a}	26.3 ± 10.8^{b}	1.7 ± 1.0^{a}	ND
Pectoral muscle	0.3 ± 0.2	1.0 ± 0.5	ND	0.04 ± 0.04

Values are means ± s.e.m.; N=4 for all species.
Different superscript letters denote significant differences between species ($P \leq 0.05$).
ND: Not Detectable.

Expression of Genes Involved in Angiogenesis

Hemoglobinless Antarctic icefishes are characterized by a higher density of blood vessels in their retinae than observed in red-blooded notothenioids (Wujcik *et al.*, 2007). Based upon this observation and the generally higher steady-state levels of NO in plasma of icefishes, compared to their red-blooded relatives, we anticipated that expression of mRNA encoding for the angiogenic growth factor, VEGF, might be expressed at higher levels in icefishes than red-blooded species. Our results are not clear-cut. VEGF mRNA expression levels in two icefish species (*C. aceratus* and *C. gunnari*) are significantly greater than in red-blooded *G. gibberifrons*, but are similar to that measured in Hb-expressing *N. coriiceps* (Figure 2.2 A). Likewise, two genes that are involved in

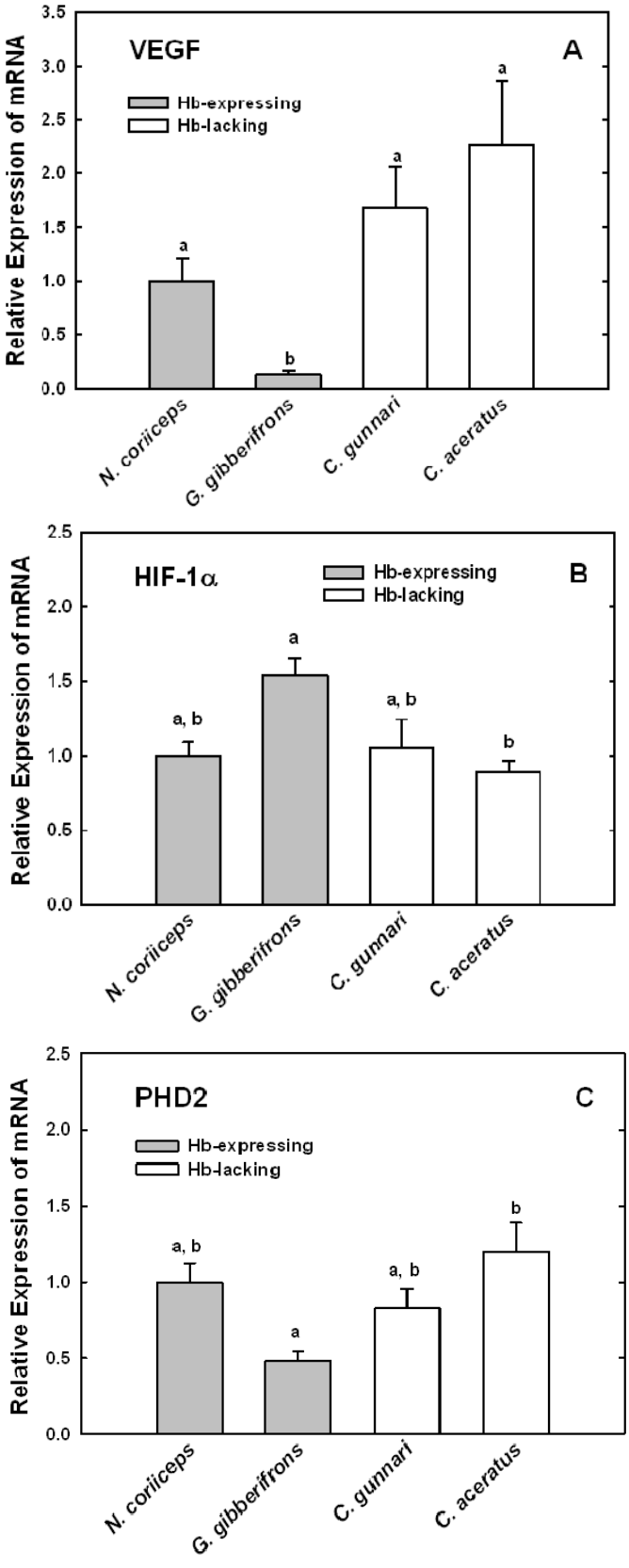

Figure 2.2. mRNA expression of genes in the angiogenic pathway in the retina of red- and white-blooded Antarctic notothenioids. Expression for VEGF (A), HIF-1α (B), and PHD2 (C) is normalized to total RNA and reported relative to *N. coriiceps*. Values are means ± s.e.m.; N= 4 for all species. Statistical significance between species denoted by different lowercase letters ($P \leq 0.05$).

upstream regulation of VEGF in response to hypoxia or NO stimulation, HIF-1α and PHD2, show no consistent differences in expression between red- and white-blooded notothenioids (Figures 2.2 B, C). Thus, comparison of adult icefish tissues with those of red-blooded notothenioid relatives reveals no indication of ongoing hypoxic challenge in icefishes, despite the much lower oxygen-carrying capacity of their blood. In addition, although vascular densities are much higher in icefishes than their red-blooded relatives, the active angiogenic poise of icefish retinae is not greater than that of hemoglobin-expressing species.

Discussion

The mechanism underlying formation of dense vascular patterns in retinae (and presumably other oxidative tissues) of hemoglobinless fishes currently is unknown. We investigated NO-mediated angiogenesis as a potential signaling pathway for establishing the extensive vasculature seen in these animals. We hypothesized that Hb-lacking icefishes would have higher levels of NO than closely-related red-blooded notothenioids because loss of Hb results in elimination of the primary route of NO degradation in vertebrate animals. As a first step, we assessed an index of plasma NO levels to determine if absence of Hb affects the steady-state concentration of the NO pool. We then evaluated the potential contribution of synthesis to extant NO levels by measuring catalytic capacity of NOS, the enzyme responsible for NO production. Finally, we examined potential for new blood vessel growth in retinal tissue by measuring expression of genes known to be involved in the angiogenic pathway.

Hemoglobinless Icefishes Have Elevated Levels of Circulating Nitric Oxide

Measurement of NO metabolites, nitrite plus nitrate (NO_x), in plasma has been accepted widely as an index reflecting the corporeal NO pool in vertebrate animals (Miranda *et al.*, 2001; Dooley *et al.*, 2008; Williams *et al.*, 2008). Many of the methods used to measure NO_x rely on principles of the Griess reaction and include a vast array of analytical techniques such as colorimetry, spectrophotometry, chemiluminescence, gas/ liquid chromatography, electrophoresis and mass spectrometry (Tsikas, 2005). Published values of NO_x in mammals are abundant, while studies reporting NO_x data for lower vertebrates, particularly fish species, are sparse. In this study, we used a spectrophotometric protocol designed after Grisham *et al.* (1995) to quantify NO_x concentration in blood plasma of white- and red-blooded Antarctic notothenioid fishes.

We hypothesized that hemoglobinless icefishes would have greater steady-state levels of NO than red-blooded notothenioids due to elimination of the major degradative route for NO when Hb is not expressed. Our results support this prediction. Apparent NO levels, indicated by NO_x, generally are elevated in icefishes compared to Hb-expressing notothenioid species. These data indicate that a higher steady-state level of NO accumulates in icefishes because of the balance between constitutive rates of NO synthesis and absence of the primary reactant in the degradation pathway for the compound.

Hemoglobinless *C. gunnari*, at least superficially, appears to be an exception to this pattern and has an NO_x concentration similar to that of red-blooded species. However, if we take differences in blood volume among species into consideration, the mass of NO_x in circulation in all icefish species, including *C. gunnari*, clearly is greater

than their red-blooded relatives. Icefishes have a 2- to 4-fold greater blood volume than equivalent-sized red-blooded notothenioids (Hemmingsen and Douglas, 1972). Thus, the somewhat modest increase in plasma NO_x concentration in hemoglobinless fishes is much more pronounced when accounting for the *in vivo* circulatory systems of the animals.

Elevated Levels of Nitric Oxide in Icefishes Are Not Due to Increased Rates of Synthesis

We expected that icefishes would have higher levels of circulating NO than red-blooded species due to slower rates of NO degradation associated with the loss of Hb. However, this is only part of the picture because steady-state level of any biological molecule is a result of the balance between rate of production and rate of degradation of that compound. We therefore set out to determine the contribution of synthesis to the circulating NO pool by examining the catalytic capacity of NOS in several different tissues, including both neuronal and muscular types. Tissue-specificity occurs for different NOS isoforms (Andrew and Mayer, 1999; Alderton *et al.*, 2001), but we primarily were interested in the total capacity of the enzyme to catalyze production of NO and our assay did not attempt to distinguish between contributions of different isoforms.

Our data show that NOS catalytic capacity generally is greater in red-blooded species than in hemoglobinless icefishes. Interspecific differences were not detected in either heart or pectoral muscle, but that was not the case for neuronal tissue. NOS activity was elevated in brain and retina of *G. gibberifrons* compared to hemoglobinless species and, surprisingly, activity was even greater than in red-blooded *N. coriiceps*

retina. This last finding was unexpected because of similar NOS activities in other non-retinal tissues. Elevated rates of NO synthesis in *G. gibberifrons* tissues may explain the moderately high NO_x concentration found in this species compared to other red-blooded notothenioids.

Why is NOS activity elevated in the retina of *G. gibberifrons* compared to *N. coriiceps*? One possible explanation may be attributed to differences in oxygen transport systems of the two fishes. *G. gibberifrons* has a lower blood oxygen-carrying capacity than *N. coriiceps* due to lower hematocrit and hemoglobin concentrations (Table 2.5). Compared to *N. coriiceps*, *G. gibberifrons* also has a markedly reduced choroid rete, a vascular structure that aids oxygen supply to the retina (Eastman, 1993; di Prisco *et al.*, 2007). Taken together, it is possible that these blood and vascular characteristics of *G. gibberifrons* limit oxygen supply to the highly aerobic retina and necessitate greater vasodilatory tone, *i.e.*, enhanced NOS activity-NO production, to compensate for an oxygen deficiency.

Lower levels of NOS activity in icefishes compared to red-blooded notothenioids suggest that the enzyme may be downregulated in hemoglobinless animals, either by a decrease in expression, an increase in degradation, or through inactivation/inhibition of existing catalyst. Indeed, it is well established that regulation of NOS is key to control of NO synthesis and it seems reasonable that these animals might decrease NOS expression and/or activity to preclude additional buildup of the NO pool. We did not investigate NOS control strategies in our research, but there are many reported mechanisms for regulation of activity, including: posttranslational modifications, protein-protein interactions, and alternate mRNA splicing (Alderton *et al.*, 2001). Additionally, NOS

can be subject to feedback inhibition by NO through formation of nitrosyl compounds (Abu-Soud *et al.*, 1995; Hurshman and Marletta, 1995). It is currently unknown whether notothenioids regulate NOS activity in the same manner as higher vertebrates. Of most importance, however, the underlying message of our data is that elevated levels of circulating NO in icefishes compared to red-blooded notothenioids do not appear to be due to increased rates of NO synthesis.

Angiogenic Poise is Similar in Retinae of Red- and White-Blooded Notothenioids

The pathway of angiogenesis has been studied extensively under both physiological and pathophysiological conditions (Folkman and Shing, 1992; Moncada, 1997; Gariano and Gardner, 2005). One objective of our study was to examine the status of signal for new blood vessel growth, *i.e.*, angiogenic poise, in retinal tissues of red- and white-blooded notothenioids. We chose to examine the genes of three factors known to be highly involved in the molecular pathway of angiogenesis and determined level of mRNA expression for each (Kimura *et al.*, 2000; Appelhoff *et al.*, 2004; Hirota and Semenza, 2006; Fong and Takeda, 2008).

We anticipated that expression of VEGF mRNA would be greater in hemoglobinless species than in red-blooded notothenioids given the extensive vascular network seen in the eyes of icefishes (Wujcik *et al.*, 2007). Our data do not support this prediction. Expression levels were similar in white- and red-blooded species with one exception; *G. gibberifrons* had a lower level of VEGF mRNA than all other species. It may be that *G. gibberifrons* is able to compensate for changes in oxygen levels by increasing vasodilatory tone via the NOS-NO system (see above) and that mechanism

alone is sufficient for adequate oxygenation of the retina without requirement for additional vascular proliferation that occurs through VEGF signaling. Indeed, other studies have documented vasodilation as a first response to low oxygen conditions (Lau *et al.*, 2000; Grange *et al.*, 2001) and angiogenesis as a second step when additional blood flow is needed to deliver adequate oxygen to the tissue (Ikeda *et al.*, 1995; Richardson *et al.*, 1999; Wagner, 2001).

Our assertion that angiogenic poise is similar among red- and white-blooded species is consistent with results reported by Morla and colleagues (Morla *et al.*, 2003). Morla et al. (Morla *et al.*, 2003) examined expression of VEGF at the protein level in skeletal muscle of five icefish species and six red-blooded notothenioids, including overlapping species from our study, *C. gunnari* and *G. gibberifrons*. Their data showed that VEGF protein expression was similar in all of the species studied. Morla et al. (Morla *et al.*, 2003) also found that NOS protein expression was greater in icefishes than in red-blooded species, which seems to contradict our NOS activity results. However, it is conceivable that icefishes have greater NOS abundance than red-blooded notothenioids and then regulate enzyme activity via mechanisms described above.

Expression analysis of HIF1-α and PHD2, two hypoxia-sensitive gene factors upstream of VEGF, did not reveal evidence for hypoxic insult to retinal tissue among the species examined. Historically it was believed that HIF-1α upregulation occurred exclusively at the protein level; however, some studies have reported tissue-specific changes in mRNA expression under hypoxic conditions (Ton *et al.*, 2003; Law *et al.*, 2006). Expression of PHD2 mRNA, on the other hand, is known to have a robust response to hypoxic insult in mammals (Metzen *et al.*, 2005). Although this may initially

seem counterintuitive because of the role of PHD2 in facilitating the breakdown of HIF-1α, this activity is oxygen-dependent and remains latent until oxygen is resupplied to the system. Thus, hypoxia-induced expression of PHD2 can be viewed as a "priming" of the system for rapid shutting off of the hypoxic signal upon reoxygenation. Measurement of PHD2 and HIF-1α mRNA thus permits us to evaluate whether icefish live in a continuously hypoxic state. At best, there was a very modest difference in expression levels between *G. gibberifrons* and other species, namely *C. aceratus*. A slight elevation in HIF-1α expression might suggest that retinal tissue of *G. gibberifrons* experiences a mild hypoxic situation. It is reasonable to postulate then, that *G. gibberifrons* adjusts for this low-level oxygen deprivation by maintaining a higher vasodilatory tone as described earlier. This explanation is consistent with the high NOS activities and the low level of expression of VEGF in this tissue in *G. gibberifrons*.

Taken as a whole, we conclude that no significant difference exists in expression of factors in the pathway of NO-mediated angiogenesis between icefishes and red-blooded species, despite marked differences in vascular densities between adults of these groups. This suggests to us that the maintenance of stable vascular structures attained in these adult animals does not require differences in angiogenic tone. Our results do not preclude, however, that the predicted differences in angiogenic poise may occur in earlier ontogenetic stages. Indeed, NO has been reported to play a role in early development of cardiovascular systems in fishes (Fritsche *et al.*, 2000; Eddy, 2005).

Summary and Conclusions

The goal of this study was to evaluate whether NO-mediated angiogenesis plays a role in forming the extensive vascular patterns seen in hemoglobinless icefishes. We established that icefishes have greater levels of plasma NO_x, and presumably circulating NO, than closely-related red-blooded notothenioids. These levels do not appear to be a result of greater NO synthesis, as indicated by lower NOS catalytic capacity in white-blooded animals. They do, however, appear to be linked to the absence of the primary degradative pathway for NO brought about by loss of Hb. Analysis of expression of genes involved in the angiogenic pathway revealed no significant differences between white- and red-blooded species, thus indicating that increases in angiogenic factors are not necessary to maintain vascular patterns in the adult retina. Nonetheless, elevation of angiogenic poise must be patent in early developmental stages of icefishes, leading to the markedly greater vascular densities that are stabilized in adult animals. Recent results from our laboratory demonstrate that an NO-mediated pathway of angiogenesis indeed remains present in notothenioid fishes (Borley *et al.*, 201X). Thus, it is still reasonable to postulate that the absence of Hb leads to elevation in NO that may induce vascular proliferation, at least in the early developmental stages of icefishes.

