Antarctic Fish: Thermal Specialists or Adaptable Generalists?

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Abstract

Antarctic fish from the suborder Notothenioidei inhabit what is perhaps the most thermally stable ocean environment on Earth. Evolutionary theory suggests that by specialising for this environment, Antarctic fish have traded-off their ability to respond to variations in temperature, and like their environment, have become extremely stenothermal. However, previous research has revealed that the Antarctic notothenioid fish *Pagothenia borchgrevinki* is not as thermally limited as evolutionary theory might predict, and is capable of acclimation to 4 °C during a one month period. The purpose of the current research was to investigate the physiological mechanisms that underpin this remarkable acclimatory ability. *P. borchgrevinki* were acclimated for one month to 4 °C and changes in oxygen consumption, prolonged swimming ability, cardiovascular function, enzyme activity and haematology were measured. Significant changes in resting oxygen consumption rate and prolonged swimming ability occurred during the acclimation period, and these changes were mediated by adjustments of enzyme activity and specific aspects of the haematology. By monitoring resting oxygen consumption and prolonged swimming ability over a much longer, six month, acclimation period it was confirmed that the adjustments evident during one month at 4 °C were sustainable in the long-term, and were not short-term compensatory mechanisms. Interestingly, fish infected with x-cell gill disease did not possess the same ability to acclimate as was demonstrated by healthy *P. borchgrevinki*. *P. borchgrevinki* are unusual among the notothenioids, possessing an active, pelagic lifestyle which differs from the sedentary, benthic lifestyle of most other species within the suborder. Therefore, it was hypothesised that the acclimatory ability demonstrated by this species may also be unusual among the notothenioids. To test this hypothesis, the acclimation ability of three sedentary, benthic notothenioids (*Trematomus bernacchii, T. hansoni* and *T.* pennellii) was investigated. Results confirmed the hypothesis, with all three species demonstrating very poor survival at 4 °C and absolutely no capacity for acclimation. Such results present a disturbing scenario for the future of Antarctic notothenioid fish in Earth's rapidly warming climate, and highlights the need for continued research combined with immediate action to combat the warming which currently threatens Antarctic marine biodiversity.

Chapter One

Introduction

Antarctica – the Continent

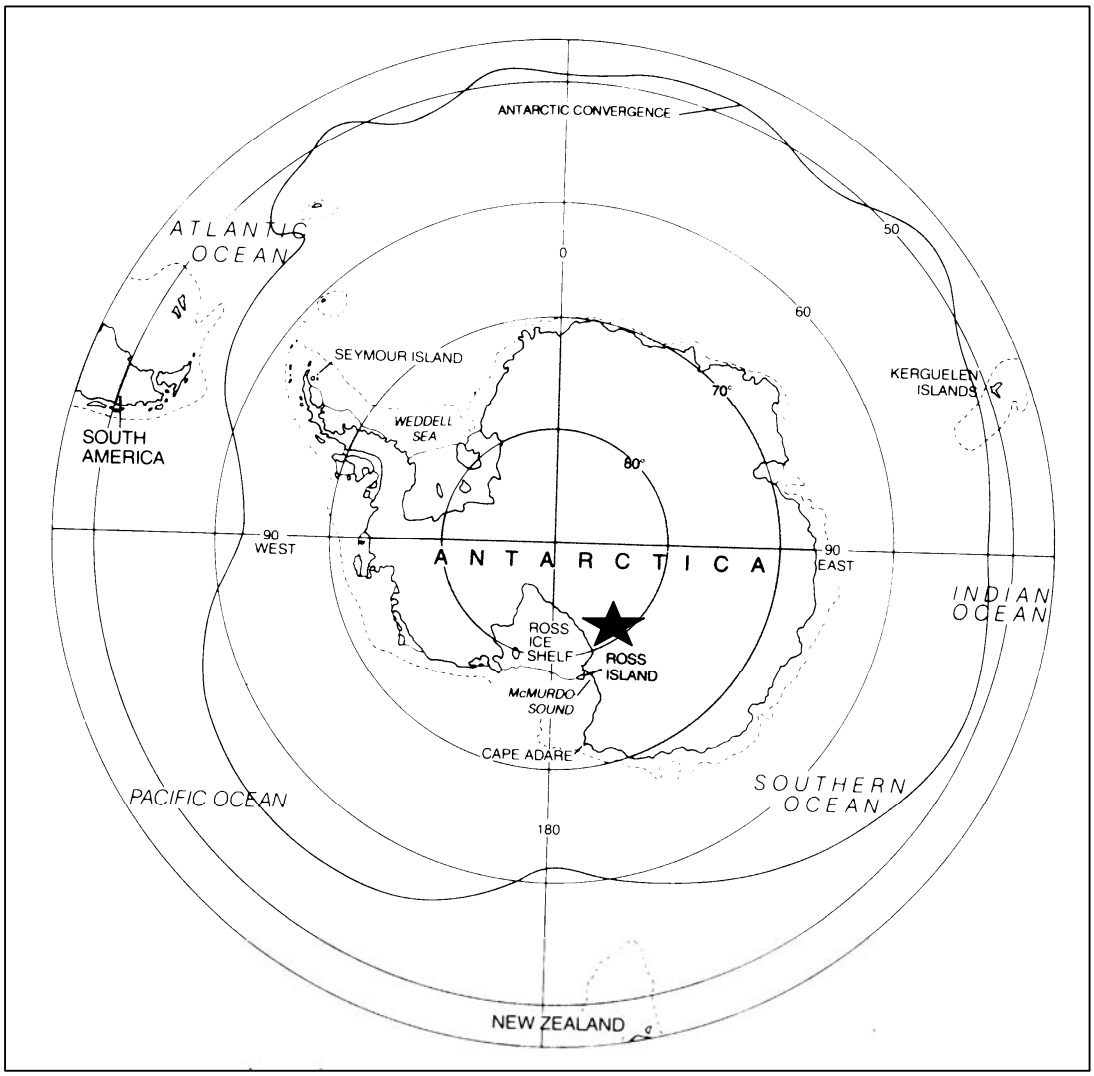
Beyond the 'Screaming Sixties' (60° S) is the immense and desolate Antarctic region. This region includes most of the Southern Ocean, scattered islands and the enormous ice-covered continent, Antarctica (King and Turner 1997) (Fig. 1.1). The Antarctic continent is vast and high, covering some 14 X 10^6 km^2 and reaching altitudes of more than 4000 m in some places (Bonner and Walton 1985; Maxwell et al. 1998). The land itself is hidden below a thick ice cap which links two geologically distinct land masses, East Antarctica and West Antarctica. East Antarctica is a large landmass with a thick crust of around 30 to 40 km depth. In contrast, West Antarctica consists of a group of mountainous islands with a crustal thickness of only 10 km in some places. These landmasses converge beneath the Ross Sea and the Ross Ice Shelf (Hatherton 1990; Waterhouse 2001).

Because of its southerly location, Antarctica has an unusual seasonal, rather than diurnal, cycle of photoperiod. Around the location of Scott Base, for around four months in winter, there is no sunlight, while the four summer months receive 24 hours of sunlight (Maxwell et al. 1998). These seasons are separated by two transition periods of increasing or decreasing day length, each around two months long (Eastman 1993).

The geological origins of Antarctica lie in the southern hemisphere super-continent, Gondwanaland, which also included present-day South America, Africa, India, Australia and New Zealand (Knox 1994). Gondwanaland remained intact for approximately 375 million years (Eastman 1993), but began to separate about 180 million years ago (Waterhouse 2001). In the course of this separation, Antarctica

broke its last bonds with Gondwanaland and has been physically isolated from other southern continents ever since (Eastman 1993).

Fig. 1.1. Antarctica and the surrounding Southern Ocean. The location of the Antarctic Convergence is indicated. The location of the study areas (McMurdo Sound and Ross Island) are also marked ★ (modified from Eastman 1993).



Today, Antarctica is characterised by cold. It is well known as the coldest, windiest, highest and driest continent on Earth (Mullan and Sinclair 1990) and air temperatures mostly remain below freezing year-round (Maxwell et al. 1998). However, ancient Antarctica experienced oscillations between warm and cold climates and was at times ice-free (Eastman 1993). During warm epochs, vast gymnosperm forests, a variety of land animals, and temperate marine fauna thrived, and these are evident in the fossil record (Bradshaw 1990). This changed about 50 million years ago when a cooling trend began to drive Antarctica towards its modern-day climate. The cooling trend resulted from Antarctica's increasing isolation from other landmasses and the formation of the Antarctic Circumpolar Current (this will be described in more detail in the section on the Southern Ocean) (Eastman 1993).

The Antarctic Circumpolar Current (ACC) developed about 22.8 million years ago (Pfuhl et al. 2004) as a result of two key events – the opening of the Tasmanian Seaway (or Gateway) between Australia and Antarctica (Cande and Stock 2004; Robert 2004); and the opening of the Drake Passage between South America and West Antarctica (Pfuhl et al. 2004). The ACC reduced heat exchange from the north and caused the gradual cooling of air temperatures and expansion of the Antarctic ice sheet. The first continent-wide ice sheet appeared in Antarctica approximately 34 million years ago, and this became a permanent (rather than ephemeral) ice sheet about 15 million years ago (Barrett 2003). Environmental feedback mechanisms associated with ice expansion and air cooling further increased the rate of temperature change (Kennett and Exon 2004), so that the ice sheet reached its present volume about five million years ago (Mullan and Sinclair 1990) and ocean temperatures fell from about 15 ℃ to less than 0 ℃ (Eastman 1993).

Antarctica – the Marine Environment of the Southern Ocean

The Antarctic continent is the southern boundary of the Southern Ocean, which is the habitat of modern Antarctic fishes (Eastman 1993). It is considered separate from its nearest neighbours, the Pacific, Indian and Atlantic Oceans, because its circulation is

mainly circumpolar, which contrasts with the anti-cyclonic gyres of the other three oceans (Lutjeharms 1990). The Southern Ocean, like the continent it surrounds, is cold and windy. Ocean temperatures near to Antarctica generally remain close to -1.8 ℃ and there is very little thermostratification (Eastman 1993). Research in McMurdo Sound suggests that near-shore shallow water sites may experience some fluctuations in temperature, including some warming between January and March. However, this warming trend only results in maximum temperatures of around -0.5 ℃ (Hunt et al. 2003). These cold temperatures largely result from the functioning of the Antarctic Circumpolar Current (ACC) (Young 1991).

The ACC (or West Wind Drift) is an extensive eastward flowing current driven by the action of westerly winds and thermohaline effects (Zambianchi et al. 1999). It extends to 1000 km wide in some areas and is unusual because it reaches the sea floor in places (Eastman 1993). Much of the ACC is made up of a thick layer of water that is about 0 – 2 ℃ and is saline, oxygen poor and macro-nutrient rich; this is termed Circumpolar Deep Water (CDW). The CDW originates at low latitudes and moves southwards until it eventually joins the surface mixed layer of the ACC. The Southern Boundary of the CDW is an important ecological zone containing large concentrations of krill and an associated cascade of organisms including blue whales (*Balaenoptera musculus*), humpback whales (*Megaptera novaeangliae*), and fin whales (*Balaenoptera physalus*) (Tynan 1998).

While the ACC dominates the flow pattern of much of the Southern Ocean, closer inshore the Antarctic Coastal Current (or East Wind Drift) drives water in a westerly direction (Young 1991). Between the ACC and the Antarctic Coastal Current, there is a complex pattern of water masses and oceanic fronts. These fronts are also associated with abrupt changes in ocean temperature, salinity and the distribution of nutrients and are therefore important determinants of the distribution of marine organisms (Lutjeharms 1990).

Within the ACC is one of the most important features of the Southern Ocean, the Antarctic Polar Front (or Antarctic Convergence) (Zambianchi et al. 1999) (Fig. 1.1). The Front is circumpolar but occurs at different latitudes around the continent, about

50° S in the Atlantic Ocean sector, 50 – 55° S in the Indian Ocean sector and 60° S in the Pacific Ocean sector (Eastman 1993). At the Antarctic Polar Front, cold, northward moving Antarctic waters sink beneath warmer sub-Antarctic waters and this results in a sharp change in ocean temperature (3 - 4 °C). It is both a thermal barrier and a physical barrier to fish migration in either direction and also affects the distribution of phytoplankton, zooplankton and sea birds (Lutjeharms 1990).

The Ross Sea

In the Pacific sector of the Southern Ocean there is a large embayment, extending to nearly 78 °S, named the Ross Sea after explorer James Clark Ross (Eastman and Hubold 1999). The Ross Sea has an area of about 500 000 km^2. Its boundaries are the line from Cape Adare to Cape Colbeck in the north, the Ross Ice Shelf to the south, Victoria Land on the west and Edward VII Land to the east (Picco et al. 2000). The continental shelf upon which the Ross Sea sits is wide and deep, which is unusual in the Antarctic where the continental shelf is usually narrow or absent. The shelf break (where the continental shelf emerges from the deep sea bottom) is very deep, at about 800 m. Beyond the break, ocean depths of over 2000 m are common. The sea floor of the Ross Sea Shelf was shaped by past glacial action and is covered with glacial sediments, sand, silt, gravel and occasional boulders (Knox et al. 2001).

The Ross Sea has peculiar physical, chemical and biological characteristics (Catalano et al. 2000). The temperature (-1.86 °C) and salinity (34.8 parts per thousand) are very stable year-round (Picco et al. 1999) and biological productivity is very high. This is largely because the Ross Sea contains some of the highest concentrations of nutrients in the Antarctic region, especially silicate, phosphate and nitrate (Lutjeharms 1990). One of the most important physical characteristics of the Ross Sea is the presence of seasonal sea ice. Sea ice formation in autumn, and the summer melt are important determinants of seasonal variability in the region. During the winter the sea ice extends to cover up to 85 % of the Ross Sea (Picco 2000).

Sea Ice

Antarctic sea ice is one of the most important climatic features in the Antarctic region and also the Southern Hemisphere, affecting heat exchange between the ocean and the atmosphere. The sea ice is an important habitat for many Antarctic organisms, providing a substrate for the growth of algae and a refuge for its associated food chain, including amphipods, euphausiids and several important fish species (Wadhams 1991). Sea ice melt is important in providing vertical stability in the water column (Knox 1994). Associated with seasonal melting is the seasonal recession of the ice edge. This occurrence is important because it releases high concentrations of biological matter into the ocean. This biological matter includes dissolved or particulate organic matter, living cells or aggregations of dead and living cells and the faecal pellets of grazers. The release of this material has a significant effect on biogeochemical cycling, bentho-pelagic coupling and the sequestration of organic carbon into the sediments (Knox 2007).

