THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON THE THERMAL TOLERANCE OF ANTARCTIC NOTOTHENIOID FISHES

BY

KEVIN BILYK

DISSERTATION

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Doctoral Committee:

Professor Arthur L. DeVries, Chair, Director of Research
Associate Professor Chi-Hing Christina Cheng
Assistant Professor Rebecca C. Fuller
Associate Professor Kurt Kwast

Abstract

The evolution of the Antarctic notothenioid fishes in the chronically cold waters of the Southern Ocean has resulted in a remarkably narrow thermal tolerance shared among most extant species. While most of these fishes have the capacity to accommodate the physiological challenges of low temperature, they show a greatly reduced tolerance to heat relative to fishes native to warmer waters. Though predominantly distributed in the Southern Ocean, today the Antarctic notothenioids are largely divided by their geographic distributions between three regionally endemic ichthyofaunas in the high-latitude High Antarctic Zone (HAZ), the lower-latitude Seasonal Pack-ice Zone, and cold-temperate waters of southern South America and the South Island of New Zealand. However, the extent to which regional differences in ice abundance and temperature affect the thermal tolerance of their endemic fishes is poorly understood.

Within the Southern Ocean the Antarctic notothenioids are largely split between the constantly freezing High-Antarctic Zone (HAZ) and the more thermally variable Seasonal pack-Ice Zone (SPZ). For these fishes, the lower boundary of their thermal tolerance is effectively determined by their capacity for freeze avoidance and limited by their circulating levels of antifreeze proteins (AFPs). To investigate the differences in freeze avoidance between these regions' endemic ichthyofaunas blood serum freezing points were measured in 11 of the 14 species of the Antarctic icefishes (family Channichthyidae). While the icefishes are a small monophyletic group within the suborder Notothenioidei, they mirror the larger group's divisions in geographic distribution and lifestyle making this family a useful system for understanding the larger group. Within this family, blood serum freezing point was negatively correlated with the latitude of species' geographic distributions with the three SPZ species showing the highest freezing points. Either equal to or higher than the freezing point of seawater, these serum freezing points left the SPZ species with insufficient protection from freezing to survive in the ice-laden waters

inhabited by the HAZ species. When the contributions to freeze avoidance of serum osmolytes, antifreeze glycoproteins (AFGPs), and the antifreeze potentiating protein was assessed, the higher freeze avoidance of high-latitude HAZ icefishes was found to result predominantly from increasing antifreeze activity from the AFGPs.

The reduced severity of some SPZ habitats which allows the survival of species with high freezing points may also be reflected in the development of notothenioid freeze avoidance during ontogeny. While prior work on larval *Gymnodraco acuticeps* has shown that HAZ notothenioids can reach adult levels of serum antifreeze activity within months of hatching, antifreeze activity has never been previously studied in any of the SPZ species. To do so, I investigated the freeze avoidance in juveniles of the high freezing point SPZ icefish *Chaenocephalus aceratus* which had the highest serum freezing point among the adult Antarctic icefishes. These maintained sub-adult levels of serum antifreeze activity through the 2+ year class, which were the oldest collected, and if the observed rate of increase was sustained they would not attain adult levels of antifreeze activity until 4.2 years after hatching.

Though most of the Antarctic notothenioids share low freezing points, the absence of selection for heat tolerance in the chronically cold Southern Ocean has over evolutionary time resulted in their modern limited heat tolerance. To determine whether differences in environmental temperature between the HAZ and SPZ are also correlated with the heat tolerance of regionally endemic fishes, the critical thermal maximum (CTMax) was determined in 11 species of Antarctic fishes including six from the HAZ and five from the SPZ. Like freeze avoidance, heat tolerance among the Antarctic fishes could be separated according to their geographic distribution with the CTMaxs of five of the HAZ species significantly below those of the SPZ species when acclimatized to their natural freezing water temperatures. However, while these shared low heat tolerances in the eight species available in

numbers allowing further study, all retained plasticity in their heat tolerance with significant and often proportionally large increases in CTMax following warm acclimation to 4°C.

The Antarctic notothenioids also include at least 16 secondarily temperate species with origins in Antarctic waters but that are now permanent residents of the warmer waters around New Zealand and South America. Presumably these once shared the limited heat tolerance found currently among endemic Antarctic fishes however, it is not known whether this polar trait continues to exert an influence on their modern heat tolerance. To investigate, I determined the CTMax in two species of New Zealand notothenioids, the secondarily temperate *N. angustata* and the basal thorn fish *Bovichtus variegatus* that does not share the former's evolutionary origins in polar waters but which inhabits a similar modern thermal environment. While *N. angustata* had significantly higher heat tolerance than its endemic Antarctic congener *N. coriiceps*, it showed significantly lower heat tolerance than *B. variegatus*, suggesting the continued presence of its presumed ancestral loss of heat tolerance.

These studies showed species' levels of freeze avoidance and heat tolerance are not homogeneous throughout the Antarctic notothenioids, but reflect regional differences even within the Southern Ocean where water temperatures range only between -1.9 and 3°C. Freeze avoidance was lowest among species restricted to the more northerly waters of the SPZ where they presumably survive in the perennially ice free waters found within this region, but which also likely limits their distributions to waters outside the more severe HAZ. The relaxed selection for freeze avoidance in some SPZ waters also allows for the slow onset of adult antifreeze activity in at least one species of icefish, *C. aceratus*. Similarly, while the Antarctic notothenioids largely share low heat tolerances when acclimatized to their natural freezing water temperatures, these show notable regional variation and a surprisingly high level of plasticity in response to warm acclimation. However, the comparably limited levels of heat tolerance in the secondarily temperate nototheniid, *N. angustata*, suggests that there may be substantial

retention of its ancestral low heat tolerance which may impairing the Antarctic notothenioid's ability to adapt to the rising water temperatures predicted from global climate change.

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CHAPTER 1: GENERAL INTRODUCTION

The Southern Ocean is home to a highly endemic ichthyofauna dominated by five families from the perciform suborder Notothenioidei (Eastman 1993; Eastman 2005). Their evolution in the chronically cold waters surrounding Antarctica has resulted in the highly cold-adapted clade of Antarctic notothenioids which are largely divided by geographic distribution into three regionally endemic groups (Kock 1992; Eastman 2005). Though these fishes are exposed to differences in temperature and ice abundance that are small by comparison to the variation found in temperate waters, these differences still appear to have had a notable effect on the thermal tolerances of the Antarctic species.

The majority of the benthic notothenioids are predominantly distributed within the high-latitude High-Antarctic Zone (HAZ). This region consists of the chronically frigid waters surrounding much of the continental coast, the conditions of which have been best studied in McMurdo Sound (77°S). There, local waters are commonly at their freezing point throughout the year with only brief periods of warming limited to surface waters (< 100 m) during the austral summer. Even so, such warming appears spatially variable as surface water temperatures in some areas of McMurdo Sound remain continually at their freezing point, while in others, short lived seasonal peaks of up to 0.5°C have been recorded (Art DeVries and C.-H.C. Cheng, personal communication)(Hunt et al. 2003).Where this surface warming occurs it has been noted to decay rapidly with depth, and below 100 m waters are chronically near freezing (Carmack and Foster 1973; Hunt et al. 2003; Clarke et al. 2009).

Besides low temperatures, HAZ waters are also characterized by an abundance of ice. The region's waters are underneath near-constant ice cover, and in some areas the winter months can bring suspended masses of minute ice crystals that fill the water column giving rise to the platelet layer beneath the surface fast ice(Penrose et al. 1994; DeVries and Steffensen 2005; Leonard et al. 2006). In McMurdo Sound such platelet ice commonly forms on lines to a depth of 30 m and is responsible for

masses of ice on the benthos known as anchor ice (Dayton et al. 1969). Though platelet ice is normally restricted to shallow waters, it has also been found at depths of 250 m in the high latitude embayments of McMurdo Sound and the Weddell Sea. This deep water ice is likely generated by freezing seawater exiting from beneath the thick neighboring ice shelves which becomes supercooled as it is advected towards the surface (Dieckmann et al. 1986).

Also within the Southern Ocean, a less diverse group of Antarctic notothenioids is restricted to the more northerly Seasonal Pack-ice Zone (SPZ). This region includes waters along the Western Antarctic Peninsula (WAP), the South Shetland Islands, and some of the Scotia Arc Islands. At the southern extent of the SPZ winter surface waters are as cold as those in the HAZ, however peak temperatures are notably higher ultimately rising several degrees above freezing during the austral summer (Everson 1977; Barnes et al. 2006). While HAZ waters are near freezing at depth, the intrusion of circumpolar deep water (CDW) in some areas of the SPZ can warm waters below 100 m up to 1°C (Clarke et al. 2009). Unlike the HAZ, ice cover is limited and seasonal, extending several hundred km from the coast during the winter but it mostly disappears by the summer. Without nearby ice shelves to generate freezing water at depth, ice formation is restricted to the first few meters of the surface waters.

Finally, at least 16 species with inferred origins among the Antarctic notothenioids are now permanent residents of the cold-temperate waters surrounding New Zealand, South America, and the sub-Antarctic Islands (Eastman 2005). These waters undergo far greater seasonal temperature variation than Antarctic waters and water temperatures can remain continually above their freezing point making local habitats ice free (Barnes et al. 2006).

My dissertation research has focused on the thermal tolerance of the Antarctic Notothenioids most of which are viable over only a narrow range of temperatures when compared to temperate fishes. The lower limit of their tolerance is effectively defined by their capacity for freeze avoidance as death

from freezing occurs at a higher temperature than the cold induced failure of their physiological systems. Most also share a greatly reduced tolerance to heat, below that of cold-temperate fishes. My interest has been in investigating whether the thermal tolerance of these fishes differs with respect to their three geographic distributions.

Freeze Avoidance

Teleost fishes face a challenge in surviving polar marine environments as the osmolarity of their body fluids is about half that of seawater (Black 1951; DeVries 1982). This leaves them with a higher colligative freezing point than the surrounding seawater, and where seawater cools to its freezing point such fishes become supercooled. In the presence of ice, supercooling results in freezing which is lethal (Scholander et al. 1957). However, the Antarctic notothenioids, and some north temperate and arctic fishes, survive ice-laden because antifreeze proteins (AFPs) fortify their body fluids against freezing (DeVries and Wohlschlag 1969).

AFPs act to greatly depress the freezing point of a solution with a negligible contribution to its osmolarity. The hallmark of biological antifreezes is a separation between the observed temperatures of ice growth and melting termed a thermal hysteresis, which is a common measure of their antifreeze activity (DeVries 1971). When measured from blood serum or plasma the combined freezing point depression due to such antifreeze activity and colligative solutes serves as a reasonable proxy for the organismal freezing point (DeVries and Cheng 2005).

The magnitude of antifreeze activity increases with the concentration of AFPs, though eventually reaching a plateau. This may be explained by the proposed absorption inhibition mechanism of non-colligative freezing point depression by the AFPs. Individual AFP molecules are believed to irreversibly bind to ice crystals arresting the crystal's further growth by preventing the addition of more

water molecules to the ice lattice (Raymond and DeVries 1977). Based on this model, binding sites for AFPs become increasingly occupied as concentrations increase blunting the impact of further increases.

The antifreeze activity of notothenioid blood serum is predominantly due to their antifreeze glycoproteins (AFGPs) which are present at concentrations of up to 40 mg ml^{-1} in some species (Jin and DeVries 2006). Within these fishes' body fluids AFGPs are found as a heterogeneous mixture of size isoforms differing in their number of glycosolated Ala-Ala-Thr tripeptide repeats (DeVries and Cheng 2005). The smallest size isoforms with four and five repeats are most prevalent in serum, but they are less effective, producing only two thirds of the hysteresis as the larger size isoforms at equivalent concentrations (Schrag et al. 1982; DeVries 1986).

Notothenioid antifreeze activity in the blood is enhanced through a much less abundant, second antifreeze, the antifreeze potentiating protein (AFPP). AFPP shows little antifreeze activity by itself, and is typically present at concentrations of only 2 – 3 mg ml^{-1}, but it aids in reducing freezing point by an additional 1 °C or more, by potentiating the activity of the larger AFGP size isoforms (Jin 2003). The presence of the AFPP in notothenioid serum helps explain how measured serum antifreeze activity in some species can exceed the plateau level of purified AFGP activity, as well as the strong cooling rate dependence of antifreeze activity measured in native serum not observed in pure mixtures of AFGPs.

Though AFGPs have been studied longer than the recently discovered AFPP, their route into blood serum remains a persistent mystery since they are synthesized in the pancreas and exported only to the gut. There is no detectable synthesis in the liver and thus far there is no evidence that they are transported from the gut to the blood (Cheng et al. 2006). Additionally, the phenotypic plasticity of AFGP levels within notothenioids remains poorly understood. Though recent work on the HAZ nototheniid *Pagothenia borchgrevinki* found AFGP levels to decline over four months of warm acclimation to 4 °C, this occurred almost entirely in the small size isoforms and it remains unclear how much this decline reflects decreasing levels of synthesis or increased degradation (Jin and DeVries 2006).

Given that freezing is fatal to fishes there is a strong selective pressure for freeze avoidance to match the lowest temperatures within a species natural habitat. Prior comparative studies of AFP bearing fishes have noted that species with reduced levels of antifreeze activity tend to be restricted to warmer or ice free waters. In the HAZ waters of McMurdo Sound species' blood serum antifreeze activity show notable vertical zonation with lower levels among the notothenioids, zoarcids, and lipards restricted to deep waters (Jung et al. 1995; DeVries and Cheng 2005; Jin and DeVries 2006). Similarly the waters along the WAP are home to a number of notothenioid species with levels of blood serum antifreeze activity that would be insufficient to protect them from freezing in more severe HAZ waters (Ahlgren and DeVries 1984; Jin and DeVries 2006). These include the icefish *Chaenocephalus aceratus* and the nototheniid *Notothenia rossii* both of which have blood serum freezing points near –1.5 °C, as well as the nototheniid *Lepidonotothen squamifrons* which survives without measureable blood serum antifreeze activity.

Freeze avoidance has also been studied in four of the secondarily temperate Antarctic notothenioids which now inhabit permanently ice free waters. Cheng et al (2003) noted that the South American *Paranotothenia magellanica* as well as the two New Zealand species *Notothenia angustata* and *N. microlepidota* all continued to retain AFGP sequence in their genome, though at a greatly reduced dosage when compared to endemic Antarctic species. When blood serum antifreeze activity was measured in *N. angustata*, it was found present at very low levels which are physiologically insignificant. In one South American notothenioid, *Patagonotothen tessellata*, a genomic Southern Blot failed to reveal any AFGP sequence and subsequent investigation has found no trace of antifreeze activity in the blood.

Like other fishes, the notoththenioids undergo several discrete life stages which inhabit different portions of the water column (Loeb et al. 1993). This exposes them to different combinations of temperature and ice abundance than adults, however unlike adult notothenioids the difficulty in

procuring larval and juvenile notothenioids has meant relatively little is known about the mechanisms of freeze avoidance during these early life stages. To date the only study on these has been performed by Cziko et al (2006) who investigated larvae of three McMurdo Sound notothenioids which differ in both lifestyle and habitat. Of the three, only larvae of the cryopelagic nototheniid *Pagothenia borchgrevinki* hatched with serum antifreeze activity that approached adult levels. Larvae of the circum-Antarctic, pelagic nototheniid *Pleurogramma antarcticum* that have relatively limited antifreeze activity as adults hatched with negligible antifreeze activity. This was also true of the benthic bathydraconiid *Gymnodraco acuticeps* that have high levels of adult antifreeze activity. However, in *G. acuticeps* where sufficient larvae were available to follow the development of its freeze avoidance, blood serum antifreeze activity increased rapidly from nothing to reach adult levels within 147 days of hatching. The intervening survival of antifreeze deficient *G. acuticeps* larvae is in part due to an uncompromised integument that appears adequate to prevent inoculative freezing and allows them to maintain a supercooled state even in the presence of ice. Additionally, the delayed development of their larval gills further limits the risk of freezing, as these are an obvious site for ice entry in adults and larvae given that they are only one cell layer thick.

My interest in freeze avoidance has been in investigating how it is correlated with regional differences in habitat temperature and ice abundance. In ice-laden waters high levels of AFPs are necessary to prevent freezing despite the presumably large energy investment to maintain high serum levels in the HAZ species. However, while those notothenioids inhabiting continually ice free waters may survive with reduced or no AFPs, this likely limits their southward geographic distributions.

To study regional differences in freeze avoidance, I first systematically examined serum freezing points within the notothenioid icefishes (Channichthyidae), a small monophyletic family which mirrors many of the divisions in geographic distribution and lifestyle found throughout the Antarctic notothenioids. This study investigated the relative importance of serum osmolarity, AFGPs, and the

AFPP in generating the difference in freeze avoidance between notothenioid species. In my second chapter I investigated juveniles from one of the high freezing point SPZ icefish species, *Chaenocephalus aceratus*, to determine whether the development of freeze avoidance during ontogony may also be sensitive to differences in environmental severity.

Heat Tolerance

The upper limit to an animal's thermal range is determined by the temperature at which critical biological functions either fail or become impaired. This notably differs between taxa, and like freeze avoidance it can act as a critical physiological constraint on species' geographic distributions (Somero 2002). Heat tolerance appears heavily driven by selective pressure from peak or extreme temperatures that occur in a species' environment, as even brief exposure to temperatures that impair important biological functions may ultimately lead to death.

The selective pressure by peak temperature was ably demonstrated by Pörtner and Knust (2007) during their investigation into the eelpout *Zoarces viviparous* in the Wadden Sea. Populations of this species showed a marked decline following years when summer temperatures impaired their ability to perform activity or reproduce even though they were not high enough to cause death directly. Similarly, a study by Stillmann and Somero (2000) noted acute heat tolerance in porcelain crabs from the genus Petrolisthes showed a strong positive correlation with their maximum microhabitat temperature. Study of the evolutionary response to differing thermal conditions in the bacteria *Escherichia coli* by Mongold et al. (1996) found similarly increased fitness of experimental populations cycled between 32 and 42°C and held constantly at 42°C when assayed at 42°C which were both above the fitness of those of populations held at lower static temperatures.

While heat tolerance represents a gross measure of physiological temperature limits, easy comparisons between and even within species is confounded by a number of factors. Tolerance can

differ within a species according to a specimen's recent thermal history and the length of their exposure (Fry 1971). Heat tolerance is modified through acclimation with respect to an individual's recent temperature history, shifting to match environmental conditions. However, the extent of this plasticity is limited and varies between species. Heat tolerance also notably varies with exposure time, with longer exposures equating to lower tolerances as they allow more slowly developing thermal injuries to become apparent. Additionally, while death is the most severe consequence of exposure to high temperatures, many critical biological functions including growth, reproduction, and activity are impaired at lower temperatures.

Two common measures of heat tolerance have been developed, the upper incipient lethal temperature methodology (UILT) of Fry (Fry 1947) and the critical thermal maximum (CTMax) (Hutchison 1961; Paladino et al. 1980). UILT measures tolerance as the temperature at which median mortality ceases to be time dependent for a preset length of time, usually over week. The CTMax is a more acute measure of tolerance determined by gradual warming until specimens become so disoriented by the effects of temperature that they lose the ability to escape the thermal insult. While both equate tolerance to temperature, and are highly repeatable when determined from equivalently acclimated specimens, these measures cannot be directly compared as determinations of CTMax will exceed UILT when taken from equivalently acclimated specimens of the same species owing to the longer exposure times of the UILT.

The long evolution of Antarctic notothenioids in chronically freezing waters has resulted in the apparent loss of their heat tolerance which are today well below those of cold temperate fishes. This was first investigated by Somero and DeVries(1967)who measured a shared UILT between 5 and 7°C in the three HAZ nototheniids collected from McMurdo Sound: *Trematomus bernacchii*, *T. hansoni*, and *P. borchgrevinki* when acclimatized to their natural freezing water temperatures (–1.9 °C). Examining the heat tolerance of nototheniids collected from the more northerly WAP, Fanta et al. (1989) slowly

warmed the three species *T. bernacchii*, *N. neglecta*, and *N. rossii* at 1 °C hr^{-1} up to 10 °C. While no specimens died from this more acute exposure, specimens of both *T. bernacchii* and *N. rossii* showed sporadic losses of equilibrium above 4.5 °C.

Similar low heat tolerances have been noted in Antarctic invertebrates acclimatized to their natural freezing water temperatures suggesting that the loss of heat tolerance is a shared attribute of those organisms that have evolved in the chronically cold Antarctic waters. Peck et al. (2004) have noted an extreme sensitivity to heat in a number of SPZ Antarctic invertebrates including the scallop *Adamussium colbecki* which lost the capacity to swim below 2 °C during acute warming. While slightly more robust, the bivalve mollusk *Laternula elliptica* and the limpet *Nacella concinnaare* showed a loss of borrowing activity and righting response respectively at a temperature just above 5 °C. Study of chronic heat tolerance in the Antarctic brittlestar *Ophionotus victoriae* noted that this species could not survive prolonged exposure to 2 °C with specimens showing a mean survival time of only 42 days when held at this water temperature (Peck et al. 2009). When six representative species from four invertebrate phyla were warm acclimated to 3 °C for 60 days only one species showed an increase in their acute heat tolerance suggesting a broad loss of plasticity in heat tolerance among Antarctic invertebrates (Peck et al. 2010).

While early study of the metabolic response to warm acclimation in the HAZ nototheniid *T. bernacchii* suggests a similarly limited capacity to increase heat tolerance among the notothenioid fishes (Somero et al. 1968), more recent work has shown that some of these fishes do have a capacity to increase heat tolerance. Podrabsky and Somero(2006) found that the two HAZ nototheniids *T. bernacchii* and *T. pennellii* shared the ability to extend heat tolerance through acclimation. When transferred to 14 °C water, which is fatal to these fishes within minutes, specimens acclimated to 4 °C for four to six weeks survived for significantly longer than those held at their environmental water temperatures. Examination of cardiac function in *P. borchgrevinki* by Franklin et al. (2007) has also noted

that warm acclimation to 4 °C for four to five weeks leads to compensation in cardiac function during acute increases in temperature. These fishes also seem capable of surviving chronic exposure to elevated temperature as holding at 4°C for six months produced no ill effects in healthy *P. borchgrevinki* (Robinson and Davison 2008b).

With increasing appreciation for the thermal plasticity that is retained within Antarctic species my interest has been to understand the extent to which stenothermy is shared among the Antarctic notothenioids given the differences between their thermal environments. Understanding their evolutionary responses to the different thermal regimes is increasingly important for predicting winners and losers if the rise in the Southern Ocean temperature predicted from global climate change occurs (Clarke et al. 2007). To this end, my third chapter investigates whether SPZ species maintain greater heat tolerance compared to those from the HAZ through a comparison of five SPZ and six HAZ species. This also offered an opportunity to compare the capacity of many of these fishes to increase heat tolerance through warm acclimation providing a direct measure of its plasticity. My fourth Chapter studies heat tolerance of the secondarily temperate Antarctic notothenioid *N. angustata* to determine whether these show reduced levels of heat tolerance associated with their presumed ancestral stenothermy. If so, then the limited heat tolerance in many Antarctic notothenioids may exert a continuing influence which could impede future adaptation to rising water temperatures.

CHAPTER 2: FREEZING AVOIDANCE OF THE ANTARCTIC ICEFISHES (CHANNICHTHYIDAE) ACROSS THERMAL GRADIENTS IN THE SOUTHERN OCEAN[1]

Abstract

Biogeographic studies separate the Antarctic Notothenioid fish fauna into high- and low-latitude species. Past studies indicate that some species found in the high-latitude freezing waters of the High-Antarctic Zone have low-serum hysteresis freezing points, while other species restricted to the low-latitude seasonal pack ice zone have higher serum hysteresis freezing points above the freezing point of seawater (−1.9 °C), but the relationship has not been systematically investigated. Freeze avoidance was quantified in 11 species of Antarctic icefishes by determining the hysteresis freezing points of their blood serum, in addition, the freezing point depression from serum osmolytes, the antifreeze activity from serum antifreeze glycoproteins (AFGPs), and the antifreeze activity from serum antifreeze potentiating protein were measured for each species. Serum hysteresis freezing point, a proxy for organismal freeze avoidance, decreased as species were distributed at increasing latitude (linear regression r^2 0.66, slope −0.046 °C °latitude−1), which appeared largely independent of phylogenetic influences. Greater freeze avoidance at high latitudes was largely a result of higher levels of antifreeze activity from serum AFGPs relative to those in species inhabiting the low-latitude waters. The icefish fauna could be separated into a circum High-Antarctic Group of eight species that maintained serum hysteresis freezing points below −1.9 °C even when sampled from less severe habitats. The remaining three species with low-latitude ranges restricted to the waters of the northern part of the west Antarctic Peninsula and Scotia Arc Islands had serum hysteresis freezing points at or above −1.9 °C due to

[1] This chapter appeared in its entirety in the Journal of Polar Biology and is referred to later in this dissertation as "Bilyk and DeVries 2010". Bilyk, K.T. and DeVries, A.L., 2010. Freezing Avoidance of the Antarctic Icefishes (Channichthyidae) Across Thermal Gradients in the Southern Ocean. 33(2) 203-213. This article is reprinted with the permission of the publisher and is available from http://www.springerlink.com and using DOI: 10.10007/s00300-009-0697-z

significantly lower combined activity from all of their serum antifreeze proteins than found in the High-Antarctic Zone icefish.