Chapter 3

PHENYLHYDRAZINE-INDUCED ANEMIA CAUSES NITRIC OXIDE-MEDIATED UPREGULATION OF THE ANGIOGENIC PATHWAY IN *NOTOTHENIA CORIICEPS* [2]

Abstract

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

[2] This chapter contains the text of the following article:
Kimberly A. Borley, Jody M. Beers and Bruce D. Sidell (201X) Phenylhydrazine-Induced Anemia Causes Nitric Oxide-Mediated Upregulation of the Angiogenic Pathway in *Notothenia coriiceps*." Submitted to Journal of Experimental Biology
I took the lead drafting the article.
Jody measured the hemoglobin concentration and NOS activity of treated and untreated fish.

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 nototheniid 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 al.* (submitted). QPCR primers (Table 3.1) 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.

Table 3.1. QPCR Primers

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

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. No reverse-transcriptase controls were included to ensure the samples were not contaminated with gDNA.

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 ($p<0.001$; Figure 3.1). 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 (Figure 3.2). These results demonstrate that we have successfully induced severe anemia in a red-blooded Antarctic notothenioid.

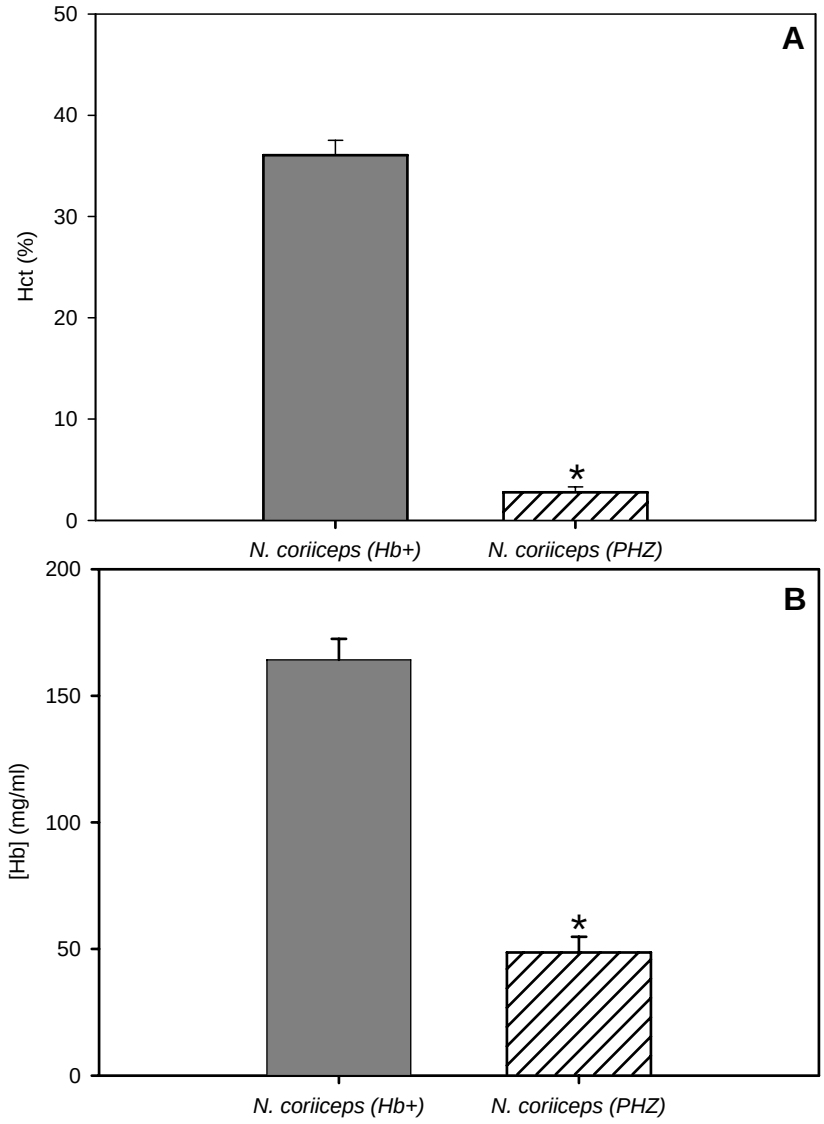

Figure 3.1. Effect of phenylhydrazine treatment upon hematocrit (A) and hemoglobin (B) concentration in *N. coriiceps*. *N. coriiceps* were treated for ten days with the hemolytic agent, phenylhydrazine (PHZ; see methods). Asterisk denotes significance between control and PHZ-treated *N. coriiceps* ($p \leq 0.05$). Values are means ± s.e.m; $N=8$ for both control and treatment groups.

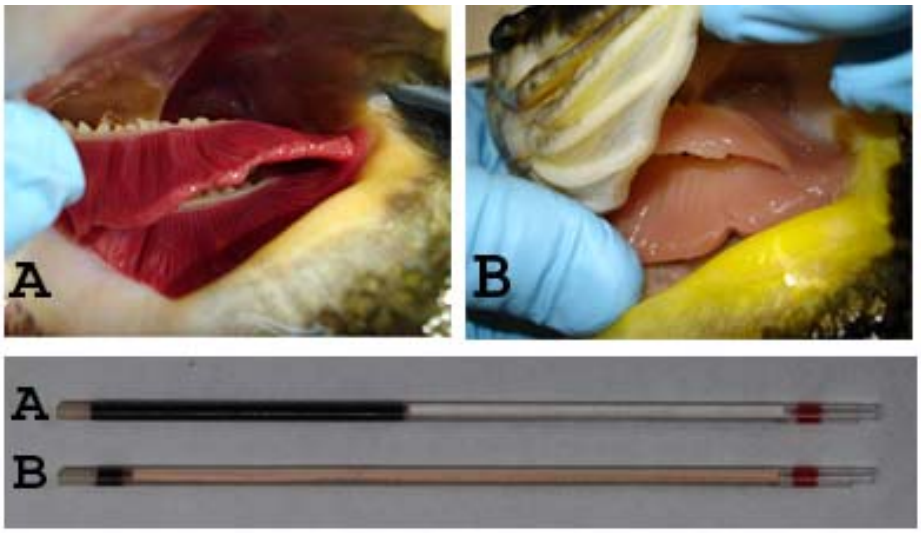

Figure 3.2. Reduction in hematocrit of *N. coriiceps* by treatment with phenylhydrazine. Photographs of gills and Hct capillary tubes from control *N. coriiceps* (A) and *N. coriiceps* treated with phenylhydrazine (PHZ) for 10 days (B). Plasma of PHZ-treated *N. coriiceps* is red-tinted due to the release of Hb when red blood cells are lysed by PHZ.

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 (Figure 3.3). 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.

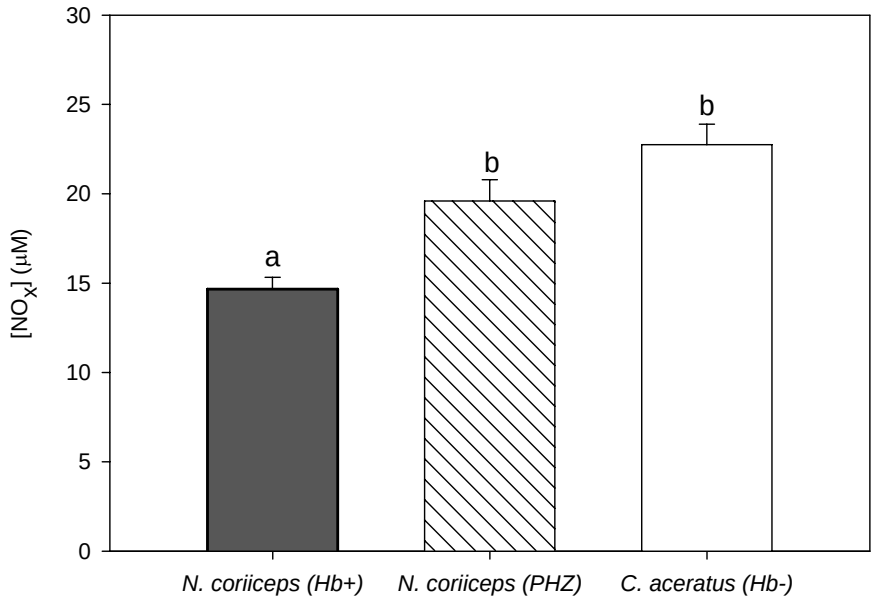

Figure 3.3. Plasma concentration of nitrate plus nitrite (NO_x) is increased in *N. coriiceps* treated with phenylhydrazine. *N. coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb) while *C. aceratus* is a species that lacks Hb. *N. coriiceps* treated with phenylhydrazine, 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.

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 (Figure 3.4). 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).

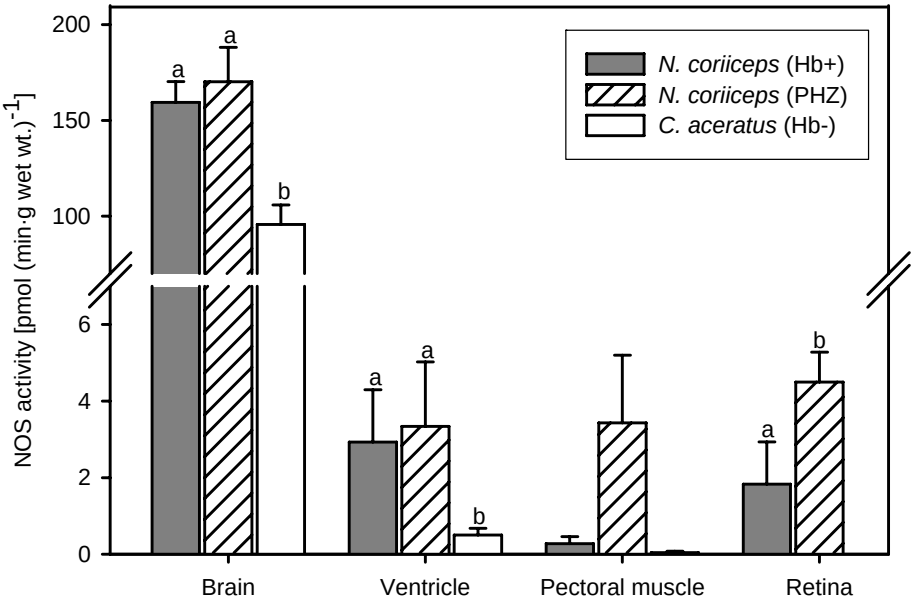

Figure 3.4. Nitric oxide synthase (NOS) activity in tissues of control and PHZ-treated *N. coriiceps*. *N. coriiceps* (Hb+) is an Antarctic notothenioid species that expresses hemoglobin (Hb) while *C. aceratus* is a species that lacks Hb (Hb-). *N. coriiceps* treated with phenylhydrazine, a hemolytic 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.

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 (Figure 3.5). 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-) (Figure 3.5). These data indicate PHZ-treatment stimulates angiogenesis in the retina of *N. coriiceps*.

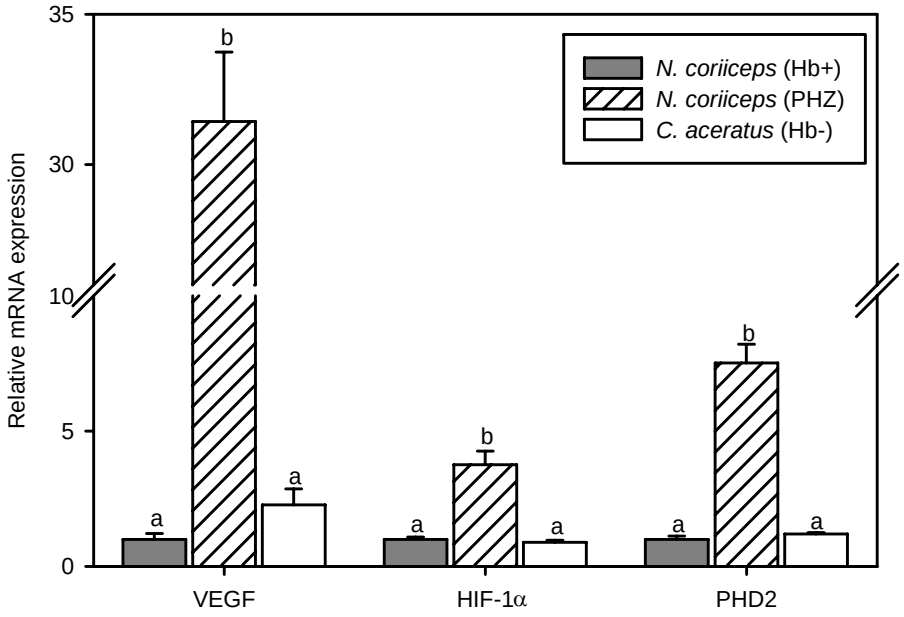

Figure 3.5. PHZ-treatment results in an increase in expression of genes associated with angiogenesis. *N. coriiceps* (Hb+) expresses hemoglobin (Hb) while *C. aceratus* lacks Hb (Hb-). *N. coriiceps* treated with phenylhydrazine, a hemolytic agent, are labeled 'PHZ.' Expression was normalized to total 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 $p\leq0.05$ within a gene. *C. aceratus* data, as reported in Beers *et al.* (submitted), are illustrated for comparison.

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 an 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 the 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 Hemoglobin 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 ancestral icefishes to compensate immediately for the lower oxygen-carrying capacity of blood due to loss of Hb expression.

Chapter 4

EVOLUTION OF THE ANTARCTIC ICEFISH (FAMILY: CHANNICHTHYIDAE) MYOGLOBIN GENE

Abstract

The Antarctic icefishes (Family: Channichthyidae) are most well known as the only adult vertebrates that do not express the circulatory oxygen-binding protein, hemoglobin. Several species of icefishes also have lost the expression of myoglobin (Mb), an oxygen-binding protein expressed in the ventricular tissue of Antarctic notothenioids. Previous studies have identified six species of icefish that do not express Mb. Sequencing of *Dacodraco hunteri's* Mb genomic DNA (gDNA) sequence identified a duplicated TATA box in the promoter that was previously reported as the mechanism leading to the loss of Mb expression in *Chaenocephalus aceratus.* Sequencing of the Mb promoter of the remaining channichthyid species identified the duplicated TATA box in 14 out of 16 species including 8 species known to express Mb. Based on the presence of the duplicated TATA box in the promoters of 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 the presence or absence of a distant regulatory element to inhibit transcription.

Introduction

Antarctic icefishes are the only known adult vertebrates that do not express hemoglobin (Hb). Hemoglobin, an oxygen-binding protein present in red-blood cells of vertebrates, is responsible for distributing oxygen throughout the body. Lack of Hb expression in icefish has been traced back to a gene deletion event that occurred approximately 8.5 Ma when the icefish are known to have diverged from the red-blooded bathydraconids (Near, 2004).

In an interesting twist in the story of icefish evolution, several channichthyid species do not express myoglobin (Mb) as well. Myoglobin is usually expressed in cardiac myocytes and oxidative skeletal muscle fibers and assists in storage and delivery of oxygen in these cell types (Ordway and Garry, 2004). In red-blooded Antarctic notothenioids and Mb-expressing icefishes, Mb is expressed in the ventricle, but is absent in the oxidative pectoral adductor profundus skeletal muscle (Sidell *et al.*, 1997). Myoglobin is functional in icefish that express the protein (Acierno *et al.*, 1997). Although Mb expression is considered beneficial, the expression of the protein has been lost on four distinct occasions within the icefish family resulting in the loss of Mb expression in six species in four genera (Fig. 1) (Sidell *et al.*, 1997; Moylan and Sidell, 2000; Grove *et al.*, 2004).

Myoglobin expression was lost by a common ancestor to the congeners *Champsocephalus esox* and *C. gunnari.* In this genus, a 5-bp duplication in exon 2 results in a shift in the reading frame resulting in a premature stop codon (Grove *et al.*, 2004). *C. gunnari* continues to express Mb mRNA. Although it has not been confirmed, it is believed that *C. esox* will express Mb mRNA due to the similarity of the sequence to

C. gunnari. *Pageotopsis macropterus* and *P. maculatus* have also lost the expression of Mb protein. mRNA has been detected for *P. macropterus* and it is presumed that Mb is also transcribed in *P. maculatus*. In this genus, myoglobin is transcribed, but not translated due to an aberrant polyadenylation sequence (Vayda *et al.*, 1997).