The Antarctic sea ice begins to form each autumn (around March / April) when the surface of the sea freezes (Knox et al. 2001). Initial ice formation occurs at the surface of the water as small ice platelets and needles, which eventually freeze together to form a continuous sheet. As the sea freezes the salt is squeezed out and increases the salinity and density of the surrounding water (King and Turner 1997). The rate of sea ice formation is mainly associated with air temperature (Maykut 1985). The sea ice is often covered with layers of blown snow and is shaped with pressure ridges caused by collision with land or other floes, and also polynyas (large and persistent openings in the ice pack) (Lutjeharms 1990). As air temperatures warm, or northward movement of the sea ice takes it to warmer latitudes, ice melt begins. Melting of the Antarctic sea ice mostly occurs at the sea-ice interface. The process of sea ice formation and melt occurs annually in most parts of Antarctica, so most Antarctic sea ice is first-year ice of around 1 to 1.5 m. However, areas in the Ross Sea, Weddell Sea and Bellingshausen Sea contain multi-year ice which may be well over 3 m thick (Wadhams 1991).

Antarctic Fish

The sea ice laden waters of the Ross Sea provide habitat for a diverse range of marine communities, including many species of fish. The fish fauna can be divided into two groups – the deep sea species of the continental slope and deep sea trenches; and the coastal species living on the continental shelf (Kock 1985). The ancestral coastal fishes probably travelled with Antarctica as it moved away from Gondwanaland. They effectively became trapped on the Antarctic continental shelf due to physical isolation and the thermal isolation caused by the Antarctic Circumpolar Current (Macdonald et al. 1987). These fish either died out, or adapted to the cooling conditions of the Antarctic over many millions of years and are now a highly endemic and specialised group.

Coastal fishes also had to adapt to the highly seasonal pattern of primary production in the Southern Ocean. The seasonality of primary production is most prominent in the high latitudes and is a direct result of the seasonal pattern of sunlight. Some high latitude fish species constrain their reproduction and growth to the light summer months when food is more available. As a result, these species often possess slow growth rates and associated low reproductive rates (Clarke 1988).

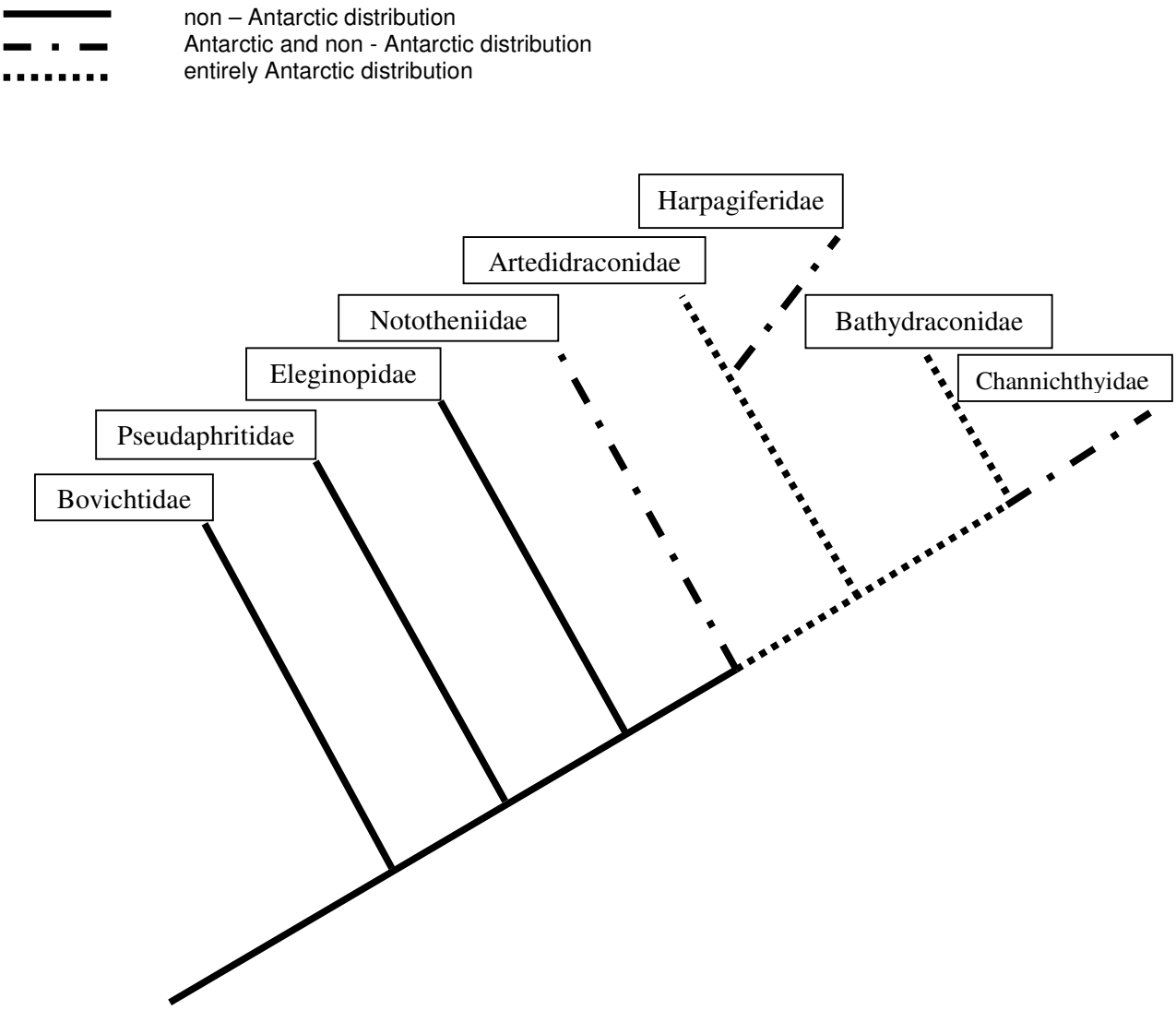
Notothenioidei

Among the coastal fishes, the most abundant and widely distributed group is the perciform suborder Notothenioidei (Eastman 1993). Notothenioid fish are a particularly important group in the Ross Sea where they dominate the shelf waters in both biomass and abundance (Eastman 1993). While there is no known fossil record for Antarctic notothenioid fish, they are presumed to have evolved from an early benthic ancestor trapped on the Antarctic continental shelf. From this benthic ancestor, lack of competition allowed the group to take over ecological niches usually occupied by taxonomically diverse species in temperate regions (Knox et al. 2001). While some species evolved adaptations for life in the water column, benthic habitats still contain a higher biomass of fish species compared with pelagic habitats (Donnelly et al. 2004). Because the notothenioids are an endemic group which have

radiated relatively rapidly in a geographically isolated area, they have been termed a 'species flock' (following the definition of Ribbink 1984), aligning them with similar radiations in freshwater lakes. Species flocks present interesting systems for biological study because they contain a disproportionate speciosity, for example the 5.6 fold difference in Antarctic compared with non-Antarctic notothenioids (excluding the Bovichtids) (Eastman and McCune 2000). The date of Antarctic notothenioid radiation has been debated and morphological analyses have produced differing results compared with more modern molecular techniques (Bargelloni et al. 2000). Modern DNA analysis suggests that the radiation of Antarctic notothenioids took place around 10 – 15 million years ago (Bargelloni et al. 1994), and this fits well with the accepted dates of Antarctic cooling (Barrett 2003). However, molecular clock analysis, which is calibrated with information from the fossil record, suggests that the Antarctic notothenioid radiation occurred about 24 million years ago (Near 2004).

The notothenioid group includes temperate water species which belong to the families Bovichtidae, Pseudaphritidae and Eleginopidae (Cheng et al. 2003). These families are believed to have emerged very early, before the cooling of Antarctic waters and before the radiation of Antarctic notothenioids. As a result, they did not evolve the cold specialisations of Antarctic nototheniods and have been confined to life in temperate water habitats (Fig. 1.2). Because of their early separation, it has been suggested that the Bovichtidae (the most basal clade) are only distantly related to other notothenioids (Bargelloni et al. 1994; Bargelloni et al. 1997; Lecointre et al. 1997). Two temperate species, *Notothenia angustata* and *N. microlepidota*, which do not belong to the basal families (Bovichtidae, Pseudaphritidae and Eleginopidae), entered temperate water habitats via a different evolutionary path. These species had an Antarctic origin, but are now endemic to Southern New Zealand. They diverged from their Antarctic ancestors around 11 million years ago, which is coincident with the northward advance of the Antarctic Convergence over New Zealand. After the southern retreat of the Antarctic Convergence, these species remained in their New Zealand habitats (Cheng et al. 2003).

Fig. 1.2. Phylogenetic relationships of the suborder Notothenioidei. Drawn using information from Lecointre et al. (1997); Hoofd and Egginton (1997) and Brodeur et al. (2003).



Ichthyologists currently recognise eight families of notothenioids (Table 1.1; Fig. 1.2). The research presented in this thesis focused on the notothenioid family Nototheniidae, which will be the subject of the next section. Notothenioid fish possess a wide range of physiological and morphological adaptations that permit their survival in the Southern Ocean. The most important of these will be briefly described below.

a. Freezing resistance

Survival in the Ross Sea requires resistance to freezing. The high sodium chloride concentrations in the blood of Antarctic fish, compared with temperate species, partly accounts for the lowering of the freezing point (Ahlgren et al. 1988). However, in the Antarctic notothenioids freezing resistance is achieved mostly by the presence of protein-sugar molecules called antifreeze glycoproteins (AFGPs) (DeVries 1988). AFGPs are synthesised by the five Antarctic notothenioid families (Nototheniidae, Harpagiferidae, Artedidraconidae, Bathydraconidae and Channichthyidae) but not in the temperate water families (Bovichtidae, Pseudaphritidae and Eleginopidae). This has been suggested as further evidence that these families diverged prior to Antarctic cooling (Cheng et al. 2003; Near 2004). Both of the New Zealand nototheniid fish *Notothenia angustata* and *N. microlepidota* synthesise AFGPs, but not at concentrations that would prevent freezing (Cheng et al. 2003). The AFGPs in notothenioid fish are a family of molecules of various sizes. They are made up of between four and 55 repeats of a basic glycotripeptide unit alanine (or sometimes proline)-alanine-threonine. Each threonine is linked to a disaccharide of galactose-N-acetylgalactosamine (DeVries 1988; Cheng et al. 2003). The molecules are present year-round in notothenioid fish, in contrast to antifreeze molecules in Arctic fish which appear seasonally (DeVries 1988). Species which more commonly come into contact with ice (for example cryopelagic species) have higher concentrations of AFGPs than those which contact ice less frequently (Jin and DeVries 2006).

AFGPs depress the freezing point of the body fluids below the freezing point of sea water (-1.86 ℃) via a non-colligative method termed adsorption-inhibition.

Table 1.1. The eight families of notothenioid fish.

Family	
Bovichtidae	Thornfish. Found to the north of Antarctica on the southern coasts of Australia, New Zealand and South America.
Pseudaphritidae	One known non-Antarctic species, with an Australian distribution.
Eleginopidae	One known non-Antarctic species, with a South American distribution, off the coast of Chile and near Tierra del Fuego.
Nototheniidae	Antarctic cods. Probably resemble the ancestral type.
Artedidraconidae	Plunderfishes.
Harpagiferidae	Spiny Plunderfishes.
Bathydraconidae	Dragonfishes.
Channichthyidae	Icefishes.

Adsorption of the antifreeze molecule onto the surface of an ice crystal prevents its further growth, thereby keeping the crystals small enough to prevent cellular damage. As a result, the freezing point of some Antarctic notothenioids is as low as -2.7 °C. The fate of the AFGPs and their bound ice crystals is not certain. The molecules are not excreted with other waste products; however, if the ice crystals are present during the entire lifetime of the fish, it is assumed that their accumulation would interfere with cellular function (DeVries 1988). Research by Hunt et al. (2003) suggests that in some near-shore areas of McMurdo Sound, water temperatures may rise above the equilibrium melting point of ice in Antarctic fishes (-1.1 °C) for up to 21 days of the year. This suggests passive melting may be a mechanism of endogenous ice removal in Antarctic fish. However, the warming trend has only been documented at a few sites in McMurdo Sound, so this cannot be confirmed as a widespread mechanism at this time (Hunt et al. 2003).

Early research suggested AFGPs were synthesised in the liver and secreted into the circulatory system for distribution to the rest of the body (O'Grady et al. 1982). However, more recent work on the Antarctic cod *Dissostichus mawsoni* has shown the pancreas to be a site of AFGP synthesis. AFGPs synthesised in the pancreas are secreted into the digestive tract. Concentrations of AFGPs are thought to be high in the digestive tract because this is the site of food and water absorption (Trinachartvanit et al. 2000). AFGPs occur in most body fluids except the

endolymph, ocular fluids and the urine. Freezing would be unlikely in the endolymph which is located deep within the skull, and the endolymph gains some protection from the surrounding tissues which contain antifreeze (DeVries 1988). The ocular fluids are protected from freezing because the head skin surrounding the ocular orbit extends over the cornea and is a physical barrier to ice entry (Turner et al. 1985). Urine does not freeze because the urethra is normally closed by a muscular sphincter and a thick layer of protective mucus (DeVries 1988).

The development of antifreeze glycoproteins has also necessitated specialisation of the notothenioid kidney. The antifreeze molecules are small enough to be lost during urine formation in the glomerulus of most fish kidneys, and this would be energetically very expensive (DeVries 1988). However, the kidneys of notothenioid fish are aglomerular, urine being formed instead by active secretion through the walls of the kidney tubules (Foster 1990).

b. Metabolic cold adaptation

Early measurements of oxygen consumption on the goldfish, *Carassius auratus*, showed an exponential decrease in respiration rate as temperature decreased. The graph of these data became known as Krogh's Normal Curve. Extrapolation from the curve suggested that at polar temperatures, metabolic rate would be too low to support the survival of fish. Krogh postulated that cold water fish must possess some form of compensation in metabolic rate to allow survival at low temperatures (Krogh 1914). Initial measurements of the metabolic rate of Antarctic fish supported the idea that the rates of Antarctic fish were raised when compared with extrapolation of data from temperate species. This elevation of metabolic rate was termed metabolic cold adaptation (Scholander et al. 1953; Wohlschlag 1960; Wohlschlag 1964).