Introduction

In both species number and biomass, the Perciform suborder Notothenioidei is the predominant taxon in the Southern Ocean's modern ichthyofauna (Eastman 1993; Eastman 2005). Today, members of this taxon are represented throughout nearly all of the Southern Ocean's ecological niches extending as far north as the coastal cold-temperate waters of South America and New Zealand (Gon and Heemstra 1990; Eastman 1993; Eastman 2005). A novel adaptation within the notothenioids, the evolution of antifreeze proteins (AFPs), is considered partly responsible for their successful colonization and adaptive radiation in the freezing waters of the Southern Ocean (Eastman 2005).

Studies of the freezing behavior of fishes have used the hysteresis freezing point (temperature of ice growth) of blood serum as a measure of their freeze avoidance (DeVries and Cheng 2005). The large depression of the serum hysteresis freezing point found in Antarctic notothenioid blood serum is due to the additive effects of elevated levels of osmolytes (DeVries 1982) and their AFPs (Ahlgren and DeVries 1984; DeVries and Cheng 2005). The serum antifreeze activity and hysteresis freezing point in the Antarctic notothenioid species examined to date have been constant under natural conditions showing no variation with seasonal temperature change (DeVries 1982). This is in contrast to some Arctic fishes that have seasonal variation in their production of AFPs (DeVries 1982).

Despite having serum osmolytes levels above those found in temperate and tropical fishes, the serum equilibrium freezing point (equivalent to the melting point) of Antarctic notothenioids remains 1 °C above the freezing point of seawater (−1.9 °C), hence the need for AFPs in their serum to depress its freezing point further where water temperatures drop below their serum equilibrium freezing point (DeVries and Cheng 2005). The AFPs contribute to the serum freezing point depression via a non-

colligative mechanism that involves their adsorption to endogenous ice and the inhibition of its growth (Raymond and DeVries 1977). The hallmark of the AFPs is their separation of the temperature at which ice melts (equilibrium freezing point or melting point) and the temperature at which ice grows (hysteresis freezing point), which is referred to as their antifreeze activity (DeVries 1971).

The antifreeze activity in Antarctic notothenioids results from the combined actions of two distinct proteins: the antifreeze glycoproteins (AFGPs), and the recently discovered antifreeze potentiating protein (AFPP) (DeVries 1982; Jin 2003; DeVries and Cheng 2005). In the red-blooded notothenioids, much of the antifreeze activity arises from the AFGPs, which circulate in the blood at concentrations of up to 30 mg ml^{-1} as a mixture of different size classes (DeVries and Cheng 2005; Jin and DeVries 2006). AFPP enhances AFGP activity by potentiating the activity of the large size classes of the AFGPs (Jin 2003; DeVries and Cheng 2005). AFPP activity has been measured in some high-latitude notothenioids where it is responsible for approximately 30% of the serum antifreeze activity despite being present at concentrations of only 2 or 3 mg ml^{-1} (Jin 2003).

Despite their status as the most recently derived family within the notothenioid suborder (Near et al. 2004), the icefish share with most of the red-blooded notothenioids similar habitats, lifestyles, and distributions throughout both high- and low-latitude Antarctic waters as in Gon and Heemstra (1990) and Kock (2005). The icefish's habitats and lifestyles span the diverse thermal and ice regimes of the Southern Ocean which have presumably greatly influenced their modern freeze avoidance and distribution.

The high latitude High-Antarctic Zone contains continental shelf habitats in the Weddell Sea and southern Ross Sea (McMurdo Sound) which are ice-covered and freezing for most of the year (Littlepage 1965; Carmack and Foster 1973; Hunt et al. 2003). In contrast, the waters surrounding the low-latitude Sub-Antarctic Islands are above freezing even during the winter and are thus ice-free (Barnes et al. 2006). Between these lies the seasonal pack ice zone (SPZ) that includes northern portions of the

Western Antarctic Peninsula (WAP) and the southern Scotia Arc Islands. The SPZ is ice covered and has freezing surface waters during the winter, but even though drifting pack ice can be present in the SPZ during the summer, the summer surface water is usually a few degrees above its freezing point (Barnes et al. 2006). Furthermore benthic water temperatures are raised in some parts of the SPZ by the presence of warm circumpolar deep-water (CDW) which is present in some regions below 100 m even during the winter (Dinniman and Klinck 2004; Klinck et al. 2004; Clarke et al. 2009). Where this warm CDW intrudes onto the continental shelf water temperatures rise above the equilibrium freezing points of notothenioid fishes.

Notothenioid inhabitants of the year round ice-laden waters of the High-Antarctic Zone require high levels of serum AFPs for survival. As expected the high latitude fishes living in close association with ice have the lowest serum hysteresis freezing points, such as the cryopelagic *Pagothenia borchgrevinki* (−2.5 °C) and shallow water benthic species inhabiting the anchor ice zone such as *Trematomus bernacchii* (−2.4 °C), *Trematomus hansoni* (−2.7 °C), and *Gymnodraco acuticeps* (−2.6 °C) (Ahlgren and DeVries 1984; DeVries and Cheng 2005; Cziko et al. 2006; Jin and DeVries 2006).

The SPZ is a less severe environment relative to the high latitude waters as freezing conditions are found only in the winter surface and coastal waters. This suggests SPZ fishes would require serum AFPs only if inhabiting the surface waters during the winter. Although the shallow water SPZ species *Notothenia coriiceps* has a serum hysteresis freezing point of −2.2 °C, several benthic and epibenthic fishes endemic to the SPZ share notably higher serum hysteresis freezing points of −1.5 °C including *Notothenia rossii*, *Notothenia larseni*, and the icefish *Chaenocephalus aceratus* (Ahlgren and DeVries 1984; Jin and DeVries 2006). Notothenioids inhabiting the Sub-Antarctic Islands where water temperatures are above freezing clearly have no need for AFPs; however, the few species that have been investigated have serum hysteresis freezing points similar to those found in the SPZ waters (Ahlgren and DeVries 1984).

In the present study, serum hysteresis freezing points were measured in 11 icefish species and the individual freezing point depressions from serum osmolytes, AFGPs, and AFPP were quantified. This study posed the question as to whether freeze avoidance of a species reflected the severity of freezing conditions found within their range, and the respective roles of serum osmolality, AFGP activity and AFPP activity in adaptation to environmental severity. Given the energetic costs of synthesizing and maintaining a constant concentration of AFPs (DeVries 1988) species inhabiting a reduced or non-existent threat of freezing would be expected to have lower levels of freeze avoidance compared with those living in freezing ice-laden waters.

Materials and Methods

Collection and sampling

Specimens of 11 icefish species were collected from the Southern Ocean between 1990 and 2008, their collection location, month, and year are summarized in Table 1.1. Of the 11 species, *Chionodraco hamatus* were collected by hook and line and *Pagetopis macropterus* by diver. The remaining species were collected by otter trawl (5-m or 33-m foot rope) at depths from 50 to 450 m with a bottom time of 30 min. Live specimens were sampled as soon as possible after their removal from the cod end of the trawl as most species failed to survive prolonged periods following trawling stress. *C. aceratus*, *P. macropterus*, and *C. hamatus* were exceptions as they were sampled at least 2 weeks following collection. Specimens were anesthetized with MS-222 (80 mg l^{-1}) then blood was drawn from their caudal vein by syringe using an 18-gauge needle. The blood was allowed to clot at 4°C for 4 h then centrifuged and the sera stored at −80 °C until analyzed.

Determination of blood serum osmolality and equilibrium freezing point

Serum osmolality was determined with a Wescor 5520 vapor pressure osmometer (Wescor Inc.). The osmometer was calibrated daily, prior to use, with Opti-Mole 1,000 and 290 mOsm standards (Wescor Inc.). The calibration of the osmometer was checked against these standards following every tenth serum measurement and if it deviated from the expected values by more than 3 mOsm the osmometer was recalibrated. Notothenioid sera appear to contain a volatile that accumulates on the thermocouple of the osmometer with repeated exposure. This accumulation decreases the precision of subsequent measurements; however, the effects of this contaminant could be minimized by regular recalibration after every tenth serum measurement.

Serum osmolality was measured in duplicate for each sample and the average taken as the sample's osmolality. If the sample's two measurements differed by more than 10 mOsm, a third measurement was performed and the two closest osmolalities were averaged. The equilibrium freezing point (equivalent to the melting point) was then determined by multiplying the serum osmolality by $-0.001858\ ^{\circ}C\ mOsm^{-1}$ (DeVries and Cheng 2005).

Determination of the total antifreeze activity present in blood serum

The total antifreeze activity present in native serum was measured with a Clifton Nanolitre Freezing Point Osmometer (Clifton Technical Physics). This is a measure of the combined freezing point depression from all of the AFPs present in the native sera of a given specimen. Prior to use, the nanolitre osmometer was calibrated with distilled-deionized water (0 mOsm) and Wescor Opti-Mole 1,000 mOsm standard (Wescor Inc.).

A mineral oil filled microcapillary pipette attached to a micrometer syringe was used to inject the serum samples into the six wells of the osmometer's sample holder which contained type-B

immersion oil. While under observation through a compound light microscope at 250×, the samples were frozen by rapid cooling to −40 °C then slowly warmed (0.05 °C min^{-1}) until only a single small crystal (5–10 μm diameter) remained.

The antifreeze activity, or thermal hysteresis, of each sample was measured as the difference between the temperatures at which this seed crystal slowly melts and the temperature at which it begins to rapidly grow (hysteresis freezing point) (DeVries 1971). The latter was determined by allowing the seed crystal to anneal for 1 or 2 min just below its melting point then cooling the sample at 0.05 °C min^{-1}, with intermittent pauses for further annealing, until unrestricted growth of the seed crystal was observed.

Antifreeze activity was determined in two to six of the sample wells for each sample and the resulting values were averaged to produce a single estimate for the combined activity of all of the AFPs present in the specimen's blood serum. The hysteresis freezing point of notothenioid serum results from the combined colligative freezing point depression of serum due to small osmolytes (equilibrium freezing point) and the non-colligative freezing point depression from all of the present AFPs. In this study, the serum hysteresis freezing point of each specimen was calculated as the sum of their equilibrium freezing point determined from the osmolality measurement obtained from the Wescor vapor pressure osmometer, and its antifreeze activity determined from the difference between the melting and freezing temperatures obtained with the nanoliter osmometer.

Measurements of the antifreeze activity of the native sera were notable for large variation within samples. This was associated with the presence of the AFPP whose potentiating activity is strongly affected by differences in seed crystal size and cooling rate (Jin 2003). In an attempt to minimize this variability, a slow rate of cooling (0.05 °C min^{-1}) was maintained, and seed crystals were melted to a uniform small size (5–10 μm), although these efforts to control variability in the measurements of serum hysteresis freezing point were not always successful.

Determining the contributions of AFGPs and AFPP to the total antifreeze activity in blood serum

The total antifreeze activity of notothenioid serum results from the combined contributions of two distinct AFPs: the AFGPs, and the AFPP (Jin 2003; DeVries and Cheng 2005). To quantify their respective roles in freeze avoidance, the individual contributions of the serum AFGPs and the AFPP to the total serum antifreeze activity were measured using a modification of the technique described by Jin (2003). This technique exploits the heat stability of the AFGPs and heat lability of the AFPP. Heating serum samples for 10 min at 100 °C causes AFPP to lose all activity leaving only the antifreeze activity from the AFGPs.

Samples of 150 µl of native serum were pipetted into 1.5 ml screw-cap microfuge tubes (Fisher Inc.) and closed with screw caps (Fisher Inc.) fitted with an O-ring to prevent evaporation. The tubes were immersed in boiling water for 10 min resulting in a thick white precipitate. After centrifugation at 10,000g for 5 min, the pelleted spongy precipitate was then well macerated with a thin spatula to ensure an equal solute distribution between the supernatant and residual fluid in the pellet. The sample was again centrifuged at 14,000 g for 10 min.

To determine whether solutes were evenly distributed between the two phases, the osmolality of the supernatant was measured on the Wescor vapor pressure osmometer and compared with that of the corresponding native serum. If the difference was more than 30 mOsm, the precipitate was mixed again with the supernatant and centrifuged at 14,000 g for 5 min before retesting. If the difference persisted then a new sample was prepared.

The antifreeze activity of the supernatant was determined with a Clifton nanolitre osmometer as previously described. The resulting average measurement was an estimate of the antifreeze activity in the serum sample from AFGPs. The difference between the antifreeze activity of the heated and

native serum of the same specimen then provided a measure of the antifreeze activity in the serum sample from AFPP.

Statistical analysis

Throughout the analysis of freeze avoidance, the serum hysteresis freezing point, osmolality, total antifreeze activity from the combined serum AFPs, AFGP activity, and AFPP activity were all considered to be independent traits belonging to each species. The above were analyzed for significant differences between species using Welch's test. Welch's test was selected as a robust alternative to the analysis of variance (ANOVA) under heteroscedasticity (Coombs et al. 1996; Wilcox 2003) which was necessary as Levene's test uniformly rejected the assumption of equal variances in these traits between species ($P < 0.05$).

The icefish examined in this study can be divided into two groups based on the geographic distribution (Kock 1992; Kock 2005). *Chionodraco rastrospinosus*, *Neopagetopsis ionah*, *Cryodraco antarcticus*, *Cryodraco atkinsoni*, *Chionodraco myersi*, *P. macropterus*, *Chaenodraco wilsoni*, and *C. hamatus* all share distributions that include the High-Antarctic Zone which is characterized as being within the minimum extent of the annual ice cover around the continent (Hart 1942; Voronina 1971; Kock 1992), while *C. aceratus*, *Champsocephalus gunnari*, and *Pseudochaenichthys georgianus* are restricted to the SPZ and waters further north. To determine whether there are significantly greater measures of freeze avoidance in the High-Antarctic Zone icefish relative to the SPZ icefish a one tailed Student's t test was performed on the absolute value of species' averages of each measured trait.

Where a significant difference was found in traits between distributions, a simple linear regression was performed against the mean latitude of their geographical range using a generalized linear model. Latitude served here as a reasonable proxy for environmental severity with species possessing more southerly distributions presumed exposed to a greater threat of freezing. The mean

distribution latitude of each species was determined as the arithmetic mean of the highest and lowest latitudes from which they have been commonly reported and which were estimated from distribution charts and tables in Gon and Heemstra (1990) and Kock (2005). A summary of the mean distribution latitudes of each species used in this study is given in Table 1.1.

To account for the possible role of phylogenetic influences, significant regressions were repeated on phylogenetic independent contrasts (PIC) computed from the consensus phylogeny proposed by Near et al. (2003) using the contrast module of the PHYLogeny Inference Package (Felsenstein 1989). Regressions on these contrasts exclude the effects of phylogeny and allow environmental influences on character traits to be independently explored (Felsenstein 1985). Blood serum was not available for all of the Channichthyid species presented in the phylogeny and those taxa were pruned from the phylogeny prior to the analysis. Branch lengths were estimated from the maximum parsimony tree constructed from the mitochondrial 16S and ND2 genes in Near et al. (2003).

Specimens of *C. antarcticus* were available from the South Shetland Islands, the WAP, and the Ross Sea presumably representing populations exposed to differing environmental severity. To determine whether there were differences in freeze avoidance within this species across its range, a one-way ANOVA followed by a Tukey post hoc test was performed to assess significant differences ($P < 0.05$) in their freeze avoidance traits between specimens collected from different regions. The Tukey post hoc test was selected as it controlled for type-I error across all comparisons.

Results

Species averages for the serum hysteresis freezing point, osmolality, equilibrium freezing point, and total antifreeze activity are given in Table 1.2. The individual contributions of the AFGPs and AFPP to the total antifreeze activity present in blood serum are shown for each species in Fig. 1.1. The Welch's

test revealed significant variation between species ($P < 0.01$; Table 1.3) in their serum hysteresis freezing point, osmolality, total antifreeze activity, AFGP activity, and AFPP activity.

The average serum hysteresis freezing points of all of the High-Antarctic Zone icefish species were below the freezing point of seawater (−1.9 °C), which was also true for all of the individual specimens in all but one species. The sole exception was *C. antarcticus* where 5 out of the 15 specimens had serum hysteresis freezing points above the freezing point of seawater. Among the eight High-Antarctic Zone species their average serum hysteresis freezing points ranged from high of −1.97 °C in *C. antarcticus*, and −2.04 °C in *C. rastrospinosus*, to low of −2.49 °C in *C. myersi*, and −2.44 °C in *C. hamatus*.

Two of the icefish species restricted to the SPZ, *C. gunnari*, and *P. georgianus*, had average serum hysteresis freezing points close to that of seawater. However, in both species, there were a substantial number of individuals with serum hysteresis freezing points above the freezing point of seawater, some by as much as 0.25 °C. The third species restricted to the SPZ, *C. aceratus*, had the highest average serum hysteresis freezing point among all of the Antarctic icefish (−1.47°C) and was the only species where the serum hysteresis freezing points of all of the individual specimens were above the freezing point of seawater.

In 10 of the 11 icefish species, the total antifreeze activity from all of the serum AFPs accounted for 47–61% of the observed depression in the hysteresis freezing point of their blood serum. The AFPs contributed most heavily in *P. macropterus* where they were responsible for 61% of the freezing point depression. In *C. aceratus*, which had the highest serum hysteresis freezing point of all of the Antarctic icefish species, AFPs were responsible for only 37% of the depression in their serum hysteresis freezing point. Among all 11 icefish species, the contribution of the AFPP to the total serum antifreeze activity averaged 35%, although this varied greatly between species ranging from 15% in *C. rastrospinosus* to 55% in *C. gunnari* (Fig. 1.1).

When compared with the three species distributed in the SPZ, the eight species with a High-Antarctic Zone distribution had significantly low-serum hysteresis freezing points, and significantly greater serum osmolality, total antifreeze activity, and AFGP activity as determined by a one-tailed Student's t test (Table 1.4). AFPP activity did not significantly differ between the High-Antarctic Zone and SPZ species.

Both the simple linear regressions and the regressions on PIC found that as the mean latitude of a species distribution increased, the serum hysteresis freezing point declined, while the total antifreeze activity and antifreeze activity from AFGP present in the serum increased (Table 1.5; Fig. 1.2). The measures of both slope and significance for these were largely unchanged between linear regressions and regressions on the PIC (Table 1.5) suggesting that the difference reflects adaptation to the differing environmental conditions in the High-Antarctic Zone and the SPZ.

Antifreeze potentiating protein activity was excluded from this analysis because the difference between species with High-Antarctic Zone and SPZ distributions was non-significant. Although serum osmolality was significantly greater in the High-Antarctic Zone species, the linear regression against latitude resulted in a model with poor fit and a slope that was marginally non-significant ($r^2 = 0.27$, $P = 0.1$).

Comparison of *C. antarcticus* collected from the South Shetland Islands, the WAP, and the Ross Sea showed no significant differences in the total antifreeze activity in their blood serum. Antifreeze activity from AFGP and AFPP in the blood serum did not significantly differ between fishes collected from the South Shetland Islands and the Ross Sea; measurements were unavailable to compare fish collected from the WAP. Serum hysteresis freezing points were significantly lower, and serum osmolality significantly higher in icefish collected from the Ross Sea compared with those from the South Shetland Islands and the WAP.

Discussion

Like most red-blooded notothenioids, the icefishes rely on AFPs to depress their freezing point sufficiently to avoid freezing in the Southern Ocean's ice-laden waters. Exposed to a constant high threat of freezing, the average serum hysteresis freezing points of all of the High-Antarctic Zone icefish species were below the freezing point of seawater (−1.9 °C). The highest average freezing point in the High-Antarctic Zone icefish species was found in *C. antarcticus* where the serum hysteresis freezing point was approximately equal to the freezing point of seawater. The three low-latitude SPZ species inhabiting a less severe environment all had serum freezing points equal to or above that of seawater (Table 1.2).

Although there is a trend of greater freeze avoidance in species distributed at increasing latitude for the individual fish, it is their habitat temperature and the presence or absence of ice in their environment that determines whether they live or freeze. The low-serum hysteresis freezing points of the High-Antarctic Zone species reflect the severity of their habitats where the water is at its freezing point and there is an abundance of ice crystals in the upper part of the water column. These severe conditions result in part from the influence of nearby ice shelves located along much of the continental coast and in the southern reaches of the Ross and Weddell Seas (Foldvik and Kvinge 1974; Nicholls and Makinson 1998; DeVries and Steffensen 2005).

The seawater at the underside of ice shelves or glacier tongues is cooled to its in situ freezing point which is lower than the surface freezing point due to the effect of hydrostatic pressure (Fujino et al. 1974). When this cold water is advected towards the surface, it becomes supercooled due to an increase in its freezing point from the release of hydrostatic pressure. The resulting spontaneous ice nucleation produces "clouds" of small ice crystals even in the habitats of deep-water fishes (DeVries and Steffensen 2005). These small ice crystals are thought to grow into ice platelets when transported away from the shelf by deep currents and have been retrieved from the water column with a trawl at a depth of 250 m some distance from the ice shelf front in the Weddell Sea (Dieckmann et al. 1986). Outflow of

cold ice shelf water also results in the formation of anchor ice down to depths of 33 m in the shallow water of McMurdo Sound (Dayton et al. 1969) and other coastal areas in the vicinity of ice shelves. As a result of these conditions, the icefishes inhabiting the waters near ice shelves can be exposed to an abundance of ice crystals even at depths where ice is normally absent. This would require their serum hysteresis freezing points be equal to or lower than the in situ freezing point of seawater to avoid freezing.

Where the distribution of a species includes ice-laden freezing waters there would be substantial selective pressure to reduce serum hysteresis freezing points below the temperature of the surrounding water, which is what is observed. The serum hysteresis freezing points of the High-Antarctic Zone icefish are similar to those in the red-blooded notothenioids native to ice-laden shallow waters of McMurdo Sound and the shallow coastal waters of the continent near ice shelves (DeVries and Cheng 2005). The lowest serum hysteresis freezing points were observed in *P. macropterus* and *C. hamatus* that are consistent with their shallow water, high-latitude habitats (Gon and Heemstra 1990; Kock 2005) where the outflow of freezing ice shelf water generates an abundance of ice crystals. Similarly, low-serum hysteresis freezing points were found in the high latitude species *C. myersi* and *C. atkinsoni* which have also been collected in waters in close proximity to ice shelves (Takahashi and Nemoto 1984; Ekau 1988; La Mesa et al. 2002). The remainder of the high-latitude species also had serum hysteresis freezing points below the freezing point of seawater indicating that they probably encounter ice at some stage in their life cycle.

The three SPZ species have converged on lower serum levels of AFPs (Table 1.5) which are insufficient for survival in the severe environments prevalent in the High-Antarctic Zone. Although the serum hysteresis freezing point is only a measure of the freeze avoidance present at the time of sampling, during this study the serum hysteresis freezing points for two of these three SPZ species: *C. aceratus*, and *C. gunnari*, were measured from specimens collected in both August and March which

would expose them to different seasonal conditions. That the serum hysteresis freezing points of these species did not significantly differ between the specimens collected in August and March suggests that there may be no seasonal change in the high-serum hysteresis freezing points found in these species. In addition, recent investigation of the third SPZ species, *P. georgianus*, found specimens collected in August to have serum hysteresis freezing points above the freezing point of seawater (DeVries, unpublished) as did those measured in this study which was collected in March.

Although the SPZ surface waters are at their freezing point during the winter (DeVries and Steffensen 2005; Barnes et al. 2006) the adult SPZ icefish are in little danger of freezing even with their high-serum hysteresis freezing points because their benthic habitats are ice-free. When winter bottom water cools to the freezing point of sea water (−1.9 °C), these fish become supercooled by about a half of degree which is metastable in the absence of ice.