Two species of icefish, *Chaenocephalus aceratus* and *Dacodraco hunteri*, do not express myoglobin mRNA or protein. In *C. aceratus*, a 15 bp insertion that results in a duplication of the TATA box (TATAAAA) sequence is believed to block transcription of the gene (Small *et al.*, 2003). *D. hunteri* has also been shown to lack both protein and mRNA expression of Mb; however the mechanism by which transcription is blocked has not been determined (Moylan and Sidell, 2000). To determine the mechanism by which this species lost Mb expression, the *D. hunteri* myoglobin gene was sequenced. Like *C. aceratus*, the promoter of *D. hunteri* contains a 15 bp INDEL resulting in the duplication of the TATA box, presumably resulting in the loss of myoglobin mRNA and protein expression.

The current phylogeny of the icefish family (Figure 4.1) does not group *D. hunteri* and *C. aceratus* together. This phylogeny was based on morphological data as well the sequence of mitochondrial genes ND2 and 16S. Previous phylogenies did not always agree on the position of *D. hunteri* and *C. aceratus* within the family. Figure 4.2 shows the four previous phylogenies of the icefish family. Iwami (1985), Voskoboinikova (2000), and Balushkin (2000) used morphological characters to construct their phylogenies (Figure 4.2 A-C). These three morphological phylogenies consistently grouped *Psuedochaenichthys*, *Neopagetopsis* and *Pagetopsis*. The position of *Chaneoceophalus* and *Dacodraco* varies between the three distinct phylogenies. Chen

et al. (1998) produced the first mitochondrial DNA phylogeny. Using sequence data from the cytochrome b and D-loop genes, it was still not possible to confidently determine the position of *C. aceratus* within the phylogeny (Figure 4.2 D). The most recent and best supported phylogeny is based on the combined analysis of ND2 and 16S mtDNA sequences and the 58 character states from the morphological data collected by Iwami (1985), Voskoboinikova (2000), and Balushkin (2000) (Figure 1; Near *et al.*, 2003).

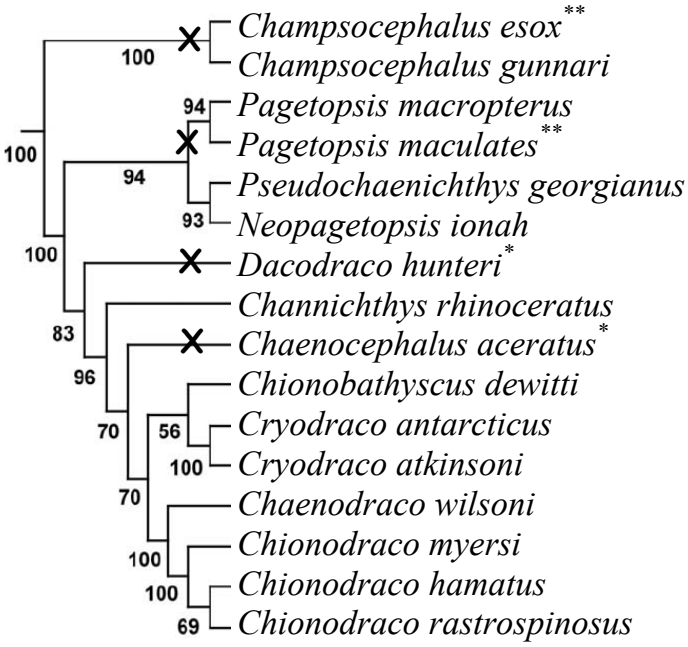

Figure 4.1. The loss of myoglobin expression has occurred on multiple occasions within the Channichthyid family. Figure adapted from phylogeny based on morphological and mitochondrial DNA data (Near *et al.*, 2003). 'X' indicates the loss of myoglobin protein expression. * indicates the loss of myoglobin mRNA expression while ** indicates that there is no data on these species for myoglobin mRNA expression. This figure portrays the results from Sidell *et al.* (1997), Moylan and Sidell (2000), and Grove *et al.* (2004).

In this chapter, the genomic sequence of *D. hunteri* is presented, demonstrating the presence of the same 15 bp INDEL in *C. aceratus* and *D. hunteri*. In order to investigate the possibility that these species might be sister species, the Mb genes from

all species of icefish plus 5 species of red-blooded Antarctic notothenioids were sequenced. Analysis of the promoter sequence from each species has revealed that the duplicated TATA box is present in species that express Mb. Based on these new data, we have rejected our initial hypothesis that *C. aceratus* and *D. hunteri* are sister species and have given more thought to the role of the duplicated TATA box in regulating the transcription of myoglobin.

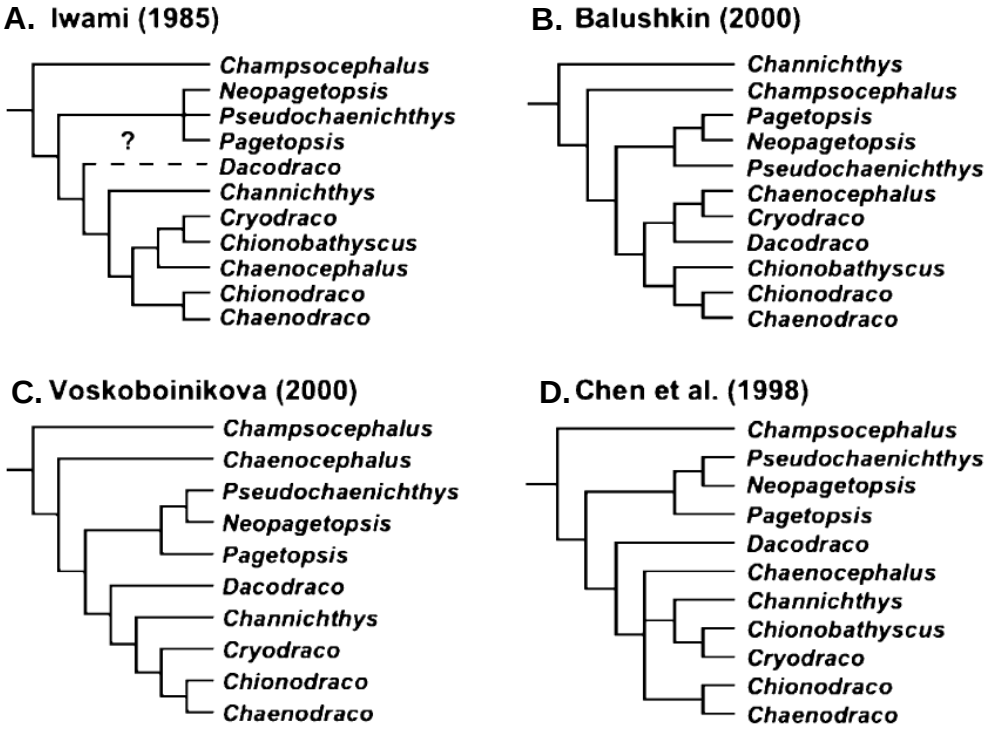

Figure 4.2. Phylogenies depicting the relationships between icefish species (Family: Channichthyidae). Phylogenies A, B, and C were developed based on morphological characters while **D** was based on cytochrome b and D-loop mtDNA sequence data. Figure from Near *et al.* (2003).

Methods

Preparation and Isolation of Myoglobin Genomic DNA

D. hunteri ventricular tissue was collected by William Detrich on the 2003 AMLAR cruise. DNA was extracted using the Qiagen DNEasy kit as described in the kit protocol. gDNA samples for the rest of the species were generously provided by Tom Near of Yale University.

Amplification of Myoglobin

The myoglobin gene was amplified from gDNA samples using primers listed in Table 4.1 and 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 then submitted for sequencing.

Table 4.1. Nucleotide sequence of primers used to amplify the myoglobin gene. Position refers to the position 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)

DNA Sequencing and Sequence Analysis

Samples were sequenced at the University of Maine DNA Sequencing Facility (Orono, ME) and submitted to tblastn (http://blast.ncbi.nlm.nih.gov) to confirm gene identity.

Sequence chromatographs were analyzed using Chromas (Technelysium Pty Ltd) and aligned using Clustal W2 (Larkin *et al.*, 2007). The alignment (Figure 4.3) is labeled with the transcription start sites, introns, exons, and putative promoter elements that were identified from Small *et al.* (1998). MEGA 4 (Tamura *et al.*, 2007) was used to calculate percent similarity between the different species.

Construction of the Phylogeny

To assess the evolutionary history of the channichthyid family, a phylogeny of the family was constructed using the Maximum Parsimony Method using the myoglobin gene sequence. Gaps in the sequence were eliminated during pair-wise comparisons. The phylogeny presented is the bootstrap consensus tree inferred from 1000 replicates. Phylogenetic analysis was conducted using MEGA4 (MEGA; Tamura *et al.* 2007).

Results

Alignment of Myoglobin Sequence

Figure A.1 contains the alignment of the Mb gene for all 21 species sequenced. With the exception of *P. maculatus*, the sequence for each species stretches from -1067 to +1366 compared to the *C. rastrospinosus* sequence. These sequence data cover 1067 bp of the promoter region upstream of the transcription start site through the first two exons

ending 54 bp into the third intron. Due to the inability of primers to bind in all species, we are missing the 3' end containing the third and final exon of the Mb gene. The *P. maculatus* sequence ends shortly after the start codon (*C. rastrospinosus* +550) due to inability to amplify the 3' end with the primers listed in Table 4.1. For the 15 icefish species and 5 red-blooded notothenioids that have been sequenced into the third intron, the sequence length ranged from 2262 bp in *C. esox* to 4789 bp in *P. macropterus* due to insertions and deletions.

Compared to the 2425 nucleotides of the *C. rastrospinosus* reference sequence, 2343 of the nucleotides are conserved among the species sequenced (with the exception of *P. maculatus* due to the incomplete sequence for this species). Table B.1 provides the percentage similarity between the different species for the myoglobin sequence. The highest similarity was seen between icefish species, *C. rhinoceratus* and *C. antarcticus* (99.76%), while the lowest percent similarity is between the red-blooded *H. antarcticus* and white-blooded *C. esox* (96.91%).

Conservation of Putative Promoter Elements

Putative promoter elements identified by Small *et al.* (2003) are marked in the alignment in Figure A.1 and summarized in Table 4.2. The TATA box (TATAAAA) is conserved in all 21 species at -25. The TATAAAA element in mammalian Mb interacts synergistically with YTAAAATATAR elements. Small *et al.* (2003) identified two possible YTAAAATATAR sites (-457 and -347). The first site is located 457 bp upstream of the transcription start site. At this site, none of the sequences match the

Table 4.2. 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.

	Mb Expression		Promoter Elements								
	mRNA	Protein	CANNTG (-663)	Duplicated TATA Box (-651)	CANNTG (-575)	WGATAMS (-478)	YTAAAATATAR (-457)	YTAAAATATAR (-347)	WGATAMS (-261)	CAAT (-129)	TATA BOX (-25)
C.rastrospinosus	+	+	cagctg	(none)	cacgtg	agataac	**ttaaaaataa**	ttaaaataa	agataag	caat	tataaaa
P.maculatus	?	-	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
P.macropterus	+	-	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
D.hunteri	-	-	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.antarcticus	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.gunnari	+	-	cagctg	**tataaaa**	cacgtg	agataac	tttaaataa	**(deleted)**	agataag	caat	tataaaa
C.esox	?	-	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	**(deleted)**	agataag	caat	tataaaa
C.dewitti	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	**(deleted)**	agataag	caat	tataaaa
P.georgianus	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.aceratus	-	-	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.wilsoni	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.rhinoceratus	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.hamatus	+	+	cagctg	(none)	cacgtg	agataac	**ttaaaaataa**	ttaaaataa	agataag	caat	tataaaa
C.myersi	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
N.ionah	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
C.atkinsoni	+	+	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
D.longedorsalis	?	?	cagctg	(none)	cacgtg	agataac	ttaaaataa	ttaaaataa	agataag	caat	tataaaa
H.antarcticus	?	?	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	**tttaaataa**	**atataat**	**ggaat**	tataaaa
P.charcoti	?	?	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	**ttatttaaataa**	acataag	**ggaat**	tataaaa
G.acuticeps	?	?	cagctg	**tataaaa**	cacgtg	agataac	ttaaaataa	**tttaaataa**	agataag	**ggaat**	tataaaa
R.glacialis	?	?	cagctg	**tataaaa**	cacgtg	agatgac	ttaaaataa	**ttattaaataa**	agataag	**ggaat**	Tataaaa

consensus sequence perfectly. *C. hamatus* and *C. rastrospinosus* have TTAAAATAA while all the other species have TTAAATAA at that site. At 347 bp upstream, none of the sequences match the consensus sequence perfectly. Most of the species encode TTAAAATAA at that site while *C. gunnari*, *C. esox*, and *C. dewitti* are missing the sequence and four out of five of the red-blooded species encode either TTTAAATAA or TTATTAAATAA.

The potential MyoD left site, CACGTG at -575, and E2A site, CAGCTG at -663, are 100% conserved in the 21 species examined. The two potential GATA4-binding elements (-261 and -478) are conserved except for in *H. antarcticus*. The CAAT box that is not present in the seal or human Mb is conserved in all the icefish species, but is not found in 4 out of 5 of the red-blooded notothenioids.

Other landmarks previously identified within the *C. rastrospinosus* sequence are also conserved. The transcription start site is 100% conserved. Also 100% conserved are the Kozak consensus sequence, the translation start codon, and intron/exon boundaries.

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 concluded to be responsible for the loss of Mb expression in *C. aceratus* (Small *et al.*, 2003) and presumed to be responsible for the loss of Mb expression in *D. hunteri* is located at -651. Interestingly, the 15 bp INDEL was found in 18 out of 21 species sequenced (Figure 4.3). The 18 species containing the INDEL consist of 4 out of 5 of the red-blooded species (INDEL is not present in *D. longedorsalis*), all six of the icefish

species lacking Mb expression, and 8 out of 10 Mb-expressing icefish species (INDEL is not present in *C. rastrospinosus* and *C. hamatus*). The *C. gunnari* INDEL contains an additional 5 bp 'aatac' in the middle of the 15 bp INDEL, but it does not interrupt the 'TATAAAA' sequence.

Figure 4.3. The Duplicated TATA Box is Present in the Majority of Icefish Myoglobin Promoters. Species are labeled Mb+ or Mb- indicating if myoglobin is expressed.

```
C.rastrospinosus(Mb+) AGCTGATGTAAAAC-------------------AAATCTTAAAAC
C.hamatus       (Mb+) AGCTGATGTAAAAC-------------------AAATCTTAAAAC
C.antarcticus   (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.dewitti       (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
P.georgianus    (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.wilsoni       (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.rhinoceratus  (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.myersi        (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
N.ionah         (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.atkininsoni   (Mb+) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
P.maculatus     (Mb-) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
P.macropterus   (Mb-) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
D.hunteri       (Mb-) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.aceratus      (Mb-) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
C.gunnari       (Mb-) AGCTGATGTAAAACCTATAAAACAAATCAAAAACAAATCTTAAAAC
C.esox          (Mb-) AGCTGATGTAAAACCTATAAAACA-----AAAACAAATCTTAAAAC
                                  ^^^^^^^^^^
                                  Duplicated TATA Box
```

Two Species Contain Large INDELs

Red-blooded *R. glacialis* has a 1918 base pair insert in the promoter region. This insert occurs at -306 in the *C. rastrospinosus* reference sequence. In order possibly to identify the origin of this stretch of DNA, the sequence was run against the NCBI nucleotide database using discontinguous megablast. The sequence showed significant similarity to three NCBI entries, two chimeric antifreeze glycoprotein/trypsinogen-like serine protease (AFGP-TLSP) sequences from *D. mawsoni* and one lactate dehydrogenase-A (LDH-A) sequence from *C. gunnari*. The results are summarized in

Table 4.3. The INDEL is most closely related to the AFGP/TLSP gene (Accession Number AF134320.1) with an E value of 4e-85. The AFGP/TLSP gene aligns with three areas of the *R. glacialis* Mb INDEL: 1→42 (AFGP/TLSP 2014→2055; 92% identity), 847→889 (2307→2265; 95% identity), 1657→1918 (2265→2010; 86% identity). Interestingly almost the exact same regions of the *R. glacialis* Mb INDEL are significantly similar to the *C. gunnari* L-LDH-A gene (Accession Number DQ314522.1) with an E value of 5e-65. The LDH-A gene aligns with the following three regions of the *R. glacialis* INDEL: 1→42 (LDH-A 525→566; 90% identity), 847→889 (818→776; 95% identity), 1657→1918 (776→521; 86% identity). The translated nucleotide sequence was run against the NCBI protein database using blastx. No significant matches were found in the protein database.

