SUMMARY

Overview: Antarctic notothenioid fishes are among the most stenothermic organisms on earth. The hemoglobinless Channichthyidae (icfish) family is even more stenothermic than red-blooded notothenioids. While low thermal tolerance of notothenioids has been well-documented, the underlying physiological and biochemical mechanisms responsible are unknown. In this proposed research, we will test the hypothesis that cardiac work is significantly greater in icefishes compared to red-blooded species and that as temperature increases, the greater cardiac work of icefishes, coupled with reduced blood O2-carrying capacity, results in cardiac failure at a lower temperature compared to red-blooded species. We also hypothesize that neuronal function limits thermal tolerance of red-blooded notothenioids. Our specific objectives are:

1. **Determine if cardiac function limits thermal tolerance** by measuring heart rate concurrently with critical thermal maximum (CT_MAX) in red- and white-blooded notothenioids. Routine and maximal cardiac work in warm-acclimated (up to 4°C) fishes will be measured at 0°C and 4°C using an in situ heart preparation, revealing thermal plasticity of cardiac function.

2. **Characterize metabolic and transcriptional responses to elevated temperature** by quantifying levels of adenylates and HIF-1α in hearts of red- and white-blooded fishes at 0°C and CT_MAX. We will determine if thermal tolerance is limited by the heart’s ability to produce ATP and if notothenioids can defend against hypoxia during warming.

3. **Determine if mitochondrial function contributes to thermal tolerance** by identifying oxidized mitochondrial proteins and phospholipids, and measuring mitochondrial respiration rates and activities of respiratory chain complexes in hearts of red- and white-blooded fishes harvested at 0°C and CT_MAX.

4. **Determine if neuronal function limits thermal tolerance in notothenioids** by quantifying behavioral responses to warming of whole animals and regional warming of the cerebellum.

5. **Determine if warm acclimation improves cardiac performance at elevated temperatures and extends thermal tolerance limits** by concurrently measuring CT_MAX, cardiac function and maximum aerobic scope in-vivo in ambient and warm-acclimated red- and white-blooded notothenioids.

6. **Identify the molecular bases of thermal tolerance and enhanced performance following warm acclimation** by measuring Arrhenius break temperatures of mitochondrial respiration, and synaptosomal and mitochondrial membranes. We will also measure maximal activities of enzymes from central pathways of oxidative metabolism in control and warm-acclimated red- and white-blooded notothenioids.

Intellectual Merit: This proposed research brings together an international team of comparative biologists with expertise in thermal biology, metabolic biochemistry, cardiovascular physiology and neuroscience. Using an integrated approach, we will identify the biochemical, cardiovascular and neuronal underpinnings of thermal tolerance and acclimatory responses in red- and white-blooded Antarctic notothenioid fishes. Our results will be of considerable interest to scholars of polar and thermal biology, and also to scientists studying climate change. The research will make significant contributions to fields as diverse (but inter-related) as comparative biochemistry, cardiovascular physiology and neuroscience. Our proposed experiments will generate the first-ever measurements of cardiac work in icefishes, resolving the long-standing controversy of whether or not the loss of hemoglobin reduces cardiac work. This will provide insight to the unique adaptations that have arisen in cold-bodied fishes.

Broader Impacts: There is no question that the region of the Western Antarctic Peninsula, home to most species of icefishes, is rapidly warming. A key and timely challenge for Antarctic scientists is to learn how individual species, and the ecosystem as a whole, will respond to both physical and chemical changes in their environment. This research will provide training opportunities for graduate and undergraduate students, building a new generation of Antarctic scientists. We will build upon our two-year collaboration with biology teacher Paula Dell of Lindblom Math and Science Academy in Chicago, which serves a largely minority student body (70% African American, 26% Hispanic). In this proposed work, 2 students from Lindblom and Dell will attend the Oceans Wide summer internship program in 2016 at the Darling Marine Center in Maine. Students will learn about the marine environment, and will construct a camera to be deployed during the 2017 field season, enabling us to learn more about the habitat of notothenioid fishes.
PROJECT DESCRIPTION

1. Overview
Antarctic notothenioids are among the most stenothermic organisms on earth. Antarctic icefishes lack hemoglobin, and are even less tolerant of acute increases in temperature than red-blooded notothenioids (2). The physiological and biochemical underpinnings of the markedly low thermal tolerance of notothenioids, including icefishes, are largely unknown. Studies in temperate fishes and invertebrates, and our own work with Antarctic fishes, indicate that cardiac function may be the weak link limiting thermal tolerance, particularly in icefishes (3-6). There remains a paucity of data regarding the cardiovascular physiology of icefishes and the capacities of these animals to adjust to gradual changes in temperature. What little we know suggests that the cardiac work of icefishes exceeds that of red-blooded species. Warming of the Southern Ocean will likely increase the demand for cardiac work in all notothenioids because as temperature increases, the requirements for oxygen will rise, mandating an increase in cardiac output. Icefishes, whose hearts may be operating closer to their limits of maximal performance than red-blooded fishes, may be particularly at risk because they possess a blood oxygen carrying capacity only 10% that of red-blooded notothenioids. Studies proposed herein will bring together an international team of experts in fish physiology and biochemistry, employing integrative approaches to address the following central hypotheses:

Cardiac function limits thermal tolerance in channichthid (ice) fishes. Cardiac work is significantly greater in icefishes compared to red-blooded species. As temperature increases, the greater oxygen demand of the icefish heart, coupled with the low oxygen-carrying capacity of their blood, results in cardiac failure at a lower temperature compared to red-blooded species. We also plan to test the hypothesis that upper thermal limits of white- and red-blooded notothenioids involve different physiological underpinnings: cardiac failure in icefishes versus neuronal failure in red-blooded notothenioids.

2. Results from prior NSF support  O’Brien and Crockett are currently supported by grants from IOS, as well as OPP. We are currently in year 2 of a no-cost extension of ANT 0741301 (KMO); $556,600 and ANT 0739637 (ELC); $203,218. (the latter originally awarded to Bruce Sidell) Sept 1, 2008 – Aug. 31, 2011; Collaborative research: Linkages among mitochondrial form, function and thermal tolerance in Antarctic notothenioid fishes. The overarching goal of this project is to determine if changes in mitochondrial architecture, correlated with the loss of Hb and Mb in channichthid icefishes, impact mitochondrial function and thermal tolerance. We are also in year 2 of a second award, Collaborative research: Redox balance in Antarctic notothenioid fishes: Do icefishes have an advantage? Sept. 1, 2011- Aug. 31, 2014; ANT 1043781 (KMO); $404,970 and ANT 1043576 (ELC); $372,072. We are deploying this spring for our first field season for this project. The first grant (0741301/0739637) is most relevant to our work proposed here and indeed, provides critical baseline studies upon which we have formulated our current hypotheses. We will focus on our results from these studies.

The major findings of this project are:
(1). Work from our collaborators Dr. Bruce Sidell (now deceased) and Jody Beers determined that there is a significant correlation between hemocrit and critical thermal maximum (CT_MAX) (2). The CT_MAX of the icefishes C. rastrospinosus (13.3±0.2°C) and C. aceratus (13.9±0.4°C) are significantly lower than the red-blooded species G. gibberifrons (15.5±0.2°C) and N. coriceps (17.1±0.2°C), and CT_MAX is correlated with hemocrit, suggesting that blood oxygen-carrying capacity may limit thermal tolerance in notothenioids. (2). Supplemental oxygen (i.e.; hyperoxia) does not increase CT_MAX in either white- or red-blooded fishes (Devor et al., in review). However, lactate levels increase 1.5-fold in hearts of icefishes but not red-blooded fishes exposed to CT_MAX under both normoxic and hyperoxic conditions. These results indicate that improving oxygen delivery does not enhance thermal tolerance, and that poor oxygen delivery may trigger anaerobic metabolism in icefish hearts, potentially contributing to their lower thermal tolerance. (3). Mitochondria from icefishes are more tightly coupled than those from red-blooded fishes and produce reactive oxygen species (ROS) at a higher rate when electron transfer is interrupted (Mueller et al., 2011). Consistent with this, there is an increase in levels of oxidized proteins and lipids in hearts of icefishes exposed to CT_MAX but not
red-blooded species, suggesting that oxidative damage to cardiac muscle may contribute to lower thermal tolerance of icefishes compared to red-blooded fishes (Mueller et al., 2012). (4). Arrhenius break temperatures of the enzyme acetylcholine esterase (AChE) are similar between red- and white-blooded notothenioids, suggesting thermal limits of neuronal function are similar among species (O’Brien and Ortego, in prep) (Fig. 1). Together, these data support our central hypothesis in this proposal that different physiological underpinnings are responsible for thermal tolerance limits in white- and red-blooded notothenioids.