Supercooling in the absence of ice is not unusual as indicated by observations that some Arctic fishes spend their entire lives supercooled by almost 1 °C on the bottom of deep fjords (Scholander et al. 1957). Antarctic red-blooded nototheniids can also supercool by as much as 4 °C in a laboratory setting for at least an hour (DeVries and Cheng 1992; Praebel et al. 2009). However, in the presence of ice supercooled adult fish will freeze at the hysteresis freezing point of their blood serum (DeVries and Cheng 2005). This is presumably because the integument is not an absolute barrier to ice entry, which is suggested by the presence of ice in the spleens of the high-latitude nototheniids (Praebel et al. 2009). Once it has entered their body, ice is a potent nucleator of further ice formation if cooled below the serum hysteresis freezing point. The SPZ icefish can survive in their benthic habitats despite having serum hysteresis freezing points above that of seawater because they do not encounter ice.

The absence of present day ice shelves in the SPZ region precludes the introduction of supercooled water and the formation of ice in deep-water habitats unlike in the High-Antarctic Zone. The benthic habitats of the WAP are also influenced by the CDW, which in many places flows onto the

shelf reaching to within a few kilometers of shore, even during the winter. The temperature of this water regardless of season is around 1 °C (Dinniman and Klinck 2004; Klinck et al. 2004) and thus the problem of freezing does not exist for icefish that inhabit it. Because of the warm CDW and the absence of local ice shelves and glacial ice tongues, the SPZ icefish exist without the danger of freezing as long as they avoid winter ice in the surface waters.

With the geographic ranges of the three SPZ icefish centered in these warmer low latitude waters they maintain insufficient freeze avoidance to survive in the more severe habitats found in the High-Antarctic Zone. It is even plausible that high-serum hysteresis freezing point of *C. aceratus* (−1.47 °C) could restrict it to the Sub-Antarctic Islands were it not for the presence of the warm CDW on the WAP shelf.

Given that both the supercooled state is metastable, and that the adult SPZ icefish inhabit ice-free water, it is difficult to explain why they have any AFP in their blood serum. It is energetically expensive to synthesize proteins as the formation of each peptide bond requires hydrolysis of two molecules of ATP and the additional attachment of sugars to the AFGP polypeptide also involves the hydrolysis of ATP (DeVries 1988). The rates of biochemical reactions are relatively slow in the freezing Antarctic waters and hence growth, development and reproduction are much slower relative to temperate and tropical fishes (Clarke 1983). Synthesis of AFPs that are unnecessary for survival is an inefficient utilization of high-energy phosphate bonds.

A possible explanation for the maintenance of serum AFPs in the SPZ icefish is that upon hatching their larval stages float or swim to the surface waters where they feed on plankton. Many aspects of the life histories of the icefishes are not known (Kock 2005), and during these early life stages, it is unknown whether they enter the surface where ice would be present, or remain a few meters below the surface where ice exposure would be minimal. If at the surface, they would require serum AFPs to avoid freezing once they develop gills in the early juvenile stage (Cziko et al. 2006). Juvenile

icefishes have been reported to often forage in krill swarms (Rembiszewski et al. 1978; Kock 2005) which can enter surface waters where ice is present. If they do require higher levels of AFPs for freeze avoidance during their early life stages, it may then be retained in the adults. Similarly, adult *C. gunnari* and *P. georgianus* are suspected of making vertical migrations (Kock 2005) which may require higher levels of serum AFPs to survive occasional exposure to ice in the surface waters.

Another possible explanation for the persistence of serum AFPs in these fish is that in the past high levels were required to avoid freezing during glacial maxima when extensive ice shelves were present and ice streams flowed across the continental shelf (Anderson 1991). High concentrations of serum AFPs would be necessary to survive the freezing water generated at the underside of these ice shelves which filled the continental shelf. Their present day lower levels of serum AFPs may be the result of expression of fewer functional AFP genes than were present during the glacial maxima, and it is possible that more evolutionary time is required for mutations to accumulate leading to further AFP gene inactivation following the recent removal of freezing selective pressure.

There are regions of overlap in the range of the High-Antarctic Zone icefish with the SPZ species. Although there are substantial differences in the freeze avoidance between the SPZ and High-Antarctic Zone species, there were no differences in the latter when collected from both zones. Specimens of the icefish *C. antarcticus* had equivalently high levels of total serum antifreeze activity regardless of whether they were collected from the High-Antarctic Zone or the SPZ. Similarly the High-Antarctic Zone icefish *C. wilsoni* collected entirely within the SPZ (Table 1.1) maintained a serum hysteresis freezing point well below the freezing point of seawater (Table 1.2). As noted in prior studies this implies that at their natural habitat temperatures the level of AFP expression in Antarctic notothenioids may be constrained (DeVries 1982).

In most fishes, NaCl is responsible for greater than 90% of the freezing point depression in their body fluids. In the polar notothenioids, the antifreeze activity from AFPs also make a substantial

contribution to the depression of the freezing point of body fluids of these fishes. Comparisons of their relative contributions to the freezing point depression in the high- and low-latitude groups revealed some differences. Based on their respective slopes (Table 1.5), total serum antifreeze activity accounts for 71% of the decrease in the serum hysteresis freezing point with increasing latitude. This was predominantly due to the greater antifreeze activity from serum AFGPs present in the High-Antarctic Zone species relative to those from the SPZ.

The relationship between icefish serum osmolality and latitude appear to be marginally statistically non-significant. This was due to the high variability in measurements of serum osmolality between species, potentially caused from trawl-induced stress in some species which result in the breakdown of ion regulation and an influx of NaCl, the magnitude of which however varies from trawl to trawl. More recent measurement of serum osmolality and ions from specimens caught by hook and line, or sampled after 2 weeks acclimation to aquarium conditions after trawl capture, indicate a clear difference between the serum osmolality of High-Antarctic Zone and SPZ notothenioids (DeVries, unpublished). Clearly, high osmolalities will further depress freezing point, but whether it can be viewed as an adaptation for increasing freezing avoidance is unclear. It may just be the result of a disproportionate temperature effect on ion pumps and membrane leaks (Hochachka 1988).

The other major contribution to serum hysteresis freezing point came from AFPP which comprises 35% of the average total serum antifreeze activity in the 11 icefish species (Fig. 1.1). Despite this substantial contribution to freeze avoidance, no systematic relationship between AFPP activity and environmental severity was noted during this study. It may be possible that the amount of AFPP that a species expresses is a characteristic unique to that species.

In summary, serum hysteresis freezing points were lower in the icefish species distributed in the High-Antarctic Zone compared with those species restricted to the SPZ. The low-serum hysteresis freezing points of the High-Antarctic Zone fishes were predominantly the result of higher serum

antifreeze activity from AFGPs, while serum osmolality and AFPP activity did not systematically differ between the High-Antarctic Zone and SPZ species. The more limited freeze avoidance of the SPZ icefishes was independent of phylogenetic influences and corresponds to the less severe conditions that can be found within the SPZ along with the high metabolic cost of AFP production.

CHAPTER 3: DELAYED ONSET OF ADULT ANTIFREEZE ACTIVITY IN JUVENILES OF THE ANTARCTIC ICEFISH *CHAENOCEPHALUS ACERATUS*[2]

Abstract

Many Antarctic notothenioid species endemic to the Seasonal Pack-ice Zone have converged on adult blood serum freezing points that are several tenths of a degree above the freezing point of seawater. While these fishes share high adult serum freezing points, the development of their freeze avoidance during ontogeny has not been studied. We investigated this in wild caught juveniles of one such species, *Chaenocephalus aceratus* (family Channichthyidae), using blood serum antifreeze activity as a proxy for their freeze avoidance. Juvenile serum antifreeze activity was significantly below that of adults through the oldest year 2+ specimens collected. This increased at an estimated rate of $0.368 \times 10^{-3} \pm 0.405 \times 10^{-4}$°C day−1 which, if sustained, would leave *C. aceratus* below their adult serum antifreeze activity levels of 0.57 ± 0.08°C until 4.2 years after hatching. Underlying the 2.7-fold increase in their serum antifreeze activity from late year 0+ juveniles to adults was an even greater 10.4-fold increase in the concentration of their serum antifreeze glycopeptides, which increased proportionally across all of their serum AFGP size isoforms. With insufficient antifreeze activity to avoid freezing in the ice-laden surface waters, both adult and juvenile *C. aceratus* are most likely restricted to the year round ice-free waters where a metastable supercooled state can be maintained.

Introduction

As adults, high-latitude Antarctic notothenioids survive in perennially freezing and ice-laden waters by maintaining high serum concentrations of antifreeze proteins (AFPs). These fishes' AFPs, along

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with elevated serum levels of sodium chloride and small organic osmolytes (O'Grady and DeVries 1982; Raymond and DeVries 1998) can depress their serum freezing points a few tenths of a degree below that of seawater (−1.9 °C) preventing their freezing (DeVries and Cheng 2005). While the freeze avoidance of adult notothenioids has been broadly surveyed, few studies of freeze avoidance of the larval and juvenile life stages have been carried out.

Recently Cziko et al. (2006) investigated freeze avoidance in the larvae of the coastal high-latitude notothenioids *Pagothenia borchgrevinki*, and *Gymnodraco acuticeps*. Larvae of the cryopelagic *P. borchgrevinki* hatched with high levels of serum antifreeze activity comparable to their adults, suggesting similar high concentrations of serum AFPs. Unlike *P. borchgrevinki*, larvae of the benthic *G. acuticeps* hatched with very little serum antifreeze activity resulting in larval serum freezing points above the freezing point of seawater. The freeze avoidance of their larval and early juvenile stages was instead attributed in part to their intact integument which served as an effective barrier to ice entry, preventing freezing in their natural environment where they were supercooled by 1 °C and exposed to ice crystals.

Compared to the severe freezing conditions found throughout high-latitude Antarctic waters, milder conditions can be found in the more northerly Seasonal Pack-ice Zone (SPZ) including portions of the Western Antarctic Peninsula (WAP) and the southern Scotia Arc Islands (DeVries and Steffensen 2005; Barnes et al. 2006). As a result of the region's less severe conditions it is home to representative species from several notothenioid families that survive as adults with blood serum freezing points several tenths of a degree above that of seawater (Ahlgren and DeVries 1984; Jin and DeVries 2006; Bilyk and DeVries 2010). Unlike the high-latitude notothenioids, freeze avoidance during the early life stages of these high freezing point fishes has not been studied.

The icefish (family Channichthyidae) *Chaenocephalus aceratus* belongs to this group of SPZ notothenioids with high adult freezing points (Jin and DeVries 2006; Bilyk and DeVries 2010). Hatching

from demersal eggs (Detrich et al. 2005), this fish then begins poorly understood larval and juvenile developmental stages that are thought to last from 3 to 4 years (Loeb et al. 1993; La Mesa and Ashford 2008). Unlike the benthic adults, the planktonic larvae and pelagic juveniles of *C. aceratus* can be found in the water column (Kock and Kellermann 1991; Kock 2005) where they may be exposed to the seasonally freezing conditions found in the SPZ surface waters.

As in the other Antarctic notothenioids, the freeze avoidance of *C. aceratus* is predominantly the result of antifreeze activity from circulating antifreeze glycopeptides (AFGPs) (DeVries 1971). AFGPs, like other AFPs, depress serum freezing points with a negligible contribution to the serum osmolality by means of an adsorption–inhibition mechanism (Raymond and DeVries 1977; Knight et al. 1991). These can be found in notothenioid serum at concentrations up to 30 mg ml^{-1} as a mixture of size isoforms which differ by their number of glycosylated alanine-alanine-threonine repeats (Cheng 1996; DeVries and Cheng 2005). While the smaller size isoforms with 4–11 repeats are the most abundant in blood serum, they depress the non-colligative freezing point with only two/thirds the effectiveness of the larger size isoforms on a mass basis (Schrag et al. 1982). Additionally, the recently discovered antifreeze potentiating protein (AFPP) increases the serum antifreeze activity of notothenioid sera by boosting the activity of only the large AFGP size isoforms (Jin 2003). As a result the antifreeze activity present in notothenioid blood serum is influenced by both the concentration of AFGPs as well as the proportions of their size isoforms (Jin and DeVries 2006).

In the present study, we investigated the freeze avoidance of juvenile *C. aceratus* by comparing measurements of serum antifreeze activity in wild caught year 0+ to year 2+ juveniles to those made in adults. To then investigate the changes in circulating AFGPs underlying those occurring in serum antifreeze activity with age, we measured the serum concentration of AFGPs in juveniles and adults, and compared the electrophoretic profiles of their serum AFGPs.

Materials and Methods

Specimen collection and sampling

Adult and juvenile specimens of *C. aceratus* were collected along the WAP during July and August of 2008 by bottom trawls at depths from 70 to 120 m aboard the R/V L.M. Gould. Juveniles were collected from Dallmann Bay, and the adults from Dallmann Bay, the Gerlache Strait, and near Snow Island (Fig 2.1). Specimens were identified according to Gon and Heemstra (1990) for adults and both Gon and Heemstra (1990) and Kellermann (1989) for juveniles.

Blood samples were drawn from juvenile specimens immediately after capture from the caudal vein using an insulin syringe with an attached 28 gauge needle. These were allowed to clot on ice for 4 h, centrifuged at 10,000 g, and the sera stored at −80°C until analyzed. The total length (TL) of each juvenile specimen was then measured to the nearest tenth of a cm and used to estimate their individual year class using the instantaneous growth rate reported by La Mesa and Ashford (2008).

Adults were transported in running seawater to Palmer Station where they were maintained in flow through aquaria at −1.5 °C. Following 3–4 weeks of recovery from capture and transport stress the adults were anesthetized with MS-222 (80 mg l^{-1} seawater) and blood samples collected from the caudal vein using a syringe fitted with a 21 gauge needle. Following the same protocol as the juveniles, blood samples were allowed to clot on ice, centrifuged, and the sera stored at −80 °C until analyzed.

Measuring the buoyancy of year 0+ juveniles

Buoyancy was measured in six juvenile specimens estimated to be between 307 and 380 days post-hatch (dph) (TL 11.8–13.5 cm). Although morbid, these specimens were tested soon after collection and showed no signs of desiccation or decomposition. Following the protocol of Eastman and DeVries (1982) and Eastman and Sidell (2002), specimens were weighed in air (W_{air}) and in ambient temperature seawater (W_{water}) to the nearest 0.01 g using a Mettler Toledo top-loading electronic balance (Mettler

Toledo Inc.). W water was measured by suspending the specimens with 5–0 silk suture from a stiff rod fastened to the pan of the balance. Specimens were attached to the suture by a small barbless hook inserted through the skin on the tip of their lower jaw. Before measuring their W water all air bubbles were cleared from a specimen's opercular and oral cavities. Buoyancy (B) was then expressed as:

$$\%B = (W_{water} / W_{air}) \times 10^2$$

Determination of serum melting point

Blood serum osmolality was determined for each specimen using a Wescor 5520 vapor pressure osmometer (Wescor Inc.) as described in Bilyk and DeVries (2010). Each serum sample's colligative melting point was then calculated by multiplying its measured serum osmolality by 0.001858 °C $mOsm^{-1}$.

Determination of serum antifreeze activity

Serum antifreeze activity was measured using a Clifton nanoliter freezing point osmometer (Clifton Technical Physics Inc.) following the method described in Bilyk and DeVries (2010). The antifreeze activity in a specimen's blood serum is equated to the thermal hysteresis which is the difference between the temperature at which a single small seed crystal (5–10 μm diameter) slowly begins to melt, and the temperature at which it begins to rapidly grow (DeVries 1971). Prior to use the nanoliter osmometer was calibrated with distilled-deionized water (ddH_2O; 0 mOsm) and a Wescor Opti-Mole 1000 mOsm standard (Wescor Inc.).

Determination of serum AFGP concentration by HPLC

Serum AFGP concentrations were quantified following the recently developed HPLC method of Jin (2003) and Jin and DeVries (2006). Concentrations were measured in six adult specimens, two year

2+ juveniles, three year 1+ juveniles, and four year 0+ juveniles. 50 µl of 5% trichloroacetic acid (TCA) was added to 50 µl of each serum sample in a 1.5 ml Eppendorf microcentrifuge tube which was then immediately vortexed and centrifuged at 14,000g. 50 µl of the resulting TCA supernatant was then diluted in the column running buffer (100 mM sodium phosphate in 300 mM NaCl, pH 7.6) as follows 1:10 for adults, year 2+ juveniles, and year 1+ juveniles and either 1:2 or 1:5 for year 0+ juveniles. These different dilutions were necessary in order to obtain an equivalent response from the year 0+ juveniles on the HPLC detector. The diluted samples were then injected into a 20 µl sample loop of an isocratic HPLC system.

The HPLC system was comprised of a Model 110B single piston pump (Beckman Instruments), a Bio-Sil SEC 125-5 size exclusion column (300 mm × 7.8 mm; MW range 5,000–100,000; particle size 5 µm, Bio-Rad), and an ISCO V4 variable wavelength absorbance detector. The pump flow rate was 0.6 ml/min and the absorbance was recorded at 220 nm. The output signal from the absorbance detector of the HPLC system was recorded and analyzed on a computer using an E-Lab Chromatography System (OMS-Tech) which was used to integrate the peak areas of the major AFGP size classes.

Concentrations of the serum AFGP size classes were obtained by comparing their peak areas to those of known concentrations of purified large AFGPs (size isoforms 1–5) and small AFGPs (size isoforms 7 and 8). The concentration of the intermediate AFGP 6 size isoform was determined using AFGP 7 and 8 as a standard as AFGP 6 elutes as a shoulder on the profile of AFGP 7 and is clearly separated on the elution profile from the smallest of the AFGP 1–5 group.

Analysis of serum AFGPs by polyacrylamide gel electrophoresis

The electrophoretic profiles of the serum AFGP size isoforms were characterized from representative juvenile and adult specimens using two polyacrylamide gel electrophoresis (PAGE) gels. The first gel controlled for differences in AFGP concentrations between samples allowing investigation

of variation in the proportion of AFGP size isoforms with age. The second was performed to investigate the concentration of the individual AFGP size isoforms at physiological concentrations with age.

To compare the profiles of serum AFGP size isoforms between ages at nearly equivalent concentrations, purified AFGP samples were prepared from a representative adult and year 2+ juvenile, along with a pool of 800 µl comprised of serum from year 1+ and year 0+ juveniles. It was necessary to pool serum from the youngest year classes due to their low serum concentration of AFGPs and the small quantity of blood collected from each specimen. To examine differences in physiological concentration between age classes, six samples of 300 µl serum were prepared for purification, two each from adults and year 2+ juveniles, and two from independent pools from year 0+ juveniles. For both sets of samples serum pools were prepared from serum of specimens with comparable TL and antifreeze activity.

Each of these serum samples and pools were treated with an equal volume of 5% TCA which precipitates most serum proteins but leaves AFGPs in solution (Ahlgren and DeVries 1984). These samples were immediately vortexed then centrifuged at 14,000g. The entire supernatant for each sample was then dialyzed in Pierce Slide-A-Lyzer mini dialysis units (Pierce Biotech. Inc.) with a 3,500 Da molecular weight cut off. The dialysis was performed over 24 h at 4 °C first against deionized water then against two changes of ddH2O after which the samples were lypholyzed.

For the comparison at equal concentrations the lypholyzed material from each of the three age classes was redissolved to 50 mg ml^{-1} in ddH2O, and for the comparison of physiological concentrations the lypholyzed samples were redissolved in 30 µl of ddH2O which was one-tenth of their original serum volume. PAGE was then used on both sets of samples to compare the concentration of individual AFGP size isoforms, and to examine size isoform homogeneity across samples of different ages. AFGP samples were electrophoresed based on the technique described in Chen et al. (1997). 5 µl of each AFGP sample was mixed with 5 µl of sample buffer (0.9 M boric acid, in 30% glycerol, pH 8.6) then fluorescently labeled by adding 2.5 µl of fluorescamine (Fluram: Roche Diagnostic) in acetone (4 mg ml^{-1}). 2 µl of

loading dye (0.315 M boric acid, 1% bromphenol blue, in 40% glycerol) was then added to each sample before loading them onto a 10–20% non-denaturing, gradient polyacrylamide gels which were run at 34 V cm^{-1} for 8 h at 4°C.

Five microliters of total serum AFGPs purified from *Dissostichus mawsoni* were run alongside the *C. aceratus* samples in both gels as standards with a known size isoform profile. These were purified from serum by TCA extraction as earlier described for *C. aceratus* samples then redissolved in ddH2O to a final concentration of 50 mg ml^{-1} for the constant concentration gel, and to 75 mg ml^{-1} for the physiological concentration gel. As previously described these were then mixed with sample buffer, labeled with fluorescamine, and mixed with loading dye before loading them onto the gels.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to test for significant effects of year class on the serum antifreeze activity, total AFGP concentration, and colligative melting point among representative samples of *C. aceratus* adults and juvenile year classes. This ANOVA was performed directly on measurements of serum antifreeze activity and colligative melting point, however, Levene's test rejected the assumption of equal variances among measurements of total AFGP concentrations which was resolved by $\log_{10}$ transforming these values.

Serum antifreeze activity, colligative melting point, and the $\log_{10}$ transformed serum AFGP concentration values were then compared among the year classes using a post hoc Tukey test at a 0.05 level of significance. The Tukey test was chosen here for its ability to control the probability of type I error across all comparisons. To then estimate the rate at which juvenile serum antifreeze activity increased, a simple linear regression analysis was performed on serum antifreeze activity against their TL which served as a proxy for age. Finally, to test whether serum osmolality and antifreeze activity significantly covary, which could indicate that the reduced antifreeze activity was resulting from dilution

of blood serum, their Pearson's correlation coefficient was calculated among all of the collected juvenile samples.

Results

Serum samples were collected from a total of 27 juvenile and 6 adult *C. aceratus*. The juveniles ranged in TL from 10.3 to 27.5 cm and their ages were estimated from 240 to 990 dph. The buoyancy of juveniles measured from six specimens between 307 and 380 dph (TL 11.8–13.5 cm) was found to be 3.22 ± 0.174% making them negatively buoyant in seawater.

Average measurements of serum colligative melting point, antifreeze activity, and AFGP concentration for adults and representative specimens of the juvenile year classes are presented in Table 2.1. The one-way ANOVA found a significant effect of age on the serum antifreeze activity of these representative *C. aceratus* ($F_{3,10} = 31.34$, $P < 0.0001$, $r^2 = 0.90$), with the subsequent Tukey's test showing significantly greater serum antifreeze activity with increasing age (Table 2.2).

While the juveniles had significantly lower levels of serum antifreeze activity than adults, this increased with TL in the juvenile specimens (simple linear regression: slope $1.60 \times 10^{-2} \pm 0.176 \times 10^{-2}$ °C cm^{-1}, $P < 0.0001$, $r^2 = 0.767$; Fig 2.2) showing an increase with age. Estimated from the instantaneous growth rate of 0.23 mm/day for juvenile *C. aceratus* reported in La Mesa and Ashford (2008), the antifreeze activity in juveniles increased at a rate of $3.68 \times 10^{-3} \pm 0.405 \times 10^{-4}$ °C day^{-1}.

Corresponding to their increasing serum antifreeze activity with age was an even greater increase in serum concentration of AFGPs (Figs 2.3, 2.4). As with serum antifreeze activity, the one-way ANOVA found a significant effect of age on the concentrations of serum AFGPs ($F_{3,10} = 44.65$, $P < 0.0001$, $r^2 = 0.93$), with the subsequent Tukey's test showing significantly greater concentrations with increasing age (Table 2.2).

Unlike either serum antifreeze activity, or AFGP concentration, the one-way ANOVA on the serum colligative melting point was marginally non-significant ($F_{3,10} = 3.48$, $P = 0.0584$, $r^2 = 0.51$) when compared between adults and juvenile year classes. When the serum colligative melting point and antifreeze activity were compared across all 27 juvenile samples these were not significantly correlated (Pearson's correlation coefficient $r = -2.31$, $P = 0.244$) suggesting that the reduced serum antifreeze activity of younger juveniles was not the result of serum dilution from seawater intrusion.

When the lyophilized material from adult, year 2+ juveniles, as well as pooled year 0+ and year 1+ juveniles were analyzed by PAGE at equal concentrations (50 mg ml^{-1}), all three of these age classes showed the same banding pattern with nearly equal band intensities (Fig 2.5a). Despite being run at equivalent concentrations the electrophoretic profile of the two serum pools combining year 0+ and year 1+ specimens had slightly lower overall band intensity and greater background noise which is likely an artifact of the much greater volume of serum that had to be concentrated to produce these samples. While serum samples from the youngest juveniles had low concentrations of AFGPs the remaining TCA soluble constituents of their blood serum did not appear similarly affected by age. As a result these contaminants represented a larger proportion of the lyophilized material in samples from younger specimens. When similar quantities of lyophilized material were then loaded onto the gel the samples from younger specimens had less AFGP and more contamination relative to those from the older *C. aceratus* leading to overall reduced band intensity.