Table 4.3. Blast Results for *R. glacialis* Mb INDEL. Discontinguous megablast results from NCBI. AFGP/TLSP : Chimeric antifreeze glycoprotein/trypsinogen-like serine protease precursor. L-LDH-A: L-Lactate dehydrogenase A

Accession Number	Description	Max Score	Total Score	Query Coverage	E value	Max Identity
AF 134320.1	*D. mawsoni* chimeric AFGP/TLSP precursor, gene, partial cds	325	458	18%	4e-85	95%
AF 134321.1	*D. mawsoni* AFGP/TLSP precursor, gene, partial cds	320	449	18%	2e-83	95%
DQ 314522.1	*C. gunnari* L- LDH-A gene, exon 5, intron 5, exon 6 and partial cds	259	387	20%	5e-65	88%

White-blooded *P. macropterus* contains a 2,293 nucleotide INDEL located at +665 in the *C. rastrospinosus* reference sequence placing the INDEL within the second intron of myoglobin. The nucleotide and translated amino acid sequences were run against the 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 top five hits from the blastn analysis are listed in Table 4.4. LIM domains are composed of two zinc finger domains separated by 2 hydrophobic residues. LIM domains mediate protein-protein interactions. Proteins containing LIM domains are known to play a role in controlling gene expression or are involved in different aspects of maintaining cytoskeletal function (Kadrmas and Beckerle, 2004).

Table 4.4. Blast Results for *P. maculatus* Mb INDEL. Blastn results from NCBI. LIMD1: LIM domains containing protein 1

Accession Number	Description	Max Score	Total Score	Query Coverage	E value	Max Identity
XM 686167.3	Predicted: *D. rerio* similar to LIMD1, mRNA	129	240	10%	8e-26	84%
XM 002196989.1	Predicted: *T. guttata* LIMD1, mRNA	116	197	10%	5e-22	77%
XM 0015113490.1	Predicted: *O. anatinus* similar to LIMD1, mRNA	109	179	11%	7e-20	75%
XM 418800.2	Predicted: *G. gallus* LIMD1, mRNA	100	177	9%	4e-17	80%
CR 385534.1	Predicted: *G. gallus* LIMD1, mRNA	100	100	4%	4e-17	80%

Construction of an Icefish Phylogeny Based Upon Myoglobin Sequences

A Maximum Parsimony phylogeny was constructed using the myoglobin gene for all species of icefish with the exception of *P. maculatus*. Five red-blooded Antarctic notothenioids were included to root the channichthyid phylogeny. Figure 4.4A shows the bootstrap consensus tree with the bootstrap values at the branches. Figure 4.4B shows

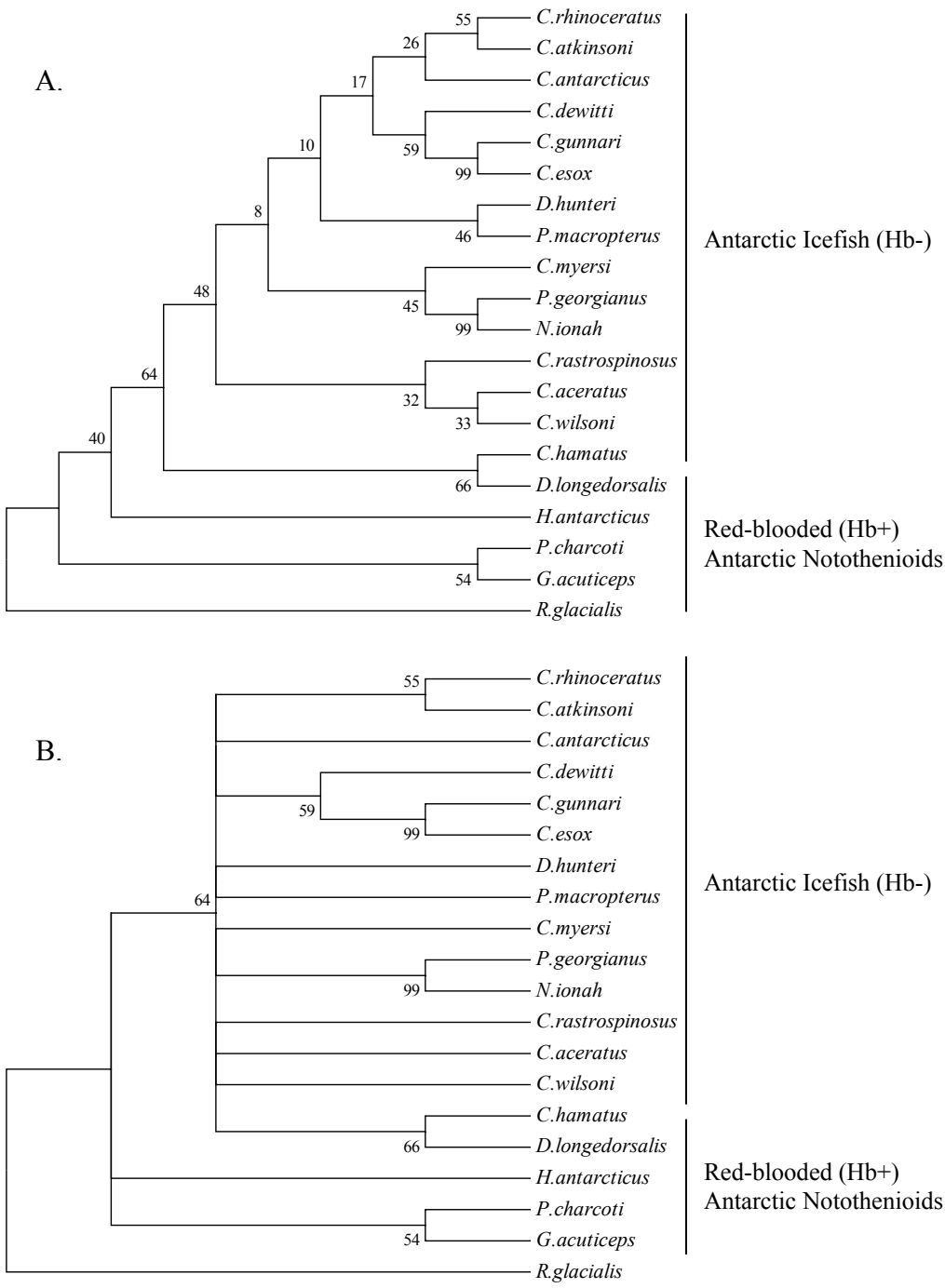

Figure 4.4. Myoglobin-based Channichthyid Phylogeny. Phylogenies constructed using Maximum Parsimony analysis with MEGA4 (Tamura *et al.*, 2007). (A) Bootstrap consensus tree inferred from 1000 replicates. The numbers indicate the percentage of trees containing that branching pattern. (B) Bootstrap consensus tree collapsed at any branch with a bootstrap value less than 50%.

the bootstrap consensus tree collapsed at any branching point with a value less than 50%. The support for the tree is weak with only 7 out of 17 branch points having bootstrap values over 50%.

Discussion

The myoglobin gene is typically well conserved among the notothenioids sequenced with percent similarity between the species ranging from 96-99%. However, there have also been some drastic changes to the sequences in some of the species. Large INDELs are present in the promoter of *R. glacialis* and second intron of *P. macropterus.* BLAST results indicate the *R. glacialis* insert is similar to the antifreeze glycoprotein gene sequence. The position of this 1918 nt insert, approximately 300 bp upstream from the transcription start site, could affect transcription of the myoglobin gene by isolating upstream promoter elements from the TATA box and promoter elements located close to the transcription start site. It is not known if *R. glacialis* expresses myoglobin. The *P. macropterus* 2,293 nt insertion in the second intron shares sequence similarity with proteins containing a LIM-domain. Based on the partial cDNA sequence available for this species, we can deduce that the insertion does not affect splicing of the Mb mRNA. Although it is located in an intron, the *P. macropterus* insertion may affect transcription of the myoglobin gene. Introns are no longer believed to be 'junk DNA.' Cis-acting regulatory elements have been identified within introns. In *P.macropterus,* only a very low level of transcript was detected in ventricular tissue via PCR screening (Vayda *et al.*, 1997). The low level of transcript in this species could be due to a decrease in transcription or an increase in mRNA degradation. The ~2 kb insertion could potentially

disrupt transcription of Mb resulting in the documented low levels of transcript in this species. Another hypothesis, presented by Vayda *et al.* (1997), attributes the low transcript to an aberrant polyadenylation signal present in the Mb mRNA sequence. The presence of a poly(A) tail stabilizes mRNA through the binding of the poly(A) binding protein (Bernstein *et al.*, 1989). The aberrant poly(A) signal in the *P. macropterus* sequence may result in the reduction of Mb transcript by decreasing the mRNA stability (Vayda *et al.*, 1997).

The sequencing of the *D. hunteri* Mb gene identified a 15 bp INDEL in the promoter region. This 15 bp INDEL was found to be identical to an INDEL previously identified as a duplication of the TATA box (TATAAAA) in *C. aceratus* (Small *et al.*, 1998; Small *et al.*, 2003). Small *et al.* (2003) demonstrated that a 41 bp oligonucleotide containing the 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 the promoter region of *C. rastrospinosus* is sufficient for stimulating transcription while the promoter region of *C. aceratus* is not. They proposed the duplicated TATA box results in loss of Mb transcription. They hypothesized that proteins binding at the duplicated TATA box inhibit the ability of E2A to bind upstream resulting in loss of Mb expression. The presence of the same 15bp INDEL in *D. hunteri* suggested this mechanism might also be responsible for the lack of Mb mRNA expression in *D. hunteri* as well. 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.

Upon sequencing the Mb promoter from 16 icefish species and 5 red-blooded species, it is clear the presence of the duplicated TATA box alone cannot determine if the gene will be transcribed. The 15 bp INDEL originally identified in *C. aceratus* is present in 18 of the 21 species sequenced. Of the 14 species containing the INDEL, 8 of the species are icefish known to express Mb. Four out of five of the red-blooded notothenioids sequenced also contain the 15 bp INDEL. These new data led us to two possible conclusions about the loss of Mb expression in *C. aceratus* and *D. hunteri*. Either the loss of Mb expression in *C. aceratus* and *D. hunteri* is completely independent of the TATAAAA sequence duplication or the duplication of the TATAAAA sequence requires the appropriate "context" to inhibit transcription.

To investigate the possibility that the loss of Mb expression in *C. aceratus* and *D. hunteri* has occurred through a mechanism independent of the duplicated TATA box, the Mb sequences of these species were compared to the sequences of species known to express Mb. No simple explanation for the loss of transcription was identified for either species. Promoter elements and transcription start sites identified by Small *et al.* (1998) do not differ between species that do express Mb and those that do not. While the E-box is conserved in *D. hunteri,* this species is missing eight nucleotides immediately upstream of the putative E-box, which could affect binding of E2A to the putative promoter element. The *C. aceratus* and *D. hunteri* sequences were also checked for an early RNA cleavage site or an early translation stop codon, but neither was identified.

It is possible that 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 could potentially inhibit transcription of the Mb gene.

Despite the fact that the promoters of *C. aceratus* and *C. rastrospinosus* are 99% conserved, the promoters 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 something in the *C. aceratus* promoter is capable of inhibiting transcription. The duplicated TATA box was identified as the best candidate. In light of the new data, I propose testing some additional chimeric reporter gene constructs. In particular, I propose inserting the duplicated TATA box from *C. aceratus* into the *C. rastrospinosus* promoter and removing the TATA box from the *C. aceratus* construct. If the duplicated TATA box can block the transcription of the reporter gene, then removal of the duplicated TATA box should restore transcription of the *C. aceratus* reporter construct and the insertion of the duplicated TATA box into the *C. rastrospinosus* reporter construct should inhibit transcription. In order to determine why Mb is not transcribed in *D. hunteri,* I propose a similar set of experiments testing reporter constructs containing the *D. hunteri* Mb promoter with and without the duplicated TATA box.

If further experiments confirm the TATA box inhibits transcription in the transient transcription experiments, then how is it that many of the species containing the duplicated TATA box continue to transcribe Mb? Previous papers have confirmed the expression of Mb mRNA in 10 of the species that we have now identified as having the duplicated TATA box (Sidell *et al.*, 1997; Vayda *et al.*, 1997; Moylan and Sidell, 2000). The simplest explanation is that it depends on the genomic context of the Mb gene. The reporter gene construct simplifies the process of transcription. It allows us to study the transcriptional activity of a promoter with all of the necessary binding factors present, but removes the genomic context of the promoter. Scientists have been aware for over thirty years that distant regulatory elements can control transcription of a gene, but techniques have not been available to study a gene within the appropriate genomic context. 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 an unrelated gene (Sexton *et al.*, 2009). There are two possible mechanisms by which the duplicated TATA box may be responsible for the lack of Mb transcription in *C. aceratus* and *D. hunteri*. The genomes of *C. hunteri* and *D. hunteri* could contain a distant regulatory element that is absent from the genomes of the other icefish species that enables the binding of TFIID to the duplicated TATA box resulting in the loss of transcription. The other explanation is that the genomes of *C. aceratus* and *D. hunteri* could lack a distant regulatory element that in other icefish species blocks the ability of TFIID to bind to the duplicated TATA box, maintaining transcription of Mb in these species.

The 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. The sequencing of the Mb gene from the other members of the channichthyid family shows this INDEL is not unique to *C. aceratus* and *D. hunteri*. The construction of a channichthyid phylogeny using the Mb gene sequences does not group *C. aceratus* and *D. hunteri* together. Based on this information and the previous phylogenies constructed using mtDNA sequences and morphological data, I reject the hypothesis that the *C. aceratus* and *D. hunteri* are sister species.

If *C. aceratus* and *D. hunteri* are not sister-species, it is likely that the loss of Mb transcription in these species is through two distinct mechanisms. Without the data showing the mechanism responsible for the loss of Mb expression in these fish, we cannot determine conclusively that two independent mechanisms are responsible for the loss of Mb expression in these species. Previous research examining the evolution of hemoglobin in the channichthyid family determined that a derived icefish, *Neopagetopsis ionah*, has maintained a "genomic fossil" through lineage sorting (Near *et al.*, 2006). It is possible that a mutation responsible for the loss of Mb in *C. aceratus* and *D. hunteri* was present prior to the divergence of the lineages leading to *C. aceratus* and *D. hunteri* and through random sorting was maintained in the genomes of these two species. Further research is needed to attempt to determine the exact mechanism responsible for the loss of Mb in these species. In addition, the Mb gDNA sequences will be used along with other nuclear gene sequences to continue to improve the resolution of the channichthyid phylogeny.

Chapter 5

CONCLUDING REMARKS

"Nothing in Biology Makes Sense Except in the Light of Evolution"

- Theodosius Dobzhansky

Historically, most of the research on Antarctic Notothenioid fishes has focused on how these animals are able to survive in an extreme environment. However, these animals are offer amazing opportunities for study of evolutionary questions. The geological history of the Antarctic continental-shelf has created, in essence, an "island" allowing the Antarctic notothenioids to evolve in geographic isolation (Eastman, 2000). Geographically isolated populations will diverge due to random genetic drift as well as natural selection. As described in Chapter 1, the Antarctic notothenioids evolved during a geographically and environmentally tumultuous time in Antarctica's history, followed by a period of relaxed natural selection. During this period, the Southern Ocean cooled and a subsequent loss of habitat occurred as the continental shelf was covered by sea ice. Notothenioids survived these conditions by adapting to the cold temperatures and finding areas of refugia. After sea ice retreated, the animals experienced a period of relaxed competition allowing them to radiate into open niches (Eastman, 2005). This unique set of circumstances has resulted in the evolution of 96 species of Antarctic notothenioids. In contrast, there are only 26 species of non-Antarctic notothenioids inhabiting the coasts of Australia and South America (Eastman and Eakin, 2000).