Training and Development: This grant has supported the training of two Ph.D. students and one M.S. student. Dr. Jeff Grim, mentored by Crockett, defended his Ph.D. dissertation in Aug., 2010, and was awarded an OPP Postdoctoral Fellowship in Polar Regions Research to work with Dr. Bill Detrich at Northeastern University. He now holds a tenure-track position as Assistant Professor at Presbyterian College. Irina Mueller, mentored by O’Brien, was awarded her Ph.D. in 2012 and is now a postdoctoral researcher at the University of California, Irvine. Devin Devor, also mentored by Crockett, completed his M.S. in Jan., 2013. Undergraduates Kristen Dullen, a native Alaskan, and Jeff Hennigh, both at UAF, were also involved in research. Dullen presented her research at the meeting for the Society for Integrative and Comparative Biology (SICB) in Jan., 2013. At Ohio University, undergraduate Elizabeth Evans has been working on the project and will present a poster at SICB in Jan., 2014.

Graduate students presented their research for a total of 5 presentations (3 at SICB and 1 each at a Gordon Conference and the meeting of the Scientific Committee for Antarctic Research).

Other Broader Impacts: Paula Dell, a high school teacher at Lindblom Math and Science Academy in Chicago, Ill participated in our field season in 2011 as a PolarTREC teacher and is deploying with our field team this spring, 2013. Two Lindblom students, Oloade Olowale (Nigerian) and Isabele Raymundo (Hispanic), spent 3 weeks in O’Brien’s laboratory at UAF during the summer of 2012, studying differences in expression of heat shock proteins between red- and white-blooded fishes. These data will be included in a manuscript in preparation for Polar Biology. Our collaboration with Paula continues. She is currently working with her students to construct a tethered camera that we will deploy from the ARSV Laurence M. Gould in June, 2013 to study fishing grounds and learn more about the ecology of notothenioid fishes. Crockett recently presented a seminar on the biology of Antarctic fishes and demonstrated a laboratory exercise she developed for a professional development day aimed at high school teachers (Boat-of-Knowledge in the Science Classroom).

Publications supported by ANT-0741301 and ANT-0739637
Listed here are publications from O’Brien and Crockett’s laboratories (* indicates graduate students; # undergraduate students).

3. Introduction and background

The composition of the Antarctic fish fauna has been strongly influenced by the distinctive oceanographic features of the Southern Ocean, the waters of which are delineated by the Antarctic Polar Front (APF), located between 50°S and 60°S, depending on ocean sector and season (7). Steep gradients in physical and chemical properties (i.e., temperature and salinity) are found across the APF, which restrict the dispersal of many marine organisms. Water temperatures north of the APF are 4-8°C higher in the summer and 1-3°C higher in the winter than waters south of the front, where water temperatures are usually less than 0°C and vary little on a seasonal basis (8). Inhabiting this nutrient-rich environment, with relatively few predators, the notothenioids underwent rapid speciation following opening of the Drake Passage 20-40 MYA and cooling of the Southern Ocean (9). Today, the perciform suborder Notothenioidei dominates the fish fauna of the Southern Ocean, accounting for 91% of the fish biomass on the Antarctic shelf and 45% of the benthic fish species (9).

Antarctic notothenioid fishes provide an unparalleled opportunity for studying biochemical and physiological adaptations that have enabled ectothermic organisms to thrive in the cold. The distinctive features of the Southern Ocean: its freezing temperatures, isolation, and limited competition have provided suitable conditions for the evolution of several unique characteristics among the Antarctic fish fauna. Best known is the expression of antifreeze glycoproteins, which prevent body fluids from freezing (10). Others include cold-stable microtubules (11), the absence of a heat shock response (12), and perhaps most extraordinary, the loss of expression of the oxygen-binding proteins hemoglobin (Hb) and myoglobin (Mb) in the family Channichthyidae (icefishes) (13).

Loss of Hb expression in channichthid icefishes makes them unique among vertebrate animals, and yet potentially vulnerable to the impacts of climate change. A central feature of our proposed work is to build on previous results, which implicate cardiac function as limiting thermal tolerance of icefishes. We will examine how cardiac function is affected by elevated temperature over both acute and acclimatory time scales. We also intend to expand our studies, and investigate the potential role of neuronal function in limiting thermal tolerance of notothenioids, as first suggested in landmark studies by Somero and DeVries (1967) (14). We propose an integrative approach to determine the physiological and biochemical bases of thermal tolerance of Antarctic notothenioid fishes, spanning the molecular to organismal levels of biological organization.

3a. Antarctic icefishes do not express the oxygen-binding protein hemoglobin. In 1954, the Norwegian scientist Johan Ruud confirmed that the blood of icefishes was devoid of hemoglobin (Hb), establishing icefishes as the only vertebrates on earth, that as adults do not express the circulating oxygen-binding protein Hb (13). There are 16 species within the family Channichthyidae. The loss of expression of Hb is due to a deletion of the β-globin gene and partial deletion of the α-globin gene in most icefish species (15). Phylogenetic analyses using mitochondrial DNA sequences and molecular clock methodology predict icefishes diverged from the notothenioid lineage between 5.5 and 2 MYA (16). This was long after the cooling of the Southern Ocean (8, 17). Because oxygen solubility is inversely related to temperature, the icy-cold temperature of the Southern Ocean was likely crucial to the survival of the ancestral notothenioid possessing the genetic (Hb) lesion.

The loss of Hb reduces the oxygen-carrying capacity of icefish blood to less than 10% that of red-blooded notothenioids. Oxygen transport to tissues is enhanced by multiple modifications in the cardiovascular system that occurred prior or subsequent to the loss of Hb. The blood volume of icefishes is 2-4 times greater than red-blooded teleosts (18). High cardiac output is maintained by a large, slow-beating heart, which functions as a volume rather than pressure pump (19-21). Most all notothenioids have a type I heart, characterized by a spongy myocardium and lack of coronary circulation (21, 22). The trabeculated nature of the heart provides a large surface area for oxygen
diffusion into cardiomyocytes, and the extent of trabeculation is greater in icefishes compared to red-blooded species (23). Blood pressure is minimized by large bore vessels, with diameters 2-3 times larger than red-blooded species (24). These large-bore vessels allow blood to circulate at a high velocity, maintaining a steep partial pressure gradient for oxygen between vessels and tissues, which facilitates the diffusion of oxygen into tissues (20).

3b. Is cardiac work greater in icefishes compared to red-blooded notothenioids? There are two opposing views regarding the impact of the loss of Hb expression on cardiac performance in icefishes. The loss of erythrocytes (with Hb) reduces blood viscosity, which has led to the suggestion that the absence of Hb may be advantageous because it reduces work of the icefish heart (25-27). On the other hand, the loss of Hb is correlated with 2-5-fold increase in blood volume (28, 29). Back-of-the-envelope calculations estimate that the cost of circulating this large volume of blood increases work of the icefish heart compared to red-blooded notothenioids (30). Actual cardiac work cannot be estimated because venous return pressure has not been measured in icefishes. However, cardiac power development, the product of stroke volume, heart rate, and mean ventral aortic pressure can be estimated from published literature (19, 31). Mean ventral aortic pressures are only ~ 1.6 fold higher in red- blooded fishes compared to icefishes, yet cardiac output is on average 3.3-fold higher, resulting in an estimated 2-fold increase in cardiac power output in icefishes compared to red-blooded fishes (30) and a 5-fold increase in energy expenditure compared to temperate teleosts (28). In experiments we propose here, we will directly measure for the first time, routine and maximum cardiac output, along with ventral aortic and venous return blood pressure, calculate cardiac work in red- and white-blooded notothenioids, and resolve this long-standing controversy.