When physiological concentrations were used the serum AFGP size isoforms of year 0+ juveniles uniformly showed much lower concentrations as indicated by reduced fluorescence relative to older *C. aceratus* (Fig 2.5b). However, the same electrophoretic profile is still visible in the year 0+ juveniles as in older fish.

Discussion

Adult *C. aceratus* have a serum freezing point of −1.5°C which is the highest measured among the Antarctic icefishes. This is in marked contrast to the low serum freezing points of high-latitude notothenioids which are below the freezing point of seawater (−1.9°C) (Jin and DeVries 2006; Bilyk and DeVries 2010). In the presence of ice, adult notothenioids will freeze at their blood serum freezing point making this an important constraint on the geographic range and habitats of these species (DeVries and Cheng 2005). The high serum freezing point found in adult *C. aceratus* therefore likely limits their distribution to the waters of the northern WAP, the Scotia Arc, and the Sub-Antarctic Islands where the combination of freezing temperatures and ice are limited to the winter surface and coastal waters.

In this study, juvenile *C. aceratus* were found to have levels of serum antifreeze activity that were significantly below the already limited levels of adults. The serum antifreeze activity of the year 0+ juveniles averaged only 37% of the mean adult levels, and while this increased to 72% in the year 2+ juveniles this remained significantly below their adult serum antifreeze activity (Table 2.2). If the rate of increase in serum antifreeze activity observed from year 0+ juveniles to year 2+ juveniles is sustained until reaching adult levels, then these are not reached until they have grown to a TL of 35 cm corresponding to an estimated age of 4.2 years after hatching (Fig 2.2).

While the serum antifreeze activity of Antarctic notothenioids results from both AFGPs and the AFPP, the latter plays only a small role in the freeze avoidance of *C. aceratus* (Jin 2003; Bilyk and DeVries 2010). This, combined with the low measured serum antifreeze activity in juvenile specimens, led us to focus on the serum AFGPs to explain the development of their freeze avoidance from juvenile to adult.

The 2.7-fold increase in serum antifreeze activity between year 0+ juveniles and adult *C. aceratus* results from a corresponding 10.4-fold increase in their serum AFGP concentration (Table 2.1). That this increase in concentration was not matched in antifreeze activity is a result of the hyperbolic

relationship between antifreeze activity and AFGP concentration, with antifreeze activity increasing more slowly than the concentration of AFGPs above 4 mg ml^{-1} (DeVries and Cheng 2005).

The HPLC chromatograms from which this increase in concentration was determined (Fig 2.3) were similar in their overall profile to those of other notothenioids previously reported in Fig 2A and B of Jin and DeVries (2006). However, they differ from this previous study both in having a more rapid elution and in showing a more clearly defined peak for the small AFGP 7. The former results from our use of a 30 cm rather than a 60 cm column (Jin 2003) while the increased resolution may be explained in part by the higher concentrations of our serum samples as only 1:10 or smaller dilutions of the TCA treated serum were used. Additionally, some differences in the shape of the chromatogram likely result from interspecies differences in the concentration of AFGP size isoforms.

In *C. aceratus*, the increase in serum AFGPs underlying their increase in serum antifreeze activity occurred through a proportional increase across all of their serum AFGP size isoforms (Fig 2.5a). All of the same size isoforms are found in *C. aceratus* serum from juvenile to adult, with no change in their relative proportions over this time. This apparent constraint on their synthesis of AFGPs during development may be further evidence for a genomic basis of the proportions of AFGP size isoforms (Cheng 1996).

If the low levels of serum antifreeze activity and AFGP concentration found in the late year 0+ juvenile *C. aceratus* are indicative of those found prior to metamorphosis then their larvae may possess little or no AFPs. Such low serum antifreeze activity in larval and early juvenile *C. aceratus* is puzzling as both have been collected in the plankton (Loeb et al. 1993; Kock 2005). This distribution in the upper part of the water column has the potential to bring these early life stages into close contact with ice in the winter surface waters exposing them to a greater threat of freezing than the benthic adults. Not only could this ice cause their poorly protected blood serum to freeze but it also suggests that other body fluids would be similarly susceptible to freezing as they would also have low levels of antifreeze.

This presents a particular challenge in preventing freezing of hypo-osmotic fluids in their digestive tract which could be nucleated by ice ingested during feeding (Cheng et al. 2006).

To survive ice exposure the planktonic larva of *C. aceratus* may employ a similar strategy as noted in the larvae of the McMurdo Sound dragon fish, *G. acuticeps*. These can supercool by as much as 2.6°C below their hysteresis freezing in the presence of ice without freezing during laboratory experiments. They accomplish this in part from their uncompromised integument and limited larval gill surface area that together act as a physical barrier to ice entry into their supercooled body fluids (Cziko et al. 2006).

An intact integument appears sufficient to protect the dragonfish until they begin producing their own AFPs even in the ice-laden and freezing seawater of the sub-ice platelet layer that they inhabit immediately after hatching (Evans et al. 2005). Presumably antifreeze deficient larval *C. aceratus* would be similarly protected against freezing by the physical barrier provided by their integument if they enter the icy winter surface waters of the SPZ.

The absence of AFP production in larval *C. aceratus*, and its slow accumulation in juveniles may not be detrimental given the less severe conditions in their habitat. Unlike the larval dragon fish that are found in continually freezing and ice-laden waters, larval *C. aceratus* are exposed to only a single winter season before metamorphosis (Loeb et al. 1993). This exposure to winter conditions happens immediately after hatching which may be timed so that larvae are exposed to these severe conditions when it has its least exposed surface area as their gills have not yet fully developed. When the seasonal freezing conditions in the SPZ surface waters then subside larvae and juveniles would no longer be in danger of freezing unlike the early life stages of *G. acuticeps* and *P. borchgrevinki* which are under constant threat of freezing in high latitude waters. For *C. aceratus* the physical barrier may thus be sufficient to fully protect their larvae over this shorter period of exposure to freezing conditions, removing the need for early AFPs, and explaining the low levels observed in the year 0+ juveniles.

Following metamorphosis juvenile *C. aceratus* are assumed to begin a pelagic lifestyle which lasts from 2 to 4 years (Loeb et al. 1993; Kock 2005; La Mesa and Ashford 2008). While the integument alone may succeed in protecting recently hatched larval notothenioids from freezing this likely no longer offers sufficient protection for the juvenile and adult life stages. Not only does exposed surface area rapidly increase after the development of the gills, but we can infer from the rapid synthesis of AFGPs noted in *G. acuticeps* larvae that exposure to physical insults in the environment and parasites both begin to compromise the physical barrier leading to sites for ice entry (Cziko et al. 2006).

Additionally the common presence of endogenous ice within several species of McMurdo Sound nototheniids (Tien 1995; Praebel et al. 2009) suggests the presence of lesions in the integument or gill lamellae that allow ice entry, and which are presumably common in adults and juveniles as well. Without a reliable physical barrier and in the absence of sufficient serum osmolytes or AFPs to depress their freezing point below that of seawater (Table 2.1), the results of this study suggest these juveniles can survive only by avoiding contact with ice.

A behavioral freeze avoidance strategy is consistent with the prolonged juvenile stage of *C. aceratus* that suggests the slow accumulation of AFGP is related to a lack of a stringent requirement for freeze avoidance. Along the WAP, which is the southern extent of this species range, vertical temperature profiles taken in Ryder Bay (68 °S 30□) south of our collection site (Fig 2.1) show freezing waters and thus ice are restricted near the surface even during the winter. As reported by Meredith et al. (2004) the winter mixed layer reached depths of 200 m during an ENSO year but was otherwise restricted to the upper 50 m. However, even within the mixed layers reported water temperatures were several tenths of a degree above their freezing point, and by 150 m the influence of the warm CDW was noted with temperatures rising above 0 °C.

The downward projection of surface ice crystals into these waters appears constrained by more than just unfavorable temperatures. Experimental study of the wave-driven turbulence of surface water

found that air bubbles are only propelled downward to depths of three to seven times the significant wave height (Toba and Kawamura 1996) and positively buoyant ice crystals would be expected to show a similar limitation. Furthermore, surface ice formation (grease ice) significantly reduces wave height and the wave driven turbulence propelling ice into the water column. Thus by remaining sufficiently deep to avoid contact with ice crystals in the upper part of the water column, juvenile *C. aceratus* can avoid freezing even when water temperatures fall below the juvenile freezing point.

Even where water temperatures fall below the juvenile's freezing point as long as they avoid ice found in the surface waters *C. aceratus* can maintain a supercooled state which is metastable since the degree of supercooling is small. It is well known that some Arctic fishes remain supercooled their entire lives by inhabiting the bottom of deep fjords where there is no ice present to nucleate freezing (Scholander et al. 1957). It has also been shown that several McMurdo Sound nototheniids can be supercooled by 6 °C in the absence of ice and remain so for at least an hour indicating the absence of nucleating structures within the body of the fish (A. L. DeVries, unpublished data). Similarly the liparid fishes of McMurdo Sound remain in a permanently supercooled state in their natural habitat at depths of 500–700 m (Jung et al. 1995). Even though this fish is in close proximity to the Ross Ice Shelf presumably no ice crystals are present at that depth to cause nucleation.

The extended pelagic phase of the juveniles is probably related to the availability of krill in the surface and mid waters, and perhaps avoiding predation by large benthic fishes. There may be a substantial benefit in terms of food resources as well as predator avoidance for the species to inhabit waters where the potential for freezing exists. This prey resource benefit must also apply to energetic considerations because the juvenile icefish are as negatively buoyant as the adults. The buoyancy of late year 0+ and early year 1+ juveniles was similar to the 3.73% reported for large adult *C. aceratus* which are clearly benthic (Eastman and Sidell 2002).

The onset of adult levels of antifreeze activity corresponds to an age of about 4.2 years post-hatch and a length of about 35 cm. *C. aceratus* is reported to transition to a benthic life style and feed primarily on other fishes and only occasionally on krill at this age and length (Flores et al. 2004). This timing suggests that these fish do not attain their full adult freeze avoidance until they also transition to an adult diet, lifestyle, and habitat.

The limited freeze avoidance of adult and juvenile *C. aceratus* may reflect a lack of selection for surviving freezing conditions if they remain in habitats that are above their freezing point or that allow for a stable super cooled state. The delayed onset of adult antifreeze activity in *C. aceratus* indicates it is not required during the juvenile stage, where if freeze avoidance were more strongly required, the juveniles would be unlikely to survive. Part of the geographic range occupied by *C. aceratus* is also home to at least one nototheniid, *Lepidonotothen squamifrons*, without antifreeze activity (A. L. DeVries unpublished data; C.-H. C. Cheng, personal communications). This suggests that there are habitats within the WAP waters that do not present a significant risk of freezing as both species are relatively abundant in the WAP waters. The delayed onset of serum antifreeze activity in juveniles may then reflect a lack of necessity for antifreeze activity in adult *C. aceratus* and signal that this trait is no longer under positive selection.

CHAPTER 4: HEAT TOLERANCE AND ITS PLASTICITY IN ANTARCTIC FISHES [3]

Abstract

The adaptive radiation of the Antarctic notothenioid ancestral benthic fish stock within the chronic freezing waters of the Southern Ocean gave rise to five highly cold adapted families. Their stenothermy, first observed from several high-latitude McMurdo Sound species, has been of increasing recent interest given the threat of rising polar water temperatures from global climate change. In this study, we determined the heat tolerance in a geographically diverse group of 11 Antarctic species as their critical thermal maximum (CTMax). When acclimatized to their natural freezing water temperatures, environmental CTMaxs ranged from 11.95 to 16.17 °C, well below those of fishes endemic to warmer waters. There was a significant regional split, with higher CTMaxs in species from the more northerly and thermally variable Seasonal Pack-ice Zone. When eight of the Antarctic species were warm acclimated to 4 °C, all showed a significant increase over their environmental CTMaxs, with several showing plasticity comparable in magnitude to some far more eurythermal fishes. When the accrual of heat tolerance during acclimation was followed in three high-latitude McMurdo Sound species, it was found to develop slowly in two of them, which was correlated with their low metabolic rates.

Introduction

Antarctica's coastal fishes are divided between largely non-overlapping geographic distributions in the High-Antarctic Zone (HAZ) and the Seasonal Pack-ice Zone (SPZ) (Kock 1992). Both lie within the chronically cold Southern Ocean where water temperatures range between − 1.9 and 3 °C (Deacon

[3] This chapter appeared in its entirety in the Journal of Comparative Biochemistry and Physiology Part A and is referred to later in this dissertation as "Bilyk and DeVries 2011". Bilyk, K.T. and DeVries, A.L., 2011. Heat tolerance and its plasticity in Antarctic fishes. 158(4) 382-390. This article is reprinted with the permission of the publisher and is available from http://www.sciencedirect.com and using DOI: 10.1016/j.cbpa.2010.12.010

1984; Eastman 1993). Despite this narrow temperature range, these region's endemic ichthyofaunas are exposed to distinct temperature conditions which may have led to the differentiation of their respective heat tolerances.

The HAZ encompasses much of the continental coast where water temperatures remain at or near their freezing point throughout the water column and for most of the year (DeVries and Cheng 2005). In the HAZ waters of McMurdo Sound, where seasonal variation in water temperatures has been investigated, the upper 50 m of the water column rises above its freezing point during summer peaking at − 0.5 °C for a few weeks in January (Hunt et al. 2003). The more northerly SPZ includes the waters along the Western Antarctic Peninsula (WAP) and the southern Scotia Arc Islands. While the winter surface waters here can be as cold as those in the HAZ, they show a comparably larger temperature range with increases of 3 °C along the WAP during the summer months (Barnes et al. 2006).

Furthermore, in some places along the WAP, warm circumpolar deep water (CDW) periodically intrudes onto the continental shelf raising deep water temperatures up to 1 °C (Clarke et al. 2009). While the seasonal changes are small relative to those in temperate environments, there still appears to be a strong correlation between these small temperature differences and certain physiological/biochemical traits in the Antarctic notothenioids. For example, the warmer and mostly ice free habitats in the SPZ region have been noted to correspond with a reduction in freeze avoidance relative to fishes endemic to the HAZ (Bilyk and DeVries 2010) and a similar correlation may exist with their heat tolerance.

The heat tolerance of Antarctic fishes was first studied by Somero and DeVries (1967) who determined the upper incipient lethal temperature (UILT) in three HAZ nototheniids. This technique, based on lethal dosage methodologies, measures heat tolerance as the temperature at which median mortality is no longer time dependent. They found that the species *Pagothenia borchgrevinki*, *Trematomus bernacchii*, and *Trematomus hansoni* shared UILTs between 5 and 7 °C when acclimatized

to the water temperature in McMurdo Sound (− 1.9 °C). Given that the lower boundary of their tolerance is dictated by the temperature at which they freeze (− 2.2 °C), this results in a remarkably narrow thermal range when compared to temperate and tropical fishes (Elliot 1981).

Most animals do not have a static heat tolerance; rather it changes in response to their recent thermal history through acclimation. However, given the long residence of Antarctic fishes in constant freezing seawater, this plasticity had long been thought either lost or marginal (Brett 1970). Recently though, Podrabsky and Somero (2006) noted that the two McMurdo Sound nototheniids *T. bernacchii* and *Trematomus pennellii* both shared the ability to extend heat tolerance through acclimation. When transferred to 14 °C water, which is fatal to these fishes within minutes, specimens acclimated to 4 °C for four to six weeks survived for significantly longer than those held at their environmental water temperatures. While the ability to extend heat tolerance in these two species which are endemic to particularly cold stable Antarctic waters suggests that this ability is widespread throughout the Antarctic ichthyofauna, the study's use of survival time at a single temperature as a measure of heat tolerance makes it difficult to compare either tolerance or the ability to extend it between Antarctic, temperate, and tropical fishes.

The critical thermal maximum (CTMax) is a commonly used technique equating an animal's heat tolerance to the temperature at which it loses the ability to escape from constant rapid warming (Paladino et al. 1980). While this technique has been used extensively in temperate and tropical fishes it has never been used to determine heat tolerances in Antarctic fishes. Though it is a measure of acute tolerance, it has proven correlated with longer term measures of tolerance, and in some cases to a species' physiological optima (Kilgour and McCauley 1986; Garland Jr. et al. 1991; Bennett et al. 1998). Combined with its ease of determination, these make it a useful tool for identifying species' relative sensitivity to long term increases in local water temperatures.

However, caution must be applied when comparing CTMaxs as they have been shown sensitive to experimental parameters. Warming rate, which often differs between studies, is positively correlated with CTMax even in specimens that are held and tested under otherwise identical conditions (Becker and Genoway 1979; Terblanche et al. 2007). Despite this, when consistent heating rates are used the CTMaxs of species are repeatable with little intraspecies variability and thus can be used for comparisons between species and experimental groups.

In this investigation we used the CTMax methodology to survey heat tolerance in a geographically diverse group of 11 species of Antarctic fishes acclimatized to the cold water temperatures of their natural habitats. These include representatives of nototheniid species endemic to both the HAZ and SPZ allowing us to examine regional differences in heat tolerance as well as two zoarcid fish species. CTMaxs were also determined in eight of the species following warm acclimation to 4 °C, which when compared to their environmental CTMaxs provided a measure of the plasticity of their heat tolerance. Finally, the accrual of heat tolerance during this warm acclimation was followed over three weeks in three HAZ species.

Materials and Methods

Collection of fishes

Specimens of the four HAZ nototheniid species were collected from the shallow waters of the ice-covered McMurdo Sound (77 °S) by hook and line during the Austral spring (Oct and Nov) before warming of the surface waters. The two species of zoarcid fishes were trapped between September and December of 2007 at a depth of 500 m where the water is a constant − 1.9 °C throughout the year (Littlepage 1965). Following collection, specimens were transported in aerated insulated coolers at − 1.9 °C to the Crary laboratory's aquarium facility at McMurdo Station. CTMaxs were then either immediately determined, or if this was not possible, the fish were placed into 7000 L aquaria where they were held

for up to 21 days in a constant flow of local seawater which ranged in temperature from − 1.5 to − 0.9 °C. The McMurdo aquaria had windows and thus the fish were exposed to 24 h of daylight. At Palmer Station the aquaria also had widows and specimens thus were exposed to 10 h of subdued light. No attempt was made to control the lighting during the determination of the CTMax as from our experience it seems to have little effect on the behavior in the aquarium. Most would feed with interior lights turned on.

Specimens of the five notothenioid SPZ species were collected along the WAP near Anvers Island (64 °S) during the Austral winter months of July and August of 2008. Most were collected aboard the R.V. L.M. Gould by bottom trawl and trapping with additional specimens of *Notothenia coriiceps* collected by hook and line in 2 m of water from the shore at Palmer Station. Most ship trawls and trap lines were set in 100 m or less where the temperature was less than − 1 °C. Some *N. coriiceps* and *Lepidonotothen squamifrons* were captured at 300 m where the water temperature was 1 °C. Specimens were transported in flow-thru sea water aquaria for two to four days until they could be transferred to Palmer Station. Since surface waters were freezing, tank temperatures were held at − 1.5 °C using submersible 300 W aquarium heaters as several of the SPZ species were at risk of freezing in the ice-laden sea water supply.

The SPZ specimens were transferred to aquaria at Palmer station which received a constant flow of local seawater from Arthur Harbor ranging between − 1.7 to − 1.0 °C, the variation depending upon whether Arthur Harbor was ice covered and wind direction. CTMaxs were determined immediately for shore collected specimens, while those collected aboard the research vessel were allowed three to seven days to recover from collection stress.

Determination of the environmental CTMaxs of Antarctic fishes

Environmental CTMaxs were determined for the 11 Antarctic species following the methodology of Paladino et al. (1980). As these had been caught or held at temperatures below − 0.9 °C this served as a measure of the heat tolerance at their habitat temperatures. Specimens were transferred into 40 or 80 L plexiglas aquaria at the same water temperature as their holding tanks and which were large enough to allow free movement. Specimens were allowed several minutes to adjust to their new surroundings and then they were warmed at 0.3 °C min^{-1} through the activation of submerged stainless steel aquarium heaters.

This rate allows core body temperature to closely track the surrounding water temperature while fast enough to avoid the onset of substantial warm acclimation (Becker and Genoway 1979; Lutterschmidt and Hutchison 1997b). During warming the tank was vigorously aerated to prevent thermal stratification and maintain oxygen saturation. Water temperature was monitored once a minute using a DiGi sense model 8525 thermistor thermometer (Eutech Instruments Inc.) with an attached YSI series 401 temperature probe (Measurement Specialties Inc.) suspended in the aquaria.

Warming was continued until a persistent loss of equilibrium was observed as the inability of the fish to right itself for one minute after rolling on its side. While other investigators have argued for the onset of respiratory tremors or muscle spasms as signifying the CTMax (Lutterschmidt and Hutchison 1997a; Lutterschmidt and Hutchison 1997b), only a persistent loss of equilibrium was clearly observed in all of the Antarctic species. The temperature at which the specimen's persistent loss of equilibrium was first observed was then taken as their CTMax. They were then removed from the warming tank, quickly weighed to the nearest 0.1 g, then returned to their holding aquarium at its original temperature for observation during recovery.

Most specimens were tested following a brief period in the research station's holding aquaria which could be up to 0.5 °C warmer than local surface waters. To gauge whether any significant warm acclimation occurred during their residence in the holding aquaria, CTMaxs were determined on freshly

caught and aquaria held specimens of *P. borchgrevinki* and *N. coriiceps* then compared between groups by species using a two-tailed Student's t-test.

Log_{10} transformed CTMaxs were then compared among all specimens for significant differences between species using a one-way analysis of variances (ANOVA). This transformation was necessary to achieve the homogeneity of variance necessary for an ANOVA that was rejected by Levene's test in the untransformed CTMaxs. Significant groupings among the Antarctic species were then determined using a Student Newman–Keuls multiple range test performed on the log_{10} transformed CTMaxs. Finally a two-tailed Student's t-test was performed on species' average CTMaxs to compare heat tolerance between the HAZ and SPZ groups.

The effect of 4 °C acclimation on CTMax

Eight species were warm acclimated to 4 °C. This temperature was selected as it is above the seasonal temperature high at both collection sites (Barnes et al. 2006), but does not cause mortality in these fishes during extended acclimation (Somero and Devries 1967; Jin and DeVries 2006; Robinson and Davison 2008a). The tank temperature was maintained between 3.95 and 4.05 using a 1000-Watt immersion stainless steel heater and a temperature activated solenoid valve controlling the addition of cold seawater. If the water temperature rose above the temperature set point, the controller opened the solenoid valve and cold seawater entered the acclimation aquarium until the temperature dropped below the set point. This configuration also ensured regular turnover of the water within the 1000 L aquaria with complete replacement occurring in 48 h.

Independent samples of each species were warm acclimated except for *Trematomus pennellii* where there were insufficient specimens. For this species the same specimens used to determine their environmental CTMax were afterwards acclimated to 4 °C. However, they were allowed one week of recovery at –1.5 °C prior to warm acclimation which is sufficient to allow the transitory heat hardening

resulting from their CTMax to pass in temperate lower vertebrates (Maness and Hutchison 1980). The six McMurdo Sound species were warm acclimated for 21 days while limitations on tank space and field time at Palmer Station meant that the WAP nototheniids *Gobionotothen gibberifrons* and *N. coriiceps* could be warm acclimated for only 7 and 14 days respectively. Over the acclimation period detritus was periodically removed from the tank to avoid fouling and the notothenid fishes were fed notothenid white muscle once per week to satiation while the zoarcids refused food in captivity. Following acclimation, CTMaxs were determined using the previously described method. Species' acclimated CTMaxs were then compared to their environmental CTMaxs using a two-tailed Student's t-test except for *T. pennellii* where a paired t-test was more appropriate. Acclimated CTMaxs were then compared between species using a one-way ANOVA followed by a Student Newman–Keuls multiple range test, both performed on log10 transformed values.

Where both environmental and warm acclimated CTMaxs were available for the same species, their acclimation response ratio (ARR) was determined. This is a comparative measure of the sensitivity of heat tolerance to acclimation calculated as the difference between CTMaxs divided by the difference between their respective acclimation temperatures (Claussen 1977). For these species the denominator was taken to be the difference between 4 °C and their assumed water temperature of their habitats (–1.9 °C). The assumed environmental temperature seems reasonable given the similarly cold surface water temperatures during the collection of these fishes in the WAP during the winter and in McMurdo Sound during the early spring. At worst, this would understate the sensitivity of their heat tolerance to warm acclimation making this a conservative measure of their capability for acclimation.