This unique evolutionary history has resulted in a combination of advantageous and disadvantageous traits in modern day notothenioids. Some advantageous traits identified in these fish include the evolution of antifreeze glycoproteins (Chen *et al.*, 1997a), and adaptation of proteins to function in an extremely cold environment (Sidell, 1998; Detrich *et al.*, 2000; Sidell, 2000; Kawall *et al.*, 2002). In this dissertation, I have focused on two disadvantageous traits: the loss of Hb and myoglobin (Mb) in the channichthyid family. Research on the anatomy, physiology and biochemistry of these animals has also identified cardiovascular characteristics that compensate for the loss of Hb and Mb (Moylan and Sidell, 2000; O'Brien and Sidell, 2000; O'Brien *et al.*, 2000; Wujcik *et al.*, 2007). Maintenance of a disadvantageous trait at first seems to challenge the core principles of evolution by natural selection. However, when the disadvantageous trait is viewed in the historical context in which channichthyids evolved, it is clear that these fishes do not disprove natural selection. Icefish demonstrate how relaxed natural selection can lead to maintenance of a disadvantageous trait and subsequent adaptation to compensate for the disadvantageous trait.

In chapters 2 and 3, I proposed a mechanism that may be responsible for one or more of the modifications to the icefish cardiovascular system. I demonstrated that loss of Hb can trigger an intrinsic NO signaling pathway leading to the growth of new blood vessels. This mechanism could theoretically have allowed ancestral icefish immediately to compensate for the loss of Hb and may still contribute to ontogenetic development of the cardiovascular system of modern icefishes. This research presents a mechanism by which a species can adapt to a physiological or environmental condition in the absence of genetic modifications.

In order to use the Antarctic notothenioids as a model in which to study diverse biological questions, we need to understand how the different species are related. In chapter 4, I presented new information on the evolution of the icefish family. A common genetic lesion in *C. aceratus* and *D. hunteri* believed to be responsible for the loss of Mb expression led to the hypothesis that these species may actually be sister-species. This hypothesis was subsequently rejected due to the presence of this same genetic lesion in 14 out of 16 species. While these new data provide us with more information on the evolution of the Mb gene in icefish, further research will be required to determine why Mb is not expressed in *C. aceratus* and *D. hunteri*.

The Antarctic notothenioids are truly a remarkable suborder of fishes. The icefish in particular are a group in which we can study questions we cannot ask in any other organism; however, there are limitations to working with a 'non-model' organism. For example, there is no sequenced genome for an Antarctic notothenioid which restricts questions that can feasibly be studied. With the invention of rapid and affordable genomic sequencing techniques, it will become possible to study some questions that have historically been out of reach, allowing us to gain further insight into the evolution, physiology and biochemistry of the Antarctic notothenioids.

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APPENDIX

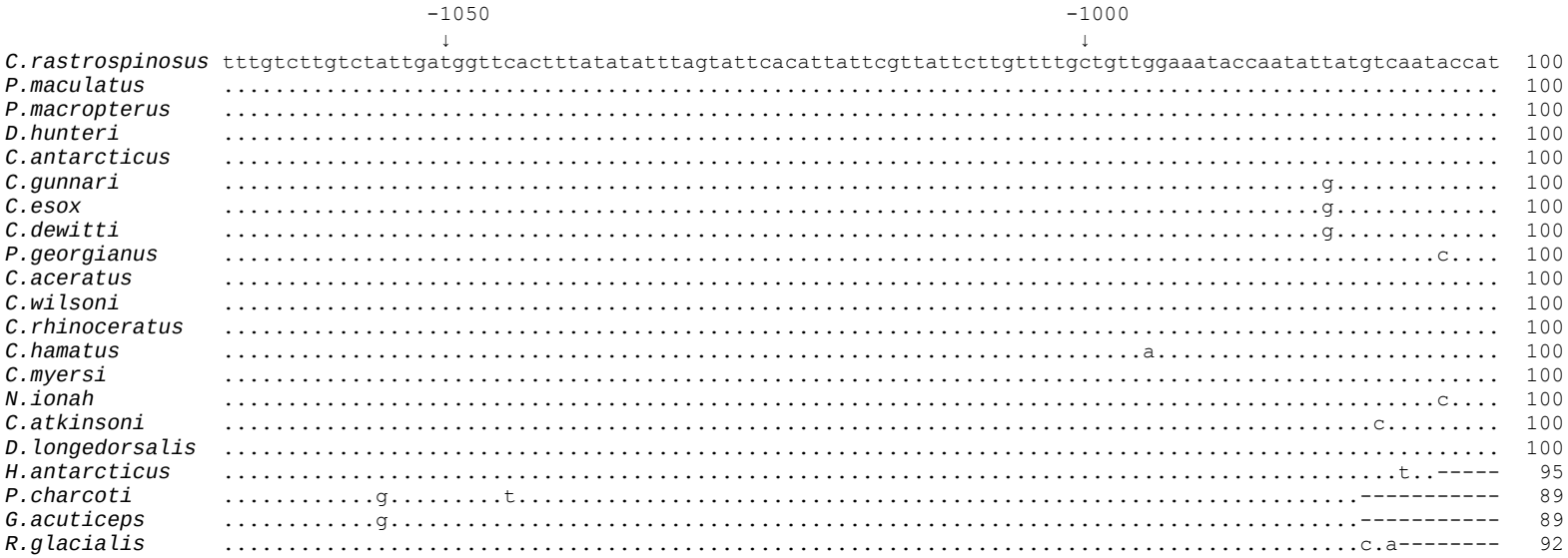

Figure A.1. Alignment of myoglobin gene sequences from 21 Antarctic Notothenioids. Alignment of the sequence was obtained using CLUSTAL X. Residues identical to the *C. rastrospinosus* sequences are indicated with a dot (.). Deleted bases are indicated by a dash (-). Numbering based on *C. rastrospinosus* reference sequence with +1 marking the putative transcription start site. Large INDELS in *R. glacialis* and *P. macropterus* are in italics. Capital letters indicate protein-coding nucleotides. Putative promoter elements are in red bold-faced type. The 15 bp INDEL that contains a duplication of the TATAAAA sequence is in blue bold-faced type. Only the 5' 1599 bp of the *P. maculatus* myoglobin gene is included in this alignment.

Figure A.1. Continued

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                                -950                                                  -900
                                  ↓                                                     ↓
C.rastrospinosus tacctatcttatccctatgtctgttcactcatacatgccctgtattgttattacagttgtcattcacacacatgcactaatttaactctaaaaaa-catc  199
P.maculatus      ..................................................a.........t.............................-....  199
P.macropterus    ..................................................a.........t.............................-....  199
D.hunteri        ..................................................a.....................................----------  189
C.antarcticus    ..................................................a.........t.............................-....  199
C.gunnari        ..................................................a.....................................-....  199
C.esox           ..................................................a.....................................-....  199
C.dewitti        ..................................................a.....................................-....  199
P.georgianus     ..................................................a.....................................-....  199
C.aceratus       ..................................................a.....................................-....  199
C.wilsoni        ..................................................a.....................................-....  199
C.rhinoceratus   ....c.............................................a.....................................-....  199
C.hamatus        ..................................................a.....................................-....  199
C.myersi         ..................................................a.....................................-....  199
N.ionah          ..................................................a.....................................-....  199
C.atkinsoni      ..................................................a.....................................-....  199
D.longedorsalis  ..................................................a.....................................-....  199
H.antarcticus    -..........t.....................................a.....................................a....  194
P.charcoti       ---........c..t...........t.....................a.a.....................................-....  185
G.acuticeps      ---..........t.....a.....................................a.....................----------  175
R.glacialis      ..............t.....................................a.....................................a....  192
                                -850                                                  -800
                                  ↓                                                     ↓
C.rastrospinosus tgaaattctactgacatgtaaacacgattcatttaactgttttctcttctgttacacgctgtcctggttttggaataaacactcatgtttctccattaat  299
P.maculatus      ..................................................................................................c..  299
P.macropterus    ..................................................................................................c..  299
D.hunteri        -..c..............................................................................................c..  288
C.antarcticus    ..................................................................................................c..  299
C.gunnari        ..................................................................................................c..  299
C.esox           ......g...........................................................................................c..  299
C.dewitti        ..................................................................................................c..  299
P.georgianus     ..................................................................................................c..  299
C.aceratus       ..................................................................................................c..  299
C.wilsoni        ..................................................................................................c..  299
C.rhinoceratus   ..................................................................................................c..  299
C.hamatus        ..................................................................................................c..  299
C.myersi         ................a.................................................................................c..  299
N.ionah          ..................................................................................................c..  299
C.atkinsoni      ..................................................c...............................................c..  299
D.longedorsalis  ..................................................................................................c..  299
H.antarcticus    ......g...........................................................................................c..  294
P.charcoti       ..................................................................................................c..  285
G.acuticeps      ---------------------------------.................g.....................c.....a.............c..  241
R.glacialis      ..................................................................................................c..  292
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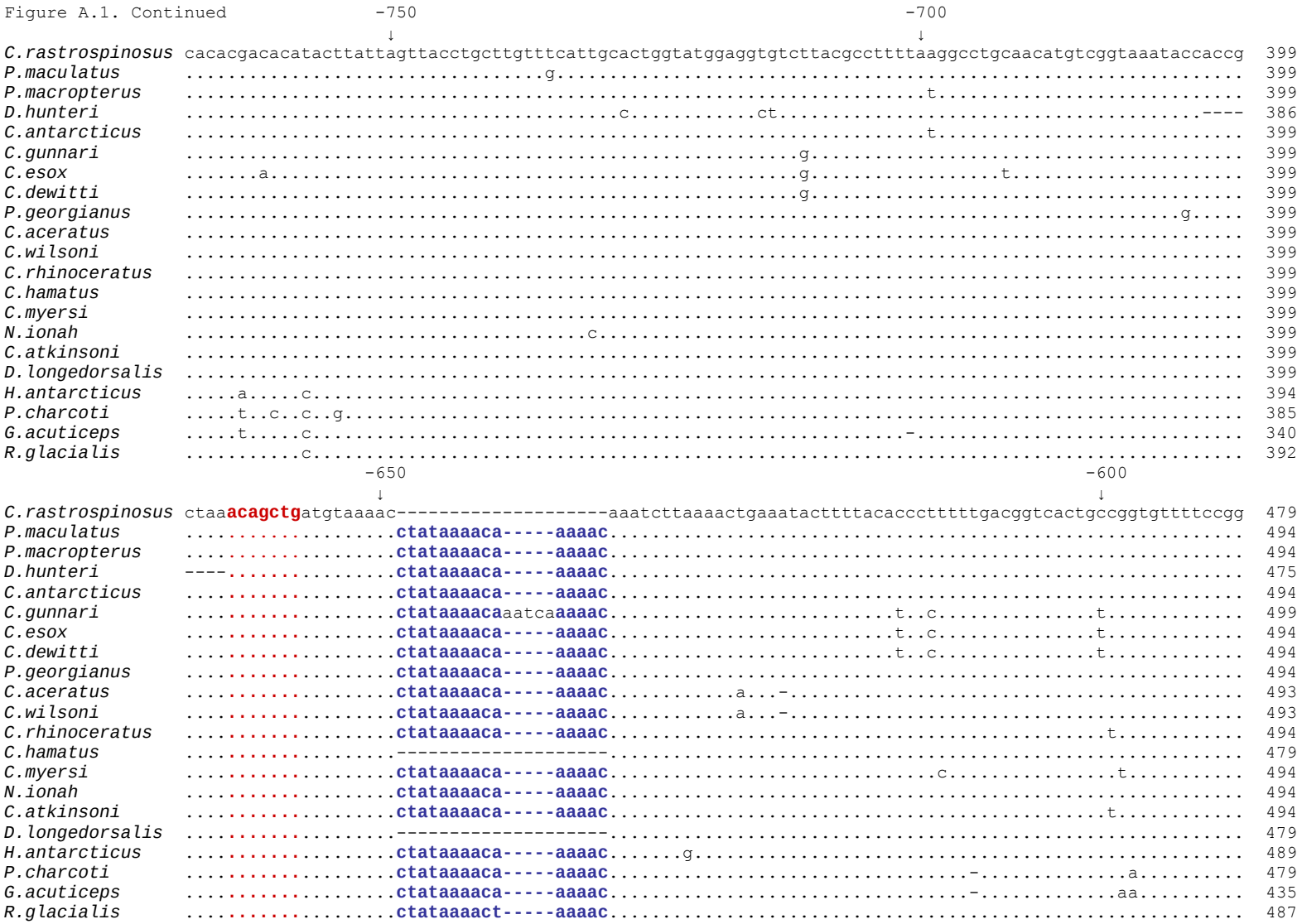
Figure A.1. Continued
-750
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C.rastrospinosus
P.maculatus
P.macropterus
D.hunteri
C.antarcticus
C.gunnari
C.esox
C.dewitti
P.georgianus
C.aceratus
C.wilsoni
C.rhinoceratus
C.hamatus
C.myersi
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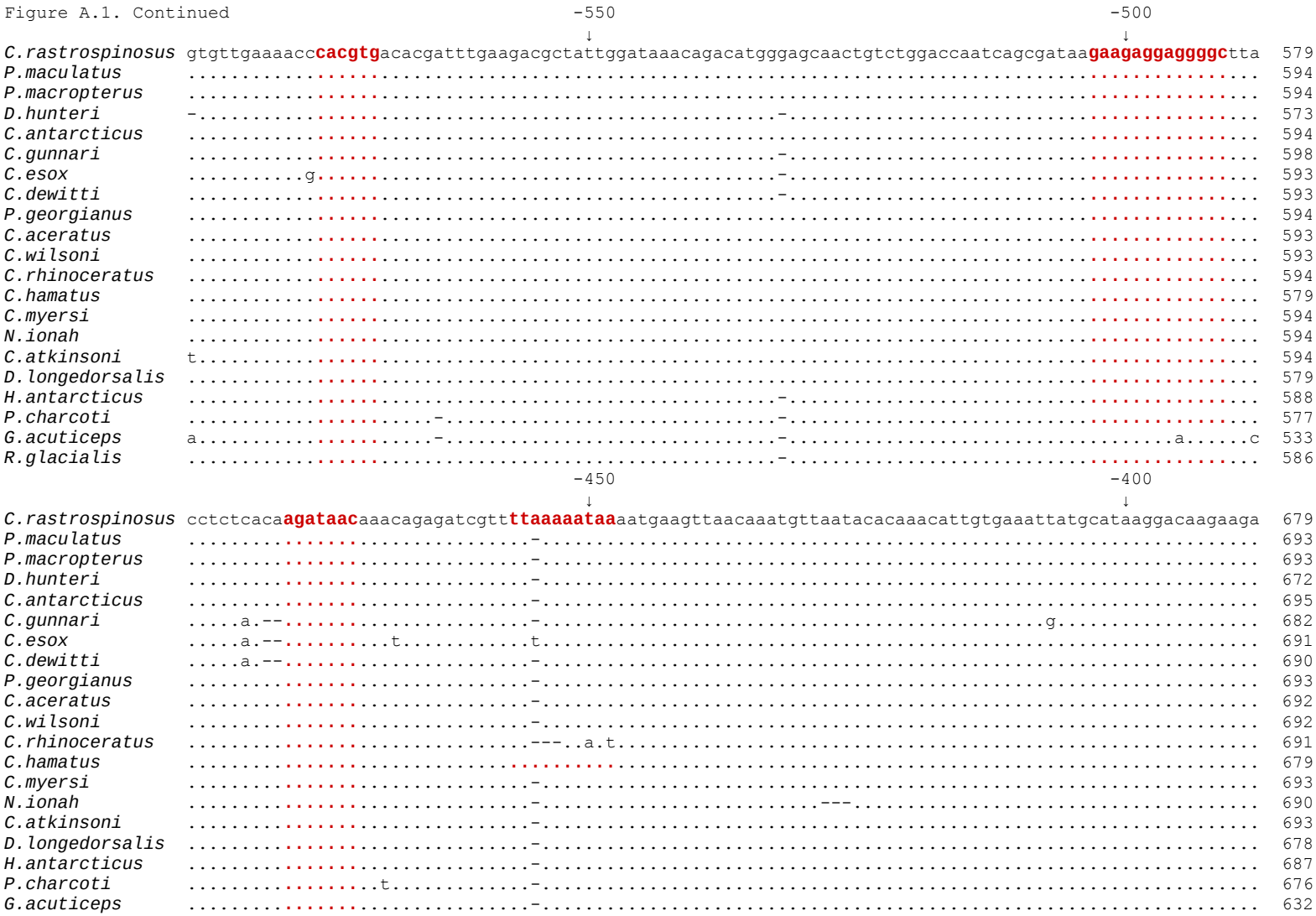
P.charcoti
G.acuticeps
R.glacialis