The concept of metabolic cold adaptation was challenged by Holeton (1974) who suggested that MCA was an artefact resulting from poor experimental technique and interpretational problems. Holeton criticised the short acclimation times to experimental temperatures and equipment, suggesting 48 hours as an appropriate duration, compared with the half to one hour acclimation time used in earlier

experiments. Holeton also criticised the theoretical background to the concept of MCA suggesting that high maintenance costs of metabolism and the resulting reduced metabolic scope for activity would in fact be detrimental for these animals (Holeton 1974). If resting metabolic rates are raised, little energy is left over for growth and reproduction (Montgomery and Wells 1993). Holeton's own careful measurements of metabolic rate in 11 species of polar fish found no evidence for MCA.

Since Holeton (1974), a wealth of experimental data and discussion has accumulated which both disputes (for example Clarke 1980; Forster et al. 1987; Davison et al. 1990; Clarke 1991; Bushnell et al. 1994; Steffensen et al. 1994; Hop and Graham 1995; Clarke and Johnston 1999; Drud Jordan et al. 2001) and supports MCA (for example DeVries and Eastman 1981; Torres and Somero 1988a; Torres and Somero 1988b; Somero 1991). Some authors suggest that MCA becomes evident in situations of high energy turnover, such as during exhaustive exercise, but not in the resting metabolic rate (Hardewig et al. 1998). Other authors suggest that the upward adjustment of ion pump densities in Antarctic fish is the causal basis of MCA. The upward regulation of ion pump densities acts to maintain channel/pump flux ratios at unity, and this presumably results in higher maintenance metabolic costs (Hochachka 1988).

The concept of MCA still attracts research attention and debate. However in general, the metabolic rates of Antarctic fish are much lower than those of tropical or temperate fish at the normal environmental temperature. For example, the metabolic rate of sedentary tropical species at 26 °C may be up to four times higher than in sedentary polar species at 0 °C (Johnston et al. 1991).

c. Haematological properties

Increased viscosity of the blood is a potential problem for Antarctic fish. At low temperatures, molecular movement is slowed and this results in increased viscosity of fluids (Eastman 1993). Increased blood viscosity at cold temperatures creates extra work for the cardiovascular system and can impair oxygen delivery to the

tissues. Because of this, notothenioid fish possess fewer circulating red blood cells than temperate species with similar lifestyles, thereby reducing their blood viscosity and decreasing the resistance to blood flow. In channichthyids, the reduction in viscosity is taken to extremes with the complete lack of circulating erythrocytes. The loss of erythrocytes is compensated for in these fish with an increase in the size of the heart and the blood volume (Egginton 1996). As in other teleost fish, the spleen is used as a storage place for red blood cells and these can be released into the circulation in times of need, such as during exercise. The release of red blood cells increases the haematocrit and thus the oxygen carrying capacity of the blood. In many teleost species splenic contraction occurs in response to increased sympathetic nervous activity and the release of circulating catecholamines. However, in notothenioid fish the mechanism of control is different. Stress results in splenic contraction, but does not change the level of circulating catecholamines. Instead, control of the spleen is cholinergic, while control of the splenic arteries is adrenergic. Therefore, cholinergic autonomic innervation leads to splenic contraction in these fish (Nilsson et al. 1996). The reduction in red blood cell number is partly compensated for by the high concentration of oxygen in the cold waters of the Antarctic. Water in the Ross Sea contains about 1.6 times as much oxygen as water at 20 ℃, this factor, combined with the excellent mixing of Antarctic waters means that it is unusual for Antarctic fish to experience hypoxic conditions (Eastman 1993).

d. Enzymes

The enzymes of Antarctic fish have several adaptations that appear to be related to function at cold temperatures. While most enzymes have an optimum temperature range between 20 and 40 ℃, those of Antarctic fish function best near 0 ℃ (Foster 1990; Somero 1991). The cells of these fish also possess high concentrations of enzymes, especially those involved in aerobic metabolism. The catalytic activity of the enzymes is improved via an increase in the number of catalytic sites on each enzyme, as well as higher catalytic activity at each active site (Somero 1991).

The function of both citrate synthase and lactate dehydrogenase in the brain of Antarctic fish appear to show compensation for low temperature. Citrate synthase

(CS) is an indicator of citric acid cycle activity, and thus aerobic metabolism. Lactate dehydrogenase (LDH) is an indicator of potential for anaerobic energy production. The activity of CS and LDH varies widely between species depending on the general level of locomotory activity. However, both of these enzymes appear to be cold adapted in at least eight species of Antarctic notothenioid fish, showing increased activity at low temperatures compared with tropical and subtropical species. This increased enzymatic activity may be due to higher concentrations of enzymes or more efficient enzymes or some combination of these mechanisms (Kawall et al. 2002).

e. Buoyancy

All adult notothenioids lack a swim bladder and most are heavy-bodied benthic species, presumably reflecting the lifestyle of the ancestral species (Eastman 1993). However, some notothenioids have evolved to occupy ecological niches within the water column. The niche occupied by a species seems to be correlated with their buoyancy (Table 1.2). Species that occupy niches in the water column have greater buoyancy and comparatively light skeletons compared to species that occupy niches on the sea floor (Eastman 1993). Pelagic species also possess other specialisations to counteract the lack of the swim bladder. These include reduced mineralization of the scales and skeletons and the incorporation of oily lipids into the tissues to reduce density (Foster 1990). *Pleuragramma antarcticum* and *Dissostichus mawsoni* are neutrally buoyant, permanent members of the pelagic community. Both species have lipid stores which persist during the winter and help to produce static lift. In *P. antarcticum*, the lipid is stored in large subcutaneous and intermuscular sacs. In *D. mawsoni*, the lipid is stored in adipose cells in subcutaneous and muscular deposits. In both species, the lipid is composed primarily of triacylglycerols (Eastman 1988).

There is a correlation between the buoyancy of notothenioid fish and their burst swimming speed. Franklin et al. (2003) measured the burst swimming speed and buoyancy of the cryopelagic *Pagothenia borchgrevinki* and three benthic/demersal notothenioids (*Trematomus bernacchii*, *T. nicolai* and *T. hansoni*) as well as the bathydraconid *Gymnodraco acuticeps*. There was a relationship between burst

swimming speed and buoyancy in these species. *P. borchgrevinki* (the most buoyant species) attained the highest burst swimming speed, while the most negatively buoyant species, *G. acuticeps*, demonstrated the lowest burst swimming speed.

Table 1.2. The buoyancy and ashed skeletal weight of notothenioid fish occupying benthic, epibenthic and cryopelagic niches. Pelagism seems to be associated with increased buoyancy and low ashed skeletal weight (data from Eastman 1993).

	Benthic *Trematomus bernacchii*	Epibenthic *Trematomus loennbergii*	Pelagic *Pleuragramma antarcticum*
Buoyancy (wt. water / wt. air X 100)	3.37	2.28	0.57
Ashed skeletal weight (% body weight)	1.08	0.76	0.34

f. Lipids

As well as a function in improving buoyancy for some notothenioids, lipids also have other roles related to life in the cold. Lipids are important determinants of membrane fluidity, which is important for efficient cell function. At low temperatures, such as those experienced by notothenioid fish, the fluidity of membrane lipids may be compromised. However, the membrane lipids of notothenioid fish are more fluid at low temperatures than those of temperate species due to an increase in the unsaturation of membrane phospholipids (Eastman 1993). This also provides for unimpaired function of the nervous system at low temperatures (Foster 1990).

It has also been suggested that intracellular lipids may play an important role in oxygen transport through cells at low temperatures. Part of the acclimatisation response to cold conditions in fish involves hypertrophy of the slow skeletal muscle cells. In turn, this may impair oxygen exchange between capillaries and mitochondria. Increased intracellular lipid concentration is thought to reduce the problems associated with diffusive exchange of oxygen by assisting with intracellular transport of oxygen and by acting as an intracellular storage site for oxygen. Lipid may be more important in this role than myoglobin, because myoglobin function is impaired at low temperatures (Hoofd and Egginton 1997; Egginton et al. 2002), and

is absent in the oxidative muscle tissues of some species of notothenioids (Sidell et al. 1997).

Nototheniidae

The notothenioid family Nototheniidae is the most diverse family with respect to both habitat and morphology. It is also the most widely distributed, diverse and abundant family of larval, postlarval and juvenile fishes in the Ross Sea (Vacchi et al. 1999). The family includes benthic species as well as species adapted for life in the water column. The retention of larval characteristics in the adult form (paedomorphosis) is thought to have assisted colonisation of the water column in these fish (Eastman and DeVries 1985; Eastman 1993). Nototheniids are thought to have diverged relatively recently with the divergence of the genus *Trematomus* about 3.4 million years ago (Eastman 1993). The research documented in this thesis involves four species of notothenid fish. *Pagothenia borchgrevinki* (Boulenger 1902) was the main study species and results from this species were compared with those for three other species – *Trematomus bernacchii* (Boulenger 1902), *T. pennellii* (Regan 1914) and *T. hansoni* (Boulenger 1902) (Fig. 1.3).

a. *Pagothenia borchgrevinki* (Bald Notothen)

P. borchgrevinki is a cryopelagic species, usually living within the upper 30 m of the water column on the coasts of the Ross Sea, Davis Sea, Weddell Sea and the Antarctic Peninsula (Gon and Heemstra 1990). However, the presence of *Pleuragramma antarcticum* in the diet of *P. borchgrevinki* suggests that it can also reach greater depths (Knox 1994). *P. borchgrevinki* usually swims beneath the platelet ice, but does occasionally enter the ice to feed or shelter from predators. Some studies have also reported the presence of *P. borchgrevinki* far out to sea (Andriashev 1970). Because this lifestyle is very different from that of its benthic ancestors, *P. borchgrevinki* has substantial morphological adaptations that set it apart from these fish, including higher concentrations of antifreeze, specialised

camouflage (silvery reflective layers or strata argentea beneath the skin) and changes to the body shape (including streamlining and drag reduction) (Eastman and DeVries 1985). The active lifestyle of this species also necessitates adaptations to the oxygen transport system, such as low blood oxygen affinity (Eastman 1993). *P. borchgrevinki* reaches maximum lengths of around 28 cm (Dewitt et al. 1990).

The diet of *P. borchgrevinki* varies with locality and season, suggesting an opportunistic feeding strategy. Prey items include polychaetes such as *Harmothoe sp.*, molluscs such as *Clione antarctica*, copepods such as *Calanoides arctus*, amphipods such as *Hyperiella macronyx*, euphausiids such as *Euphausia crystallorophias* and fish larvae such as *Pleuragramma antarcticum* (Knox 1994). The thecosomatid pteropod *Limacina helicina* is also an important prey item for *P. borchgrevinki* (Foster et al. 1987) as are other juvenile *P. borchgrevinki* and juvenile icefish (W. Davison unpublished observation). *P. borchgrevinki* is itself an important food source to predatory Antarctic vertebrates including Weddell seals (*Leptonychotes weddellii*; Davis et al. 1999); emperor penguins (*Aptenodytes forsteri*, Cherel and Kooyman 1998) and south polar skua (*Catharacta maccormicki*, Mund and Miller 1995).

b. *Trematomus bernacchii* (Emerald Notothen)

T. bernacchii is a circum-Antarctic species which can be found living on the sea floor in shallow waters and up to depths of 550 m. It is present at some offshore islands including Peter I, South Shetland, Elephant and Orkney Islands (Gon and Heemstra 1990). It is a relatively inactive species, with a heavy skeleton and lack of buoyancy adaptations. Like *T. pennellii* and *T. hansoni*, its body is dorsoventrally depressed and lacks streamlining. Support on the substrate is achieved by the modified pelvic and anal fins (Eastman 1993). It is a mottled brown fish providing excellent camouflage in its sea floor habitat where it pursues a territorial lifestyle. There are two colour morphs, one brown and one with a white blotch (or three white stripes) on the head. Both males and females are generally long-lived, females living around 10 years and males about five years. The fish reach lengths of around 35 cm for females and 28 cm for males (Dewitt 1990).

The diet of *T. bernacchii* varies with the location and the season, making these fish feeding generalists. A hunt-and-peck strategy is employed to capture prey such as polychaetes like *Barrukia cristata*, bivalves such as *Admussium colbecki* and euphausiids (Vacchi et al. 2000).

c. *Trematomus pennellii* (Sharp-spined Notothen)

T. pennellii is a circum-Antarctic benthic species, usually found in waters less than 200 m deep (DeWitt et al. 1990). Like *T. bernacchii*, it is a relatively inactive, heavy and dorsoventrally depressed species. Perching is a common habit, supported by the modified pelvic and anal fins. The sponges covering the sea floor provide useful perching and hiding places (Eastman 1993). The diet of *T. pennellii* includes polychaetes, especially *Amithas membranifera*, *B. cristata* and *Aglaophamus ornatus*, as well as some amphipods and fish eggs (Vacchi et al. 2000).

d. *Trematomus hansoni* (Striped Notothen)

T. hansoni is a circum-Antarctic species which lives on the sea floor in shallow waters and up to 550 m (Dewitt 1990). It is slightly more active than *T. bernacchii* and *T. pennellii*, but still much less active than *P. borchgrevinki*. This species can perch vertically using its pectoral and pelvic fins (Eastman 1993). *T. hansoni* is a mainly piscivorous, hunt-and peck-predator, usually preying upon juvenile fish. Fish eggs, polychaetes and decapods form a secondary food source (Vacchi et al. 2000).

Fig. 1.3. The four study species: a. *Pagothenia borchgrevinki.* b. *Trematomus bernacchii.* c. *T. hansoni.* d. *T. pennellii.*

a.

b.

c.

d.

Climate Change and Antarctica

The pre-industrial atmospheric concentration of carbon dioxide was about 280 parts per million (ppm). By 1991, this value had risen to 350 ppm due to the burning of fossil fuels and changing land use (Ellis 1991). Recent measurements put atmospheric carbon dioxide concentration around 379 ppm (IPCC 2007). This concentration has not been exceeded for at least the past 420 000 years, and probably not the past 20 million years (Watson et al. 2001). Carbon dioxide absorbs the infrared radiation reflected back from the Earth's warming by the sun and redirects it to Earth. This leads to warming of the Earth's surface and the troposphere (Kukla and Gavin 1981; Wood 2001; Zorita et al. 2005). This process is often termed the 'Greenhouse Effect'. Other gases also absorb infrared radiation, including methane, nitrogen oxides, ozone and halogenated hydrocarbons, and many of these gases are also increasing in concentration in the atmosphere (Ellis 1991; Watson et al. 2001; IPCC 2007). Increased concentrations of these gases have already resulted in an observed global warming trend in Earth's climate (Wood 2001; IPCC 2007). Out of the 12 years between 1995 and 2006, 11 ranked among the hottest for global surface temperature since the beginning of the instrumental record in 1850 (IPCC 2007).