3c. Cardiac performance may define upper thermal limits of Antarctic notothenioids. At current Antarctic temperatures, the potentially high workload of the icefish heart is not problematic. However, the Western Antarctic Peninsula (WAP), home to many white- and red-blooded species is experiencing the most rapidly rising temperatures in the southern hemisphere and is among the most rapidly warming regions in the world (32, 33). A recent study has documented a 2-fold longer summer “melt season” associated with the Peninsula compared to melt seasons sixty years ago (34). Warming of the Southern Ocean is of particular concern for nototheniid fishes, since their upper thermal limits are relatively low compared to temperate and Arctic species (2, 14, 35-37). Considering the narrow thermal windows of icefishes (2), as well as their reliance on cold and oxygen-rich waters, some researchers have expressed concern regarding the ability of channichthyids to respond to projected increases in temperature (38, 39).

As temperatures rise, whole-animal oxygen demands are likely to increase. Given our current knowledge of temperature effects on fish (40), cardiac work will increase to meet greater tissue oxygen demand. Icefish hearts may not be capable of meeting this challenge, given their already high cardiac energetic demand (28), poor oxygen supply (18), and in some species, lack of Mb, leading to reduced tissue oxygenation compared to red-blooded and red-hearted notothenioids (41). We predict that with warming, icefish hearts may become hypoxic, more so than in red-blooded fishes. Indeed, our studies thus far support this conjecture. Levels of lactate increase nearly 2-fold under normoxic conditions during warming in hearts of icefishes but not red-blooded notothenioids (5).

Our results with notothenioid fishes are consistent with mounting evidence that the upper thermal limits of ectotherms are set by their ability to maintain aerobic metabolic scope, which is intrinsically linked to cardiac function (3, 6). Aerobic metabolic scope represents the difference between routine and maximum rates of oxygen consumption (i.e., V\text{O}_2 \text{max} – V\text{O}_2 \text{min}) . As temperature increases, both measures of metabolic rate increase concurrently, yet V\text{O}_2 \text{max} plateaus at a lower temperature than V\text{O}_2 \text{min} (Fig. 2) (42) . The temperature at which the greatest difference between V\text{O}_2 \text{max} and V\text{O}_2 \text{min} occurs (i.e.; greatest aerobic metabolic scope) represents the temperature optimum of the organism (\text{T}_\text{opt}). The temperature at which aerobic scope declines to zero is the critical temperature (\text{T}_\text{crit}). Studies have shown that organismal performance declines at temperatures above \text{T}_\text{opt}. For example, rates of successful sockeye salmon migrations in Canada plummeted to less than 11% when water temperatures exceeded \text{T}_\text{crit}. In contrast, 77% of returning salmon successfully migrated when water temperatures were at \text{T}_\text{opt} (40). Similarly, when 5-year average temperatures in the Wadden Sea rose above \text{T}_\text{opt}, abundance and growth rates of the eelpout Zoarces viviparous declined (43).
Loss of aerobic scope at high temperatures is closely linked to failure of cardiac function. To maintain or increase aerobic metabolic scope as temperature increases, cardiac output, a function of heart rate and stroke volume, must increase. Most fishes increase cardiac output in response to warming by increasing heart rate (i.e; 44, 45). At temperatures above $T_{\text{opt}}$, heart rate does not increase, resulting in a decline in aerobic scope. Consequently, heart rate is highly correlated with $T_{\text{opt}}$ and whole organismal performance (3, 43). It is generally accepted that Antarctic notothenioids, like temperate teleosts, increase cardiac output largely by increasing heart rate rather than stroke volume, and that the cardiovascular system is characterized by a low heart rate due to a high cholinergic (vagal) tonus on the cardiac pacemaker (31). These observations, however, are based on a few red-blooded species where considerable interspecific variability exists (46). It remains unclear whether this view is applicable to channichthyids. Most likely it is not, given the vast differences in the cardiovascular systems between icefishes and red-blooded notothenioids. Moreover, the ability to increase cardiac output in response to stress is likely reduced in icefishes because of their lower blood buffering capacity (47, 48) and reduced capacity to synthesize catecholamines, which regulate oxygen delivery in response to stress (49). Studies we propose here will (1) empirically test the hypothesis that cardiac function limits thermal tolerance in notothenioids (2) characterize cardiac performance in response to thermal stress in icefishes, filling major gaps in our knowledge of cardiac physiology of icefishes, and (3) determine thermal plasticity of cardiac function in both red- and white-blooded notothenioids.

3d. Mitochondrial function and ATP production in cardiac muscle of icefishes may be compromised as temperature increases. Our results suggest that cardiac function limits thermal tolerance of icefishes, yet the biochemical underpinnings of reduced cardiac function at elevated temperatures in notothenioids or other teleosts are largely unknown. We will determine if cardiac function is impaired in channichthyids due to a reduction in energy charge (i.e., ATP levels), which may result from oxidatively damaged mitochondrial proteins and/or phospholipids.

As oxygen supply becomes more limited at elevated temperatures, cardiac function must be maintained by adjustments in metabolism. Teleost hearts oxidize fatty acids and carbohydrates to produce ATP under aerobic conditions (50). Under hypoxic conditions, creatine phosphate (CrP) stores can be tapped as an energy source, and glycogen and glucose can be catabolized anaerobically (51, 52). Similarly, exposure to elevated temperatures can lead to hypoxia, resulting in a shift towards anaerobic metabolism in both temperate and polar fishes (53, 54). Yet, cardiac muscle of most teleosts has limited glycolytic capacity and its function cannot be sustained by anaerobic metabolism (55). Consequently, upper temperature limits of teleosts may be constrained by the heart’s ability to produce ATP as oxygen supply declines. Indeed, $T_{\text{opt}}$, heart rate and increased cardiac anaerobic metabolism (as indicated by increased levels of AMP-activated protein kinase) have been shown to be highly correlated in both rainbow trout and coho salmon (56), suggesting as others have, that the upper thermal limits of heart rate and thus aerobic scope, are delineated by the ability to sustain aerobic metabolism in the heart (57, 58).

Little is known about how, or if, cardiac metabolism is altered in Antarctic notothenioid fishes in response to elevations in temperature. Lactate increases in hearts of icefish Chaoencephalus aceratus in response to warming, but it is unknown if an increased reliance on anaerobic metabolism corresponds with a decline in energy charge (5). Moreover, lactate levels do not increase in hearts of the red-blooded Notothenia coriiceps in response to warming (5). This difference in physiological responses among red- and white-blooded notothenioids may stem from several factors, including a heart that may be sufficiently well oxygenated in the red-blooded species (even with warming), or differences in lactate clearance. We will assess changes in the energetic status of cardiac muscle in
response to elevated temperatures by measuring levels of the adenylates (ATP, ADP, AMP) as well as creatine (Cr) and CrP, in order to calculate and compare energy charge in hearts of animals held at ambient temperature and exposed to their C\textsubscript{TMAX}. If ATP and/or CrP levels remain stable despite cardiac failure, we can infer that compromised cardiac function is not due to an inability to maintain ATP production at elevated temperatures but rather perhaps, a decline in intracellular pH resulting from elevated lactate might be contributing to cardiac failure, or even alterations in calcium handling. Tissue pH will be measured, but evaluating calcium homeostasis is beyond the scope of this project.

We have determined that mitochondria from icefishes produce more ROS than red-blooded species when electron transfer is disrupted by chemical inhibitors that mimic hypoxia (59). Consistent with this, levels of oxidized proteins and lipids increase in hearts of icefishes (but not in the red-blooded species) exposed to C\textsubscript{TMAX}, suggesting mitochondrial function is impaired at elevated temperatures (4). Elevated levels of lactate in hearts of icefishes exposed to C\textsubscript{TMAX} may arise from the combined effects of hypoxia and mitochondrial dysfunction. In studies proposed, we will determine if mitochondrial proteins and phospholipids become oxidatively damaged in hearts of notothenioids during warming. We will quantify levels of, and identify oxidized mitochondrial proteins and activities of individual components of the respiratory chain in animals held under ambient conditions and exposed to C\textsubscript{TMAX} temperatures. This will enable us to determine if mitochondrial function is, in fact, compromised during warming.

We would also like to focus our attention on the dimeric phospholipid cardioliaptin (CL). CL is largely restricted to the inner mitochondrial membrane where it interacts with a large number of proteins (60). Although CL is “promiscuous” with its interactions among proteins, CL appears to be particularly important in the function of many proteins or protein complexes that are associated with oxidative phosphorylation, including complexes I, III, IV, the F\textsubscript{1}F\textsubscript{0}ATP synthase, cytochrome c, and the ADP/ATP carrier protein, as well as the pyruvate carrier and creatine kinase (60, 61). There is increasing evidence that CL is not only an essential factor in mitochondrial respiration but also a “green” phospholipid because it promotes efficiency during oxidative phosphorylation (62).