2.4. The accrual of heat tolerance during acclimation to 4 °C.

The increase in CTMax during warm acclimation was followed in the three HAZ species, *P. borchgrevinki*, *T. bernacchii*, and the zoarcid fish, *Lycodichthys dearborni*. These were chosen as they

were available in large numbers and showed different thermal tolerance responses after three weeks of warm acclimation. CTMaxs were determined in independent samples of 6–10 specimens at 2, 4, 7, 14, and 21 days of acclimation to 4 °C. The exceptions to this were *L. dearborni* where insufficient specimens were available for a measurement at two weeks, and *P. borchrevinki* where the large number of specimens allowed an additional determination at 28 days.

The increase over environmental CTMaxs during acclimation was then analyzed for each species by fitting an exponential regression model with parameters estimated by the Gauss–Newton Method. To assess their goodness of fit a Pseudo-R^2 was calculated for each species as 1 − (Residual Sum of Squares / Total Corrected Sum of Squares) (Kutner et al. 2004).

Measuring the thermal lag between fish and water

The range of mass of the 11 Antarctic species spanned nearly two orders of magnitude which suggest large interspecies differences in thermal lag. To test whether this could account for the observed differences between species' CTMaxs, the core body and external water temperatures were monitored during warming in *P. borchgrevinki*, *T. bernacchii*, and *N. coriiceps*. The former two were selected as they were representative in mass and body shape of the smaller nototheniids, while the latter was a representative of the larger species.

Three specimens each of *P. borchgrevinki* and *T. bernacchii*, and six *N. coriiceps* were anesthetized in 80 mg L^{-1} tricanemethanesulfate (MS-222) until nonresponsive. These were then weighed on a top loading balance and a thin thermistor was implanted into their dorsal muscle mass. This was positioned near the vertebral column beneath the first dorsal fin and secured by suturing it to the skin.

Each fish was then allowed 30 min to recover from anesthesia at which point the aquarium water was heated at a rate of 0.3 °C min^{-1}. Both water temperature and the fish's core body

temperature were recorded simultaneously once a minute for 45 min. The thermal lag of each specimen was then calculated as the average difference between each measurement of external water temperature and core body temperature following its initial stabilization.

Results

The environmental CTMaxs of the 11 Antarctic species ranged from a low of 11.95 °C in the HAZ notothenid *P. borchgrevinki*, to a high of 16.17 °C in the SPZ notothenid *N. coriiceps* (Table 3.1, Fig. 3.1). Determinations of CTMax resulted in low levels of mortality. Approximately one in 20 specimens died, and no net increase was observed during recovery from the CTMax when compared to untested fishes. Neither *P. borchgrevinki* (*P* value 0.32) nor *N. coriiceps* (*P* value 0.41) showed any significant difference in CTMaxs between freshly caught and aquaria held specimens according to a two-tailed Student's t-test. The lack of difference in CTMax strongly suggests the absence of warm acclimation during their limited holding periods at temperatures ranging from − 0.8 to − 1.5 °C.

The eight warm acclimated species universally showed a significant ($P < 0.05$) increase in their CTMaxs over environmental specimens (Table 3.1, Fig. 3.1). This induced increase ranged from 3.24 °C in *P. borchgrevinki* to 1.22 °C in *N. coriiceps* corresponding to ARRs ranging from 0.55 to 0.21 (Table 1). Additionally, none of these species showed distress when returned to water temperatures between − 0.8 °C and − 1.7 °C indicating that their gain in heat tolerance did not come at the expense of tolerance to their environmental temperatures.

During the warm acclimation of *P. borchgrevinki*, *L. dearborni*, and *T. bernacchii* all showed an initial sharp increase in CTMax that began to level off within seven days (Fig. 3.2). The regression function for CTMax in *P. borchgrevinki* was:

$$\text{CTMax}=-3.03\times e^{-0.38}\times\text{days}+14.98 \quad (\text{Pseudo-}R^2{:}0.62, P<0.0001),$$

in L. dearborni:

$$CTMax = -2.14 \times e^{-0.12} \times days + 15.67 \quad (Pseudo\text{-}R^2: 0.42, P < 0.0001),$$

and in T. bernacchii:

$$CTMax = -1.11 \times e^{-0.41} \times days + 14.80 \quad (Pseudo\text{-}R^2: 0.15, P < 0.01).$$

The weak support for this last species likely reflects their small overall increase in CTMax combined with low precision in its determination.

Significant variation was found between species in both their environmental CTMaxs (one way ANOVA $F_{10,110} = 29.8$, $r^2 = 0.73$, $P < 0.0001$) and warm acclimated CTMaxs (one way ANOVA $F_{7,70} = 12.54$, $r^2 = 0.55$, $P < 0.0001$). Groupings for both determined by the Student Newman–Keuls multiple range test are presented in Fig. 3.1. When species' averages were compared between the SPZ and HAZ species, those from the SPZ had significantly higher environmental CTMaxs (two-tailed Student's t-test $P = 0.0004$). Limitations in time and resources prevented us from warm acclimating sufficient SPZ species to make a similar comparison among acclimated Antarctic fishes, however the SPZ notothenioids *N. coriiceps* and *G. gibberifrons* continued to have significantly higher CTMaxs than all HAZ species except the zoarcid *P. brachycephalum* following warm acclimation (Fig. 3.1).

As expected, thermal lag was much greater in the larger SPZ notothenioid *N. coriiceps* than in either of the McMurdo Sound notothenioids *P. borchgrevinki* and *T. bernacchii* (Table 3.2). However, the greater thermal lag present in *N. coriiceps* was insufficient to account for their higher CTMax than those of the McMurdo Sound fishes, or the high CTMaxs of the equally large WAP notothenioids, *G. gibberifrons* and *Notothenia rossii*.

Discussion

Thermal tolerance and plasticity vary between species and often reflect differences in their environments (Cossins and Bowler 1987). While this is most apparent when comparing animals between climatic regions, it can also often be observed between species within biomes. In fishes heat tolerance has commonly been determined as either their UILT or CTMax. While both measure tolerance in terms of temperature and produce closely correlated results, differences between their end points and the length of exposure mean that they cannot be directly compared (Kilgour and McCauley 1986).

The UILT measures heat tolerance as the highest temperature at which the median mortality is no longer dependent on the length of exposure (Fry 1971). However, since this must be extrapolated from the mortality of independent samples held at a series of static temperatures for set periods, this technique is costly in terms of time, aquarium space, and specimens.

The CTMax is an increasingly used alternative which determines heat tolerance as the temperature at which the effects of rapid warming become so severe that the organism is no longer capable of escaping further increases in temperature. As the temperature approaches the CTMax in fish tremors and muscle spasms are often observed followed by a persistent loss of equilibrium (Paladino et al. 1980). This technique is far more economical than the UILT in both time and numbers of specimens required, and since a rapid rate of warming is used this avoids the complication of acclimation during the determination of heat tolerance which may not be true of UILT's given the latter's longer exposure times (Becker and Genoway 1979; Lutterschmidt and Hutchison 1997b).

As a measure of acute tolerance, CTMax temperatures consistently exceed the UILT for equivalently acclimated specimens of the same species (Kilgour and McCauley 1986; Bennett et al. 1998). However, the CTMax may be of more relevance for understanding the stresses in a species' natural environment (Lutterschmidt and Hutchison 1997b). Some caution is warranted though when comparing CTMaxs. As a cumulative effect of time and temperature they are sensitive to the rate of

warming, with higher CTMaxs resulting from faster rates in equivalently acclimated specimens (Becker and Genoway 1979; Terblanche et al. 2007).

The environmental CTMaxs of Antarctic fishes

With our warming rate of 0.3 °C min^{-1}, the highest environmental CTMax among the Antarctic fishes was 16.17 °C (Table 3.1), well below those reported from fishes endemic to warmer waters. The low environmental CTMaxs of the Antarctic fishes are even slightly lower than that of the polar cod, *Boreogadus saida* (17 °C) (DeVries, unpublished) which inhabits a thermal environment similar to the SPZ Antarctic species. The CTMaxs of temperate and tropical fishes acclimated near their environmental water temperatures often exceed 30 °C and cold temperate species above 20 °C (Otto and Ohararice 1977; Becker and Genoway 1979; Bennett et al. 1998; Currie et al. 1998; Fangue and Bennett 2003; Carveth et al. Comparison of upper thermal tolerances of native and nonnative fish species in Arizona). Though these studies applied a variety of warming rates ranging from 0.1 to 0.5 °C min^{-1}, the difference in CTMax due to variable rates of warming were small. Becker and Genoway (1979) investigated the effect of warming rate on the CTMax of the two freshwater fishes, *Oncorhynchus kisutch* and *Lepomis gibbosus*, and found the CTMaxs of both species varied by only 2 °C over this range of rates. Increasing the rate of heating from 0.07 to 0.75 °C min^{-1} in specimens of the Antarctic fish *T. hansoni* ranging from 61 to 105 g resulted in a similar increase of just 2.3 °C (DeVries, unpublished), to small to explain the large difference between cold temperate and polar species. A fraction of this increase in CTMax would be due to a corresponding increase in thermal lag observed as the warming rate is elevated.

The comparably low CTMaxs of the Antarctic fishes indicate a limited heat tolerance consistent with earlier UILT of 5 to 7 °C in HAZ nototheniid species indicating a low upper thermal tolerance (Somero and Devries 1967). They are also similar to the limited heat tolerance noted in Antarctic invertebrates. Peck et al. (2010) found acute tolerances below 15 °C in five species of SPZ Antarctic

invertebrates from four phyla, and only the mollusk *Yoldia eightsi* reaching a temperature near 20 °C. An earlier investigation noted an even more marked intolerance to chronically elevated temperatures in the Antarctic invertebrate *Ophionotus victoriae* which is incapable of acclimating to temperatures as low as 2 °C (Peck et al. 2009). This widespread sensitivity to heat among the Antarctic marine fauna further supports the argument that the removal of positive selective pressure for heat tolerance from their exposure to constant freezing water temperatures over evolutionary time has lead to a reduction in tolerance.

As a fish approaches its CTMax during warming hyperactivity, loss of coordination, then loss of equilibrium occurs in sequence. These are similarly induced by direct heating of a fish's cerebellum at equivalent temperatures (Friedlander et al. 1976) suggesting that a break down in the integration in the central nervous system (CNS) limits the Antarctic species' capacity to withstand acute heat stress. The CNS has been shown to be particularly temperature sensitive failing at lower temperatures than organ systems, cells, enzyme activities and peripheral nervous conduction (Roots and Prosser 1962; Prosser and Farhi 1965), and within the nervous system the site of this temperature limitation has been localized at the synaptic junctions (Prosser and Nelson 1981).

Evidence for the failure of conduction at the CNS synapses in Antarctic fishes' at relatively low temperatures comes from two studies on the nototheniid *P. borchgrevinki* (Hochachka and Somero 2002). In specimens acclimatized to their freezing local water temperatures, a temperature driven increase in the release of the neurotransmitter acetylcholine occurs at neuromuscular junctions, peaking sharply between 12 °C and 14 °C (Macdonald and Montgomery 1982), at a temperature near this species CTMax. This temperature response is likely true of cholinergic synapses in the CNS as well, and it occurs in conjunction with a sharp temperature driven decline in the activity of the enzyme acetylcholine esterase, which is responsible for breaking down this neurotransmitter (Baldwin 1971). The consequence of these temperature effects would be the accumulation of excess acetylcholine in the

synaptic cleft undoubtedly disrupting conduction and integration in the CNS, causing the observed symptoms associated with warming to the CTMax.

Interspecies variation in environmental CTMaxs

While the Antarctic fishes have a narrow range of environmental CTMaxs, they also show significant interspecies variation most of which is associated with their geographic distribution. SPZ species have significantly higher environmental CTMaxs than all but one of the HAZ species, the zoarcid *Pachycara brachycephalum* (Fig. 3.1). While it is possible that this split could result from either the SPZ species acclimatization to warmer local water temperatures or due to their greater mass, this appears instead to show an innately greater tolerance to heat.

Some specimens of the SPZ species, *L. squamifrons* and *N. coriiceps* were collected from the warm CDW and thus were acclimatized to higher water temperatures. However, these specimens were held for 10 days at near freezing water temperatures including transport and residence time in the Palmer station aquaria. It is likely that this holding period would have eroded most of any heat tolerance due to acclimatization to the 1 °C. Furthermore, no significant difference was found between the CTMaxs of *N. coriiceps* collected from either the freezing winter SPZ surface waters, or specimens collected from the CDW, which may suggest that acclimatization at 1 °C in CDW had no detectable effect on heat tolerance.

The higher environmental CTMaxs of the SPZ species could also be an effect of their average higher mass (Table 3.1). Greater mass requires more time for the propagation of heat from the periphery into the temperature sensitive core that could inflate CTMaxs when determined at rapid warming rates. However, the difference in the thermal lag between the HAZ species *T. bernacchii* and *P. borchgrevinki* and the SPZ species *N. coriiceps* could not account for the observed difference in their environmental CTMaxs (Table 3.2). Further support that the difference is real comes from the

observation that even the smallest of the SPZ species, *L. nudifrons* and *L. squamifrons* had higher environmental CTMaxs than the HAZ species of comparable mass.

The CTMaxs of the SPZ species could have also been underestimated due to seasonal differences in collection times of the SPZ and HAZ fauna. While the HAZ species were collected and tested during the austral spring and early summer, the SPZ species were winter acclimatized at their time of testing. The effect of season on the heat tolerance of temperate fishes has been well established (Fry 1967), and recent observations of seasonally changing activity levels in the SPZ notothenid *N. coriiceps* suggest this may be true of the Antarctic ichthyofauna as well (Campbell et al. 2008).

The greater heat tolerances found in the SPZ species is probably associated with their evolution in a slightly warmer environment. Summer surface water temperatures are seasonally higher in the SPZ and continually warmer in the deep waters influenced by CDW (Barnes et al. 2006; Clarke et al. 2009). This is in contrast with HAZ habitats such as those in McMurdo Sound where water temperatures, remain close to their freezing point (− 1.9 °C) through most of the year with the exception of the surface water (upper 50 m) which warms to only − 0.5 °C for a few weeks in January (Hunt et al. 2003). Differences in peak environmental temperatures have previously been reported to drive intra-species thermal adaptation in fruit flies and killifish (Davidson 1988; Fangue et al. 2006) and inter-species adaptation in Porcelain crabs (Stillman and Somero 2000) which may hold true for Antarctic fishes as well.

Outside of the regional differences in heat tolerance, inter-species variation in the CTMaxs was largely muted (Fig. 3.1) with the exception of the HAZ notothenid *P. borchgrevinki* and the zoarcid *P. brachycephalum*. *P. borchgrevinki* had the lowest CTMax significantly differing from all of the other species except their fellow benthic shallow-water notothenid *T. pennellii* (Fig. 3.1). The particularly low heat tolerance of *P. borchgrevinki* may be a consequence of its adaptations to a cryopelagic lifestyle

which brings it into regular contact with super-cooled seawater and cold brine just below the sub-ice platelet layer (Andriashev 1970).

At the other extreme, *P. brachycephalum* was the only HAZ species whose environmental CTMax overlapped with some SPZ species despite being collected from the high-latitude constantly cold deep water (− 1.9 °C) of McMurdo Sound (Fig. 3.1). This corroborates previous work on this species' physiology and ecology that have shown notable thermal flexibility. These include minimal cellular oxygen demand at higher temperatures than other HAZ species (Mark et al. 2005), a geographic distributions into warmer waters (Brodte et al. 2006), and a continued capacity to reduce whole organ aerobic capacity through warm acclimation (Lanning et al. 2005). The zoarcid *P. brachycephalum* is relatively phylogenetically distant from the nototheniids which belong to the Perciformes suborder Notothenioidei. This species' greater heat tolerance may in part be associated with the fact that the Zoarcidae is a cold temperate water family and some have invaded the Southern and Arctic Oceans.

Effect of 4 °C warm acclimation on CTMax

Though most animals have the ability to adjust their heat tolerance in response to their recent thermal history, the capacity of Antarctic fishes to adjust their heat tolerance through acclimation to elevated temperatures has been poorly studied. Recently, warm acclimation experiments performed on two nototheniids by Podrabsky and Somero (2006) have indicated a significant capacity for these fishes to elevate their heat tolerance through acclimation. In our experiments, acclimation to 4 °C for one to three weeks also produced a significant increase over the environmental CTMaxs for species from both regional groups (Table 3.1, Fig. 3.1), indicating that this thermal plasticity is universal throughout the Antarctic ichthyofauna.

However, despite the increases, the CTMaxs were less than 18 °C, well below that of temperate fishes cold acclimated to 5 °C. The CTMaxs of two temperate fishes *Carassius auratus* and *Cyprinodon*

variegatus were 30.8 °C and 34.6 °C respectively when acclimated to 5 °C (Bennett et al. 1998; Ford and Beitinger 2005). When the two cold-temperate species *Cottus cognatus* and *O. kisutch* were similarly cold acclimated, their CTMaxs were 23.5 °C and 25.3 °C (Otto and Ohararice 1977; Becker and Genoway 1979). Even accounting for differences in warming rates these species had far greater CTMaxs than the Antarctic species at comparable acclimation temperatures. That the Antarctic species continue to show this comparative shortfall in heat tolerance with warmer water fishes even when similarly acclimated shows an innately lower heat tolerance.

Despite the low CTMaxs of the Antarctic fishes acclimated to 4 °C, the responsiveness of their heat tolerance to this 5.9 °C increase in acclimation temperature measured as their ARR was comparable to that in some far more eurythermal species. Though neither the relationship between ARR and overall thermal range, nor the homogeneity of the ARR within a fish's acclimatory range have been well studied, more eurythermal species have often shown a greater ARR than stenothermal ones (Beitinger and Bennett 2000).

This degree of thermal flexibility in Antarctic fishes stands in contrast to many of the Antarctic invertebrates. Peck et al. (2010) noted that five of six invertebrate species from four phyla showed no increase in acute heat tolerance in response to 60 days of warm acclimation to 3 °C. The lack of thermal flexibility was even more marked in the WAP brittle star *O. victoriae* which failed to acclimate to temperatures of just 2 °C suggesting an extreme lack of capacity for warm acclimation (Peck et al. 2009).

The ARR of the Antarctic species exceeded 0.4 in four species (Table 3.1) which is similar to those calculated using CTMaxs with similar heating rates in the far more eurythermal goldfish *C. auratus* (0.43) over its entire thermal range (Ford and Beitinger 2005), as well as in the pumpkin seed sunfish *Lepomis gibbosus* (0.51) and the channel catfish *Ictalurus punctatus* (0.38) over a 10 °C increase in acclimation temperature (Becker and Genoway 1979; Carveth et al. 2006). By comparison the relatively more stenothermal species show less thermal flexibility with ARRs in the salmonids *O. kisutch*,

Onorhynchus mykiss, and *Salmo salar* of 0.34, 0.18 and near zero respectively over a 10 °C increase in acclimation temperature (Becker and Genoway 1979; Elliot 1981; Currie et al. 1998). While the comparably low CTMaxs noted at environmental temperatures and at 4 °C suggests that the capacity of these fishes to increase their heat tolerance will quickly be outstripped with increasing acclimation temperature, the relatively high ARR of some of the species suggests that they may ultimately be capable of acclimating to higher temperatures than previously thought given their long evolution in chronic freezing water temperatures.

While the ARR for the two SPZ species *N. coriiceps* and *G. gibberifrons* were lower than those of the HAZ species this must be carefully interpreted as both could be understated. In part this is due to the unavoidably shorter acclimation periods applied to these species which may not have allowed enough time for their full warm acclimation. It also reflects our use of − 1.9 °C as their environmental water temperature even though some of these fishes may have remained partly acclimated to deep warm, waters in the SPZ where temperatures in some areas are near 1 °C (DeVries and Steffensen 2005). Finally, it may also have been reduced by seasonal effects which could have suppressed the capacity for acclimation in winter SPZ specimens.

The Antarctic species increase in heat tolerance from warm acclimation comes despite their apparent loss of the ability to induce an increase in some cellular heat shock proteins (HSP70) in response to either acute (Hofmann et al. 2000) or chronic elevated temperatures (Clark et al. 2008; Buckley and Somero 2009). While HSPs are commonly believed responsible for mitigating cellular damage that occurs at increased temperature, they have never been causally linked to the increases in heat tolerance that occur in the whole animal with acclimation (Jensen et al. 2010). Instead, organismal tolerance may be dictated by the temperature at which physiological systems fail, which occurs below the temperatures where the tissues, cells, and proteins that comprise them are affected (Prosser and

Nelson 1981). The increased acute heat tolerance of Antarctic fishes following warm acclimation may thus reflect acclimatory changes within their CNS which make it more robust to the effects of heat.

Accrual of heat tolerance during acclimation to 4 °C

While the accrual of heat tolerance during warm acclimation has been noted to occur rapidly in some species, this appears to be dependent both on the temperature of acclimation, and its position within a species' acclimatory range (Brett 1946; Bennett et al. 1998). Acclimation occurs more rapidly at high temperatures where some studies have shown heat tolerance can become fully manifested within a few days (Doudoroff 1942). Warm acclimation of organisms inhabiting low temperature environments requires notably more time, often extending over weeks, most likely because of the rate depressing effects of low temperature on the biochemical reactions involved in the acclimatory changes (Cossins and Bowler 1987).

The accrual of heat tolerance in *P. borchgrevinki*, *L. dearborni*, and *T. bernacchii* followed a hyperbolic curve with an initial rapid buildup that quickly tapered off (Fig. 3.2). Extended residence times at 4 °C were necessary for both *P. borchgrevinki* and *L. dearborni* to fully acclimate, while *T. bernacchii* appeared to acclimate more rapidly. However, the small induced increase in CTMax in the latter species combined with the low precision of these determinations may mask a substantially slower development of heat tolerance during acclimation.

The slow response of *P. borchgrevinki* and *L. dearborni* was expected given their low acclimation temperature which would result in only a limited increase in their metabolic rate. However, the paucity of acclimation studies at these temperatures makes it difficult to judge whether acclimation in the Antarctic species shows any compensation for low temperature relative to temperate species which might be expected given their adaptation to chronic cold.

Conclusion

The Antarctic species show the lowest recorded CTMaxs among examined fishes, even lower than the Arctic polar cod which inhabits a similar thermal environment. This held true even when comparably acclimated indicating an innate shortfall in the heat tolerance of these fishes. Differences between the Antarctic species' CTMaxs suggest though that there is a particular sensitivity to heat among the high-latitude HAZ species which may put them at risk from rising water temperatures. However, despite their low CTMaxs, all the Antarctic species maintained the capacity to increase their heat tolerance through warm acclimation. When this capacity was quantified using the ARR, this showed a surprising level of thermal plasticity at low temperatures which was surprising given the presumed loss of selection for thermal flexibility that has long been assumed in this fauna. Given the future predicted increases in water temperatures in the Southern Ocean from global climate change, understanding the heat tolerance of Antarctic fishes and its plasticity is critical for understanding the threat to this cold adapted fauna. The CTMax methodology proved useful in exploring the thermal tolerances of this fauna. It generated rapid and comparable measures of acute heat tolerances, which were previously unexplored.

CHAPTER 5: HEAT TOLERANCE OF THE SECONDARILY TEMPERATE ANTARCTIC NOTOTHENIOID, *NOTOTHENIA ANGUSTATA*

Abstract

Though most of the notothenioid fishes have geographic distributions restricted to the Southern Ocean, several species with Antarctic origins have come to inhabit the warmer waters around New Zealand and southern South America. Presumably these secondarily temperate Antarctic notothenioids once shared the low heat tolerance now found throughout endemic Antarctic species. However, it is unknown whether their polar ancestry continues to exert an influence on their modern heat tolerance. Here, we investigate the heat tolerance of one such secondarily temperate species, the black cod (*Notothenia angustata*), which is now endemic to the waters around the South Island of New Zealand. Using the critical thermal maximum (CTMax) we determined the heat tolerance of specimens acclimatized to their winter water temperatures, averaging 7.9 °C, then warm acclimated to 15 °C, near the summer water temperatures in Otago harbor. These CTMaxs were then compared to those determined from equivalently acclimated specimens of the basal New Zealand notothenioid *Bovichtus variegatus*, and specimens of *N. angustata*'s Antarctic endemic congener *N. coriiceps* warm acclimated to 10 °C. Though *N. angustata* had consistently lower CTMaxs than *B. variegatus* at both water temperatures, these New Zealand notothenioids shared significantly higher CTMaxs than *N. coriiceps* even when the latter was acclimated to a slightly higher water temperature. This shows greater heat tolerance in the secondarily temperate *N. angustata* than endemic Antarctic species, however, it also suggests that some of its ancestral intolerance to heat persists.