There are many reports of recent changes in the temperature of the Earth's surface. Between 1906 and 2005, the best estimate is for a linear trend in surface temperature warming of 0.74 ℃ (IPCC 2007), although this figure varies regionally (Watson et al. 2001). The warming trend is predicted to continue into the coming centuries. Estimates of future changes in global surface temperatures are complicated and are based on a variety of climate models and scenarios. However, most models predict that the rate of warming in the future is likely to be accelerated compared to current rates of change. Predictions of globally averaged surface temperature change range between increases of 1.4 and 5.8 ℃ by 2100. Many models predict that the rate of change is likely to be greater in polar-regions (Watson et al. 2001; Wood 2001).

Estimating temperature change in the Antarctic is difficult. Meteorological stations are far apart and usually coastal (Stearns et al. 1993). Also, records are short, usually only for time periods dating from the 1960s (Phillpot 1985; King and Harangozo 1998). However, data from occupied Antarctic stations between 1949 and 1996 illustrates on average a warming trend of 1.2 °C (Jacka and Budd 1998). In some areas this increase has been more significant, such as around the Antarctic Peninsula (King and Harangozo 1998), while some areas have exhibited cooling trends (Maxwell et al. 1998). The rise in temperature on the western Antarctic Peninsula has been reported as 3 °C, making this one of the most rapidly changing regions in the southern hemisphere (Meredith and King 2005).

The ocean has been a dominant sink of this increased global heat (as well as carbon dioxide), absorbing more than 80 % of the heat added to the climate system (Levitus et al. 2001; Levitus et al. 2005; IPCC 2007). Between 1950 and 1993, a global average increase in sea surface temperature of 0.1 °C per decade was recorded (Watson 2001). Warming is greatest at the surface and in the top 700 m. This warming is outside the range of natural ocean variability and has been attributed to anthropogenic causes (Pierce et al. 2006). It has been suggested that the presence of natural and anthropogenic aerosols in the atmosphere have delayed the rate of global ocean warming. However, these aerosols have a short lifespan in the atmosphere, so this situation could change rapidly and lead to increased rates of oceanic warming (Delworth et al. 2005).

The Southern Ocean has also experienced a warming trend. Between the 1950s and 1980s, Southern Ocean mid-depth temperatures (700 – 1100 m) have risen by 0.17 °C. This increase has been largely concentrated in the Antarctic Circumpolar Current (Gille 2002). In the ocean adjacent to the western Antarctic Peninsula summer surface temperatures have risen by more than 1°C since 1951 and this has resulted in a strong salinification of the upper layer (Meredith and King 2005). Warming is predicted to cause serious effects to Southern Ocean circulation patterns (Hirst 1999; Bi et al. 2001). In some models, the thermohaline circulation is predicted to cease altogether, resulting in a warming of the entire ocean by about 7 °C over several hundred years (Bi et al. 2001). Some models predict a cooling trend for

small localised areas north of the Ross Sea (Kim et al. 2005), which may already have occurred as a result of El Niño events (Bertler et al. 2004).

Changes to Southern Ocean temperature and circulation patterns are likely to impact Antarctic sea ice. It is difficult to determine if changes in the sea ice have already begun because interannual variation in sea ice extent is large and the available data are limited (Kukla and Gavin 1981; Jacka and Budd 1998; King and Harangozo 1998; IPCC 2007). Satellite data have shown no apparent trends in sea ice extent since 1978 (Watson 2001). However, data extrapolated from early whaling records, recording the location of the ice edge between 1930 and 1987, suggest a decline in the extent of Antarctic sea ice between the 1950s and 1970s (de la Mare 1997). A decline in sea ice close to the western Antarctic Peninsula has also been reported (Meredith and King 2005). Predictions for the 21st Century are for global sea ice cover to shrink (IPCC 2007).

Changes in ocean temperature, water currents and sea ice extent will impact marine communities. While some species may shift their range further south, this is not possible for all species, particularly those already living at the limits of the Southern Ocean. A loss of productivity is likely to occur in communities dependent on the sea ice for survival (Young 1991). Reduced deep water exchange and the associated nutrient loss may also cause an overall reduction in marine productivity (Maxwell 1998). Changes are already being observed in the distribution and abundance of the Antarctic krill *Euphausia superba*, an important primary food source in the Southern Ocean. Krill density over a wide area of the Atlantic sector of the Southern Ocean (which contains more than 50 % of Southern Ocean krill stocks) has declined since the 1970s and these organisms have been replaced largely by Antarctic salps, *Salpa thompsoni*. These changes have had important consequences for the Antarctic food web (Atkinson et al. 2004). The fate of the temperature sensitive Antarctic notothenioids may also be particularly precarious, for reasons explained below.

Antarctic Notothenioid Fish and Stenothermality

In ectothermic animals, such as Antarctic fish, body temperature changes with the ambient temperature. Such changes affect the rates of internal biological processes and influence the animal's performance. An increase in ambient temperature (and therefore body temperature) usually results in an increase in the animal's metabolic rate, until an upper critical temperature is reached; whereas a decrease in temperature will produce a decrease in the animal's metabolic rate, until a lower critical temperature is reached (Cossins and Bowler 1987). The critical temperatures of organisms (both upper and lower) are variable and depend on the species, its state of thermal acclimatisation and its geographical location (Pörtner et al. 2000). Above and below the critical temperatures, biological processes become increasingly impaired by the effects of temperature until death of the animal occurs (Cossins and Bowler 1987).

The mechanisms that lead to thermal death are difficult to identify, as it is complicated to differentiate between the direct causes of death and their secondary and tertiary consequences. In the case of heat death, a variety of primary mechanisms have been suggested. These include a loss of nervous system integrity and failure of neuronal function (Cossins and Bowler 1987; van Dijk 1999; Pörtner et al. 2000); excessive membrane fluidity (Cossins and Bowler 1987); a failure of oxygen supply (and thus aerobic energy production) to meet demand (Hardewig et al. 1999a; Pörtner et al. 2000; Peck 2002; Peck et al. 2004); as well as loss of enzyme function due to the unfolding of tertiary and quaternary protein structure (Somero 1991; van Dijk 1999; Pörtner et al. 2000; Seebacher et al. 2005).

Ectothermic animals living in many different habitats experience variations in temperature on both a daily and seasonal basis. In order to offset the impairment of performance during seasonal changes in temperature, many of these organisms possess an impressive ability to adjust their physiology in response to temperature changes. These adjustments are changes in the phenotype of an organism, the scope of which is controlled by the genotype. They act to maintain the ability of an organism to carry out essential biological processes such as predator escape,

reproduction and prey capture (Hammill et al. 2004). Changes which occur in response to just one variable, such as temperature, are referred to as acclimation. However, these situations are usually restricted to laboratory settings, and in nature a change in temperature is usually accompanied by changes in other variables, such as photoperiod. Physiological adjustments of an organism to a change in several factors are termed acclimatisation (Cossins and Bowler 1987).

Organisms inhabiting seasonally variable habitats are thought to possess greater acclimatory ability than those inhabiting more stable environments. In fluctuating environments, selection pressures are thought to favour strong acclimatory abilities and phenotypic plasticity to offset the effects of seasonal temperature variation. By broadening the phenotype to cover a range of temperatures, however, the organisms are thought to reduce their performance at an optimum temperature. This concept develops from the 'Principle of Allocation' (Levins 1968) which states that phenotypes which are extended to cover a wide range of environments have reduced ability to perform in an optimum environment. The Principle of Allocation was related to temperature in a hypothesis presented by Huey and Hertz (1984) that a 'Jack-of-all-temperatures is a master of none'. In contrast, organisms inhabiting thermostable environments are able to specialise their performance for an optimum temperature (which is the prevalent environmental temperature). In the process of this specialistaion, they are considered to trade-off their phenotypic plasticity and acclimatory abilities (Johnston and Temple 2002).

Antarctic marine organisms inhabit one of the most thermostable environments on Earth and are considered the ultimate thermal specialists (Peck et al. 2004). This specialisation to a narrow temperature range has resulted in a reduction in the upper critical temperature of Antarctic marine organisms (Pörtner et al. 2000; Peck 2002), making them some of the most stenothermal organisms on Earth. The upper lethal temperature of several notothenioid fish has been reported at 6 °C or less (Wohlschlag 1962; Wohlschlag 1964; Somero and DeVries 1967; Somero 1991; van Dijk 1999), one of the lowest upper critical temperatures reported for any organism. Adaptation to the thermostable environment of Antarctic waters has also reportedly affected the evolution of the heat shock response (HSR) in these fish, thereby

contributing to their stenothermal nature. The HSR is a highly coordinated, nearly universal response to thermal stress in organisms. In the HSR, a set of genes which code for the synthesis of heat shock proteins (HSPs) are activated (Hofmann et al. 2000; Hofmann et al. 2005). The HSPs protect cellular proteins from the increased likelihood of unfolding and aggregation at high temperatures. Early work on notothenioid fish reported that HSPs were produced at a very low temperature of between 5 and 12 °C (Maresca et al. 1988). However, more recent work suggests that some notothenioid fish may lack the HSR (Hofmann et al. 2000; Place et al. 2004). The loss of the HSR in these fish is thought to have occurred as a result of evolution in cold and stable conditions, rendering a HSR unnecessary. The genes for HSP production are constitutively expressed in the notothenioid fish *Trematomus bernacchii* and cannot be upregulated in times of temperature stress (Buckley et al. 2004). The temperate water notothenioid *Bovichtus variegatus* still possesses a HSR, and this gives support to the idea that evolution in cold stable environments has resulted in loss of the HSR in Antarctic notothenioids. The Antarctic 'escapee' *Notothenia angustata*, which now inhabits temperate waters, also possesses a HSR. This species may have left Antarctic waters before the loss of the HSR, or may have regained this capacity during its evolution in the temperate New Zealand seas (Place et al. 2004; Hofmann et al. 2005).

The stenothermal nature of these organisms makes them vulnerable to the effects of global climate change. However, some Antarctic marine organisms are more robust than others (Peck 2002). Mobile marine organisms are thought to be less vulnerable than sedentary species (Pörtner et al. 2000). For example the Antarctic starfish *Odontaster validus* can survive up to 12 °C (personal communication from T. Hill to L. S. Peck in Peck 2002), compared with Antarctic limpets and bivalves which begin to lose their righting, reburying and swimming ability between 2 and 5 °C (Peck et al. 2004). Antarctic marine vertebrates are also thought to be less sensitive than marine invertebrates (Hardewig et al. 1999a). It is therefore evident that the effects of global climate change are likely to vary considerably between species, even among related groups such as the notothenioid fish. This research considers the ability of four notothenioid fish to withstand and acclimate to increased temperature.

Thesis Aims

This thesis addressed two broad research questions:

1. Has specialisation to a cold, stable environment resulted in a trade off in thermal plasticity in notothenioid fish?

2. In view of the results for question 1, what are the likely physiological effects of global climate change and specifically increased ocean temperature on these fish?

In more specific terms, the effects of temperature were examined using fish acclimated for one month to 4 °C and acclimatory ability was assessed in the following:

1. oxygen consumption
2. sustained swimming ability
3. the cardiovascular system
4. enzyme activity
5. haematological parameters
6. thermal preference
7. indicators of fitness

Long term acclimatory ability was assessed in fish acclimated for six months to 4 °C and the ability of fish with x-cell gill disease to acclimate to 4 °C was measured.

The main research species was the active, cryopelagic nototheniid fish *Pagothenia borchgrevinki*. The active, cryopelagic lifestyle of *P. borchgrevinki* may be considered atypical for Antarctic notothenioids, so results from this species were compared with three sedentary, benthic/demersal species - *Trematomus bernacchii*, *T. pennellii* and *T. hansoni*. These species possess a lifestyle that is more common among the notothenioid fish.

Chapter Two

General Experimental Methods

Collection and Containment of Research Specimens

Research was undertaken at two research facilities, one at Scott Base, McMurdo Sound (Antarctica); the second at Canterbury University, Christchurch (New Zealand). Fish used in experiments at Canterbury University were collected in McMurdo Sound, held in the research facility at Scott Base and then transported to New Zealand.

Fish Collection in McMurdo Sound

Fish were collected between October and December in the 2004 - 2005, 2005 - 2006 and 2006-2007 Antarctic summer seasons. All four species of fish were collected using hand-lines with small pieces of fish used as bait on barb-less hooks. Fishing holes close to Scott Base (New Zealand Science Support Base) were drilled using a 10 cm diameter Jiffy Drill and then melted-out to approximately 1.2 m diameter using a custom-built melt finger. A fishing hut was pulled across the hole to provide insulation and prevent it from freezing over (and to insulate the fishing scientists). At sites further away from Scott Base (such as at Cape Evans, Fig. 2.1), 10 cm diameter fishing holes were created using the Jiffy Drill. Fishing was conducted through these smaller holes without the use of a fishing hut (Fig. 2.2).

Different locations were targeted for each species. For *P. borchgrevinki*, locations which provided sea ice cover less than 4 m thick, plentiful platelet ice, water depths of between 400 - 600 m, shallow or absent snow cover and distance from seal activity were favoured. Such conditions were generally found within the previous season's United States Antarctic

Program ice breaker channel, close to McMurdo Station (United States Science Support Base) (Fig. 2.1). Fish were usually caught within 1 – 2 m of the lower surface of the sea ice, although in some cases greater depths proved to be more successful.

For the benthic species, fishing was concentrated at Cape Evans in McMurdo Sound (Fig. 2.1). All three species were captured using baited hooks lowered close to the sea floor at water depths of around 20 – 40 m. Some specimens were captured close to Inaccessible Island, near Cape Evans.

Wet Laboratory Facilities at Scott Base

After capture, fish were carefully removed from the hook and quickly placed in insulated containers to prevent ice damage resulting from prolonged exposure to air. Fish were then transported to the Wet Laboratory facilities at Scott Base. At Scott Base, fish were held in flow-through aquaria with seawater temperature maintained at -1.0 ± 0.3 °C for at least three days of recovery before experimental use. Fish used in experiments at Scott Base were not fed during the experimental period. A 24 hour light regime was maintained for all experiments, to model the summer conditions in McMurdo Sound. Selection of specimens for experimental purposes was random, however *P. borchgrevinki* individuals with x-cell disease (Franklin and Davison 1987; Davison 1998) were not selected (apart from experiments in chapter seven, where acclimation to 4 °C was attempted in fish with x-cell disease). Fish were weighed and measured, and labelled for identification using coloured suture thread attached to the dorsal fin.