CL, with four acyl chains, includes a diverse array of molecular species because of the large number of potential combinations of chains varying in chain length (18 carbon fatty acids are most common) and degree of unsaturation (polyunsaturated fatty acids are typically abundant). The particularly high complement of polyunsaturated fatty acyl chains in CL, along with its proximity and involvement with complexes of the respiratory chain, make CL a likely target for lipid peroxidation (63). Generally, the more double bonds in a fatty acyl chain, the more susceptible that particular lipid is to lipid peroxidation (64, 65). Peroxidation of phospholipids can be accompanied by a decrement in membrane function, because of changes in the physical and chemical properties of biological membranes that ultimately influence function (66). Cardioliaptin hydroperoxide (CLOOH), in particular, is associated with mitochondrial dysfunction (67) and release of cytochrome c, a key step in apoptosis (68). We will test the hypothesis that white-blooded species have higher unsaturation indices in CL (thus potentially explaining the increase in lipid peroxidation at elevated temperatures), and elevated levels of cardioliaptin hydroperoxides (CLOOH) with warming, than red-blooded species.

\textit{3e. Antarctic notothenioids may be unable to induce protective mechanisms against thermal stress.}

At a critical temperature, onset and increased reliance on anaerobic metabolism represent a threshold, beyond which a molecular cascade is triggered to protect against, or minimize damage accompanying heat stress. Protective mechanisms increase in levels of heat shock proteins (Hsps), antioxidants, and the transcription factor, hypoxia-inducible factor-1α (HIF-1α) (69). Results indicate that evolution in the chronically cold, oxygen-rich waters of the Southern Ocean has diminished the capacity of Antarctic notothenioids to mount a robust defense against thermal stress (ie; 12, 54).

The first indication that Antarctic notothenioids possess a blunted response to thermal stress came from studies of the heat shock response (HSR). Upon exposure to elevated temperatures, levels of Hsps typically increase to prevent thermal denaturation of three-dimensional protein structure (70). Antarctic notothenioids have proven to be one of the few exceptions to the paradigm that HSR is conserved across all taxa. While constitutive levels of Hsps are high in Antarctic notothenioids, Hsps are not induced in response to heat stress (12, 71).

More recent studies also hint at the possibility that other defenses against thermal stresses may be limited in Antarctic notothenioids. Heat stress can trigger an increase in oxidative damage of
macromolecules and typically, antioxidant defenses rise to match and counter those changes (72-75). While heat stress increases levels of oxidized proteins and lipids in icefishes, it does not lead to a concurrent increase in activities, or mRNA levels, of the major enzymatic antioxidants, superoxide dismutase or catalase, in either red- or white-blooded notothenioids (4). While the antioxidant defense system is extensive and includes both enzymatic and low molecular weight antioxidants, we cannot rule out the possibility that one or more of these other antioxidants might respond to thermal stress. Due to the complexities of the antioxidant defense system, we will focus our studies on another ubiquitous, critical component of the stress response: hypoxia inducible factor (HIF).

HIF is considered the master regulator of the hypoxic response (76). HIF is a heterodimer, composed of constitutively expressed α and β subunits. In an oxygen and iron-dependent reaction, catalyzed by prolyl hydroxylase, the α subunit is hydroxylated at proline 402 and/or 564 (77). Hydroxylation targets the α subunit for ubiquitination and degradation by the proteasome, thereby suppressing HIF DNA binding under normoxic conditions. Hypoxia prevents hydroxylation of the proline residue(s), resulting in stabilization of the α subunit, assembly of the heterodimer, translocation to the nucleus and binding to the highly conserved hypoxia response element (HRE), present in over 100 genes essential for surviving low oxygen conditions. HIF binding stimulates a shift towards anaerobic metabolism, angiogenesis, erythropoiesis and decreased mitochondrial respiration (78). Although Antarctic notothenioids are unlikely to encounter hypoxic conditions in their environment, tissue-level hypoxia might warrant stabilization and activation of HIF, particularly during periods of elevated oxygen demands (e.g., in a warming climate), yet whether notothenioids have the ability to increase HIF activity in response to thermal stress remains equivocal.

Mixed results have been obtained on HIF-1α expression and HIF activity after acute or acclimatory changes in temperature in notothenioids. We have found that HIF-1α mRNA levels are 6.6-fold higher in hearts of red-blooded notothenioids compared to icefishes but remain unchanged in the icefish C. aceratus or the red-blooded N. coriiceps in response to temperature ramping and/or hyperoxia (5). In contrast, others have found that HIF-1α mRNA levels increased in hearts of N. coriiceps and decreased in pectoral adductor in C. rastrirostris in response to exposure to CT_MAX (2). Similarly, HIF DNA binding to the human erythropoietin enhancer increased in liver extracts from Pachycara brachycephalum that were warm-acclimated to 4°C (79). However, warm-acclimation of P. brachycephalum to 5°C did not result in an increase in HIF-1α mRNA levels despite a shift from fatty acid to glycolytic metabolism, typically regulated by HIF in mammals (80). Additionally, HIF-1α expression was unchanged in gill of Trematomus bernacchii following a 4hr heat shock at 4°C despite an apparent shift towards glycolytic metabolism (54). Transcriptome analysis of liver tissues from Harpagifer antarcticus exposed to a longer heat stress of 6°C for 48 hr also failed to detect an increase in HIF-1α, yet increases in the expression of antioxidant genes typically regulated by HIF were detected (81). What appear to be conflicting results may be due to differential effects of temperature on HIF protein and mRNA levels and tissue-specific responses. While HIF-1 is typically not transcriptionally regulated in mammals (82), it is in teleost fishes (83, 84). We hypothesize that the heart is the most likely tissue to become hypoxic during warming, and that protein levels and DNA binding activities of HIF will increase, particularly in the icefish heart. The experiments we propose will reveal capacities of notothenioids to increase HIF and enhance HIF DNA binding in response to thermal stress.

3f. Neuronal function may limit thermal tolerance, particularly in red-blooded notothenioids.

Temperature has profound and differential effects on many aspects of neuronal circuitry (85-88). Sensitivity of the neuronal system to warming can also be a major factor in the thermal tolerance limits of ectotherms (e.g., 89, 90) and most likely in Antarctic fishes as first suggested by Somero and DeVries (14). In our own studies, we have observed marked changes associated with warming in the behavior of the red-blooded N. coriiceps, much more so than for the icefish C. aceratus. N. coriiceps displays hyperactivity and uncoordinated swimming at temperatures below CT_MAX. These are behaviors very similar to those observed after warming the cerebellum in goldfish, which results in progressive neuronal failure (89). Because none of the behavioral attributes associated with cerebellar heating were observed under hypoxic conditions in goldfish (89), these observations further support our hypothesis that the physiological underpinnings associated with thermal tolerance limits in red-
blooded notothenioids may not be related to hypoxia, but instead are more likely to be governed by
thermal sensitivities of the neuronal system. We will directly test this hypothesis by conducting an
experimental heat ramp (whole body warming as we have done in the CT_MAX trials) and also specific
warming of the cerebellum of red- and white-blooded notothenioids. We will closely monitor
temperature thresholds of behavior for both treatments. We anticipate that behaviors in both
treatments will be temperature-matched for the red-blooded N. coriceps but not in the white-blooded
C. aceratus. We expect icefish to succumb to cardiac hypoxia with whole animal warming, at a
temperature below which there will be signs of neuronal failure from warming of the cerebellum.

Membrane integrity may also be a key player in resistance adaptation and an organism’s
susceptibility to heat stress (90, 91). Our preliminary results indicate that the red-blooded N.
coriceps accrues an approximately 10% rise in serum osmolarities at CT_MAX compared with animals
at ambient temperatures (Crockett and O’Brien, unpublished). We did not, however, find a similar
result in the icefish C. aceratus at its CT_MAX, suggesting that there is no loss of membrane integrity in
the icefish at its lower CT_MAX. The change in serum osmolarity in N. coriceps is similar to what was
reported for another Antarctic notothenioid, P. borchgrevinki, when exposed to an acute heat stress
(10°C) (92). These data indicate the barrier function of biological membranes likely becomes
compromised with warming, and may contribute to thermal tolerance limits in red-blooded but not
white-blooded species. We will investigate this further by comparing physical properties and
Arrhenius break temperatures of biological membranes (cardiac mitochondria and brain
synaptosomes) in both red- and white-blooded notothenioids.