Introduction

The evolution of the Antarctic notothenioid fishes in the chronically cold waters of the Southern Ocean has resulted in a marked and shared intolerance to heat (Somero and Devries 1967; Bilyk and DeVries 2011). Though most extant members of the suborder Notothenioidei inhabit the Southern Ocean, a few species are only found in the warmer waters around New Zealand and southern South America. Divided into two distinct groups, these include both the members of the three basal notothenioid families, which diverged prior to the origin of the cold-adapted Antarctic notothenioid clade, and at least 16 species nested within the Antarctic notothenioids but now permanently found outside of Antarctic waters (Eastman 2005). Unlike the basal species, the ancestors of this latter group of secondarily temperate species presumably once shared the reduced heat tolerance noted among the endemic Antarctic species. However, it is unknown whether their Antarctic ancestry continues to constrain their modern heat tolerance.

The shallow coastal waters of southern New Zealand are home to one such secondarily temperate species, the black cod (*Notothenia angustata*). Despite a residence time in cold-temperate waters that may reach back as far as 11 MYA, *N. angustata* continues to exhibit a number of cold-adaptations characteristic of the endemic Antarctic species. While inhabiting continually ice-free waters, this species still has some functional genes for antifreeze glycoprotein (AFGP) not found among the basal notothenioid families (Cheng et al. 2003). At their environmental water temperatures, they also show intermediate levels of both cellular membrane lipid saturation (Logue et al. 2000) and ubiquitin conjugated proteins (Todgham et al. 2007) between cold-temperate and endemic Antarctic species. However, whether whole organism traits, like heat tolerance, show a similar pattern remains unknown.

Heat tolerance in fishes has typically been determined using two irreconcilable methodologies, either the measurement of their upper incipient lethal temperature (UILT), or their critical thermal

maximum (CTMax) (Kilgour and McCauley 1986). The UILT, based on lethal dosage methodologies, measures tolerance as the temperature at which median mortality first becomes independent of the length of exposure, typically determined from exposure times ranging between a day and a week (Fry 1947; Cossins and Bowler 1987). The first study of heat tolerance in the Antarctic notothenioids used this methodology, found UILTs between 5 and 7 °C in three species of high-latitude nototheniids acclimatized to their natural freezing water temperatures (-1.9 °C), which were the lowest UILTs then recorded (Somero and Devries 1967).

The CTMax is an increasingly used alternative, which determines tolerance as the temperature at which a specimen becomes incapacitated when rapidly warmed at a given rate (Hutchison 1961; Paladino et al. 1980). As a measure of acute tolerance, CTMaxs always exceed the UILT for equivalently acclimated specimens (Kilgour and McCauley 1986). CTMaxs also differ according to experimental parameters, most notably warming rate and starting temperature (Becker and Genoway 1979; Terblanche et al. 2007). However, when care is used to ensure identical experimental conditions, CTMaxs are highly repeatable and useful for making comparisons within and between species. Additionally, this latter approach benefits from greater economy of specimens, time, and space when compared with the UILT, while producing results that still appear correlated to more chronic measures of heat tolerance. When the CTMaxs of 11 species of Antarctic fishes were recently determined from specimens acclimatized to their natural freezing water temperatures, these ranged from 11.95 to 16.17 °C which, like measures of UILT, were well below CTMaxs reported from cold-temperate and temperate fishes (Bilyk and DeVries 2011).

Despite the polar ancestry of *N. angustata*, this species largely shares its present day geographic and bathymetric distributions with a basal notothenioid, the New Zealand thornfish (*Bovichtus variegatus*) (Paulin et al. 1989). In their modern thermal range these species inhabit water temperatures continually above those experienced by the endemic Antarctic notothenioids. In Otago Harbor, where

the specimens used in this study were collected, water temperatures range between monthly peaks ranging from 14.2 to 18.2 °C and lows between 5.6 and 8.6 °C (Bev Dickson, personal communications). The goal of this project was to compare CTMaxs between similarly acclimated specimens of the secondarily temperate *N. angustata*, the continually cold-temperate *B. variegatus*, and the endemic Antarctic *N. coriiceps*. This was done to determine whether the heat tolerance of the secondarily temperate Antarctic notothenioid has increased sufficiently over levels in endemic Antarctic species to match the presumptive notothenioid levels prior to their cold-specialization as indicated by *B. variegatus*.

Materials and Methods

Collection of Specimens

Specimens of the black cod, *N. angustata*, were purchased in June of 2010 from the Portobello Marine Laboratory located on the Otago Peninsula. These had been collected with baited crayfish traps placed on the rocky bottom in shallow waters (10 m) around the Otago Heads. After collection, they were held in a large outdoor aquarium (10 x 10 m by 2 m deep) with a constant flow of local seawater. Specimens of the thornfish, *B. variegatus*, were collected by hand net and by hook and line off the rocky shore and from tide pools along the Otago Peninsula and in the vicinity of the mouth of Bull Creek during June and July of 2010. Prior to experimentation both species were moved into 390 L shallow indoor aquaria for one to two weeks where they received a constant flow of local seawater ranging in temperature from 6.7 to 9.0 °C and averaging 7.9 °C, mirroring the conditions of their winter environment. While in captivity specimens of *B. variegatus* were isolated in covered plastic mesh cages to prevent escape and predation. Specimens of both species were held and treated in accordance with protocol 39/09 administered through the University of Otago Animal Ethics Committee.

Specimens of the Antarctic nototheniid, *N. coriiceps*, were collected during July and August of 2008 by traps deployed from the R.V. L.M. Gould at sites along the Western Antarctic Peninsula (WAP) and by hook and line from the shore of Anvers Island (64 °S) near Palmer station. Those collected aboard the ship were transported to Palmer Station in aquaria with a constant flow of ice laden surface waters but kept above – 1.5 °C through the use of a 300 W aquarium heater. At Palmer station all specimens were allowed several days to recover from collection stress before the start of warm acclimation.

Prior to experimentation all specimens of *N. coriiceps* and *B. variegatus* were weighed to the nearest tenth of a gram, and *N. angustata* to the nearest 10 g. To track individual specimens of *N. coriiceps* and *N. angustata* Floy tags (Floy Tag & Mfg., Inc.) were inserted into the muscle just behind the first ray of the second dorsal fin. Due to their small size *B. variegatus* could not be tagged and instead individuals were kept separated by tank partitions.

Determination of Environmental CTMax

Following a brief adjustment period to captivity, environmental CTMaxs were determined for specimens of both of the New Zealand notothenioids. The environmental CTMax of a specimen is intended as a measure of the heat tolerance present while acclimatized to their natural environmental temperatures, which were only determined for winter acclimatized specimens of *N. angustata* and *B. variegatus* in this study.

CTMaxs were determined according to the protocol of Bilyk and DeVries (2011) adapted from Palidino et al. (1980). To briefly summarize, specimens were transferred to a test aquaria at their holding tank temperature and after 10 minutes they were warmed at 0.3 °C min^{-1} until the onset of a persistent loss of equilibrium. The temperature of this endpoint was taken as the specimen's CTMax and species' environmental CTMaxs were calculated as the arithmetic mean across all specimens.

Following a specimen's persistent loss of equilibrium they were immediately returned to their original water temperature and their recovery monitored over 24 hours. On account of the large size of *N. angustata*, all determinations were made in a 60 L aquarium which afforded even the largest specimen's sufficient space for free movement. Temperature homogeneity within this aquarium was maintained through vigorous aeration and the use of a recirculation pump.

Warm acclimation of *N. coriiceps* to 10 °C

Eight specimens of *N. coriiceps* were warm acclimated at 10 °C for three weeks. As attempts to directly transfer specimens from their environmental water temperatures (– 1.5 to – 1.0 °C) to 10 °C resulted in notable mortality, these were instead warmed in steps up to their final acclimation temperature. First, specimens were held at 6 °C for five days, then 8 °C for two days, and finally at 10 °C for three weeks after which their CTMaxs were determined as previously described. Water temperature was maintained using a 300 W submersible heater and temperature controller activated solenoid valve which allowed the addition of cold local seawater only when their tank water temperature rose above the set point. During acclimation, their tank water was vigorously aerated to maintain oxygen saturation, specimens were fed white nototheniid muscle every three days to satiation with any excess removed, and the tank was cleaned of detritus daily with the sides brushed every three days to prevent fouling.

Warm acclimation of *N. angustata* and *B. variegatus* to 15 °C

Following two to five days to recover from the determination of their environmental CTMaxs, specimens of *N. angustata* and *B. variegatus* were placed at 15 °C. This water temperature was maintained using two 300 W aquarium heaters activated when water temperatures fell below their set point and a submersible pump added cold seawater from a reservoir tank when the water temperature

rose above its set point. Over the next three weeks water temperatures ranged from 14.6 to 15.4 °C cycling every 6 to 38 minutes. After three weeks CTMaxs were again determined as previously described. During acclimation, white fish muscle was provided twice per week with any uneaten food removed immediately. To reduce fouling, tanks were cleaned of detritus every three days.

Statistical Analysis

CTMaxs of *N .angustata* and *B. variegatus* were compared using a two way ANOVA testing for significant differences between species, acclimation temperature, and any interaction between the two. The CTMaxs of 10 °C acclimated *N. coriiceps* were compared with the environmental CTMaxs of *N. angustata* and *B. variagatus* using a one way ANOVA with species as the independent variable, followed by a post-hoc Tukey's test. For both, the ANOVA assumption of homoscedasticity was confirmed using Levene's test and the residuals were tested for normality.

Results

During this study all specimens remained healthy, though none of the *B. variegatus* were observed to feed. While direct transfer of *N. coriiceps* from their environmental water temperatures (– 1.5 to – 1.0 °C) to 10 °C resulted in some mortality after 3 or 4 days, neither stepped acclimation of *N. coriiceps* to 10 °C, nor direct acclimation of the New Zealand species to 15°C resulted in any mortality or visible distress.

In addition to the warm acclimation of the two New Zealand species, an attempt was made to cold acclimate these fishes to 4 °C which would have allowed for a direct comparison to the CTMaxs of eight Antarctic species previously reported by Bilyk and DeVries (2011). While three *B. variegatus* specimens readily tolerated 4 °C, six *N. angustata* showed increasing pallor, refusal of food, and declining health ultimately leading to the abandonment of this part of the experiment. However,

specimens of *N. angustata* have previously been cold acclimated to 2 °C without issue over several weeks by DeVries (unpublished), and it is unclear whether the observed decline during this study was due to sub-optimal holding conditions or from stepping them down to 4 °C too rapidly.

Environmental and warm acclimated CTMaxs for all three species are reported in both Table 4.1 and Fig. 4.1. Though some caution is warranted in comparing CTMaxs between acclimatized and acclimated specimens, the one way ANOVA found significant variation between the environmental CTMaxs of *N. angustata*, *B. variegatus*, and 10 °C acclimated *N. coriiceps* ($F_{2,17} = 90.38$; $P < 0.0001$; $r^2 = 0.91$), with the post-hoc Tukey test noting the lowest CTMaxs in *N. coriiceps*. The two-way ANOVA on *N. angustata* and *B. variegatus* found a significant effect of both species ($F_{1,20} = 53.53$; $P < 0.0001$) and acclimation temperature ($F_{1,20} = 40.15$; $P < 0.0001$) on CTMax, though no significant interaction between the two ($F_{1,20} = 0.0004$; $P = 0.9718$). The absence of an interaction between species and acclimation temperature indicates a similar increase in CTMax in both species following warm acclimation despite the lower CTMaxs of *N. angustata*.

With the exception of a single specimen out of eight *B. variegates*, there were no fatalities following the determination of CTMaxs and all of the New Zealand specimens readily recovered within several hours of their CTMax. Unlike the New Zealand species, 10 °C acclimated *N. coriiceps* were directly returned to their environmental water temperatures after their CTMax and 50% failed to recover. This reduction in survivorship may suggest that their gain in heat tolerance from warm acclimation came at a notable expense in cold tolerance raising it above the freezing point of seawater.

Discussion

Heat tolerance often reflects a species' thermal history. The selection for this tolerance appears to come from exposure to the highest temperatures in a species' environment (Mongold et al. 1996). For the Antarctic notothenioids, the removal of positive selective pressure for heat tolerance during

their evolution in the cold stable waters of the Southern Ocean has been suggested as a cause for their modern low heat tolerance (Somero et al. 1998). While the ancestors of the secondarily temperate Antarctic notothenioids once presumably shared the low heat tolerance noted among modern endemic Antarctic notothenioids, their presence in cold-temperate waters suggests that they now have greater heat tolerance than species that have continually inhabited Antarctic waters. However, it has been unclear whether the upper thermal tolerances of these species are similar to fishes of non-Antarctic origin with which they now share their thermal habitat.

Comparing heat tolerances between the secondarily temperate *N. angustata* and the basal notothenioid *B. variegatus*

Though they inhabit the same cold-temperate waters, the CTMax of *N. angustata* was significantly less than that of *B. variegatus*, both when compared between winter acclimatized specimens and between specimens warm acclimated to 15 °C (Table 4.1, Fig 4.1). However, it is likely that the difference in heat tolerance between these species is understated when measured by CTMax as this fails to account for the effects of thermal lag on the much larger black cod.

Thermal lag is the time required for heat to be conducted from the periphery of an animal to the heat sensitive tissues in its body core. As it is the latter that limits organismal heat tolerance, thermal lag serves to inflate acute measures of heat tolerance such as the CTMax in larger species relative to smaller ones (Fry 1971). When this was directly compared between three species of Antarctic nototheniids warmed at 0.3 °C min^{-1}, core temperatures in *N. coriiceps* (791 g ± 414) were 0.79 °C cooler than *P. borchgrevinki* (112 g ± 39), and 1.11 °C cooler than *T. bernacchii* (114 g ± 42) (Bilyk and DeVries 2011). The difference in mass between the *N. angustata* and *B. variegatus* in this experiment was greater yet, with *N. angustata* specimens averaging 24 times the mass of *B. variegatus* (Table 4.1) suggesting that

our determinations of CTMax are overstating the already low heat tolerance of *N. angustata* relative to *B. variegatus*.

While sharing modern thermal environments, the lower CTMaxs of *N. angustata* may reflect the persistence of low heat tolerance from its polar ancestor and which is consistent with the presence of several biochemical and cellular cold-adaptations. As previously noted, at its environmental water temperatures this species shows incomplete homeoviscous adaptation of cell membranes (Logue et al. 2000) and intermediate lability of their cellular protein pool values which fall between cold-temperate New Zealand and endemic Antarctic fishes (Todgham et al. 2007). It also retains circulating AFGPs found in most endemic Antarctic notothenioids, though at vestigial levels insufficient to provide protection from freezing conditions (Cheng et al. 2003). Unlike the endemic Antarctic species, *N. angustata* shows a modest inducible cellular heat shock response which is believed to have been lost in the endemic Antarctic species (Hofmann et al. 2000; Clark et al. 2008) but less than that of *B. variegatus* (Hofmann et al. 2005). Overall, it appears that there has been insufficient evolutionary time in the warmer New Zealand waters for these polar traits, including reduced organismal heat tolerance to completely disappear.

Despite the lower CTMax of *N. angustata* compared to *B. variegatus,* both species showed an equivalent capacity to increase their CTMax in response to warm acclimation (Fig 4.1). Recent investigations of endemic Antarctic species have shown notable organismal and systems level capacity for warm acclimation even with their comparably low heat tolerance (Podrabsky and Somero 2006; Franklin et al. 2007; Robinson and Davison 2008c; Bilyk and DeVries 2011). As such, it is not surprising the *N. angustata* appears to show a similarly unimpaired capacity to increase heat tolerance through warm acclimation. However, the plasticity of heat tolerance in *N. angustata* and *B. variegatus* was only tested over a small temperature range and it remains unknown whether these species share an equivalent response across their full acclimatory ranges.

Comparing the heat tolerance of the secondarily temperate Antarctic notothenioid *N. angustata* and the endemic Antarctic species *N.coriiceps*

Though *N. angustata* is found in the same cold-temperate waters as *B. variegatus*, it is more closely related to endemic Antarctic species including its congener *N. coriiceps*. The latter is restricted to Antarctic waters below 54 °S (Gon and Heemstra 1990) which remain continually below 4 °C throughout the year (Barnes et al. 2006). When the environmental CTMaxs of winter acclimatized (7.9 °C) *N. angustata* were compared to the CTMaxs of 10 °C acclimated *N. coriiceps*, those of the *N. angustata* were significantly higher despite the slightly warmer acclimation temperature of *N. coriiceps*.

Averaging 3.3 °C, this difference is larger than that observed between *N. angustata* and *B. variegatus* though it is likely partly overstated due to greater thermal lag in the larger *N. angustata* (Table 4.1). However, unlike the comparison between *N. angustata* and *B. variegatus*, that fraction of the CTMax difference due to thermal lag is less because of the smaller difference in body size between *N. angustata* and *N. coriiceps*. A CTMax intermediate to cold-temperate and polar fishes is consistent with the presence of some polar characteristics, which are reduced relative to the endemic Antarctic species *N. coriiceps.* The intermediate heat tolerance of *N. angustata* between *B. variegatus* and *N. coriiceps* remains despite an apparent lengthy period under selection for higher heat tolerance from the warmer water temperatures shared by the continually cold-temperate *B. variegatus*.

Though the method of dating the origin of the secondarily temperate Antarctic notothenioids remains in question, the divergence of *N. angustata* from endemic Antarctic species likely extends to at least the early Pliocene. The prevalent hypothesis for the appearance of Antarctic notothenioids in New Zealand waters is that their ranges extended northward during glacial maxima (Petricorena and Somero 2007). During prior maxima, the Antarctic circumpolar front which forms the boundary of the Southern Ocean has shifted northward by up to 5 to 10 degrees of latitude in open ocean areas (Nelson and

Cooke 2001). This could have brought the Antarctic Front near New Zealand allowing for the dispersal of the eggs and larva of *N. angustata*'s progenitor. Given the persistence of Southern Ocean fronts between New Zealand and Antarctica and the associated temperature gradient, once *N. angustata*'s progenitor arrived in New Zealand waters it would have experienced continually warmer water temperatures than Antarctic species (Kennett 1982; Nelson and Cooke 2001; Sikes et al. 2002).

Using the divergence in the mitochondrial ND2 gene between the two New Zealand nototheniids *N. angustata* and *N. microlepidota* Cheng et al. (2003) estimated the time of their exit from Antarctic waters at 11 MYA. Though this places their origin near a glacial maximum, the reliance of this analysis on universal substitution rates may not allow it to withstand future scrutiny and other investigators have instead suggested an origin during the late Miocene or early Pliocene (Eastman and McCune 2000; Petricorena and Somero 2007).

Comparing the Heat Tolerance of 10 °C Acclimated *N. coriiceps* to Other Antarctic Species

Most prior studies of increased heat tolerance from warm acclimation in the endemic Antarctic notothenioids have been limited to acclimation temperatures of 4 to 5°C. This has been in deference to the apparent stenothermy of high-Antarctic species, where chronic exposure to temperatures as low as 5 to 7°C can prove fatal in specimens acclimatized to their natural freezing water temperatures (-1.9 °C) (Somero and Devries 1967). However, comparably greater heat tolerance is present among Antarctic fishes endemic to the WAP and more northerly Scotia Arc Islands such as *N. coriiceps* which may more readily acclimate to warmer temperatures (Bilyk and DeVries 2011).

While 10 °C acclimated *N. coriiceps* have lower CTMaxs than comparably acclimated cold-temperate fishes (Otto and Ohararice 1977; Becker and Genoway 1979; Fangue and Bennett 2003), including the winter acclimatized New Zealand notothenioids, these showed a notable increase in CTMax over environmental and 4 °C acclimated specimens (Table 4.1). At 10 °C they similarly exceed the

CTMaxs of seven other 4 °C acclimated notothenioids, predominantly from high-latitude waters (Bilyk and DeVries 2011). Both their survival and continued normal activity at this temperature shows a broader thermal range than anticipated even among the fishes found in the relatively more thermally variable waters along the Western Antarctic Peninsula.

Summer acclimatized specimens of the nototheniid *Leptonotothen nudifrons*, which is restricted to more northerly Antarctic waters, warmed at 1 °C per day resulted in the mortality of most upon reaching 8 to 9 °C (Hardewig et al. 1999). Similarly, Van Dijk et al. (1999) noted a cellular shift to anaerobic metabolism at 9 to 10 °C in the phylogenetically distant Antarctic Zoarcid, *Pachycara brachycephalum* when also warmed at 1°C per day, which suggests the limits of their heat tolerance lies near this temperature. However, while these studies warmed specimens slowly they may not have allowed sufficient time for the physiological and cellular processes that occur during acclimation to fully track rising temperatures as some high-latitude Antarctic species required up to two weeks to fully manifest their increased organismal heat tolerance during warm acclimation at 4 °C (Bilyk and DeVries 2011). The failure of *N. coriiceps* to directly acclimate to 10 °C in this study would also suggest that while acclimating notothenioids to cold-temperate water temperatures is possible it may require very slow rates of warming or staged increases.

Despite the survival of *N. coriiceps* at 10 °C, it is unclear whether this species could survive the even warmer summer water temperatures experienced by the secondarily temperate species. If it could survive the summer upper temperature extremes, its capacity for activity, growth and reproduction may be inhibited as these processes are typically under more stringent thermal constraints (Fry 1971).

Notable though is the inability of *N. coriiceps* to directly acclimate to 10°C and the mortality of these warm acclimated specimens when directly returned to their environmental freezing water temperatures. In other fishes, gains in heat tolerance through warm acclimation cause a rapid reduction in cold tolerance (Bennett et al. 1998). The inability of warm acclimated specimens to recover when

returned to ambient water temperatures would suggest their cold tolerance was increased above freezing Antarctic water temperatures and continues to argue for a relatively narrow thermal range in this and other endemic Antarctic species.

Conclusion

Evolution in chronic cold has led to reduction of heat tolerance in the Antarctic notothenioids. While the Antarctic *N. coriiceps* shows a surprising ability to warm acclimate to 10 °C its heat tolerance continued to lag equivalently acclimated cold-temperate and temperate fishes. Several members of the Antarctic notothenioids are now permanently distributed outside Antarctic waters even though their ancestors presumably once shared the reduced heat tolerance now common throughout endemic Antarctic species. This evolutionary history has been noted to continue to affect the biochemical and cellular temperature response of one such secondarily temperate Antarctic notothenioid, *N. angustata* , as well as their heat tolerance. Heat tolerance can serve as an important physiological constraint on geographic distribution (Hochachka and Somero 2002) and the capacity of the Antarctic notothenioids to adapt to rising water temperatures will likely be important for their future survival in the Southern Ocean in light of predicted future impacts of global climate change on the Antarctic ecosystem (Clarke et al. 2007).