Fig. 2.1. Ross Island, showing the location of the fishing sites at Cape Evans and adjacent to McMurdo Sound. Adapted from Hatherton (1990).

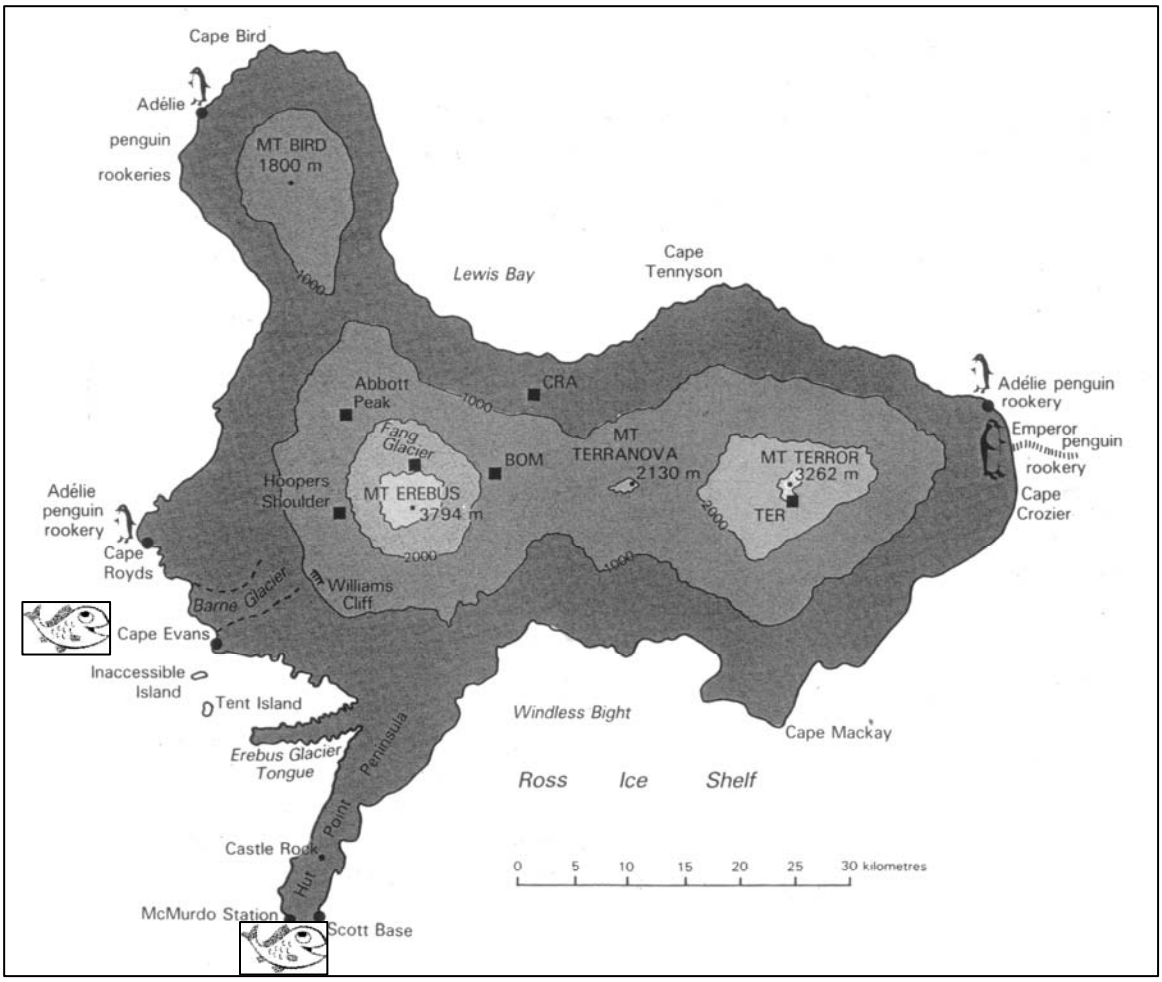
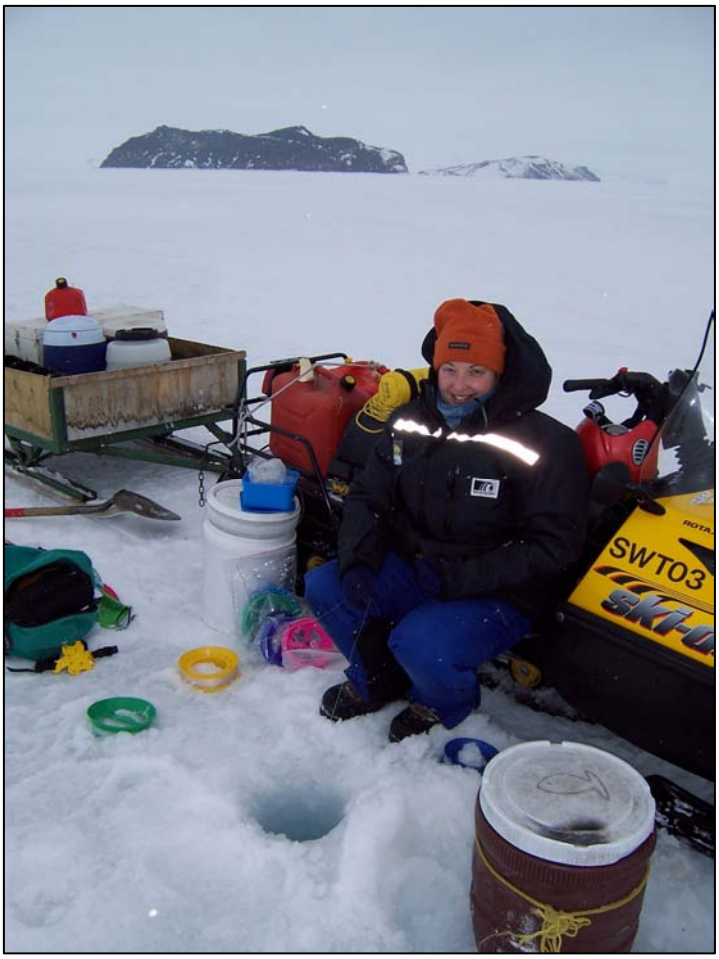


Fig. 2.2. Fishing in McMurdo Sound. a. Fishing close to Scott Base was undertaken in a fishing hut which provided insulation. b. Fishing at sites further away from Scott Base was conducted using 10 cm diameter holes drilled with a Jiffy Drill.

a.

b.



Acclimation to 4 °C at Scott Base

Fish for acclimation to 4 °C were randomly selected. Fish were placed into 65 L static water tanks in groups of no more than 12 individuals. Sea water in these tanks was maintained at 4 ± 0.3 °C using two heat exchangers linked to an adjacent fresh water tank containing a thermostatically controlled water heater. In order to maintain water quality, 20 L of water was replaced twice daily without significant alteration of water temperature. Specimens of *P. borchgrevinki* were transferred directly from the cold water system into the 4 °C water. For the benthic species, temperature was raised by 1 °C every 24 hours until the 4 °C acclimation temperature was reached.

Temperature Controlled Facilities at Canterbury University

Near to the end of each summer season fish were transported from Scott Base to the temperature controlled facility at Canterbury University. Fish were transported in insulated bins containing aerated, iced seawater. The flight to New Zealand took around eight hours in a C130 Hercules aircraft. A 100% survival rate of transported specimens was achieved each season. The fish were placed in the temperature controlled (0.0 ± 0.3 °C) recirculating seawater system where a 24 hour light regime was maintained. Water quality was monitored and maintained using a protein skimmer, biological filter and regular water changes. Fish were fed with mussels (*Perna canaliculus*) once a week except during long-term (six month) acclimation experiments involving measurements of oxygen consumption and sustained swimming ability. In these experiments, fish were fed following a routine which avoided measurement of oxygen consumption during the period of specific dynamic action of feeding (SDA). This protocol is explained in the appropriate chapter. All experimental animals were weighed, measured and identified by the attachment of coloured suture thread to the dorsal fin.

In order to acclimate fish to 4 °C, individuals were randomly selected and transferred to a smaller recirculating seawater system in a temperature controlled room. Air temperature was maintained at 4 °C and sea water temperature was regularly checked and held at 4 ±

0.3 °C. A 24 hour light regime was maintained in this room. Water quality was monitored and maintained with the regular exchange of pre-cooled sea water.

Collection, Transport and Storage of Blood and Tissue Samples

Blood and tissue samples were collected at the Scott Base Wet Laboratory. Air temperature in this laboratory was constantly below 5 °C. Prior to tissue and blood collection, fish were individually anaesthetised for five minutes in a 0.1 g L^{-1} solution of MS222 (ethyl m-aminobenzoate methanesulphonate) dissolved in sea water. Blood samples were immediately taken and the fish then killed with an acute blow to the head.

Blood samples were obtained using cardiac puncture. Approximately 0.5 mL was drawn into a heparinised syringe (ammonium heparinate, Sigma Ltd.) using a 25-gauge needle. Some of this blood was used immediately for the determination of haematocrit (Hct). Blood was drawn into a capillary tube which was then sealed at one end with Hct clay and centrifuged at 20 000 g for 90 seconds. Hct measurement was made directly after the tube was removed from the centrifuge.

A 5 µL sample of whole blood from the cardiac puncture was placed into an eppendorf tube. The blood was frozen in liquid nitrogen and transported to New Zealand where it was used in haemoglobin measurements. Finally, the remaining blood from the cardiac puncture was centrifuged at 3000 g for two minutes and the resulting separated blood plasma was collected. Blood plasma was frozen in liquid nitrogen and transported to Christchurch for use in glucose and osmolarity measurements. Samples were transported to New Zealand in an insulated container containing dry ice and then stored for up to two months at -80 °C prior to analysis.

Tissue samples were collected from fish following blood collection. Required tissues were removed from animals using standard dissection techniques under sterile conditions. These samples were then placed into labelled vials and immediately frozen in liquid

nitrogen. Samples were transported to New Zealand in an insulated bin containing dry ice and then stored for up to two months at -80 ℃ prior to analysis. Blood collection and tissue removal were completed as efficiently as possible to prevent deterioration of specimens.

Chapter Three*

The effect of warm acclimation on the oxygen consumption of *Pagothenia borchgrevinki*

Introduction

The total use of chemical energy by an organism is referred to as its energy metabolism. Metabolic rate refers to the energy metabolism per unit time (Schmidt-Nielsen 1975). The energy expenditure of organisms has been divided into two categories by Jobling (1993). First, is the energy expenditure required for 'service functions', which include for example, the energy utilised by the respiratory and circulatory systems to supply oxygen to the various body tissues. The second category is termed 'cellular maintenance functions'. These include the energy requirements for the synthesis and turnover of cellular constituents such as proteins and lipids, as well as the energy required for basic cell processes such as ion transport. These categories are not mutually exclusive. Two of the most energy consuming processes in most organisms are maintaining the activities of Na^+ K^+ ATPases and protein synthesis (Jobling 1993).

Metabolic rate is tightly controlled by organisms. Control mainly occurs in response to the concentration of adenosine triphosphate (ATP). ATP is the immediate source of chemical energy for cellular processes (Schmidt-Nielsen 1975; Brett and Groves 1979). When the concentration of ATP is high in an organism, its synthesis is reduced; but when the cellular concentration of ATP drops, the rate of its synthesis increases (Clarke and Fraser 2004). The

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metabolic rates of organisms vary between individuals of the same species and between different species. Rates are influenced by factors such as temperature, season, sex, weight / body mass, lineage, lifestyle / activity level, dietary state, maturation state, oxygen concentration of the respiratory medium and state of stress of the organism (Wohlschlag 1964; Ralph and Everson 1968; Saint-Paul et al. 1988; Somero 1991; Taylor et al. 1997; Clarke and Johnston 1999).

The metabolic rate of ectothermic animals is strongly dependent on temperature (Clarke and Johnston 1999). The internal body temperature of ectotherms rises and falls with the environmental temperature, and the metabolic rate responds to changes in the body temperature. Within physiological limits, increased environmental temperature leads to an elevation of the metabolic rate and decreased environmental temperature results in lowered metabolic rate. Changes in metabolic rate occur because the biochemical reactions which comprise the metabolic rate are temperature dependent. Changes in metabolism often occur at a fairly constant rate (Schmidt-Nielsen 1975). Generally, a 10 °C rise in temperature elicits a two or three fold rise in metabolic rate (DeVries and Eastman 1981; Jensen et al. 1993). The increase in a biological rate caused by a 10 °C rise in temperature is called the Q_{10} (Schmidt-Nielsen 1975).

When the metabolic rate of an organism changes, there is also a change in the demand for oxygen at the tissues. For aerobic metabolism, oxygen is required by the tissues to produce ATP through the oxidisation of fuel sources (Schmidt-Nielsen 1975). Oxygen is usually the final electron acceptor of the electron transport chain (Clarke and Fraser 2004). For this reason, the rate of oxygen consumption can be used as a measure of the metabolic rate of an organism (Taylor et al. 1997; Clarke and Fraser 2004) and provides a useful indicator of an organism's response to temperature change (Cech 1990; Lowe and Davison 2006), although this idea has been challenged (Clarke 1991). Because oxygen is required for the aerobic production of ATP, its supply affects all other physiological systems including muscular activity, behaviour, growth and

reproduction. It is therefore likely that impairment of aerobic performance will be the most important factor determining the fate of marine organisms in response to oceanic warming (either acclimation and survival, or relocation, or death) (Peck 2002; Peck 2004; Pörtner and Knust 2007).