3g. Can icefishes acclimate to warmer temperatures? Several studies have shown that some red-
blooded Antarctic notothenioids are capable of acclimating to warmer temperatures. Notothenioids
from both McMurdo Sound and the WAP significantly increase their CT_MAX when acclimated to 4°C
for between 7 and 21 days (35). Notothenioids from both regions are also capable of heat hardening,
meaning that upon exposure to their CT_MAX and following recovery, they significantly increase their
CT_MAX when remeasured (93). Cardiac function of notothenioids also exhibits plasticity. The factorial
scope of cardiac output is higher at temperatures between 4°C and 8°C in P. borchgrevinki acclimated
to 4°C for 4-5 weeks than those held at -1°C (94, 95). Resting metabolic rates decrease during warm
acclimation in P. borchgrevinki and by 28 days are equivalent to that of animals held at -1°C (96).

To date, thermal plasticity has only been described for red-blooded notothenioids. Whether
icefishes have a similar capacity to acclimate to warmer temperatures is unknown. Moreover, the
molecular drivers of a rise in CT_MAX following warm acclimation are unknown. We will address these
questions in our research by acclimating red- and white-blooded fishes to elevated temperatures. We
will then measure CT_MAX and quantify changes in cardiac function. We will also compare maximal
activities of oxidative enzymes, mitochondrial function, and physical properties of biological
membranes from red- and white-blooded species held at both ambient and elevated temperatures in
order to assess the capacity of channichthysids to adapt to a warming environment.

4. Proposed Studies

Objective 1: Determine if cardiac function contributes to thermal tolerance of notothenioids.
We will monitor heart rate with acute temperature ramping to CT_MAX in Notothenia coriceps (+Hb),
Chaenocephalus aceratus (-Hb) and Chaenodraco rastrospinosus (-Hb) (N= 8 for each). This will
allow us to determine if onset of heart rate variability coincides with CT_MAX. Heart rate measurements
will be led by Egginton, an expert in the cardiovascular physiology of temperate and Antarctic fishes
(47, 48, 97, 98). In parallel, Farrell will lead experiments using an in situ preparation of hearts to
measure thermal sensitivity of cardiac work; he is an expert in this technique (ie; 99, 100-102). These
experiments permit us to determine if thermal sensitivity of cardiac performance is correlated with
lower thermal tolerance of icefishes. Additionally, we will capitalize on the in situ heart preparation to
assess contributions of adrenergic and cholinergic tone to cardiac function of icefishes.

4.1a In-vivo cardiac performance during exposure to CT_max: Fish will be anaesthetized in MS222
(1:7500) dissolved in seawater and placed dorsal side up on a thoracic cradle. Gills will be irrigated
with MS222 (1:10,000) dissolved in seawater. Electrocardiogram (ECG) recording electrodes will be
inserted through the opercular septum at the base of the left fourth gill arch using a hypodermic
needle, ensuring that the pericardial cavity is not compromised. Bipolar ECG signals will be recorded
using a bioamplifier (ML 136) interfaced with a digital recording system (PowerLab, ADInstruments, USA). Fish will be allowed to recover for 24-48 hr (according to recovery of heart rate variability determined from ECG) before measuring $C_{T_{\text{MAX}}}$ as described previously (5). Briefly, fish will be placed in a 700L Xactics tank fitted with a titanium heater and equipped with flow-through seawater. Water will be aerated with Silent Giant air pumps, which also prevent thermal stratification. Water flow will be turned off and water heated at a rate of 4°C per hr. Water temperature will be recorded with a Hobo temperature logger and $C_{T_{\text{MAX}}}$ determined as the temperature at which animals lose the ability to right themselves (2, 5).

4.1b In situ cardiac performance of notothenioid fishes: The main advantage of the in situ perfused heart preparation over other in vitro models is that a maximum level of cardiac performance can be achieved that is equal to that observed in swimming fish (99, 101, 103). Thus, extrapolation from in vitro to in vivo can be performed with greater confidence, as has been done for a wide range of temperate fish species (e.g., 102). This superior performance derives from the fact that the heart is never exposed or handled until after the experiment is completed. Measurements will be made in 6-8 hearts from *N. coriiceps*, *C. rastrosinosus* and *C. aceratus* at 0°C and 4°C for fish held at ambient temperature and acclimated up to 4°C, allowing us to determine how cardiac function responds to acute thermal stress and its thermal plasticity.

**Acclimation regime.** We are uncertain if icefishes will tolerate acclimation to 4°C for as long as 4 weeks, as is routinely done with red-blooded notothenioids. We have, however, successfully acclimated the icefish *C. rastrosinosus* to 4°C for one week. Therefore, we will acclimate both *C. aceratus* and *C. rastrosinosus* to temperatures of 2°C, 3°C and 4°C for up to 4 weeks to determine the maximum duration and possible acclimation temperature we can employ for these experiments. Temperatures will be raised from ambient by 0.5°C per day until desired temperature is reached. The same acclimation regime will be used in objective 5 when we further test the capacities of icefishes and red-blooded species to acclimate to warmer temperatures.

**In situ heart preparation.** Hearts will be prepared from anaesthetised (0.1 g L⁻¹ MS222) fish. Cardiac perfusion containing a tonic level (5 nM) of adrenaline (AD) will begin immediately after a stainless steel input cannula has been secured into the sinus venosus via an exposed hepatic vein. Remaining hepatic veins will be ligated. A similar output cannula will then be secured in the ventral aorta at a point confluent with the bulbus arteriosus. Thus, flow of perfusate to and from the heart is isolated, the pericardium remains intact and the heart generates its own heartbeat, which pumps perfusate against a fixed diastolic pressure head set to achieve a routine ventral aortic blood pressure of ~2.5 kPa (but is variable if needed). The entire preparation will be immersed in a temperature-controlled bath of 0.8% saline at either 0°C or 4°C. Cardiac output (Q) will be measured in the output line with a 4.0 mm Transonic probe and flowmeter and will be set at a routine in vivo Q (red-blooded ~13 mL min⁻¹ kg⁻¹ body mass; white-blooded ~100 mL min⁻¹ kg⁻¹ body mass) using fine adjustment of the cardiac filling pressure, which varies cardiac stroke volume. The bath and perfusate temperatures will be maintained with a circulating cooling unit. Filling and output pressures will be measured at the tips of the input and output cannulae, respectively, and relative to the saline level in the bath via fluid-filled tubes connected to pressure transducers. The flow and pressure signals will be amplified and stored at a rate of 10 Hz on a Dell Latitude portable computer, interfaced via a Powerlab system using the software LabChart Pro software. The cardiovascular signals will be viewed on-line throughout the experiment and processed to generate an on-line display of myocardial power output. These inotropic characteristics will be complemented by chronotrophic readout of heart rate ($f_H$).

After a 20 min equilibration period, maximum cardiac performance will be assessed several times using different levels of adrenergic stimulation. Maximum cardiac pumping ability ($Q_{\text{MAX}}$) will be determined using incremental increases in filling pressure (i.e. a Starling response). Using $Q_{\text{MAX}}$, diastolic output pressure will then be incrementally raised until a maximum power output is generated. Routine cardiac performance will be restored prior to a new test condition. We will examine maximum performance with and without the effects of β-adrenergic simulation (varying adrenaline concentration and adding adrenergic antagonists). This range of tests will be performed in a 2×2 experimental design for two test temperatures and two acclimation temperatures. Cardiovascular variables (input pressure, output pressure, stroke volume, Q and $f_H$) will be stored using custom.
software built in LabView. Power output (mW g⁻¹ ventricle mass) will be calculated from product of [Q (mL min⁻¹) x (output pressure minus input pressure) (kPa) x 0.0167] / ventricular mass (g). Ventricular mass will be determined at the conclusion of each experiment when the cannulae are visually checked for correct positioning, and expressed relative to body mass (g). Myocardial power output will be reported as mW g⁻¹ ventricle mass and Q is reported as mL min⁻¹ kg⁻¹ body mass.