FIGURES AND TABLES

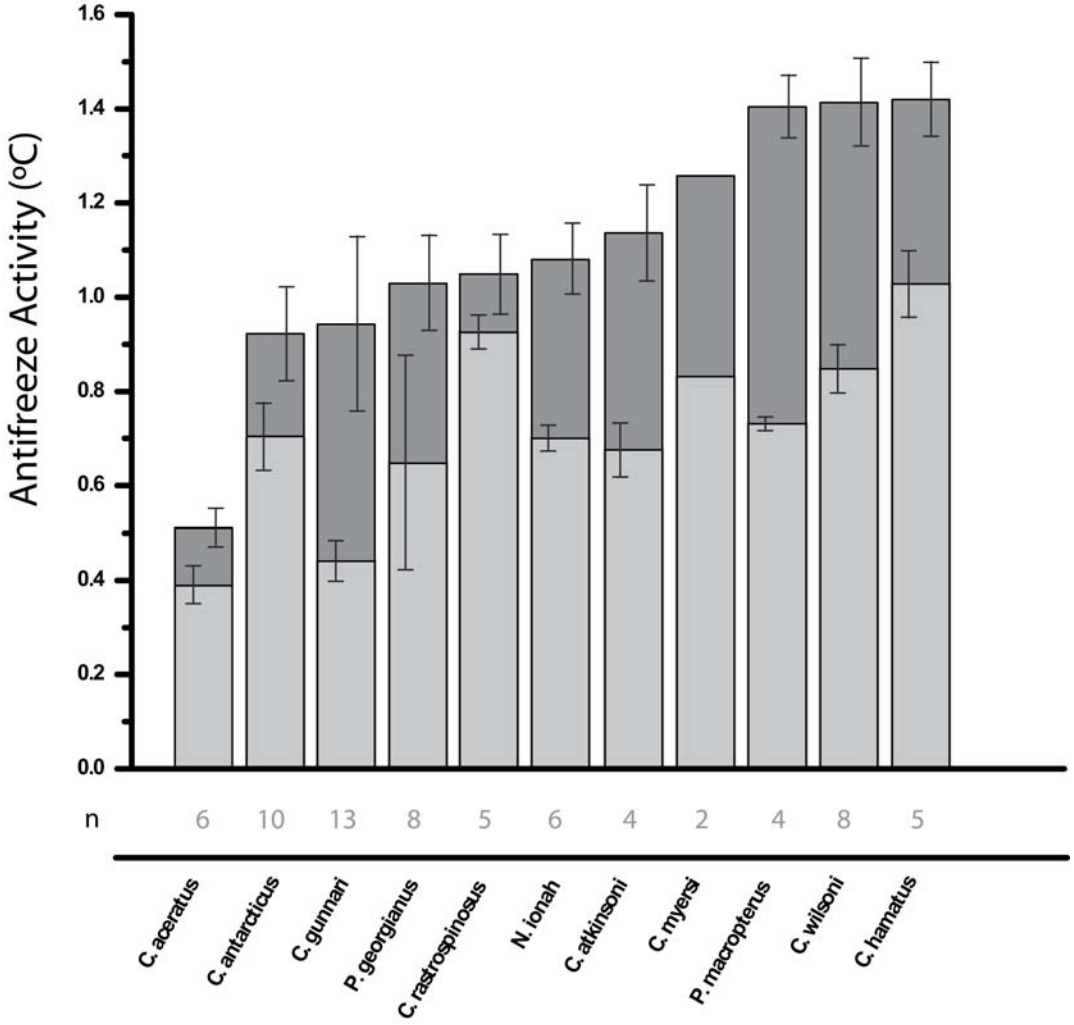

Fig. 1.1. Average species' measurements of serum antifreeze activity from AFGP (light gray), and AFPP (dark gray). The number of specimens used to determine these values is denoted by n listed above each species name. Error bars are standard deviations, which are not given for *C. myerisi* because of the small number of specimens. *C. aceratus*, *C. gunnari*, and *P. georgianus*, three of the first four species, are restricted to the waters of the seasonal pack ice zone

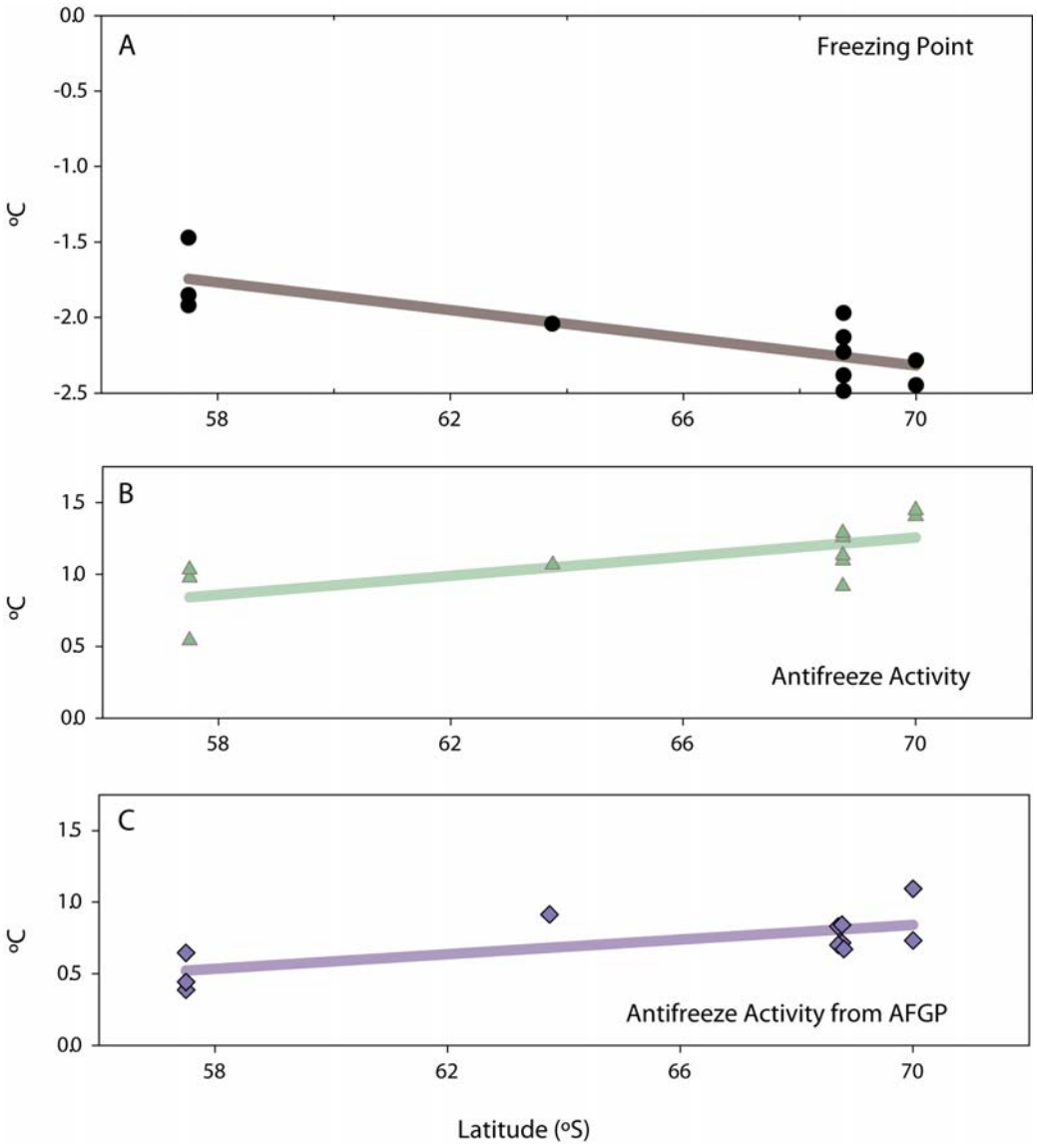

Fig. 1.2. Scatter plots and fitted regression lines for serum hysteresis freezing point, total antifreeze activity, and antifreeze activity from AFGP all plotted against the species' mean latitude of distribution. In A the Y axis denotes species' average hysteresis freezing point which is the sum of the freezing point depression from serum osmolytes and total antifreeze activity. In B and C, it is the average freezing point depression from total antifreeze activity and antifreeze activity from AFGP, respectively. In C, the position of several of the observations at 68 °45 S have been slightly offset on the X axis so all of species observations are visible

Table 1.1

Species	Catch Location	n [a]	Month and Year Collected	Catch Latitude	Mean Distribution Latitude [b]
Seasonal Pack Ice Zone Species					
Chaenocephalus aceratus	Antarctic Peninsula	15	Mar 1993, Aug 2008	65 °S	57 ° 30 ’S
	South Shetland Islands		Mar 2003	60-63 °S	
Champsocephalus gunnari	Antarctic Peninsula	18	Mar 2008	65 °S	57 ° 30 ’S
	South Shetland Islands		Mar 2003	60-63 °S	
Pseudochaenichthys georgianus	South Shetland Islands	8	Mar 2003	60-63 °S	57 ° 30 ’S
High Antarctic Zone Species					
Chionodraco rastrospinosus	Antarctic Peninsula	9	Aug 2008	65 °S	63 ° 45 ’S
	South Shetland Islands		Mar 2003	60-63 °S	
Neopagetopsis ionah	South Shetland Islands	7	Mar 2003	60-63 °S	68 ° 45 ’S
Cryodraco antarcticus	Antarctic Peninsula	5	Aug 2008	65 °S	68 ° 45 ’S
	South Shetland Islands	6	Mar 2003	60-63 °S	
	Ross Sea	4	Dec 1996	75 °S	
Cryodraco atkinsoni	Ross Sea	4	Dec 1996	75 °S	68 ° 45 ’S
Chionodraco myersi	Ross Sea	2	Dec 1996	75 °S	68 ° 45 ’S
Chaenodraco wilsoni	Antarctic Peninsula	18	Aug 2008	65 °S	68 ° 45 ’S
	South Shetland Islands		Mar 2003	60-63 °S	

Table 1.1 (continued)

Species	Catch Location	n [a]	Month and Year Collected	Catch Latitude	Mean Distribution Latitude [b]
High Antarctic Zone Species					
Pagetopsis macropterus	McMurdo Sound	4	Nov 1996	77 °50 ’S	70 °S
Chionodraco hamatus	Tera Nova Bay	12	Dec 2005	74 °S	70 °S

Table 1.1. Collection information and distribution of icefish used in this study. Species ordered by their mean distribution latitude.

[a] n is for all collection sites except for *C. antarcticus* where regional differences in freeze avoidance were considered.

[b] Mean distribution latitude defined as the arithmetic mean of the highest and lowest latitudes where the species are commonly found according to information available from Gon and Heemstra (1990) and Kock (2005).

Species	N	Serum Osmolality	Colligative Freezing Point [a]	Total Antifreeze Activity [b]	Hysteresis Freezing Point
		mOsm	°C	°C	°C
Seasonal Pack-ice Zone					
Chaenocephalus aceratus	15	499 ± 53	-0.93 ± 0.10	0.54 ± 0.11	-1.47 ± 0.12
Champsocephalus gunnari	18	468 ± 17	-0.87 ± 0.03	0.98 ± 0.23	-1.85 ± 0.24
Pseudochaenichthys georgianus	8	475 ± 27	-0.88 ± 0.05	1.03 ± 0.21	-1.91 ± 0.21
High Antarctic Zone					
Cryodraco antarcticus	15	565 ± 117	-1.05 ± 0.22	0.92 ± 0.12	-1.97 ± 0.27
Chionodraco rastrospinosus	9	524 ± 25	-0.97 ± 0.05	1.07 ± 0.08	-2.04 ± 0.08
Neopagetopsis ionah	6	555 ± 31	-1.03 ± 0.06	1.10 ± 0.08	-2.13 ± 0.08
Chaenodraco wilsoni	18	505 ± 51	-0.94 ± 0.95	1.29 ± 0.18	-2.23 ± 0.26
Pagetopsis macropterus	4	473 ± 14	-0.88 ± 0.03	1.40 ± 0.07	-2.28 ± 0.07
Cryodraco atkinsoni	4	672 ± 73	-1.25 ± 0.14	1.13 ± 0.14	-2.38 ± 0.09
Chionodraco hamatus	12	538 ± 21	-0.99 ± 0.04	1.45 ± 0.08	-2.44 ± 0.08
Chionodraco myersi	2	660 ± 134	-1.23 ± 0.25	1.26 ± 0.17	-2.49 ± 0.30

Table 1.2. Species average serum osmolality, equilibrium freezing point, antifreeze activity, and hysteresis freezing point. All measurements were taken from blood serum, species are sorted ascending by serum hysteresis freezing point, and all measurements are listed as species average ± standard deviation.

[a] The equilibrium freezing point (melting point) of blood serum is calculated as: (serum osmolality) × -0.001858°C mOsm^{-1}.

[b] Total antifreeze activity is a measure of the freezing point depression from all of the AFPs present in the blood serum.

	Test Statistic	P value
Hysteresis Freezing Point	$F_{10, 18.2067} = 52.24$	< 0.0001
Serum Osmolality	$F_{10, 17.8991} = 13.41$	< 0.0001
Total Antifreeze Activity	$F_{10, 18.1593} = 58.06$	< 0.0001
AFGP Activity	$F_{10, 15.5144} = 69.82$	< 0.0001
AFPP Activity	$F_{10, 14.9778} = 22.67$	< 0.0001

Table 1.3. Test for significant variation in freeze avoidance between icefish species**.** Comparisons were performed using Welch's test which does not assume equal variance, all measurements were taken from blood serum.

	High Antarctic [a]	SPZ [b]	P value [c]
Hysteresis Freezing Point (°C)	-2.245 ± 0.189	-1.746 ± 0.241	0.0026
Serum Osmolality (mOsm)	561.7 ± 70.6	481.1 ± 16.1	0.0452
Total Antifreeze Activity (°C)	1.202 ± 0.180	0.852 ± 0.269	0.0157
AFGP Activity (°C)	0.813 ± 0.140	0.493 ± 0.136	0.0039
AFPP Activity (°C)	0.409 ± 0.171	0.345 ± 0.222	0.3090

Table 1.4. Comparison of freeze avoidance between high Antarctic and SPZ species. All measurements were taken from blood serum.

[a] Grand mean of species average values (± Standard Deviation) for icefish with high Antarctic distributions (*C. rastrospinosus*, *N. ionah*, *C. antarcticus*, *C. atkinsoni*, *C. myersi*, *P. macropterus*, *C. wilsoni*, and *C. hamatus*).

[b] Grand mean of species average values (± Standard Deviation) for icefish with Seasonal Pack Ice distributions (*C. aceratus*, *C. gunnari*, and *P. georgianus*).

[c] Results of a one-tailed Student's t-Test for greater magnitude in the High-Antarctic Zone species.

	$F_{1,9}$	P value	r^2	Slope (°C °Latitude^{-1})
Simple Linear Regression				
Hysteresis Freezing Point	18.03	0.0022	0.66	-0.046
Total Antifreeze Activity	8.94	0.0152	0.49	0.033
AFGP Activity	8.12	0.0190	0.47	0.026
Phylogenetic Independent Contrasts				
Hysteresis Freezing Point	10.69	0.0097	0.54	-0.049
Total Antifreeze Activity	9.51	0.0131	0.51	0.038
AFGP Activity	5.37	0.0457	0.37	0.019

Table 1.5. Regression analysis on freeze avoidance against species mean latitude of distribution. All measurements were taken from blood serum, both serum osmolality and AFPP activity were excluded due to non-significant slopes in the simple linear regression.

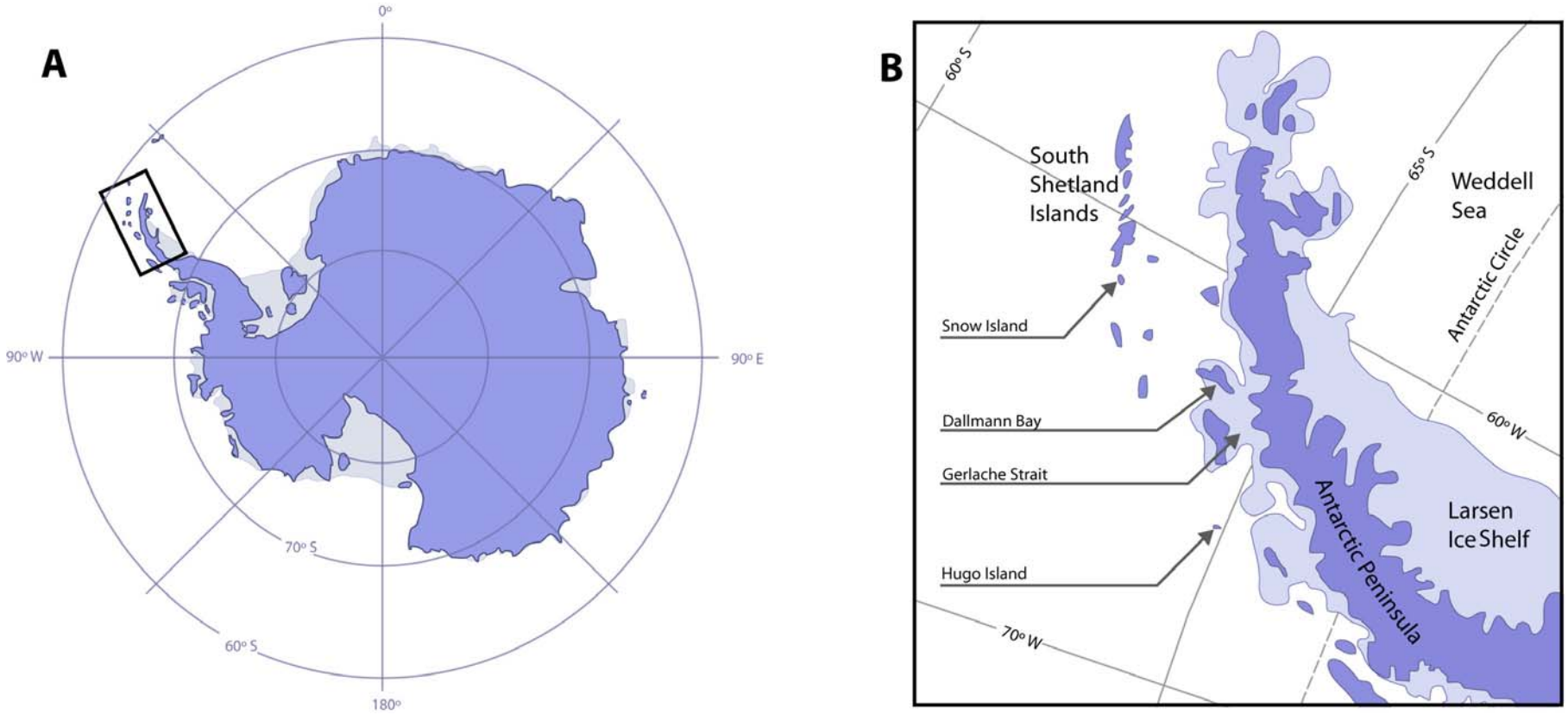

Fig. 2.1. Collection locations with respect to the Antarctic continent (a), and identified by arrows on the Antarctic Peninsula (b).

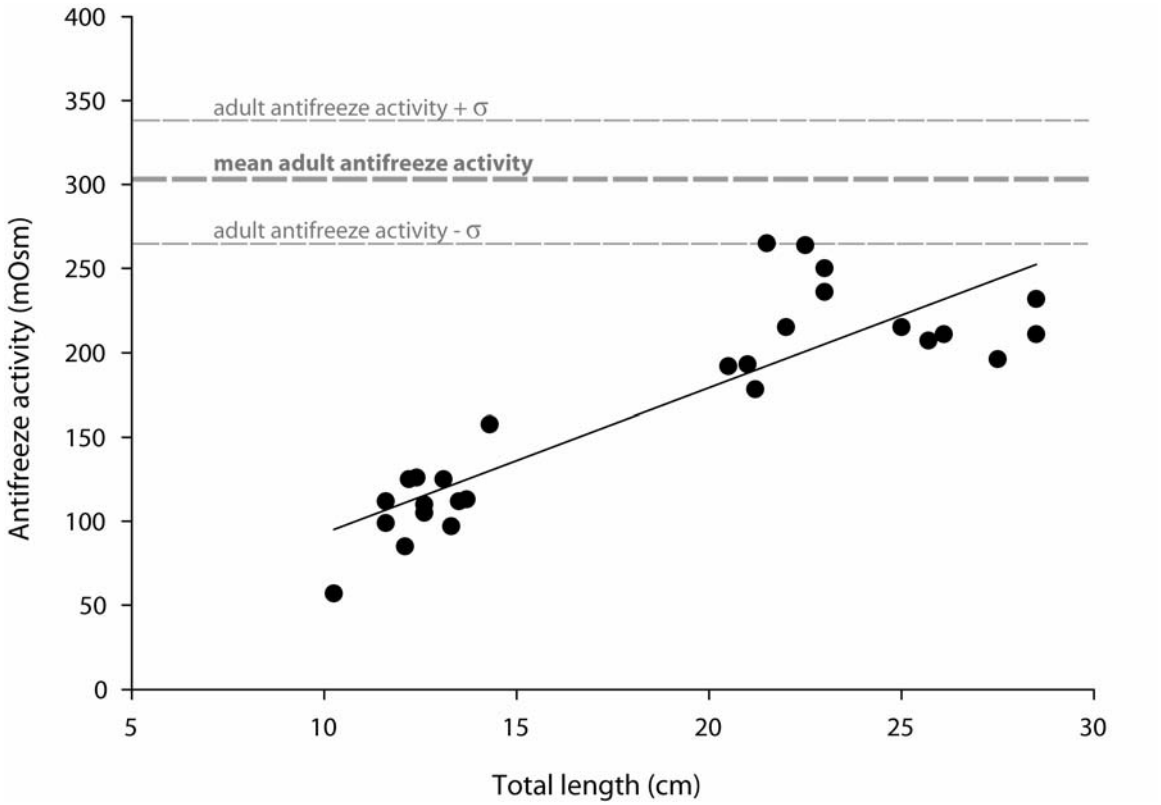

Fig. 2.2. Scatterplot of antifreeze activity in juvenile *C. aceratus* against their total length. Overlain in solid black is the simple linear regression line for antifreeze activity plotted against their total length. Mean and standard deviation for adult *C. aceratus* antifreeze activity are presented with dashed gray lines (0.57 ± 0.08°C).

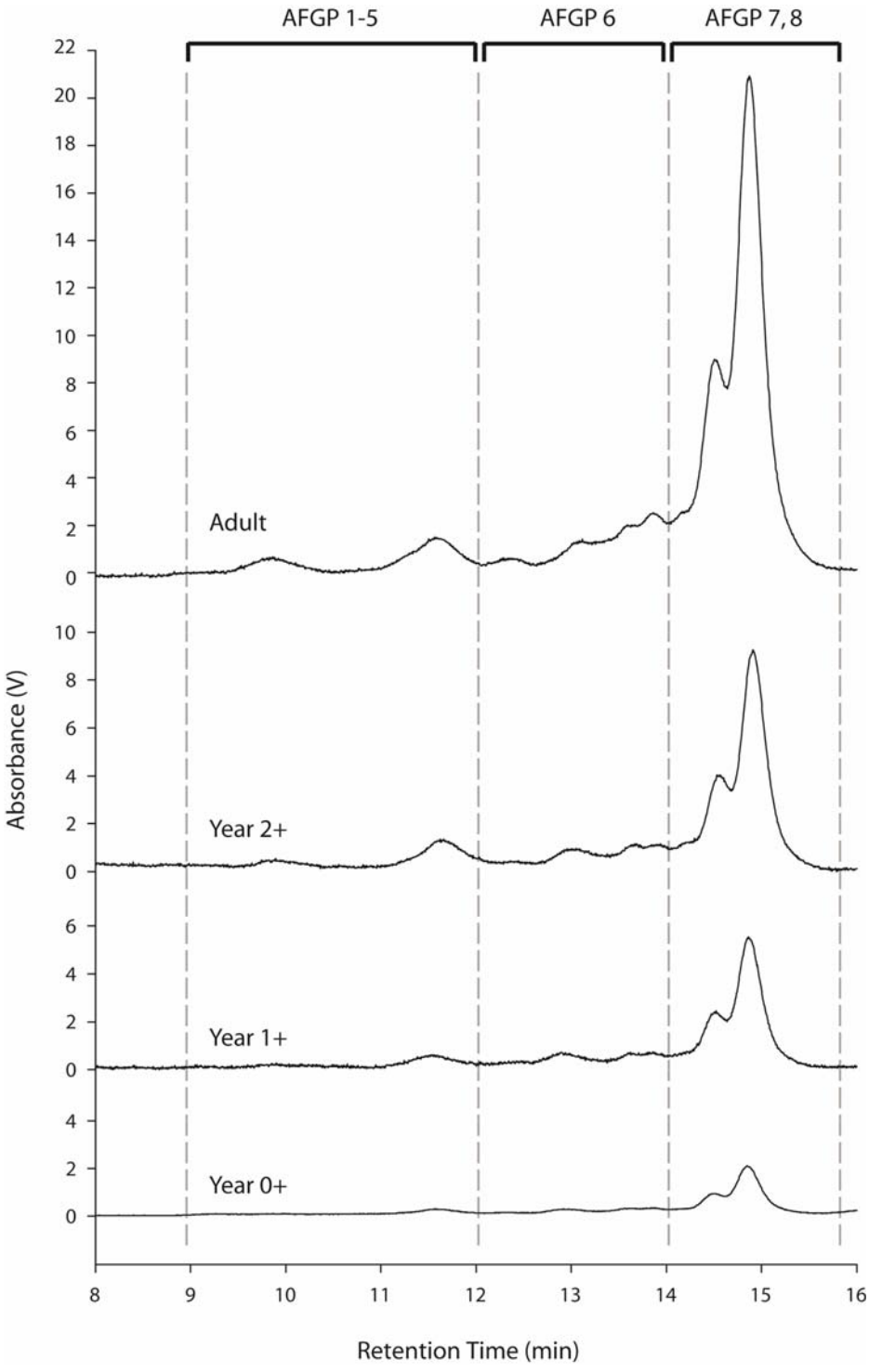

Fig. 2.3. Complete HPLC elution profiles showing absorbance at 220 nm from representative specimens of adult, year 2+, year 1+ and year 0+ juveniles, elution profiles were normalized to equivalent concentrations.