Changes in temperature may affect the ability of an organism to supply oxygen at a sufficient rate to meet the demand of the tissues. Oxygen supply becomes limiting above and below the upper and lower critical temperature of an organism. The range of temperatures between the upper and lower limits varies between species; some animals have a wide tolerance range, while others (such as Antarctic fish), have a narrow tolerance range. Variation in critical temperatures also occurs between seasons. For example, the catfish *Ameiurus nebulosus* has an upper lethal temperature of nearly 36 °C in summer, but only 28 °C in winter (Schmidt-Nielsen 1975). In some organisms, the critical temperatures also vary between different life history stages. Outside of the critical temperatures, when oxygen supply does not keep up with demand, life is supported by anaerobic metabolism, the protection given by heat shock proteins and antioxidative defences. Survival in this manner is time-limited and organisms in this situation must make acclimatory adjustments to the oxygen system or they will die (Peck 2004). For example, in the Antarctic eelpout *Zoarces viviparus*, oxygen consumption rises in an exponential way with rising temperature and eventually oxygen supply becomes limiting (Zakhartsev et al. 2003). The temperature at which oxygen supply becomes limiting is closely linked to the temperature beyond which growth, performance and abundance of this species decreases in the natural environment (Pörtner and Knust 2007).

Many species of fish (as well as other ectothermic animals), if provided with sufficient time, can compensate for changes in temperature with an adjustment of the metabolic rate (Schmidt-Nielsen 1975). Such acclimation changes act to maintain homeostasis in physiological systems, and ‘override’ the rate altering effects of temperature (Clarke 1991). Species living in thermally variable

environments are thought to possess greater acclimatory ability than species living in thermally stable environments (Johnston and Dunn 1987). This principle is a corollary of the 'Jack-of-all-temperatures is a master of none' hypothesis of Huey and Hertz (1984). The concept has also been termed the 'Thermal Specialisation Paradigm' (Seebacher et al. 2005), where thermal specialisation, such as has occurred in the notothenioids, is considered a trade off for thermal flexibility. Some experimental evidence has supported this concept. For example, the tropical whitespotted bamboo shark *Chiloscyllium plagiosum* showed an increase in oxygen consumption on exposure to increased temperatures (30 °C) and a decrease in oxygen consumption rate on exposure to lowered temperatures (15 and 20 °C). These initial changes in oxygen consumption rates were maintained after several months at the experimental temperature, indicating that this species has no ability to compensate metabolically for changed temperatures. This species inhabits reefs around the Indo-West Pacific and consequently experiences limited variation in temperature during its lifetime (Tullis and Baillie 2005). In another example, in eelpout (*Z. viviparus*) from the thermally stable environment of deep Norwegian fjords, an initial increased oxygen consumption rate with increased temperature was demonstrated. No adjustment to this rate became evident after extended time at the raised temperature (Zakhartsev et al. 2003). Following on from this theory, it might be expected that the Antarctic notothenioid fish *Pagothenia borchgrevinki*, which spends its lifetime in one of the most thermostable environments on Earth, would possess little or no capacity to acclimate its metabolic rate to changes in temperature. Testing this assumption was the aim of these experiments.

Even in species with acclimatory ability, acclimation does not necessarily fully compensate for the changes caused by temperature. The range of acclimatory changes shown by ectothermic organisms in response to acute changes in temperature have been classified by Precht et al. (1973) and are described in Table 2.1. With reference to this classification system, Antarctic ectotherms often fit with the no compensation or partial compensation definitions. For

example, the eelpout *Pachycara brachycephalum* shows little or no compensation in metabolic rate after a temperature rise (Van Dijk et al. 1999); brachiopods and limpets show partial compensation (Peck 1989), as does the bivalve mollusc *Limopsis marionensis* (Pörtner et al. 1999). The ability of the Antarctic notothenioid fish *P. borchgrevinki* to acclimate its metabolic rate has never been tested.

Table 3.1. The range of responses to acute temperature change demonstrated by ectothermic animals, as classified by Precht et al. (1973).

No compensation	Physiological rates change to new levels as a result of temperature change. The rates stay at this level thereafter.
Perfect compensation	Physiological rates change to new levels as a result of temperature change but then return to initial levels.
Partial compensation	Physiological rates change to new levels after temperature change but then return part of the way to initial levels.
Over compensation	Physiological rates change to new levels after temperature change but then rebound beyond the initial physiological rate.
Inverse acclimation	Physiological rates change to new levels after temperature change and then continue to move away from the initial rates. This is rare.

At the cellular level, the compensatory changes which occur during acclimation include changes to gene expression, contractile protein function, ion pump activity, ATP generation and synthesis of macromolecules. At a system level, the changes may include adjustment to neural activity, circulatory and respiratory processes, growth, reproduction, development and behaviour (Clarke 1991). These changes have different time courses, some occurring in less than a second, others taking more than a month. This depends on the type of organism, which physiological system is being acclimated and the scale of the temperature change (Johnston and Dunn 1987). Acclimation of the metabolic rate can take several days or weeks in fish (Jobling 1993). Time-course

measurements of the responses of Antarctic ectotherms to temperature change are rare (Peck 2002).

These experiments tested the ability of the Antarctic notothenioid fish *P. borchgrevinki* to acclimate its metabolic rate in response to raised temperature (4 °C). Measurements of resting oxygen consumption rate ($MO_{2\ rest}$) were made as indicators of metabolic rate. The effects of acute changes in temperature sometimes differ between active and resting rates of oxygen consumption (Newell 1973). Therefore, the acclimatory responses of oxygen consumption rates following exhaustive exercise and during recovery from exhaustive exercise were also measured. A time-course of acclimatory changes was determined for resting oxygen consumption rate, post-exercise oxygen consumption rate and ventilation rate.

Materials and Methods

These experiments were conducted at the Scott Base Wet Laboratory facility in McMurdo Sound. *P. borchgrevinki* were collected in McMurdo Sound and held at the laboratory as described in chapter two (General Experimental Methods). Experiments on oxygen consumption (at rest, post-exercise and during recovery from exhaustive exercise) were conducted on eight fish held in cold acclimation conditions (-1 °C) (mass 106.8 ± 4.2 g, range 92 - 126 g; total length 210.3 ± 4.5 mm, range 192 - 230 mm); and eight fish in warm acclimation conditions (4 °C) (mass 93.3 ± 3.4 g, range 83 - 110 g; total length 188.1 ± 3.7 mm, range 175 - 205 mm). There was no significant difference between the mass or length of the two fish groups. Experiments on ventilation frequency were conducted on a second group of fish with eight cold acclimating individuals (mass 42.3 ± 7.6 g, range 21 - 81 g; total length 165.8 ± 8.4 mm, range 138 - 204 mm); and eight warm acclimating individuals (mass 38.9 ± 4.3 g, range 29 - 61 g; total length 164.8 ± 6.6 mm, range 144 - 200 mm). There was no significant difference

between the mass or length of the two fish groups. Selection of experimental animals and acclimation procedures are described in chapter two (General Experimental Methods). The total acclimation time was 28 days. Fish were not fed during this period to avoid the elevation of oxygen consumption rate which results from the Specific Dynamic Action (SDA) of feeding (Johnston and Battram 1993; Boyce and Clarke 1997; Boyce et al. 2000).

Oxygen consumption at rest – time-course of acclimation

Measurements of oxygen consumption at rest ($MO_{2\ rest}$) were made at regular intervals during the four week acclimation period. Oxygen consumption was measured using closed-box respirometry techniques at the acclimation temperature of each group (-1 °C for cold fish; 4 °C for warm fish). A single fish was introduced to a cylindrical polyurethane respirometer with a perspex lid (volume 1 410 – 1 500 ml) 24 hours before oxygen consumption measurements were made. Polar organisms are known to be sensitive to handling stress (Saint-Paul et al. 1988; Boyce and Clarke 1997), but a 24 hour acclimation period is considered adequate to ensure recovery from the effects of handling stress and for fish to settle in the respirometer (Morris and North 1984; Wells et al. 1984; Wells 1987; Johnston et al. 1991). Respirometers were not covered or blacked out as continual light models the natural conditions in McMurdo Sound at the time of year experiments were conducted. Each respirometer was immersed in an aquarium tank at the acclimation temperature of the fish it contained. A continuous flow of seawater into the respirometer, from the surrounding tank, was maintained using an aquarium pump.

Immediately prior to measurement the aquarium pump was withdrawn from the respirometer. All air bubbles were removed and the respirometer was then sealed using a tight fitting respirometer bung. The fish depleted the oxygen in the respirometer for 70 minutes. The oxygen tension of the water inside the chamber was measured every 10 minutes. A 1 ml water sample was withdrawn

from the respirometer using a syringe. Suction replaced the 1 ml sample with saturated seawater from a reservoir (an attached 20 ml syringe). The water sample was injected into the measuring unit of a Strathkelvin oxygen microcell (MC100) which contained an IL 1302 oxygen electrode. The electrode was attached to a Strathkelvin oxygen meter (model 781). Readings were taken 2 minutes after the injection of the sample to allow the meter time to stabilise. The oxygen meter was calibrated before each experiment using air-saturated seawater at the same acclimation temperature as the experimental fish. The zero level was checked using a solution of 3.81 g L^{-1} sodium borate to which crystalline sodium sulphite was added and partially dissolved. Measurements were ended if the oxygen tension in the respirometer fell below 100 mm Hg before the end of the 70 minutes.

No mixing apparatus was used in the respirometer as previous studies have shown that pectoral fin and opercular movements are sufficient to ensure adequate mixing of the water (Wells 1987). Oxygen consumption measurements from a blank respirometry chamber showed negligible change in oxygen tension during 70 minutes, indicating that the biological demand of the sea water was insignificant. This finding is supported by previous Antarctic studies (for example Forster et al. 1987; Lowe and Davison 2006). No attempt was made to remove carbon dioxide from the respirometer during the 70 minutes of measurements.

Oxygen consumption following exhaustive exercise – time-course of acclimation

Measurements of oxygen consumption following exhaustive exercise were made at regular intervals throughout the four week acclimation period. Fish were swum to exhaustion individually in an 80 L Blazka-style swimming tunnel (Fig. 3.1), (Blazka et al. 1960). The clear perspex, cylindrical tunnel had a plastic mesh at each end to prevent the fish from escaping. Water flow was created by a revolving impeller and the design of the tunnel ensured uniform water flow

throughout the swimming zone. The swimming tunnel was filled with fresh, fully aerated seawater at the appropriate acclimation temperature prior to the introduction of each fish (-1 °C for cold acclimating fish and 4 °C for warm acclimating fish). The water temperature in the swimming tunnel was regularly checked throughout the experiment and did not vary by more than 0.3 °C. Fish were introduced to the swimming tunnel running at low velocity (14 cm s^{-1}), so that it was unnecessary to swim to maintain station in the water column. After a 60 minute orientation period within the tunnel, the water velocity was increased in an incremental fashion, by 6 cm s^{-1} every 10 minutes, until the fish became exhausted. Exhaustion was considered to have occurred when a fish twice fell back against the rear mesh of the swimming tunnel. At this stage, the fish was quickly removed from the swimming tunnel and placed into a respirometer filled with air saturated seawater at the appropriate acclimation temperature.

The method for determining oxygen consumption was the same as for measurement of resting oxygen consumption described above, except that readings were taken every five minutes for 30 minutes following exhaustive exercise. Transfer of the fish between the swimming tunnel and the respirometer was completed quickly, within two minutes. However, because it was impossible to measure oxygen consumption of an individual fish within the swimming tunnel (because the water volume was too large), and because there was a short break between the point of exhaustion and the first measurement of oxygen consumption, the measurement made cannot be considered the maximum rate of oxygen consumption during exhaustive exercise ($MO_{2\ max}$). For this reason, this oxygen consumption measurement will be referred to as $MO_{2\ post\text{-}exercise}$. Results for the sustained swimming speeds obtained from this experiment are presented in chapter four.

Fig. 3.1. The Blazka-style swimming tunnel used in these experiments.

Oxygen consumption after exhaustive exercise - end of the acclimation period

Methods for this experiment were the same as described above for the measurement of $MO_{2\ post\text{-}exercise}$ apart from two important differences. First, measurements were made at the end of the acclimation period (28 days), rather than throughout the acclimation period as above. Second, fish were swum to exhaustion at their normal acclimation temperature (-1 °C for cold acclimated fish and 4 °C for warm acclimated fish), as well as at the acclimation temperature of the other acclimation group (4 °C for cold acclimated fish and -1 °C for warm acclimated fish) and measurements of $MO_{2\ post\text{-}exercise}$ were made at both temperatures for both groups.

Oxygen consumption during recovery from exhaustive exercise

Following the 30 minute measurement of post-exercise oxygen consumption, the respirometer was flushed with aerated seawater for 30 minutes to restore oxygen tension to near saturation. The respirometer was then resealed for a second measurement of oxygen consumption (now 60 minutes after exhaustive exercise). The method for determination of oxygen consumption rate was the same as for measurement of resting oxygen consumption except that oxygen tension measurements were made every 10 minutes for 40 minutes. Following the 40 minute measurement period, the respirometer was flushed with aerated seawater for a further 20 minutes to restore oxygen tension. It was then resealed for measurement of oxygen consumption 120 minutes after exercise. Because the rate of oxygen consumption was lower by this stage of recovery and oxygen depletion within the respirometer was therefore occurring at a slower rate, oxygen tension measurements were made every 15 minutes over a 60 minute period. The respirometer was then flushed with aerated seawater for a further 20 minutes and a final measurement of oxygen consumption, 200 minutes after exhaustive exercise, was made using the same procedure as for measurement at 120 minutes.

Resting ventilation frequency – time-course of acclimation

Throughout the acclimation period regular measurements of ventilation frequency were made. Ventilation frequency was determined visually by counting opercular movements over a one minute period. Three counts for each fish were made over three successive minutes and a mean ventilation frequency for one minute was calculated. Measurements were made while the fish remained in their acclimation tank to prevent disturbance to the fish. If a fish moved and interrupted the count it was abandoned and returned to when stationary.

Data analysis and statistical methods

Oxygen consumption rates were determined using the following equation:

$$\frac{\Delta PO_2 \times C \times V \times 31.999}{t \times M}$$

Where:
ΔPO_2 = change in oxygen partial pressure over the measurement period (mm Hg)
C = oxygen capacitance of seawater at a given temperature (μmol L^{-1} mm Hg $^{-1}$)
V = volume of water in the respirometer (L)
31.999 = molecular weight of oxygen
t = duration of measurement (h)
M = mass of fish (g)

The oxygen capacitance value used was 2.31 μmol L^{-1} mm Hg $^{-1}$.

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance was taken at the level of $P< 0.05$. All data are presented as the mean ± standard error, unless otherwise stated. Results within acclimation groups were compared using one-way analysis of variance (ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. Results between acclimation groups were compared using repeated measure two-way analysis of variance (ANOVA) (after a Bartlett's Test confirmed homogeneity of variance) and Bonferroni post-hoc

tests. The $MO_{2\ post\text{-}exercise}$ of cold acclimated fish was compared using an unpaired T-test.