**Objective 2: Characterize metabolic and transcriptional responses to elevated temperature.** It has been proposed that an organism’s window of thermal tolerance is defined by its capacity to maintain aerobic metabolic scope (69). Metabolic rate increases concurrently with temperature, yet oxygen delivery may not match demand, resulting in a greater reliance on anaerobic metabolism. Consistent with this, we and others have found that levels of the anaerobic endproducts, lactate and succinate, increase in hearts and blood of Antarctic fishes at, or near CT_MAX (2, 5, 104). Here we will determine if adenylate levels (i.e., energy charge) and CrP are maintained in hearts of red- and white-blooded notothenioids exposed to CT_MAX. We will test the hypothesis that ATP and CrP levels decline in hearts of icefishes exposed to CT_MAX compared to those at ambient temperature but remain unchanged in hearts of red-blooded species. Additionally, increases in lactate in hearts of icefishes (5) suggest that hearts may become hypoxic during warming. We will determine if HIF-1α levels and/or HIF-1 binding activity increase in response to warming or if the ability to induce HIF has been lost during evolution at cold temperature in notothenioids. There are three HIF α genes in teleosts (105). Because HIF-1α is best characterized and ubiquitously expressed, it will be the focus of our studies. We will test the hypothesis that HIF DNA binding increases in hearts of icefishes at CT_MAX but not in red-blooded notothenioids. If it does not, then we will test the hypothesis, that one or more of the key regulatory domains in HIF-1α have been mutated, preventing dimerization and DNA binding. Hearts for these studies will be obtained from control and CT_MAX exposed animals used in objective 1 (N. coriiceps (+Hb), C. aceratus (-Hb) and C. rastrospinosus (-Hb); N=8 for each).

**Energetic status of cardiac and brain tissue.** Tissues will be harvested, freeze-clamped and stored at -80°C. Levels of ATP, ADP, AMP, CrP, creatine and lactate will be measured enzymatically (106). pH of tissue homogenates will be measured as described previously (107). Briefly, tissues will be ground in liquid nitrogen and resuspended in 150 mM KF, 6 mM disodium nitrolotriacetic acid (NTA) to inhibit metabolic reactions, centrifuged and pH of the supernatant measured using a thermostated micro-capillary electrode. Although we have quantified lactate levels in hearts of C. aceratus and N. coriiceps at ambient temperature and CT_MAX, we have not done so in hearts of C. rastrospinosus and we would like to look for correlates between levels of lactate and adenylates.

**HIF DNA binding.** DNA binding of HIF protein will be assessed by electrophoretic mobility shift assays. There are currently no HIF DNA binding sites known for fish. However, previous studies of HIF binding in teleosts, including crucian carp, rainbow trout and the Antarctic eelpout successfully employed the sense and antisense strands of the HIF-binding sites within the promoter region of the human erythropoietin gene to test HIF binding efficacy (108-110). Nuclei will be extracted from hearts of red- and white-blooded fishes held at ambient temperature and exposed to CT_MAX as described previously (111) and incubated with biotin-labeled oligonucleotides for 30 min. Controls will include excess unlabelled probe. Nuclear extracts will be mixed with loading buffer, separated on 6% native acrylamide gels and then transferred to a nylon membrane, UV crosslinked and probed with a streptavidin-HRP conjugate detectable by chemiluminescence (Thermo Scientific). DNA binding will be quantified using Image Quant v8.1 (GE Healthcare). Of concern is the ability of HIF to bind to the promoter of erythropoietin in icefish. If HIF binding is not observed we will use the promoter regions of other known HIF-responsive genes, such as the glucose transporter, GLUT 1 and/or the glycolytic enzyme phosphoglycerate kinase.

**HIF protein levels.** We will also attempt to quantify HIF protein levels using western blotting. Studies of HIF-1 protein levels in teleosts have used a HIF-1α primary antibody directed against the N-terminal domain of HIF-1α in rainbow trout (109, 110). However, this antibody did not recognize HIF in the Antarctic eelpout P. brachycephalum (79), suggesting that it will likely not work for our studies. Alternatively, we will use two primary antibodies directed against the highly conserved hydroxylated proline residues (Pro564 and Pro402) (Abcam).
**HIF-1α gene structure.** We will obtain full-length cDNA sequences of HIF-1α in *C. aceratus*, *C. rastrospinosus* and *N. coriceps*. Degenerate primers will be designed from conserved regions within the HIF-1α cDNA sequences of other teleosts (zebrafish, stickleback, carp, trout) for cloning and sequencing partial regions of HIF-1α from at least 2 individuals from each species as described (112). This will allow us to then design gene-specific primers and use Rapid Amplification of cDNA ends (RACE) PCR (Clonetech Laboratories) to obtain full length cDNA sequences. We will align the sequence with other teleosts and mammals, and determine homology, paying close attention to several important domains within HIF-1α, including a basic Helix Loop Helix domain (bHLH) for DNA binding and specificity; Per Arnt Sim (PAS) A and B domains and PAC domain, also important for dimerization; an oxygen-dependent degradation domain (ODD), critical for oxygen-dependent regulation; and two transactivation domains (TADs), important for interactions with other transcription factors (113). Sequences will be analyzed with support and software provided by the UAF Life Sciences Informatics Group.

**Objective 3: Determine if mitochondrial and membrane function contribute to thermal tolerance limits.** The increase in lactate levels in hearts of icefishes at CT_{MAX} may be due to either inadequate oxygen delivery and/or mitochondrial dysfunction, leading to an increase in anaerobic metabolism. Our data indicate that hearts of white-blooded species experience a sharp rise in lipid peroxidation (LPO) and oxidized proteins at CT_{MAX} while red-blooded species do not (4). Our goal in this objective is to determine if oxidation of mitochondrial phospholipids and proteins in hearts of icefishes leads to mitochondrial dysfunction, thereby contributing to their lower thermal tolerance compared to red-blooded species. Hearts for these studies will be obtained from the control and CT_{MAX} exposed animals (*N. coriceps* (+Hb), *C. aceratus* (-Hb) and *C. rastrospinosus* (-Hb); N=8 for each) obtained in objective 1. We hypothesize that mitochondria in hearts of white-blooded species have a higher unsaturation index in cardiolipin (CL) than red-blooded species, leading to higher levels of peroxidized CL at CT_{MAX} compared to red-blooded notothenioids. We will also test the hypothesis that exposure to CT_{MAX} results in an increase in oxidation of mitochondrial proteins and lipids causing a decline in mitochondrial function in hearts of icefishes but not red-blooded species.

**Unsaturation of cardiolipin (CL) in mitochondria.** Mitochondria will be isolated as described previously (59) from hearts of red- and white-blooded notothenioids held at ambient temperature and exposed to CT_{MAX}. Mitochondrial lipids will be extracted (114) and phospholipids isolated by ice-cold acetone precipitation and stored at -80°C under a nitrogen atmosphere in Teflon-capped vials. CL will be separated from other phospholipids by preparative thin-layer chromatography. CL will be extracted from the silica gel, solvent evaporated and samples sent to the NSF-supported Kansas Lipidomics Research Center (KLRC; PI, Dr. Ruth Welti) where samples will be analyzed. The results will provide the amount of CL at each mass (i.e., at the level of total acyl carbons: total double bonds), quantified by signal in comparison to the internal standard, and the fatty acid composition of the cardiolipins at the mass (as % of signal for each fatty acid). Unsaturation indices will be calculated for the cardiolipins. These data will be used to corroborate analyses of CL peroxidation.

**Peroxidation of mitochondrial phospholipids.** Mitochondrial lipids will be extracted as described above. PLOOH will be separated by high-performance thin-layer chromatography (HPTLC) with tetramethyl-p-phenylenediamine detection (115). Dried phospholipid samples will be re-suspended in hexane:isopropyl alcohol (IPA, 3%), and samples spotted onto glass Silica G chromatography plates (Sil60; EMD) using a Linomat5 semiAutomatic sample applicator (Camag Industries). Silica G plates will be dried at 120°C for 1 hr immediately before use. Four separate lanes containing a different amount (1, 3, 7.5, and 12 nmoles) of the PLOOH 13-hydroperoxy-9,11E-octadecadienoic (13-HpODE; Cayman Chemicals) will be included on every plate for preparation of a standard curve. We have used this method previously and have found that we can visualize, identify, and quantify five PLOOH classes (PC, PE, PS, PI, CL) plus the standard. Identification will be based on Rf values determined from HPTLC runs of pure individual samples. Image J-derived densitometric values for each PLOOH class, and for the standard, will be correlated proportionately with the amount of starting lipid.