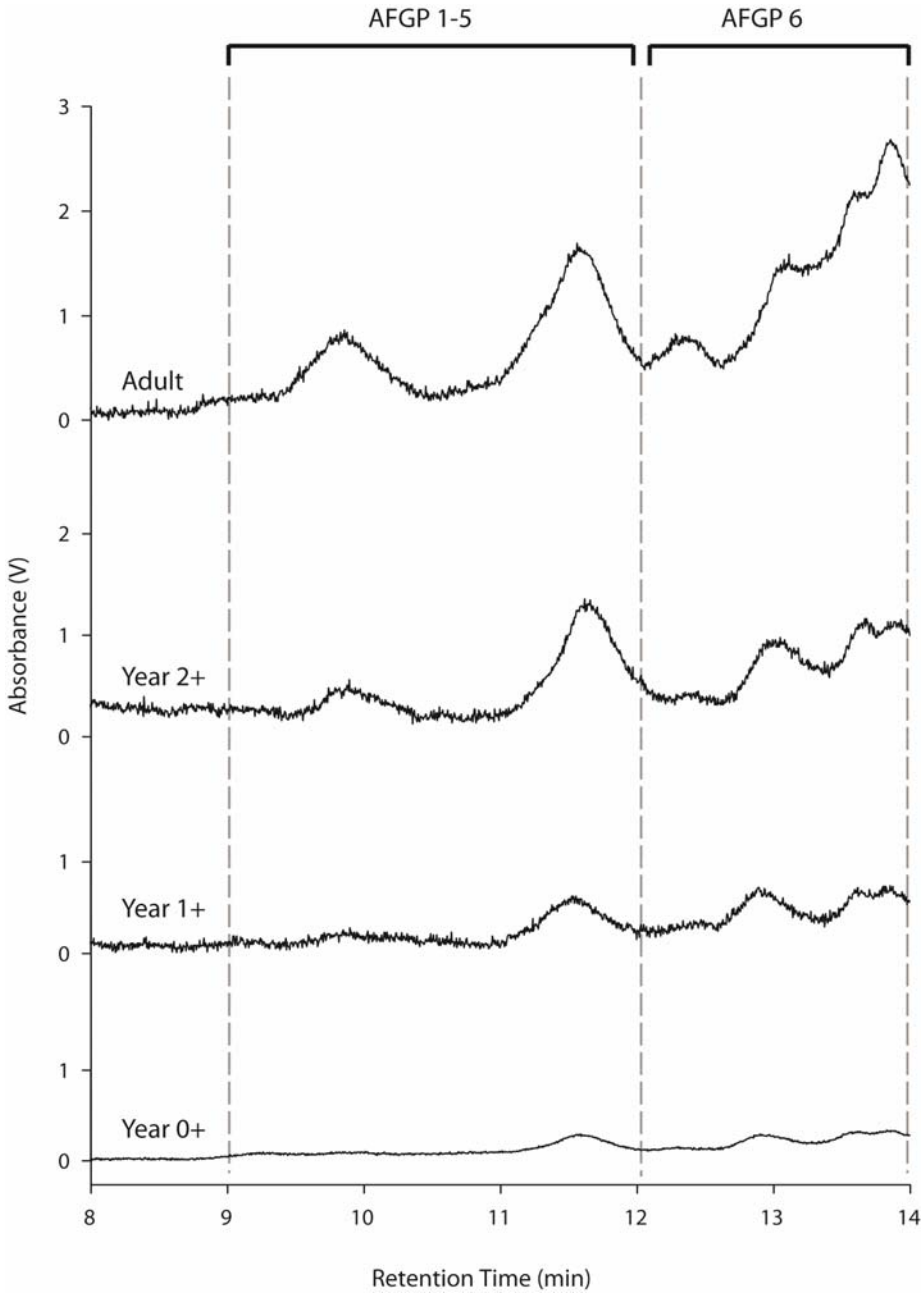

Fig. 2.4. HPLC elution profiles showing absorbance at 220 nm, expanded from Fig. 3 to show detail of the large AFGP size classes. The samples are from representative specimens of adult, year 2+, year 1+, and year 0+ juveniles.

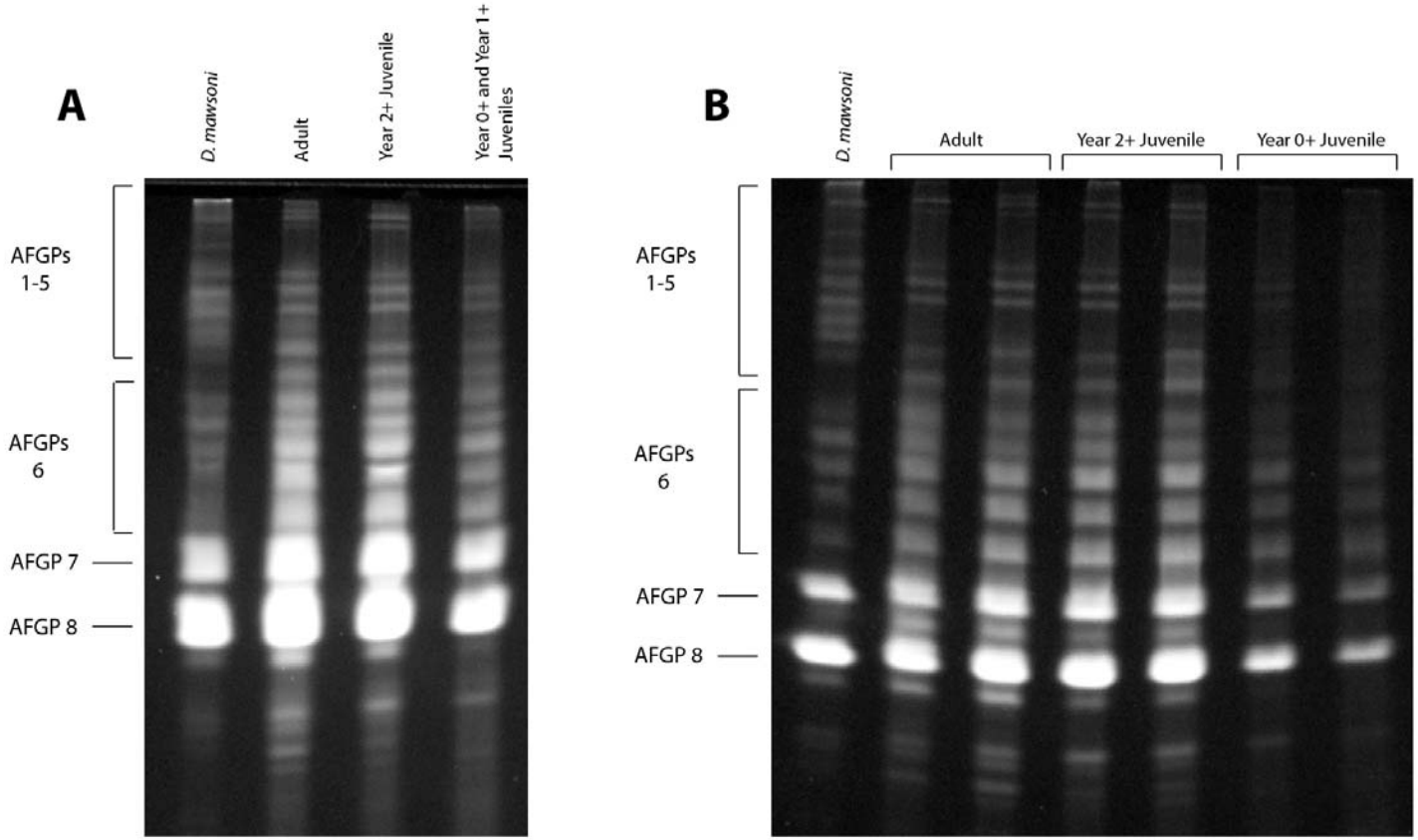

Fig. 2.5. Ten to 20% PAGE gel of purified serum AFGPs labeled with fluorescamine. The AFGPs from *C. aceratus* were run alongside 5 µl from a previously characterized sample of *D. mawsoni* as a standard which was prepared to a concentration of 50 mg ml^{-1} in (a) and 75 mg/ml in (b). In a purified serum AFGPs were redissolved to uniform 50 mg ml^{-1}. The estimated age of the year 2+ juvenile was 929 dph, the weighted average age of the four juvenile samples used to make the year 0+ and year 1+ sample pool was 359 dph. In b purified serum AFGPs were redissolved in 30 µl, one-tenth their original serum volume. The estimated ages of the year 2+ and year 0+ juvenile samples from left to right is: 750, 953, 278, 323 dph.

Age	N	TL (cm)	TL Range (cm) †	Colligative melting point (°C)	Antifreeze activity (°C)	AFGP Concentration (mg ml^{-1}) AFGP 1-5	AFGP 6	AFGP 7, 8	Total AFGP
Adult	5	NA	31 – 77	0.84 ± 0.05	0.57 ± 0.08	1.50 ± 0.89	1.44 ± 0.50	3.94 ± 1.49	6.88 ± 2.77
Year 2+ Juvenile	2	24.4 ± 1.6	22 – 30	0.91 ± 0.06	0.41 ± 0.03	0.78 ± 0.00	0.79 ± 0.06	2.21 ± 0.30	3.78 ± 0.37
Year 1+ Juvenile	3	18.4 ± 0.3	13 – 21	0.89 ± 0.02	0.35 ± 0.02	0.51 ± 0.12	0.54 ± 0.13	1.44 ± 0.07	2.49 ± 0.27
Year 0+ Juvenile	4	10.9 ± 0.5	5 – 12	0.94 ± 0.04	0.21 ± 0.02	0.16 ± 0.09	0.18 ± 0.07	0.32 ± 0.15	0.66 ± 0.28

Table 2.1. *C. aceratus* Antifreeze activity and AFGP concentration with age. Each specimen's year class was estimated from its TL, all values reported ± SD.

† Range of TL reported for each age group, adult TL as reported by Reid et al. (2007), juvenile TL ranges extrapolated from age TL relationship reported in La Mesa and Ashford (2008).

Age	n	Antifreeze Activity	Total AFGP concentration [a]
Adult	5	1	1
Year 2+ Juvenile	2	2	1, 2
Year 1+ Juvenile	3	2	2
Year 0+ Juvenile	4	3	3

Table 2.2. Grouping of *C. aceratus* age classes by their serum antifreeze activity and total AFGP concentration. Groupings were assigned using a post hoc Tukey test. Within a column the age classes that share a number do not significantly differ, an age class with only a lower number is significantly greater

[a] the comparison was made on the $\log_{10}$ of the concentration of Total AFGP concentrations measured in mg ml^{-1}.

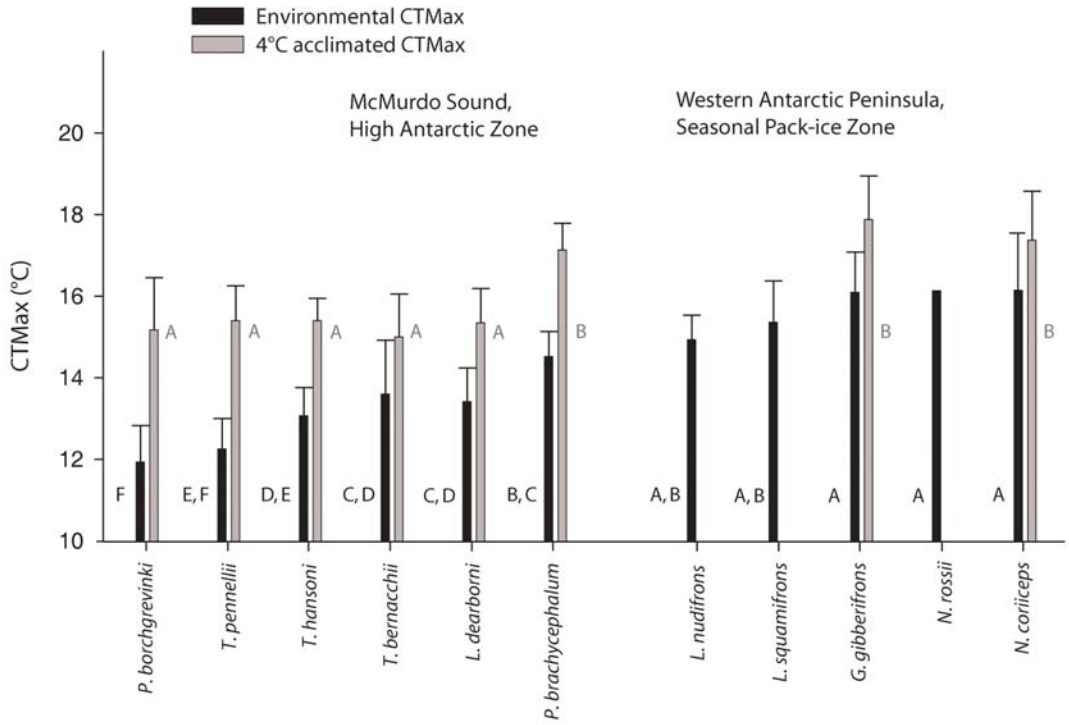

Fig.3.1. CTMax of Antarctic fishes endemic to both the High Antarctic Zone and the Seasonal Pack-ice Zone. Both environmental CTMaxs (black bars) and 4 °C acclimated CTMaxs (grey bars) are shown as their mean with brackets to illustrate standard deviations. Standard Deviation is not reported for *N. rossii* as only two specimens were available. Significant groupings determined using the Student–Newman Keuls test for both environmental and acclimated CTMaxs are displayed as the letters next to each bar. Within acclimation temperatures species that do not share a letter significantly differ, note though that environmental and acclimated CTMaxs were tested independently and overlapping letters between these do not have any meaning.

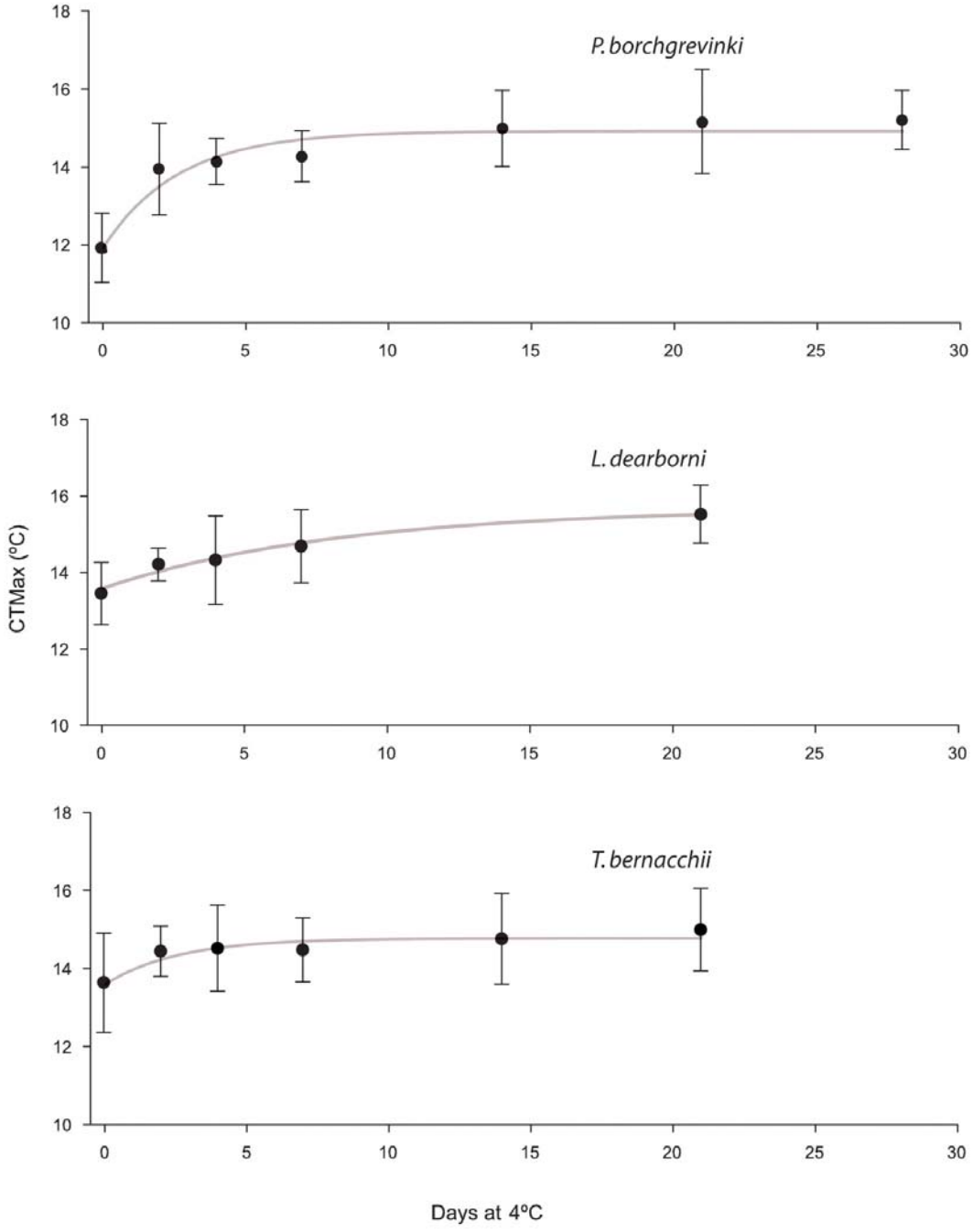

Fig. 3.2. Accrual of heat tolerance while held at 4 °C in three species of McMurdo Sound fishes. Circles denote the mean CTMax for a species at that point during acclimation and the bounding lines its standard deviation. The light grey line on each plot shows the expected CTMax from the non-linear regression analysis.

Species	Habitat	Environmental CTMax			4 °C Acclimated CTMax			ARR
		n	Mass g ± SD	CTMax °C ± SD	n	Mass g ± SD	CTMax °C ± SD	
McMurdo Sound								
Pagothenia borchgrevinki	Cryopelagic	20	124.5± 47.9	11.95± 0.90	12	101.5± 38.1	15.19± 1.28	0.55
Trematomus pennellii [a]	Shallow water, Benthic	8	74.7± 27.6	12.28± 0.74	8	74.7± 27.6	15.42± 0.85	0.53
Trematomus hansoni	Shallow water, benthic	12	157.2± 42.3	13.09± 0.69	8	169.3± 75.3	15.42± 0.55	0.39
Trematomus bernacchii	Shallow water, benthic	12	158.7± 86.8	13.62± 1.32	8	80.0± 21.8	15.02± 1.06	0.24
Lycodichthys dearborni	Deep water, benthic	9	51.4± 7.1	13.44± 0.82	13	27.5± 7.1	15.37± 0.84	0.33
Pachycara brachycephalum	Deep water, Benthic	10	51± 17.1	14.54± 0.61	9	62.2± 20.7	17.15± 0.66	0.44
Western Antarctic Peninsula								
Lepidonotothen nudifrons	Deep water, benthic	8	29.3± 9.6	15.06± 0.56	-	-	-	-
Lepidonotothen squamifrons	Deep water, benthic	10	524.4± 140.6	15.38± 1.02	-	-	-	-
Gobionotothen gibberifrons [b]	Eurybathic, benthic	20	499.0± 98.1	16.11± 0.99	10	495.3± 181.0	17.90± 1.08	0.30
Notothenia rossii	Deep water, benthic	2	620.5± 13.4	16.16± 1.34	-	-	-	-
Notothenia coriiceps [c]	Shallow water, benthic	20	479.4± 194.6	16.17± 1.40	10	345.1± 139.9	17.39± 1.2	0.21

Table 3.1. CTMaxs of Antarctic fishes. CTMaxs and mass from both environmental and warm acclimated specimens of Antarctic fishes. Species listed in ascending order by their environmental CTMax.
[a] Unlike the other species, the same specimens were used to determine warm acclimated and environmental CTMaxs in T. pennellii.
[b] *G. gibberifrons* was only warm acclimated for seven days prior to measuring its CTMax.
[c] Standard deviations could not be calculated for N. rossii on account of the small sample size.
[d] *N. coriiceps* was only warm acclimated for 14 days prior to measuring its CTMax.
ARR — acclimation response ratio.

Species	Mass	Thermal Lag	n
	g ± SD	^{o}C ± SD	
P. borchgrevinki	112.3±39	1.04±0.39	3
T. bernacchii	114±42	0.72±0.11	3
N. coriiceps	791±414	1.83±0.64	6

Table 3.2. Thermal lag between fish and water. The average difference between core body temperature and external water temperature in three Antarctic notothenioid species during warming at 0.3 °C min^{-1}. All measurements are shown ± their standard deviation.

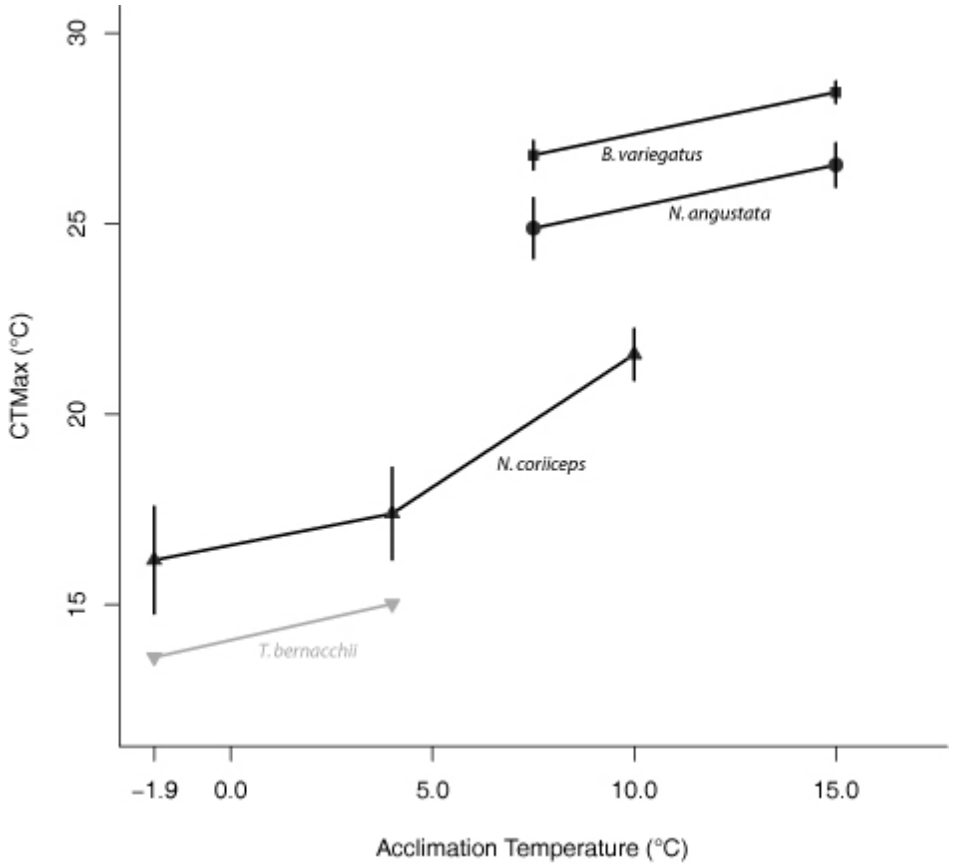

Fig. 4.1. Comparison of CTMaxs against acclimation temperature in four species of notothenioid fishes. All points represent the mean value with vertical bars denoting their standard deviation. *T.bernacchii* shown in light grey as they were adapted from Bilyk and DeVries (2011), error bars were excluded from this species as they overlap with those from *N. coriiceps*.

Species	Collection Site	n	mass	Environmental Temperature Range	Acclimation Temperature	CTMax
			g	°C	°C	°C
Notothenia coriiceps†	WAP	20	479.4 ± 194.6	-1.9 to 3	-1.9	16.17 ± 1.40
Notothenia coriiceps†	WAP	10	345.1 ± 139.9		4	17.39 ± 1.2
Notothenia coriiceps	WAP	8	404 ± 273.2		10	21.58 ± 0.67
Notothenia angustata	Otago Harbor	8	2150 ± 992.5	7 to 16	7 - 8	24.88 ± 0.79
Notothenia angustata	Otago Harbor	8	2150 ± 992.5		15	26.54 ± 0.57
Bovichtus variegatus	Otago Harbor	4	87.9 ± 42.8	7 to 16	7 - 8	26.80 ± 0.37
Bovichtus variegatus	Otago Harbor	4	87.9 ± 42.8		15	28.45 ± 0.28

Table 4.1. CTMaxs of notothenioid species investigated during this study. *N. angustata* and *B. variegatus* tested at 7-8 °C were winter acclimatized specimens while all other specimens were warm acclimated to their described temperatures.

† CTMaxs for Environmental acclimatized and 4°C acclimated *N. coriiceps* are reprinted from Bilyk and DeVries (2011).

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