Figure A.1. Continued



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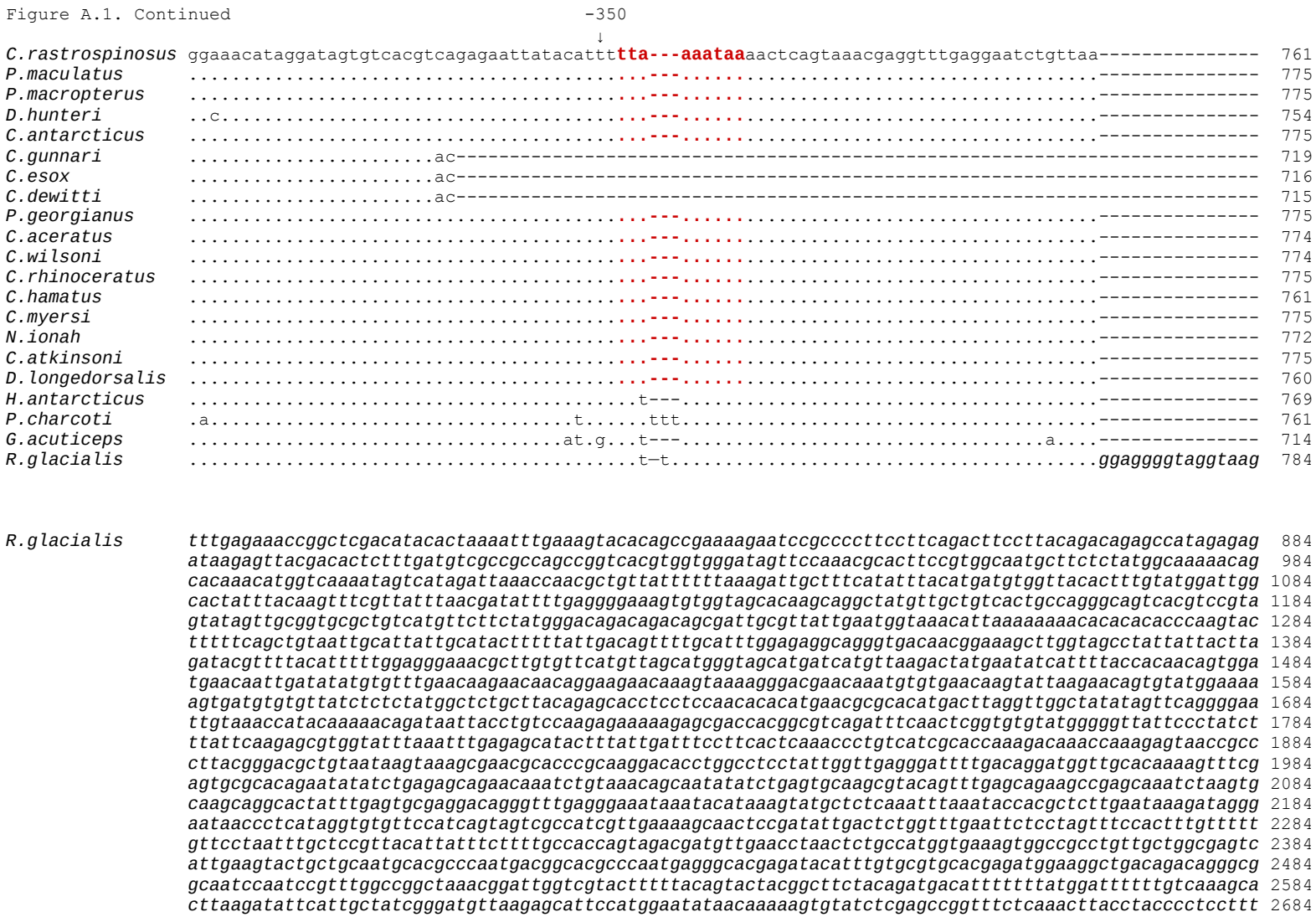

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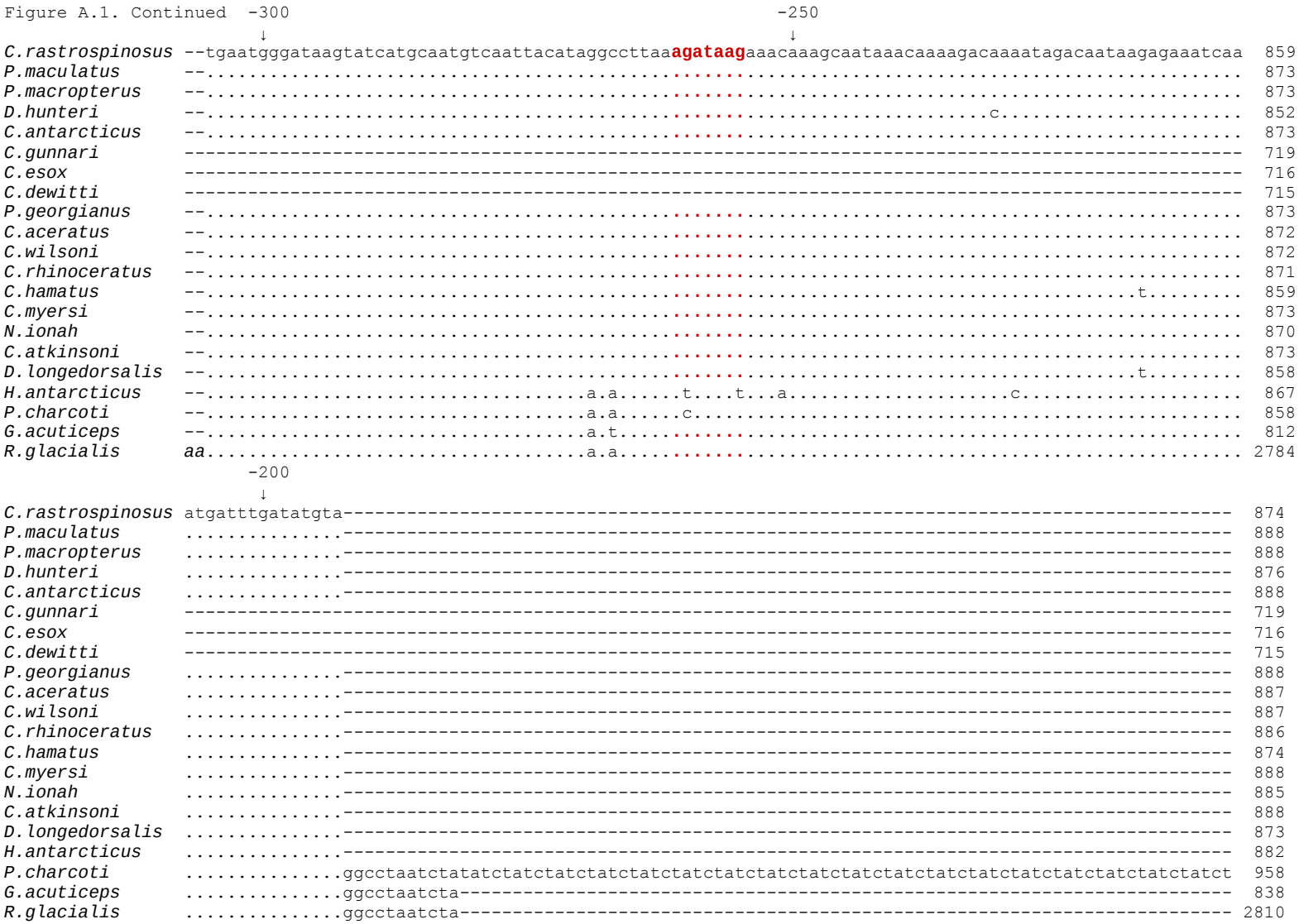

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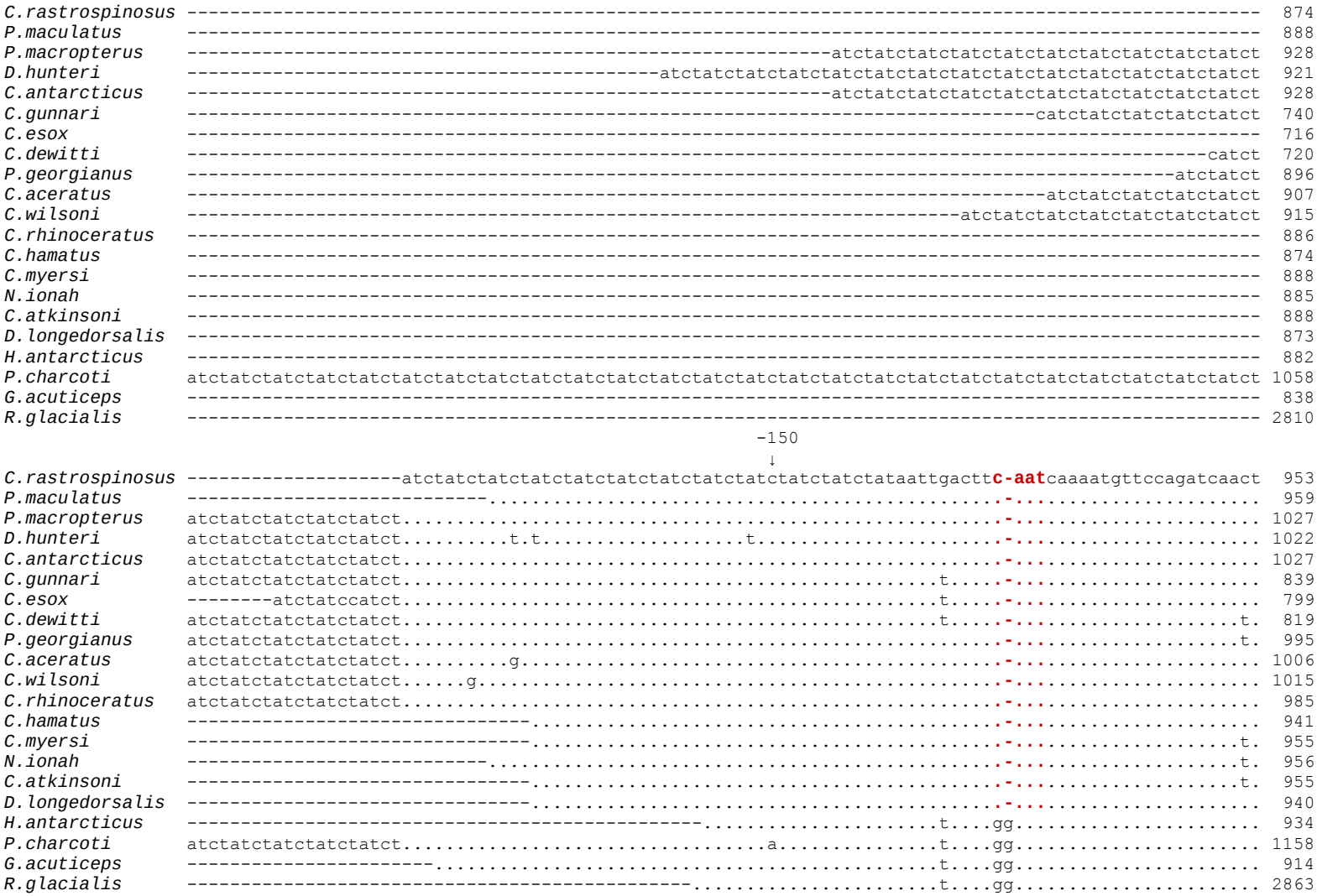

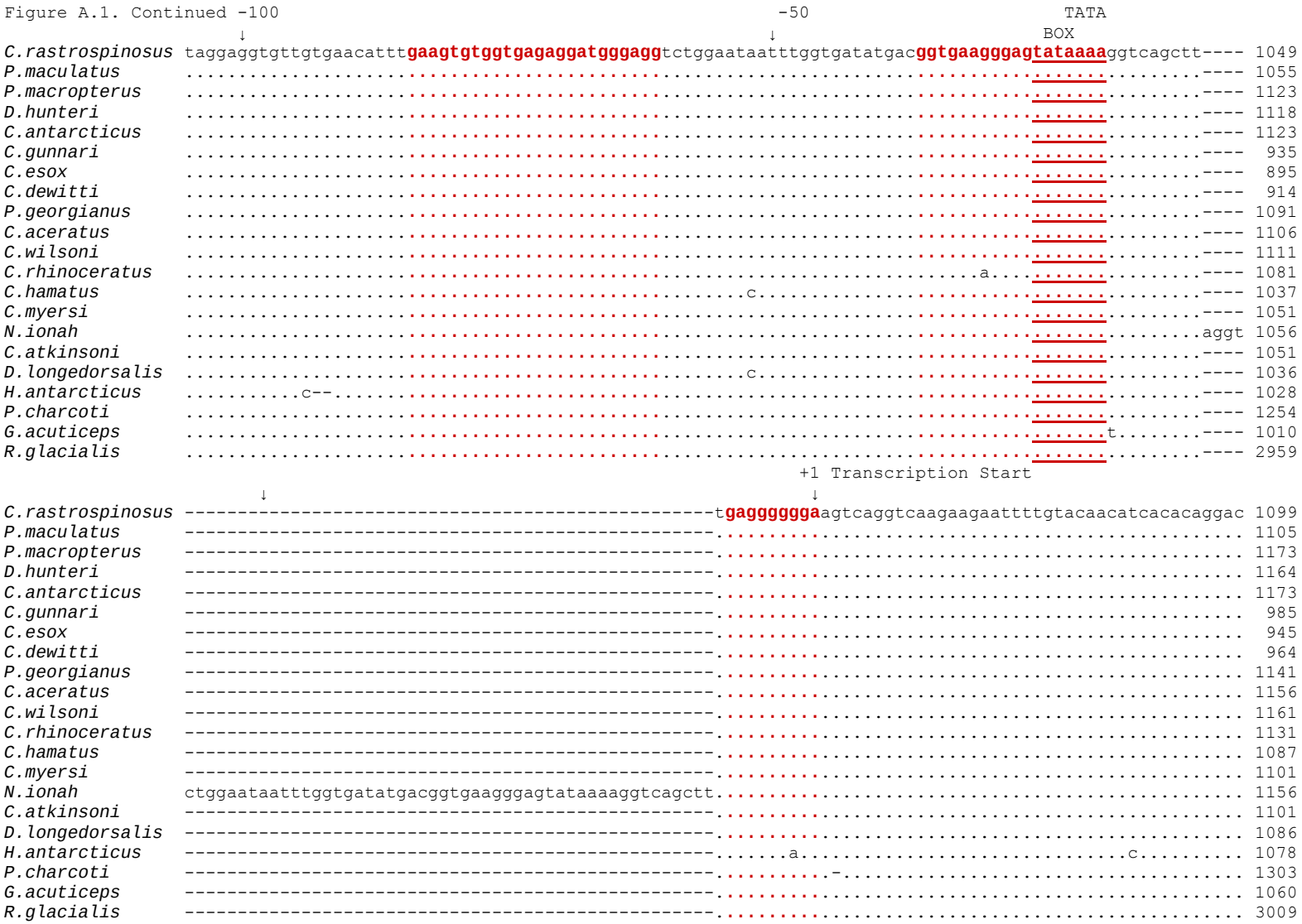
Figure A.1. Continued -100
-50
TATA
BOX
+1 Transcription Start
C.rastrospinosus
P.maculatus
P.macropterus
D.hunteri
C.antarcticus
C.gunnari
C.esox
C.dewitti
P.georgianus
C.aceratus
C.wilsoni
C.rhinoceratus
C.hamatus
C.myersi
N.ionah
C.atkinsoni
D.longedorsalis
H.antarcticus
P.charcoti
G.acuticeps
R.glacialis