Results

Oxygen consumption at rest – time-course of acclimation

The resting oxygen consumption rate ($MO_{2\ rest}$) of cold acclimating fish (-1 °C) was stable throughout the four week acclimation period (Fig. 3.2). The mean $MO_{2\ rest}$ of these fish on day one was 34.45 ± 3.12 mg O_2 kg^{-1} h^{-1}. Values obtained throughout the acclimation period were very similar to those for day one, so that the $MO_{2\ rest}$ on day one, nine, 17 and 21 were not significantly different in these fish.

The $MO_{2\ rest}$ of warm acclimating fish (4 °C) varied during the acclimation period. Rates on day one and day five were significantly higher than those for cold acclimating fish (day one, 57.80 ± 4.79; day five, 55.72 ± 3.96 mg O_2 kg^{-1} h^{-1}). These rates were also significantly higher than those for the same fish during the remainder of the acclimation period (except for day 13 where there was a small increase in $MO_{2\ rest}$). By day nine, the $MO_{2\ rest}$ of warm acclimating fish had started to fall and was not significantly different from the $MO_{2\ rest}$ of cold acclimating fish. A trend for decreasing $MO_{2\ rest}$ continued throughout the acclimation period (apart from day 13) and by day 25 the mean rate of warm acclimating fish (31.13 ± 2.3 mg O_2 kg^{-1} h^{-1}) was very close to that of cold acclimating fish (29.52 ± 3.01 mg O_2 kg^{-1} h^{-1} measured on day 21).

Oxygen consumption following exhaustive exercise – time-course of acclimation

Exhaustive exercise caused a dramatic rise in MO_2 in cold acclimating fish (Table 3.2). The mean $MO_{2\ post\text{-}exercise}$ of these fish on day six was 125.00 ± 11.41 mg O_2 kg^{-1} h^{-1}, which was about three times greater than the $MO_{2\ rest}$ measured after a similar amount of acclimation time (factorial aerobic scope 3.88 ± 0.52). By day 26, the mean $MO_{2\ post\text{-}exercise}$ had fallen from this initial value to 98.35 ± 5.04 mg O_2 kg^{-1} h^{-1}, although this was not significantly different from day six. Because each fish was swum individually in the swimming tunnel, and only one swimming tunnel was available for experimental use, measurement of $MO_{2\ post\text{-}exercise}$ was time-consuming. A decision was made to measure the $MO_{2\ post\text{-}exercise}$ on four occasions in warm acclimating fish. Unfortunately, this meant that it was only possible to measure the $MO_{2\ post\text{-}exercise}$ of cold acclimating fish on day six and day 26.

Warm acclimating fish also showed an increase in MO_2 in response to exhaustive exercise. The $MO_{2\ post\text{-}exercise}$ of these fish on day six was 133.90 ± 12.57 mg O_2 kg^{-1} h^{-1}, similar to that seen in cold acclimating fish. Because the $MO_{2\ rest}$ was high at this stage of the acclimation period (see Fig. 3.2), the $MO_{2\ post\text{-}exercise}$ was only just over double the $MO_{2\ rest}$ in these fish, so the factorial aerobic scope (2.41 ± 0.27), was significantly lower than the factorial aerobic scope of cold fish. On day 12 and 18 the $MO_{2\ post\text{-}exercise}$ of warm acclimating fish dropped to around 80 mg O_2 kg^{-1} h^{-1}, and this was significantly lower than for day six. As a result, the factorial aerobic scope was also reduced during this period (day 12: 1.85, day 18: 2.4). On day 26, the $MO_{2\ post\text{-}exercise}$ of these fish had risen again, but was still significantly different from the rate of warm fish on day six, but not the rate of cold acclimating fish on day 26. Factorial aerobic scope also increased to 3.16.

Oxygen consumption following exhaustive exercise – end of the acclimation period

The data shown in Fig. 3.3 were collected at the end of the four week acclimation period. Cold acclimated fish swum at -1 °C and 4 °C, and warm acclimated fish swum at 4 °C all showed a similar post-exercise oxygen consumption. Warm acclimated fish swum at -1 °C showed a significantly lower oxygen consumption rate of 67.7 ± 9.28 mg O_2 kg^{-1} h^{-1}.

Oxygen consumption during recovery from exhaustive exercise

The pattern of MO_2 recovery following exhaustive exercise was the same in both acclimation groups throughout the acclimation period (Fig. 3.4). Following exhaustive exercise, there was an exponential decline in MO_2 so that 60 minutes after exercise had ceased, the oxygen consumption rates were not significantly different from rates at rest. The 0 minute MO_2 of cold acclimating fish and warm acclimating fish on day six were significantly different from all other 0 minute measurements. This is because measurements of $MO_{2\ \text{post-exercise}}$ (which these data represent) were high in these groups on this day (see Table 3.2).

Resting ventilation frequency – time-course of acclimation

The ventilation frequency of cold acclimating fish was relatively stable at around 30 min^{-1} throughout the acclimation period (Fig. 3.5). There was no significant difference in the ventilation frequency of these fish on any of the days measurements were made. The inter-individual variation in ventilation frequency was high until the last measurement on day 28 of acclimation, when variation was low.

The ventilation frequency of warm acclimating fish was significantly higher than that of cold acclimating fish on days five (40.38 ± 1.61 min^{-1}) and 10 (40.38 ±

2.35 min^{-1}) of the acclimation period. After day 10, the ventilation frequency of these fish began to fall and on day 15 and 28 was not significantly higher than that of cold acclimating fish. The inter-individual variation of ventilation frequency was high in these fish throughout the acclimation period.

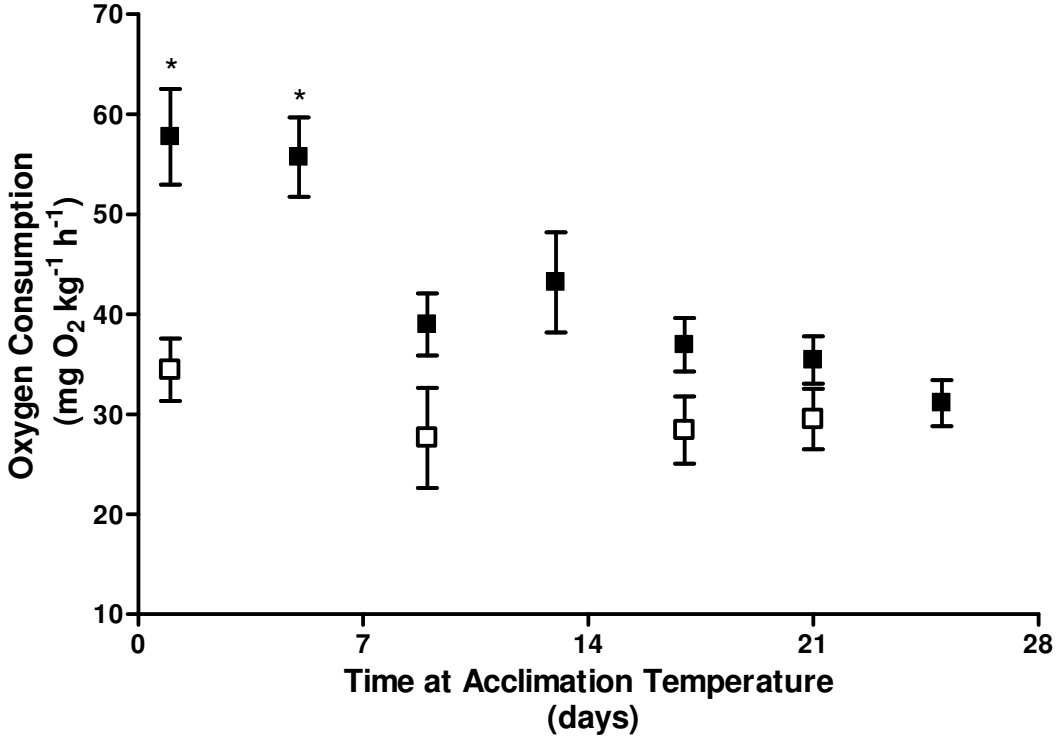


Fig. 3.2. Oxygen consumption at rest ($MO_{2\ rest}$) of *P. borchgrevinki* during four weeks acclimation to -1°C (□) and 4 °C (■). * Significantly different from the $MO_{2\ rest}$ of cold acclimating fish; and also significantly different from the $MO_{2\ rest}$ of warm acclimating fish on day nine, 17, 21 and 25. There was no significant difference in the $MO_{2\ rest}$ of cold acclimating fish on any day during the acclimation period. Note, the $MO_{2\ rest}$ of warm acclimating fish on day five has been compared with the $MO_{2\ rest}$ of cold acclimating fish on day one.

Day	6		12		18		26	
	Cold fish	Warm fish	Cold fish	Warm fish	Cold fish	Warm fish	Cold fish	Warm fish
$MO_{2\ post\text{-}exercise}$ (mg O_2 kg^{-1} h^{-1})	125.0	133.9	●	80.1*	●	88.6*	98.4	98.4♦
SEM	11.4	12.6	●	9.8	●	5.9	5.0	9.1

Table 3.2. The post-exercise oxygen consumption ($MO_{2\ post\text{-}exercise}$) of *P. borchgrevinki* during four weeks acclimation to -1°C (cold fish) and 4°C (warm fish). * Significantly different from the day six $MO_{2\ post\text{-}exercise}$ of warm fish and cold fish. ♦ Significantly different from the $MO_{2\ post\text{-}exercise}$ of warm fish on day six. There was no significant difference between the $MO_{2\ post\text{-}exercise}$ of warm and cold fish on day six; or between warm and cold fish on day 26. ● Indicates a value that was not measured due to time constraints.

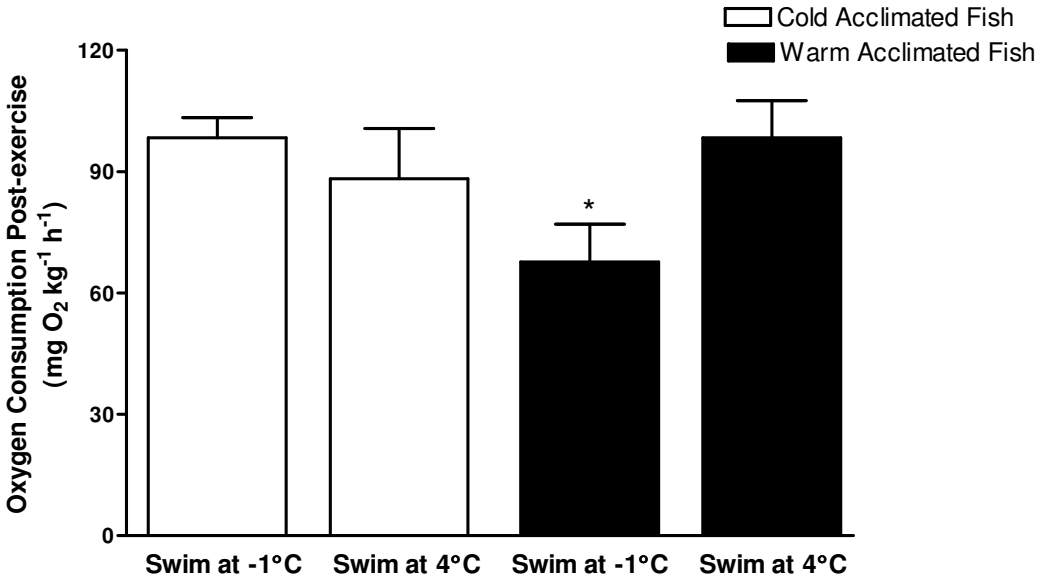


Fig. 3.3. The post-exercise oxygen consumption rate ($MO_{2\ post\text{-}exercise}$) of *P. borchgrevinki* at the end of the acclimation period. Fish were swum at the acclimation temperature (cold fish -1 ℃, warm fish 4 ℃) and the non-acclimation temperature (cold fish 4 ℃, warm fish -1 ℃). * Significantly different from the $MO_{2\ post\text{-}exercise}$ of cold acclimated fish swum at -1 and 4 ℃; and significantly different from the $MO_{2\ post\text{-}exercise}$ of warm acclimated fish swum at 4 ℃.

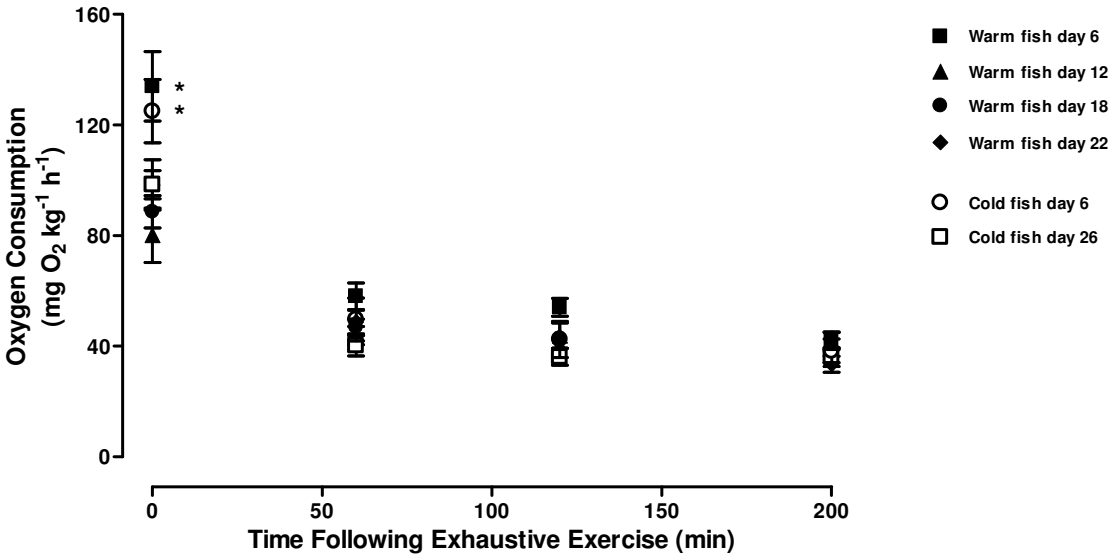


Fig. 3.4. Oxygen consumption (MO_2) of cold acclimating (-1 ℃; open symbols) and warm acclimating (4 ℃; closed symbols) *P. borchgrevinki* during 200 minutes of recovery from exhaustive exercise. 'Day' is the number of days at the acclimation temperature.
In both acclimation groups, the rate at 0 minutes was significantly higher than the resting rate. However, after 60 minutes of recovery the oxygen consumption rate had decreased and was not significantly different from the resting rate. * Significantly different from the 0 minute rate of the other groups. There was no significant difference in the 60, 120 or 200 minute rates of either acclimation group throughout the acclimation period.