**Mitochondrial protein oxidation.** Mitochondria will be isolated as described above. Mitochondrial protein carbonyl levels will be quantified by HPLC as described previously (4).
Oxidized proteins will be identified using isobaric labeling (iTRAQ) and quantitative mass spectrometry (116, 117). The 8-Plex iTRAQ labeling system (Sciex) enables us to differentially label oxidized cysteine residues from all 3 species and 2 treatments (6 conditions total) so that we can identify oxidized proteins and quantify level of oxidation of each protein relative to other species and treatments (118). Following iTRAQ labeling, samples will be mixed, trypsin digested and analyzed by LC coupled LTQ Orbitrap tandem MS at the Univ. of Nevada Proteomics Center. Proteins will be identified using MASCOT and SEQUEST.

Mitochondrial respiration and enzyme activity. Mitochondria will be isolated as described above. State III and IV respiration rates will be measured at 2°C in isolated mitochondria as described previously (59, 119). The maximal activity of each individual component of the respiratory chain will be measured spectrophotometrically at 2°C as described by Kirby et al. (120). Briefly, mitochondria will be diluted in a hypotonic buffer of 25 mM potassium phosphate, 5 mM MgCl₂, pH 7.2. Activity of complex I will be assayed by following the oxidation of NADH at 340 nm in the presence of the electron acceptor, ubiquinone. Background activity will be measured in the presence of antimycin A. Complex II will be measured as the succinate:ubiquinone oxidoreductase activity linked to the electron acceptor, DCPIP in the presence of antimycin A (inhibits complex I) and rotenone (inhibits complex III). The rate of oxidation of DCPIP is followed at 600 nm. Complex III activity will be measured as the rate of reduction of cytochrome c using the electron donor ubiquinol in the presence of KCN (inhibits complex IV) at 500 nm. Complex IV will be measured by following the oxidation of cytochrome c at 500 nm. Complex V (F₁,ATP synthase) activity is assayed in reverse as F₁-ATPase. The production of ADP by the F₁-ATPase is used to convert phosphoenolpyruvate to pyruvate (by the addition of pyruvate kinase), followed by the reduction of pyruvate to lactate (by the addition of lactate dehydrogenase) coupled to the oxidation of NADH that is followed at 340 nm.

Objective 4: Determine if neuronal function limits thermal tolerance in notothenioids. We propose a series of experiments to test the hypothesis that the physiological underpinnings responsible for limiting thermal tolerance are different in white- and red-blooded notothenioids. While we predict that cardiac hypoxia limits thermal tolerance of icefishes, we also predict that cardiac failure occurs at a temperature lower than would neuronal dysfunction. In contrast, we predict that neuronal failure limits the thermal tolerance of red-blooded notothenioids. We also anticipate that the temperature associated with neuronal failure is similar for both red- and white-blooded fishes. We can test these hypotheses directly by using an approach that will include two sets of experiments. First, we will warm the whole animal and quantify behavioral outcomes. Secondly, we will warm solely the cerebellar portion of the brain. This will allow us to warm brains of icefishes beyond their CT_MAX, enabling us to determine if neuronal failure occurs at the same temperature for red- and white-blooded species. These experiments will be led by Dr. Michael J. Friedlander, who performed regional brain warming in seminal experiments with goldfish (89).

Whole animal warming. We will systematically quantify behavioral responses to whole animal warming in red- (N. coriceps) and white-blooded (C. aceratus) fishes (N=6-8) while warming at rates of 4°C per hr. Behavior will be monitored under red light illumination, and digitally recorded (Samsung SDE-3001 4-Channel DVR with 4 Weatherproof Night Vision Cameras). We will quantify frequencies of opercular beats and pectoral fin sculling, changes in position and orientation, and spontaneous swimming movements. Behavioral endpoints will be defined as described (121) and will include hyperexcitability, loss of equilibrium, and coma. Data will be analyzed using customized software (JWatcher). In addition, regular temporally-spaced stimulation (mechano-acoustic stimuli at 20 Hz to elicit rapid flank flexion via the M-cell-spinal motoneuron pool reflex with time for recovery from habituation between stimuli and mechanical touch to the flank) will be applied to measure amplitudes and reliability of reflex responsiveness.

Effects of localized warming. Animals (red-blooded N. coriceps and the icefish C. aceratus; N=6-8) will be anaesthetized in MS222 (1:7500) dissolved in seawater and placed dorsal side up on a thoracic cradle. Gills will be irrigated with MS222 (1:10,000) dissolved in seawater. A constant-temperature thermocouple will be surgically implanted on the surface of the corpus cerebellum. This will be accompanied by the simultaneous implantation of a surrounding thermode assembly composed of 22 gauge stainless steel tubing that will have warmed solution circulated (89) for subsequent
behavioral analysis of acute localized cerebellar warming. After 6 hr recovery (and when the animal shows normal swimming, reflex and breathing behavior) the behavioral effects of localized cerebellar warming will be tested using a heat ramp similar to that described above for whole animal warming. Behavioral outcomes/thermal thresholds for cerebellar warming experiments will be monitored as described for, and compared with, whole-animal warming. At the conclusion of the behavioral experiments with thermode/thermocouple implantation, animals will be re-anesthetized in MS-222 and paralyzed with i.m. injection of d-tubocurarine to eliminate spontaneous movement while gill perfusion is maintained with MS-222 aerated seawater. Acute extracellular electrophysiological recordings will be made from individual Purkinje cells (PCs) in cerebellar cortex with tungsten microelectrodes in order to evaluate the effects of acute warming on PC spontaneous frequency and temporal patterning of spike bursts (89). This will enable us to determine if regular intervals of electrical activity (i.e., action potential bursts) become irregular with warming, and is correlated with motor behaviors (hyperexcitability, rapid swimming). We predict that behavioral endpoints associated with cerebellar warming will be similar in both species and reached at comparable temperatures (16-17°C), indicating unequivocally that neuronal failure is not responsible for reduced thermal tolerance in the icefish, C. aceratus.

**Objective 5:** Determine if acclimation to 4°C improves cardiac performance at elevated temperatures and extends thermal tolerance. Measuring cardiac work *in situ* (objective 1) allows us to experimentally manipulate pre-load and afterload pressures and determine the dynamic range of cardiac function, as well as contributions of cholinergic and adrenergic tone to cardiac function. In contrast, measuring cardiac work *in vivo*, as we will do in these experiments, allows us to assess routine cardiac function and how it responds to both acute and acclimatory changes in temperature. Additionally, we will measure how aerobic metabolic scope changes with acute increases in temperature and in animals acclimated to 4°C, allowing us to empirically test the hypothesis that the T_{OPT} of icefishes is lower than that of red-blooded notothenioids (Fig. 1). These experiments will be led by Axelson, an expert in fish cardiovascular physiology with extensive experience working on Antarctic fishes (ie; 31, 122, 123), and assisted by Egginton. This objective will focus on the acclimatory responses of *N. coriceps* and *C. rastrosinops* (N=8 for each). The acclimation regime will be determined during our first field season, as described above in objective 1 (section 41b) and these experiments will be conducted during our second field season.

*In-vivo thermal plasticity of cardiac performance.* Individual fish will be anaesthetised as described for *in situ* heart studies in objective 1. Fish will be weighed and transferred to an operating cradle where the gills can be continuously irrigated with seawater containing a lower dose of the anaesthetic (50 mg l⁻¹). To measure cardiac output (Q), the ventral aorta will be dissected free close to the base of the fourth gill arch and a Transonic 1.5SL transit-time blood flow probe (Transonic Systems Inc) will be positioned around the vessel without damaging the pericardium. To measure dorsal aortic blood pressure (Pda), the dorsal aorta will be cannulated via the third efferent branchial artery with a PE-50 catheter tipped with ~1cm of PE-10 and filled with heparinised (100 IU ml⁻¹) saline (1% NaCl). The ventral aortic blood pressure (Pva) will be measured using a P50 catheter inserted into the afferent artery in the same gill. After surgery the fish will be placed in a covered respirometer to minimize visual disturbance of the fish, and animals will be left to recover from surgery for 24-48 hr.

An intermittent flow respirometer system will be used to record oxygen consumption (MO₂). It will be placed in a larger tank supplied with a continuous flow of aerated seawater. We will increase temperature at a rate of 4°C per hr, as done for measuring C\text{MAX} but will close the respirometer and hold the temperature at 3°C intervals and measure rates of respiration. A submersible pump circulating the water in the respirometer and an oxygen optode (Firesting) will be placed in-line with the mixing pump to record oxygen levels. A second pump, controlled by a custom-built digital time relay, will be used to flush and close the respirometer at regular intervals. MO₂ will be calculated from the drop in oxygen levels in the closed state. Background oxygen consumption will be measured in the empty respirometer.