Figure A.1. Continued

```
                          +50       intron 1                                  +100
                           ↓        →                                          ↓
C.rastropsinosus attttactactctgcaggtatgagatatattttgtaattgttttaaatgtaatgagaataatagggtataaggacagagaaatgtcattgtggaaaatat 1199
P.maculatus      ..........................................................................t.............. 1205
P.macropterus    ..........................................................................t.............. 1273
D.hunteri        ..........................................................................t.............. 1264
C.antarcticus    ..................................................................................... 1273
C.gunnari        ............................g.....................................................c..t.....t.......c.. 1085
C.esox           ............................g.....................................................c..t.............. 1045
C.dewitti        ..................................................................................... 1064
P.georgianus     ..........................................................................t.............. 1241
C.aceratus       ..................................................................................... 1256
C.wilsoni        ..................................................................................... 1261
C.rhinoceratus   ..................................................................................... 1231
C.hamatus        ..................................................................................... 1187
C.myersi         ..................................................................................... 1201
N.ionah          ..........................................................................t.............. 1256
C.atkinsoni      ..................................................................................... 1201
D.longedorsalis  ..................................................................................... 1186
H.antarcticus    ..................................................t.........c.............................c 1178
P.charcoti       ................................t.................t.....................t.....t.............. 1403
G.acuticeps      ................................t.................t.....................................c 1160
R.glacialis      ................................t.................t.....................................c 3109
                        +150                                      +200
                         ↓                                         ↓
C.rastrospinosus ttttatacaaaacaatataagaaataaagcagtttttaagtgccaaggttagtattgaagaaataaaaaggtatttataaccatgtaacagcttcaagag 1299
P.maculatus      ..g.....................................c.......................................................... 1305
P.macropterus    ..g.....................................c.......................................................... 1373
D.hunteri        ..g..................................................................................... 1364
C.antarcticus    ..g..................................................................................... 1373
C.gunnari        ..g.....................................c.....................................................t.. 1185
C.esox           ..g.....................................c.......................................................... 1145
C.dewitti        ..g..................................................................................... 1164
P.georgianus     ..................................................................................... 1341
C.aceratus       ..................................................................................... 1356
C.wilsoni        ..g..................................................................................... 1361
C.rhinoceratus   ..g..................................................................................... 1331
C.hamatus        ..................................................................................... 1287
C.myersi         ..................................................................................... 1301
N.ionah          ..................................................................................... 1356
C.atkinsoni      ..g..................................................................................... 1301
D.longedorsalis  ..................................................................................... 1286
H.antarcticus    ..................................................................................... 1278
P.charcoti       ................---------...................c.....................................t.............. 1494
G.acuticeps      .....c.....................................................................................-..... 1259
R.glacialis      ............................----..................................................................... 3205
```

Figure A.1. Continued

```
                          +250                                                 +300
                           ↓                                                    ↓
C.rastrospinosus catcatctgatttcacttcacatgaattgtgcagggcttcaccaaggttacagtgcagactcagtgagggcagctgaggtgatgaagacgcctctttcta 1399
P.maculatus      ............t.....................................................a..... 1405
P.macropterus    ............t.....................................................a..... 1473
D.hunteri        ..................................................................... 1464
C.antarcticus    ..................................................................... 1473
C.gunnari        ............a........................................................... 1285
C.esox           ............a........................................................... 1245
C.dewitti        ..................................................................... 1264
P.georgianus     ..................................................................... 1441
C.aceratus       ..............................g.....................c..................... 1456
C.wilsoni        ..................................................................... 1461
C.rhinoceratus   ..................................................................... 1431
C.hamatus        ..................................................................... 1387
C.myersi         ..................................................................... 1401
N.ionah          ..................................................................... 1456
C.atkinsoni      ..................................................................... 1401
D.longedorsalis  ..................................................................... 1386
H.antarcticus    ..........................................................a.....t.t..... 1378
P.charcoti       ..................................................................... 1594
G.acuticeps      ..................................................................... 1359
R.glacialis      ..................................................................t..... 3305
                          +350                                                 +400
                           ↓                                                    ↓
C.rastrospinosus ataatgactcacacttgttcagcagtgactgcacacaatgaaacggtggatgtgtaacttgatatgatcagagacctttatttttcttggatttgtatgt 1499
P.maculatus      ..........................................................................  1505
P.macropterus    ..........................................................................  1573
D.hunteri        ..........................................................c.....t.........  1564
C.antarcticus    ..........................................................................  1573
C.gunnari        ..........................................................g.....a..g.....  1385
C.esox           ..........................................................g.....a..g.....  1345
C.dewitti        ..........................................................................  1364
P.georgianus     ..........................................................................  1541
C.aceratus       ..........................................................................  1556
C.wilsoni        ..........................................................................  1561
C.rhinoceratus   ..........................................................................  1531
C.hamatus        ..........................................................................  1487
C.myersi         ..........................................................................  1501
N.ionah          ..........................................................................  1556
C.atkinsoni      ..........................................................................a.  1501
D.longedorsalis  .......a..................................................................  1486
H.antarcticus    .......a..........................................................g.....a.....  1478
P.charcoti       .......a..........................................................g.....g.....  1694
G.acuticeps      .......a..........................................................g.........  1459
R.glacialis      .......a..........................................................g.-------.....  3398
```

Figure A.1. Continued

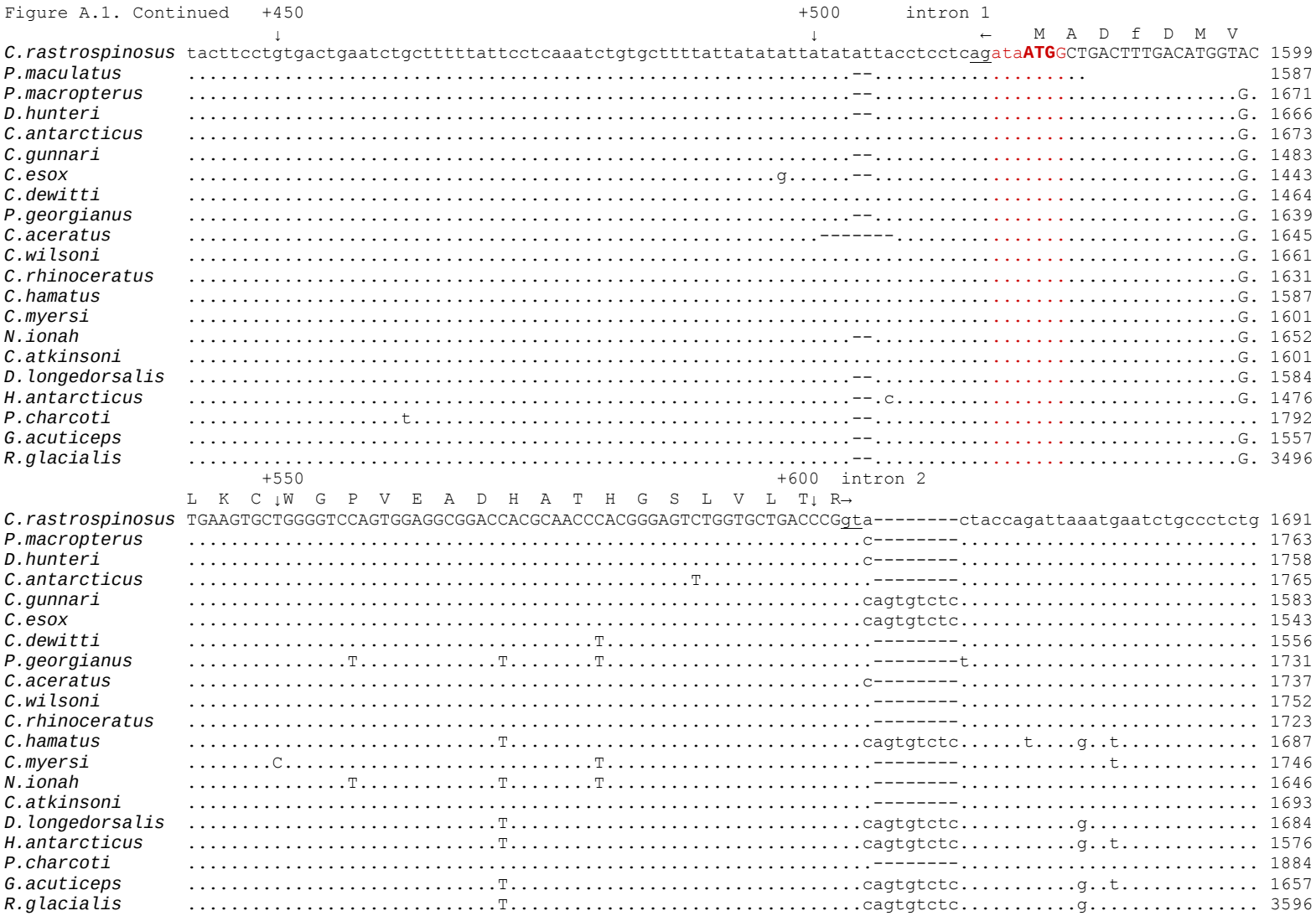

Figure A.1. Continued +650

```
                    ↓
C.rastrospinosus ggtcaggaggtttgcagggct---------------------------------------------------------------------------- 1712
P.macropterus    .....................tgtacatttttcatttagggactgaaaggaaccggctggcccaactccattgttcaatctgaccattgattgacagttt 1863
D.hunteri        ..................... ---------------------------------------------------------------------------- 1779
C.antarcticus    ..................... ---------------------------------------------------------------------------- 1786
C.gunnari        ......a.............. ---------------------------------------------------------------------------- 1604
C.esox           ......a.............. ---------------------------------------------------------------------------- 1564
C.dewitti        ..................... ---------------------------------------------------------------------------- 1577
P.georgianus     ..................... ---------------------------------------------------------------------------- 1752
C.aceratus       ..................... ---------------------------------------------------------------------------- 1758
C.wilsoni        ..................... ---------------------------------------------------------------------------- 1773
C.rhinoceratus   ..................... ---------------------------------------------------------------------------- 1744
C.hamatus        ..................... ---------------------------------------------------------------------------- 1708
C.myersi         ..................... ---------------------------------------------------------------------------- 1714
N.ionah          ..................... ---------------------------------------------------------------------------- 1767
C.atkinsoni      ..................... ---------------------------------------------------------------------------- 1714
D.longedorsalis  ..................... ---------------------------------------------------------------------------- 1705
H.antarcticus    ..................... ---------------------------------------------------------------------------- 1697
P.charcoti       ..................... ---------------------------------------------------------------------------- 1905
G.acuticeps      ..................... ---------------------------------------------------------------------------- 1678
R.glacialis      ..................... ---------------------------------------------------------------------------- 3617


P.macropterus    agaattgacctgctccatatttggggataacacactgtttgatgaatgttgacatgtctctaatattgattgagttgtagcaggtaacacagataaagaa 1963
                 Tcagtggccaaggggctgctgggagagatgtaagtccagaaattcagaactggagaggctgaccggtgagtaatgtttcaacagacaacatcatgtaact 2063
                 agcctggtaaagaagtaagagccatagactttactgtttaggtcattatggggatatacatttcatctacatttgtaatagaaagacatttgttgacagt 2163
                 ttgttggaattctgtattcatttttcagcgggataatatctaagcactgacgggtagaagcagtatcaccaggaagccattcactttgttagtattgtga 2263
                 ttttggttgtttttgaatccataacattagtgtgtttctttaccagaaacattagcagttcaaatcatatttttagatttttaatgggatctcagggatt 2363
                 tcatttaaagataaacacagatgcttggcagaaattcagagctattgaaatatgttatttagtttacctaaagatgttggggttcttatttagtgggcac 2463
                 agtccaagtattaagattctgaagctgcacaattaatttagaaaacctgtgcacagacgtctgttgttgtaagacaccccaccaacaggctccaagtggc 2563
                 cacgcactggtgggtcaaacagccgagggccccagagcccggagcgtaggcttgagggatttgtatcagatttctccacacaggttgtggatgccaaaag 2663
                 ggggacccgagacgcaggaggctgactgtcaaccccaggctctccggccccgaccgagtgacccagaggacatccaatgacgtccgcctacaaggaaagg 2763
                 gggacaactggtacacctgcgaggaccctagacgacatcaggacggatcgggctctcatcatggaggtcgactcggatgcggtcatcagcggactggagg 2863
                 agaaggacctcgaggacatccatccagcagtcgtcccaggaggcatcatcatcggaccggagaaggaagatcgggaggacatctattcagcagcgactcg 2963
                 gaagccgccatcagcaagccggaggagaaggaagacatctctctagcaggcagcccagaagccgccgtcagcggatcagaggggacaaagacacggcaag 3063
                 ccaggatggcacaggggactccactctccgctgaaacaggagtgtgtgttaagtgcaacaagacggtgtatggagccagccaggcctgccaagctatggg 3163
                 cagcctctaccatgacagctgcttcacctgcagcgcctgtagtagggaaggcgttctactatgacgcaggaagggttttctgtgaagaggactttaaaat 3263
                 ctactgtctgaaagactgccacagggtcctggcgcctaaatgtgctgcgtgtaaccagcccatcctgccctcagaggggtctgatgaaaccattcggact 3363
                 aatcaggtccaccattgaggggaaagctgcctctccccctcctgccactgtgggcctgtgtcagggagaggacgagtatggagactcagcagacaaggat 3463
                 gcagtttttgacatttttggtctccctctcattctgccatatgaaggactgtttccaaatgtttaaagggctctaaatataatgttgagagatgaaca 3563
                 cagtggagaggagcgacaatagttatgtttcaagtgccttgtgtaagttgcactcttgtttaagagtgcaaaagagggaattgtaaggaaatatttatgt 3663
                 attcatgcaaactgtgaagagacagtgagggtcagaggtgtttgtttgagatgactggaaaagacgaccagttgtccttaaactccacccccttcctgtat 3763
                 catttggtatgaaagcctatccagcttgctgttctcgctctctcttctcgcgtcgctcggactggaacgtcgtggcggcctgtgggcaggagccaggatt 3863
                 aggccaactcctgacattatacatatggtacgatatggttgtgtatggtaatgtatttgattgtattgcattgtatattaccattaaagtggattattgt 3963
                 ttaacggttaagtgtatgctctggatttccttcatgtaagataacagcttgtttagggacgcgcggagatttggaaatgcggcctagttatcatttaaat 4063
```

Figure A.1. Continued

```
                                                                                              +700
                                                                                                ↓
C.rastrospinosus    -------------gtagaaaagtttttt-gaaggcacattatttttcattaagttc--------------acattaaatgtgaggccatgacagtg---- 1780
P.macropterus       ctcctaacaggct.......t.......-..............-------......--------------..........................---- 4138
D.hunteri           -------------.g...c.tt.......-...........................--------------..........................---- 1847
C.antarcticus       -------------.......t.......-...........................--------------..........................---- 1854
C.gunnari           -------------.......t.......t...........................--------------..........................---- 1673
C.esox              -------------.......t.......t...........................--------------..........................---- 1633
C.dewitti           -------------.......t.......-...........................--------------..........................---- 1645
P.georgianus        -------------.......t.......-...........................--------------..........................---- 1820
C.aceratus          -------------.......t.......-...........................--------------..........................---- 1826
C.wilsoni           -------------.......t.......-...........................--------------..........................---- 1841
C.rhinoceratus      -------------.......t.......-...........................--------------..........................---- 1812
C.hamatus           -------------.......t.......-...........................--------------..........................---- 1776
C.myersi            -------------.......t.......-...........................--------------..........................---- 1782
N.ionah             -------------.......t.......-...........................--------------..........................---- 1835
C.atkinsoni         -------------.......t.......-...........................--------------..........................---- 1782
D.longedorsalis     -------------.......t.......-...........................ccttaacagttttattcccc....................tcct 1791
H.antarcticus       -------------.......t.......-..........................------------ttattcccc....................tcct 1771
O.charcoti          -------------...............-...........................--------------..........................---- 1973
G.acuticeps         -------------.......t.......-...........................--------------.t........................tcct 1750
R.glacialis         -------------.......t.......-...........................--------------..........................tcct 3789

                                                              +750                                              +800
                                                                ↓                                                 ↓
C.rastrospinosus    ----------------atacatcaatatagattgatataaattaatttacaagtcaacaatccatacaaatcatcatttttaagatttagaattaaacat 1864
P.macropterus       ----------------...................................................................................a 4222
D.hunteri           ----------------...................................................................................a 1931
C.antarcticus       ----------------...................................................................................a 1938
C.gunnari           ----------------...................................................................................a 1757
C.esox              ----------------........................................t..........................................a 1717
C.dewitti           ----------------...................................................................................a 1729
P.georgianus        ----------------c...............................................................................---- 1900
C.aceratus          ----------------...................................................................................a 1910
C.wilsoni           ----------------...................................................................................a 1925
C.rhinoceratus      ----------------...................................................................................a 1896
C.hamatus           ----------------...................................................................................a 1860
C.myersi            ----------------...................................................................................a 1866
N.ionah             ----------------...................................................................................a 1919
C.atkinsoni         ----------------...................................................................................a 1865
D.longedorsalis     cattaggggtgaaacg....................g.....................................a........................a 1891
H.antarcticus       cattaggggtgaaacg.................t.................................................................a 1871
P.charcoti          ----------------.................................................................................... 2057
G.acuticeps         cattaggggtgaaaca..........................................................a........................a 1850
R.glacialis         cattaggggtgaaacg...................................................................................a 3889
```

Figure A.1. Continued

```
                                                    +850
                                                     ↓
C.rastrospinosus gtcttttgaagtaaaacaggctgtttttttt-aatgtatttaatttattagattttcttttatttgtataaagaagaatatatatgtttt-------ttat 1956
P.macropterus    ..............................-t.....................................................-------.... 4314
D.hunteri        ..............................-.....................................................------t.... 2018
C.antarcticus    ............................c..-.....................................................-------.... 2030
C.gunnari        .at.......----------------------...t..............a.....................................------t.... 1827
C.esox           .at.......----------------------..................a.....................................------t.... 1788
C.dewitti        ............................c..-.....................................................-------.... 1821
P.georgianus     ..............................-.....................................g.....................-------.... 1992
C.aceratus       ............................c..-............g.........................................-----tta... 2004
C.wilsoni        ..............................-.....................................................-------.... 2017
C.rhinoceratus   ............................c..-.....................................................-------.... 1988
C.hamatus        .a............................t.........................t...a.............................-----tt.... 1955
C.myersi         ..............................--....................................................-------.... 1953
N.ionah          ..............................-.....................................................-------.... 2011
C.atkinsoni      ............................c..-.....................................................-------.... 1958
D.longedorsalis  .a............................t.....................................................--------... 1983
H.antarcticus    .a..................a.........-.....................................................--------... 1962
P.charcoti       ..............................-.....................................................-------.... 2149
G.acuticeps      .a............................-.............g.......................................aaacttt.... 1949
R.glacialis      .a............................-.....................................................--------... 3980


                 +900                                                  +950
                   ↓                                                    ↓
C.rastrospinosus atctaaatgggtctaatagttgttctaaaca------taactgaatctgttagactttgtgatatcagcaaatattgtattgatgtcaaagaataaatat 2050
P.macropterus    ............................t.t.actgaa..................................................... 4414
D.hunteri        ..............................------..................................................... 2118
C.antarcticus    ..............................------..................................................... 2124
C.gunnari        ..............................------..................................................... 1922
C.esox           ..............................------..................................................... 1882
C.dewitti        ......................c........------..................................................... 1915
P.georgianus     ..............................------.....................................................c.... 2086
C.aceratus       ..............................------..................................................... 2098
C.wilsoni        ..............................------..................................................... 2111
C.rhinoceratus   ..............................------..................................................... 2082
C.hamatus        ..............................------..................................................... 2049
C.myersi         ..............................------..................................................... 2051
N.ionah          ..............................------..................................................... 2105
C.atkinsoni      ........................---.....------..................................................... 2049
D.longedorsalis  ......................ca......------..................................................... 2077
H.antarcticus    .......................a......------.....................................tg.................. 2056
P.charcoti       ..............................------..................................................... 2243
G.acuticeps      ..............................------....g.........................a.........g.................. 2043
R.glacialis      ..............................------..................................................... 4074
```

Figure A.1. Continued

```
                  +1000                                                  +1050
                    ↓                                                      ↓
C.rastrospinosus  agaatata-----ttatcatattgtgataaacttgtgatttaaaccccttgtcctcagttgtgagtgttatttcaggtgaaccctcacatttccttttcc 2145
P.macropterus     ........-----.................................................................................... 4509
D.hunteri         ........-----.................................................................................... 2213
C.antarcticus     ........-----.................................................................................... 2219
C.gunnari         ........-----.................................................................................... 2017
C.esox            ........-----.............................................c...................................... 1977
C.dewitti         ........-----.................................................................................... 2010
P.georgianus      ........-----.................................................................................... 2181
C.aceratus        ........-----.................................................................................... 2193
C.wilsoni         ........-----.................................................................................... 2206
C.rhinoceratus    ........-----.................................................................................... 2177
C.hamatus         ........-----.g.............................................................g...................... 2144
C.myersi          ........-----.................................................................................... 2146
N.ionah           ........-----.............................................................tca.g------.............. 2194
C.atkinsoni       ........-----.................................................................................... 2144
D.longedorsalis   ........-----.................................................................................... 2172
H.antarcticus     ........-----.................................................................................T. 2151
P.charcoti        ........-----.................................................................................... 2338
G.acuticeps       ........ggcta.................................................................................... 2143
R.glacialis       ......c.-----.................................................................................... 4069

                intron2     +1100                                                  +1150
                     ← L  F ↓T  E  H  P  E  T  L  K  L  F  P  K  F  A  G  I  A  H↓ G  D  L  A  G  D  A  G  V  S  A
C.rastrospinosus  tcgcagTTTATTCACAGAGCACCCAGAAACCCTGAAGTTATTCCCCAAGTTTGCCGGCATCGCCCATGGGGACCTGGCCGGGGATGCAGGTGTTTCTGCC 2245
P.macropterus     .................................................................................................... 4609
D.hunteri         .................................................................................................... 2313
C.antarcticus     .................................................................................................... 2319
C.gunnari         ...............................T.................................................................... 2117
C.esox            ...............................T.....................................A.............................. 2077
C.dewitti         .................................................................................................... 2110
P.georgianus      .................................................................................................... 2281
C.aceratus        ........C........................................................................................... 2293
C.wilsoni         .................................................................................................... 2306
C.rhinoceratus    .................................................................................................... 2277
C.hamatus         ...................................................T................................................ 2244
C.myersi          .................................................................................................... 2246
N.ionah           .................................................................................................... 2294
C.atkinsoni       .................................................................................................... 2244
D.longedorsalis   ..a.....................................................................................T.............. 2272
H.antarcticus     ..a.....................................................................................T.............. 2251
P.charcoti        .................................................................................................... 2438
G.acuticeps       .................................................................................................... 2243
R.glacialis       .................................................................................................... 4169
```

Figure A.1. Continued

```
                          +1200                                                +1250
                  H  G  A  T  V↓ L  K  K  L  G  D  L  L  K  A  R  G  G  H  A  A↓ L  L  K  P  L  S  S  S  H  A  T
C.rastrospinosus  CACGGTGCCACAGTACTGAAAAAACTGGGCGATCTGCTGAAGGCCAGAGGCGGCCACGCTGCCCTCCTCAAACCTCTGTCCAGCAGCCACGCCAC----- 2340
P.macropterus     ..................T.....................T.....................................................----- 4604
D.hunteri         ..................T.....................T.....................................................----- 2408
C.antarcticus     ..........................................................................................----- 2414
C.gunnari         ............G....T.............................C............C.........................GCCAC 2217
C.esox            ............G....T.............................C............C.........................GCCAC 2177
C.dewitti         ..........................................................................................----- 2205
P.georgianus      ........A..........T.....................................................................----- 2376
C.aceratus        ..................T.....................................................................----- 2388
C.wilsoni         ..........................................................................................----- 2401
C.rhinoceratus    ..........................................................................................----- 2372
C.hamatus         ............G....T........T.....................C.....................................----- 2339
C.myersi          ..........................................................................................----- 2341
N.ionah           ........A..........T.....................A.....................................C.........----- 2389
C.atkinsoni       ..........................................................................................----- 2339
D.longedorsalis   ............G....T.............................C.........A.............................----- 2367
H.antarcticus     ............G....T........T.....................C.....................................----- 2346
P.charcoti        ..........................................................................................----- 2533
G.acuticeps       ............G....T.............................C.....................................----- 2338
R.glacialis       ............G....T.............................C.....................................----- 4264

                                +1300          intron 3                          +1350
                    K  H  K  I  P  I↓ I  N  F  K →                                 ↓
C.rastrospinosus  CAAGCACAAGATCCCCATTATTAACTTCAAGgtgaggttgattgacatgagggcaagttcgaacgtatctagaccattgtggttt 2425
P.macropterus     ..................................................................................... 4789
D.hunteri         ..................................................................................... 2493
C.antarcticus     ..................................................................................... 2499
C.gunnari         ..........................................c.......................................... 2302
C.esox            ..................................................................................... 2262
C.dewitti         ..................................................................................... 2290
P.georgianus      ..................................................................................... 2461
C.aceratus        ..................................................................................... 2473
C.wilsoni         ..................................................................................... 2486
C.rhinoceratus    ..................................................................................... 2457
C.hamatus         ..........................C.....................c.........t..................... 2424
C.myersi          ..................................................................................... 2426
N.ionah           ..................................................................................... 2474
C.atkinsoni       ..................................................................................... 2424
D.longedorsalis   ..................................................................................... 2452
H.antarcticus     ..................................................................................... 2431
P.charcoti        ..................................................................................... 2618
G.acuticeps       ..................................................................................... 2423
R.glacialis       ..................................................................................... 4349
```

	C.ant.	*C.rhin.*	*C.ace.*	*C.wil.*	*C.ras.*	*C.gun.*	*C.esox*	*C.dew.*	*P.cha.*	*D.hun.*	*P.geo.*	*C.myer.*	*N.ion.*	*C.atk.*	*C.ham.*	*H.ant.*	*D.lon.*	*G.acu.*	*P.macr.*
R. gla.	98.48 ± 0.22	98.48 ± 0.25	98.44 ± 0.23	98.52 ± 0.23	98.51 ± 0.23	97.89 ± 0.25	97.65 ± 0.26	98.22 ± 0.23	98.30 ± 0.23	98.12 ± 0.28	98.35 ± 0.23	98.40 ± 0.24	98.26 ± 0.24	98.39 ± 0.23	98.35 ± 0.24	98.40 ± 0.29	98.36 ± 0.27	98.47 ± 0.29	98.35 ± 0.26
P. macr.	99.52 ± 0.13	99.34 ± 0.17	99.18 ± 0.19	99.35 ± 0.17	99.25 ± 0.18	98.44 ± 0.23	98.19 ± 0.26	98.89 ± 0.18	98.11 ± 0.24	99.02 ± 0.23	99.10 ± 0.17	99.17 ± 0.19	99.00 ± 0.19	99.21 ± 0.17	98.70 ± 0.18	97.45 ± 0.33	98.61 ± 0.21	97.60 ± 0.31	
G. acu.	97.69 ± 0.30	97.69 ± 0.30	97.60 ± 0.32	97.69 ± 0.30	97.81 ± 0.29	97.16 ± 0.38	96.97 ± 0.44	97.56 ± 0.36	97.73 ± 0.28	97.34 ± 0.31	97.56 ± 0.29	97.98 ± 0.28	97.67 ± 0.28	97.85 ± 0.27	97.85 ± 0.29	97.64 ± 0.30	97.86 ± 0.28		
D. lon.	98.70 ± 0.22	98.70 ± 0.22	98.83 ± 0.21	98.91 ± 0.21	98.87 ± 0.22	97.81 ± 0.30	97.58 ± 0.33	98.28 ± 0.26	97.48 ± 0.35	98.31 ± 0.26	98.53 ± 0.24	98.62 ± 0.26	98.44 ± 0.26	98.66 ± 0.24	98.88 ± 0.20	98.20 ± 0.26			
H. ant.	97.54 ± 0.31	97.54 ± 0.31	97.53 ± 0.32	97.58 ± 0.29	97.53 ± 0.30	97.05 ± 0.36	**96.91 ± 0.38**	97.39 ± 0.31	97.30 ± 0.31	97.17 ± 0.35	97.36 ± 0.34	97.54 ± 0.32	97.27 ± 0.35	97.45 ± 0.30	97.53 ± 0.33				
C. ham.	98.79 ± 0.18	98.79 ± 0.18	98.91 ± 0.29	99.00 ± 0.16	98.96 ± 0.18	97.90 ± 0.26	97.62 ± 0.30	98.37 ± 0.21	97.48 ± 0.28	98.39 ± 0.23	98.62 ± 0.19	98.79 ± 0.18	98.57 ± 0.20	98.66 ± 0.19					
C. atk.	99.63 ± 0.12	99.71 ± 0.10	99.33 ± 0.15	99.59 ± 0.12	99.46 ± 0.14	98.27 ± 0.26	98.04 ± 0.29	99.33 ± 0.16	98.23 ± 0.25	99.04 ± 0.21	99.21 ± 0.16	99.46 ± 0.14	99.12 ± 0.18						
N. ion.	99.17 ± 0.19	99.17 ± 0.20	99.00 ± 0.20	99.21 ± 0.17	99.16 ± 0.18	97.99 ± 0.27	97.76 ± 0.30	98.92 ± 0.22	97.92 ± 0.27	98.74 ± 0.26	99.50 ± 0.13	99.25 ± 0.17							
C. myer.	99.50 ± 0.14	99.50 ± 0.13	99.29 ± 0.16	99.54 ± 0.12	99.50 ± 0.14	98.18 ± 0.25	97.95 ± 0.29	99.29 ± 0.16	98.23 ± 0.25	98.95 ± 0.20	99.34 ± 0.14								
P. geo.	99.27 ± 0.14	99.26 ± 0.17	99.10 ± 0.16	99.31 ± 0.15	99.29 ± 0.15	98.12 ± 0.27	97.83 ± 0.30	98.99 ± 0.20	98.09 ± 0.26	98.84 ± 0.22									
D. hun.	99.11 ± 0.22	99.09 ± 0.25	98.97 ± 0.22	99.10 ± 0.22	98.99 ± 0.23	98.03 ± 0.29	97.77 ± 0.29	98.67 ± 0.23	97.78 ± 0.27										
P. cha.	98.36 ± 0.23	98.33 ± 0.23	98.09 ± 0.25	98.35 ± 0.21	98.57 ± 0.21	97.38 ± 0.26	97.11 ± 0.31	98.12 ± 0.27											
C. dew.	99.34 ± 0.16	99.38 ± 0.14	98.98 ± 0.19	99.25 ± 0.17	99.20 ± 0.16	98.75 ± 0.21	98.52 ± 0.23												
C. esox	98.11 ± 0.25	98.10 ± 0.24	97.92 ± 0.27	98.06 ± 0.26	98.00 ± 0.26	99.29 ± 0.18													
C. Gun.	98.41 ± 0.23	98.43 ± 0.23	98.27 ± 0.22	98.36 ± 0.24	98.27 ± 0.24														
C. ras.	99.59 ± 0.12	99.59 ± 0.11	99.50 ± 0.13	99.75 ± 0.10															
C. wil.	99.68 ± 0.12	99.67 ± 0.11	99.59 ± 0.13																
C. ace.	99.43 ± 0.15	99.43 ± 0.14																	
C. rhin.	**99.76 ± 0.10**																		

Table A.1. Percentage Similarity of Myoglobin Between Species. Calculations of percentage similarity ± standard error were generated using the Kimura (1980) two-parameter distance matrix. MEGA: Molecular Evolutionary Genetics Analysis, Version 4.0 (Tamura *et al.*, 2007). Standard error was obtained by a bootstrap procedure (1000 replicates). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). A total of 20 species and 6986 positions were analyzed. The most similar (*C. antarcticus* vs. *C. rhinoceratus*) and least similar (*H. antarcticus* vs. *C. esox*) comparisons are highlighted in boldface type. Species names are abbreviated with the first three letters.

BIOGRAPHY OF THE AUTHOR

Kimberly Borley was born in Springfield, OH on June 24, 1982. She attended Kenton Ridge High School and graduated in June 2000. She attended Ohio University and graduated with a Bachelor's of Science in Biology in June of 2004. In July of 2004, she started entered the Biochemistry and Molecular Biology graduate program at The University of Maine. Kim is a candidate for the Doctor of Philosophy degree in Biochemistry and Molecular Biology from The University of Maine in May 2010.