The dorsal and ventral aortic catheter and the flow probe leads will be exteriorized *via* a small opening at the top of the respirometer. Pda and Pva will be recorded by connecting the catheter to a
DPT-6100 pressure transducer (pvb Medizintechnik) that is regularly calibrated against a static column of seawater. The signal from the transducer will be amplified using a 4Champ amplifier (Somedic, Hörby). The flow probe leads will be connected to a Transonic blood flow meter (Model T206, Transonic Systems Inc). Voltage signals from the recording equipment will be fed into a PowerLab system (ADInstruments Pty Ltd, Castle Hill, Australia) connected to a laptop computer running LabChart Pro software (ADInstruments Pty Ltd, Castle Hill, Australia) for data acquisition and subsequent data analysis. Heart rate will be measured as described in objective 1.

An extracorporeal loop formed by connecting the afferent and efferent branchial cannulae will be used to record partial pressure of oxygen (PO2) in the dorsal and ventral aortic blood. An oxygen electrode (type 16-73) inserted into the blood line, connected to a meter (type OM-4, Microelectrodes Inc) will be used to measure PO2. This allows direct measurement of both pre-branchial (P02) and post-branchial (P02) oxygen tensions without significantly affecting blood volume of the fish. The oxygen electrode will be submerged in the experimental chamber to keep it at the same temperature as the fish.

Objective 6: Molecular basis of differences in thermal tolerance between red- and white-blooded notothenioids and remodeling of cardiac muscle and neuronal tissue in response to warm acclimation. Experiments described here are designed to elucidate the capacity of notothenioids to remodel biological membranes and maintain mitochondrial function with warm acclimation. We will compare membrane fluidities and Arrhenius break temperatures (ABT) of synaptosomal and cardiac mitochondrial membranes, and mitochondrial state III respiration rates from C. rastrospinosus and N. coriceps held at ambient temperature and warm-acclimated. Tissues will be harvested from animals used in objective 5. We predict that membrane fluidity of synaptosomes from red- and white-blooded notothenioids will be comparable at the threshold temperatures for neuronal failure in each species (as measured when heating the cerebellum) as was shown for temperature-acclimated goldfish (121). On the other hand, we expect that the ABT for cardiac mitochondrial membranes will be lower for the icefish than for the red-blooded species. Additionally, we hypothesize that warm acclimation will increase ABTs of mitochondrial state III respiration rates and mitochondrial and synaptosomal membranes. We also predict that warm acclimation will decrease the activity of aerobically-poised enzymes in cardiac muscle.

Mitochondrial and synaptosomal membrane fluidity and Arrhenius break temperatures. Mitochondria will be isolated by differential centrifugation (119) and membranes stored frozen (-80°C). Synaptosomal membranes will be prepared using a discontinuous sucrose gradient (121). Steady-state fluorescence depolarization will be measured using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) according to (124). Fluorescence will be monitored with a Perkin-Elmer LS-50B spectrofluorometer with excitation and emission monochrometers set at 356 nm and 430 nm, respectively. Polarization values will be recorded at 2°C intervals from 2°C to at least 30°C. ABT will be determined using the statistical procedure as described (1).

Mitochondrial respiration and enzyme activity. State III respiration rates will be measured at 2°C as described in objective 3. The ABT of state III rates will be measured as describe in (119). Measurements will be made in mitochondria isolated from hearts of animals held at ambient temperature and acclimated to 2-4°C. Maximal activities of citrate synthase (CS) and cytochrome c oxidase (COX) will be measured at 2°C in hearts of control and acclimated animals. The activity of COX will be measured in fresh tissues as described by Wharton and Tzagoloff (125). Activity of CS will be assayed in tissues as described previously (126, 127).

Sample size and statistical analyses: For all experiments unless otherwise noted, we will use a sample size of 8, which we have found previously to be suitable for thermal tolerance experiments. Measurements among species and treatments will be analyzed statistically using a 2- or 3-way ANOVA where appropriate (JMP software).

5. Broader Impacts

There is no question that the region of the Antarctic Peninsula is warming at a rapid rate. A key and timely challenge for Antarctic scientists is to learn how individual species, populations, and ecosystems will respond to these changes. Our proposed research will contribute to this and in addition, will elucidate the capacities of Antarctic notothenioids to adjust physiological and
biochemical processes to a warming environment. This research will train graduate, undergraduate, and high school students in integrative and comparative thermal biology, biochemistry, cardiovascular physiology, and neuroscience. The proposed work will be of considerable interest to scholars of polar and thermal biology, and scientists interested in potential outcomes related to climate change. It will also make important contributions to the fields of metabolic biochemistry and cardiovascular physiology, as well as neurobiology. In all of these areas there is a long-standing and renewed interest in thermal effects. We also propose to continue our successful collaboration with Paula Dell, a biology teacher at Lindblom Math and Science Academy in South Central Chicago (69% Black, 26% Hispanic). Paula accompanied us on our 2011 field season and is joining us again during our 2013 field season. We propose to continue our collaboration with Paula and her students. With the support of the program Oceans Wide, Paula and her students will construct a camera system that can be deployed from the LMG for extended periods of time (up to 24 hr) and record images using time-lapse photography. In preparation, 2 Lindblom students and Paula will attend a 2-week segment of the Oceans Wide Intern Program held at the Darling Marine Center in Maine during the summer of 2016. The president of Oceans Wide, Campbell “Buzz” Scott has served as a Marine Projects Coordinator on both the LMG and Nathaniel B. Palmer. Having worked in the Antarctic, he understands the challenges of working in this remote and cold environment. He has also advised Paula and her students in constructing the tethered camera system we are deploying in 2013. Crockett will visit with students in Maine, as she spends part of her summer nearby at the Mount Desert Biological Laboratory. Crockett will also visit with students in Chicago and both O’Brien and Crockett will meet regularly with students and Paula using Skype, as we have done for the last year.

6. Management Plan and Timeline. Coordination of our collaborative research is key to successful execution and completion of the proposal’s objectives, along with communication of the results. O’Brien will oversee and coordinate the cardiovascular work, and will be responsible for the mitochondrial bioenergetics and molecular biological components of the research. Crockett will oversee and coordinate the neurophysiology and membrane biochemistry. We will foster and enhance our collaboration with a series of regular video-conferences and include students in this process. Regular communication will enable us to anticipate specific needs, and design and plan animal collections to make use (where possible) of the same animal for more than one experiment. Not only is this important to minimize the impact of our field collections on the Antarctic ecosystem, but it will allow us greater statistical and interpretative power in our analyses. We will be in more frequent communication during the fall (2014, 2016) preceding our field seasons. While at Palmer Station, the field team will meet briefly each morning to coordinate our research plans.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Yr 1</th>
<th>Yr 2</th>
<th>Yr 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vivo</em> heart rate measurements in animals exposed to CT&lt;sub&gt;MAX&lt;/sub&gt; (obj. 1; Egginton)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In situ</em> cardiac performance (obj. 1; Farrell)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energetic status of hearts &amp; HIF levels and DNA binding in animals exposed to CT&lt;sub&gt;MAX&lt;/sub&gt; (obj. 2; O’Brien)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>CL unsaturation; peroxidized mitochondrial lipids in animals exposed to CT&lt;sub&gt;MAX&lt;/sub&gt; (obj. 3; Crockett)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial function and oxidized mitochondrial proteins in animals exposed to CT&lt;sub&gt;MAX&lt;/sub&gt; (obj. 3; O’Brien)</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Contribution of neuronal function in setting thermal tolerance limits (obj. 4; Crockett &amp; Friedlander)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> cardiac performance and aerobic scope in ambient and warm acclimated animals exposed to CT&lt;sub&gt;MAX&lt;/sub&gt; (obj. 5; Axelsson &amp; Egginton)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial &amp; synaptosomal membrane fluidity and ABT from ambient and warm-acclimated animals (obj. 6; Crockett)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State III respiration rates and ABT; aerobic metabolic capacity in ambient and warm-acclimated animals (obj. 6; O’Brien)</td>
<td></td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES

5. Devor DP, O’Brien KM, & Crockett EL (in review) Hypoxia does not extend thermal tolerance limits in white- or red-blooded Antarctic notothenioid fishes.