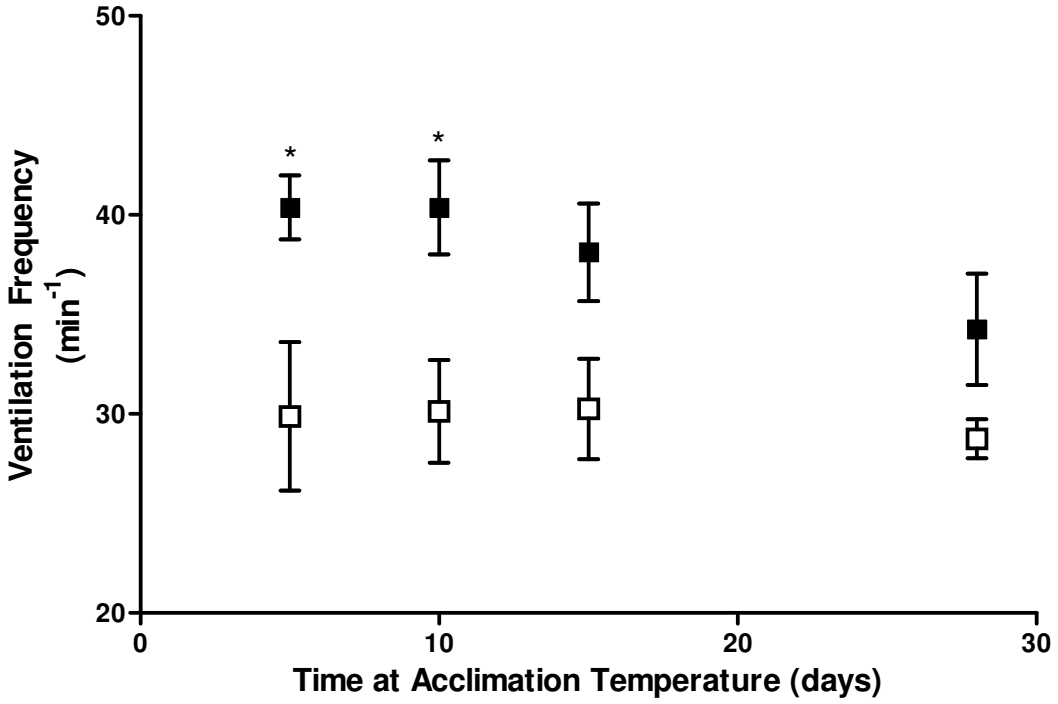


Fig. 3.5. The ventilation frequency of *P. borchgrevinki* during four weeks acclimation to -1 °C (□, cold acclimating fish) and 4 °C (■, warm acclimating fish). * Significantly different from the ventilation frequency of cold acclimating fish. There was no significant difference in the ventilation frequency of cold acclimating fish on any day during the acclimation period. Because of the high inter-individual variation, there was no significant difference between the ventilation frequency of warm-acclimating fish on any day during the acclimation period.

Discussion

ATP production which meets the demands of metabolising animal tissues is essential for the continued survival and unimpaired function of an organism (Brett and Groves 1979). Production relies on the provision of oxygen to the body cells. For this reason, metabolic rate and oxygen consumption are tightly controlled by organisms. In fish, oxygen supply to the tissues is provided by the combined processes of branchial gas exchange and blood gas transport. These processes are regulated by fish to ensure that oxygen delivery matches metabolic demand (Perry and Wood 1989). In *P. borchgrevinki*, the oxygen uptake is almost entirely by the gills (Davison 2001), even when gill function has been impaired by x-cell gill disease (Davison et al. 1990). In other notothenioid species, especially the channichthyids, the skin may act as an important site for oxygen uptake (Hemmingsen and Douglas 1977).

The metabolic rate, and therefore the rate of oxygen delivery required by the tissues is variable. There are considerable differences in the metabolic rate of teleost orders, and also between species within a single order (Clarke and Johnston 1999). The lifestyle of a species influences its metabolic rate so there are differences in the metabolic rate of organisms with pelagic or demersal habitats, between species with different feeding strategies and between species with different locomotory ability (Somero 1991). Usually, more active species have higher resting metabolic rates than more sedentary species. For example, the resting oxygen consumption rate of the active Antarctic species *Trematomus hansoni* is higher than that of the sedentary benthic species *T. nicolai* (Wells 1987). This difference in metabolic rate probably results from variations in the relative proportions of body organs associated with more or less active lifestyles. Different organs have different inherent mass specific demands for oxygen (Clarke and Johnston 1999). There is a decline in oxygen consumption rates with increased depth of occurrence in Antarctic fish. The rate of decline exceeds

that expected as a result of temperature change with depth, as polar oceans show little vertical thermostratification (Torres and Somero 1988a; b). Antarctic species with habitats at 1000 m are likely to have a metabolic rate two orders of magnitude lower than that of species with surface habitats (Torres and Somero 1988a).

Metabolic rates within a species are mass specific. Large fish generally have higher metabolic rates than small fish, for example, the rested oxygen consumption rate of the Antarctic species *Pogonophryne scotti* increases as weight of the fish increases (Saint-Paul et al. 1988). However, on a unit-weight basis, small fish have greater oxygen requirements than large fish of the same species (Jobling 1993). Many other factors also influence metabolic rates of individuals including the activity level, state of stress, feeding state, season, age, reproductive status, thermal history and so on (Schmidt-Nielsen 1975). For all species, and particularly the thermally sensitive Antarctic species, temperature is of critical importance in determining the metabolic rate. This is especially so in fish because branchial respiration causes the body temperature to closely follow the temperature of the external medium (Bouchard and Guderley 2003).

An acute change in temperature results in changes to the rate of biological processes in ectothermic animals (Peck 2002). Raised temperature raises the metabolic rate. The underlying mechanisms which contribute to the increased metabolic rate have been discussed by Clarke and Fraser (2004). Research by several authors (see Clarke and Fraser 2004 for review) suggests that metabolic rate changes are driven directly by changes in the kinetic energy of the cell, so that the higher kinetic energy of cellular molecules at higher temperatures leads directly to a change in metabolic rate. This is termed the Universal Temperature Dependence of metabolism (UTD). However, Clarke and Fraser (2004) suggest that purely temperature-driven changes in the rate of ATP production would be needlessly wasteful of cellular resources. They suggest instead that increased metabolic rates at elevated temperatures result from increased rates of protein

turnover and increased passive leakage of protons across the inner mitochondrial membrane.

To fuel increased cellular activity, extra oxygen supply is required (Taylor et al. 1997). As a result, acute exposure to raised temperatures causes an initial increase in the oxygen consumption rates of many organisms; although metabolic rate can become independent of temperature in the mid-part of an organism's temperature range (Jobling 1993). For example in the South American fish *Prochilodus scrofa* a 10 °C rise in temperature causes a four fold increase in metabolic rate (Barrionuevo and Fernandas 1998), the giant freshwater prawn *Macrobrachium rosenbergii* also shows an increase in oxygen consumption rate on exposure to elevated temperatures (Manush et al. 2004), and the rested oxygen consumption rate of *P. borchgrevinki* nearly doubles on exposure to 6 °C (Lowe and Davison 2006). Prolonged elevation of resting metabolic rate is detrimental to an organism because it potentially reduces the aerobic scope for activity, if there is no associated increase in maximum oxygen consumption rate. The scope for activity is defined as the difference between an organism's maximum metabolic rate and its resting metabolic rate, when these are measured under the same set of environmental conditions. This measurement is important because it reflects the breadth of behavioural and physiological options available to an ectothermic animal in a given environment (Fry 1947; Lowe 2004). Maintenance of a high resting metabolic rate also takes energy away from essential processes such as growth and reproduction, and extra stress is placed on the heart as heart rate and cardiac output usually increase to maintain oxygen delivery to the tissues (Jensen 1993). The increased requirement for oxygen during exposure to elevated temperature is also problematic when organisms reach their thermal limits (either upper or lower). At these limits, organisms struggle to provide oxygen to the tissues at a rate that is sufficient to fuel the cellular processes. Anaerobic metabolism takes over, but most organisms cannot support life in this manner indefinitely (Taylor et al. 1997, Peck 2002).

Many organisms are responsive to the effects of temperature change and attempt to maintain homeostasis of metabolic rate through acclimatory changes (Johnston and Dunn 1987; Clarke 1991). Acclimatory changes occur at a range of levels of biological organization and involve a variety of physiological systems, thus the time-courses of these changes are variable. Changes to the metabolic rate are likely to occur early in the acclimation period, because the supply of ATP affects all other essential processes. Early acclimation of ATP producing systems occurs in the rainbow trout *Oncorhynchus mykiss* (Bouchard and Guderley 2003). Acclimation of the metabolic rate of *P. scrofa* to a 10 °C rise in temperature also occurs relatively rapidly, within two to three days. This species migrates along rivers prior to reproduction and experiences regular, rapid changes in water temperature (including heated discharge from electrical power stations), so rapid acclimation ability is ecologically beneficial (Barrionuevo and Fernandas 1998). In contrast, organisms living in thermostable environments, such as polar seas, are thought to possess limited or no acclimatory ability (Johnston and Temple 2002; Zakhartsev et al. 2003; Tullis and Baillie 2005).

Oxygen consumption at rest – time-course of acclimation

The standard metabolic rate (SMR) is the metabolic rate of a quiet, inactive, non-stressed and post-absorptive ectothermic animal (the equivalent rate in an endothermic animal is termed the basal metabolic rate, BMR). The standard metabolic rate of an ectothermic organism can be measured as the oxygen consumption rate of a post-absorptive animal in the complete absence of activity. A large proportion of this standard metabolic rate is made up of energy requirements for protein synthesis and to maintain ionic gradients across membranes (Clarke and Fraser 2004). Measuring the standard metabolic rate provides a good indication of the maintenance energy requirements of the animal, but is very difficult to measure in pelagic fish, which typically make

spontaneous movements during measurements of oxygen consumption (Cech 1990). This is the case for *P. borchgrevinki* where spontaneous movement is commonplace during measurements of oxygen consumption. In this species, measurement of resting (or routine) metabolic rate is more accurate. This is the oxygen consumption rate of a post-absorptive animal, making spontaneous movements (Brett and Groves 1979; Johnston and Dunn 1987; Cech 1990; Clarke and Fraser 2004). Antarctic marine ectotherms characteristically possess low resting metabolic rates and low Q_{10} values (within the natural temperature range) compared with temperate or tropical marine ectotherms. When temperatures rise beyond the normal range, Q_{10} values are elevated (Peck 2002). A typical tropical fish at 30 °C has a resting oxygen consumption rate that is about six times higher than that of polar fish at 0 °C, and so must also find about six times more food to fuel the resting metabolism. It has been suggested that the major factors responsible for this lowered metabolic rate of Antarctic ectotherms are a reduction in protein turnover, and differences in cellular ion balance and membrane homeostasis (Clarke and Johnston 1999).

Resting oxygen consumption rates obtained in the current experiments compare well with previous findings. The oxygen consumption rate of cold acclimated fish was very stable during the four week acclimation period, close to 35 mg O_2 kg^{-1} h^{-1} (for fish with a mean weight of 106.8 ± 4.2 g). This is slightly higher than results obtained by Wilson et al. (2002) of around 27 mg O_2 kg^{-1} h^{-1}, but close to values obtained by Forster et al. (1987) of around 39.6 ml O_2 kg^{-1} h^{-1}, and also those of Lowe and Davison (2006) of about 33 mg O_2 kg^{-1} h^{-1}, for 0 °C acclimated fish. It is lower than early results obtained by Wells (1987) of around 48 mg O_2 kg^{-1} h^{-1}, which were used as evidence for metabolic cold adaptation in Antarctic fish. The stability of the oxygen consumption rate throughout the acclimation period in cold acclimating fish, suggests that lack of feeding and confinement in the aquarium system did not significantly affect the oxygen consumption rate of the fish. Oxygen consumption rates showed a reasonably high level of inter-individual variation which presumably occurred as a result of the effects of

spontaneous activity. While most fish spent the duration of the measurement period resting on the pectoral fins on the bottom of the respirometer, some fish had a tendency for small bouts of spontaneous movement. This problem has previously been recorded for the active *P. borchgrevinki* (Forster et al. 1987).

Following feeding, there is an increase in the metabolic rate known as the Specific Dynamic Action (SDA) of feeding, or the Heat Increment (HI) or Dietary Induced Thermogenesis (DIT). The increased metabolic rate fuels the increased energy requirements for digestion, absorption and storage of nutrients, as well as for deamination of amino acids and synthesis of excretory products (Jobling 1993). Protein synthesis has also been suggested as a major component of the SDA (Wieser 1994). Following feeding, the SDA is evident as a steep increase in oxygen consumption, which peaks at about two to three times the resting levels within a few hours. Following this peak, oxygen consumption rates gradually return to pre-feeding levels. The duration of the SDA is variable and depends on the species, the size of the organism, the temperature and the meal size (Jobling 1993; Boyce and Clarke 1997; Boyce et al. 2000). The SDA has been reported to last for up to two weeks in Antarctic fish (Wells 1987; Davison et al. 1990; Johnston and Battram 1993; Boyce and Clarke 1997; Boyce et al. 2000). The long duration of the SDA has been suggested to result from a lack of cold-adapted digestive enzymes in these fish (Boyce et al. 2000). Stable oxygen consumption rates in the present experiments, which are in agreement with measurements made by other authors, suggests that there was no significant influence of the Specific Dynamic Action (SDA) of feeding on these fish.

Acute exposure to 4 °C resulted in the expected elevation of oxygen consumption rate in warm acclimating *P. borchgrevinki*, indicating a raised metabolic rate. In the first five days of exposure to 4 °C, the resting oxygen consumption rate of these fish was more than 20 mg O_2 kg^{-1} h^{-1} higher than that of cold acclimating fish (Q_{10} = 3.35). The raised metabolic rate of *P. borchgrevinki* on exposure to acute temperature increase has been shown by

other authors. The resting oxygen consumption rate of this fish was shown to increase by more than 250 % between -1 and 4 °C (Wilson et al. 2002), and increase significantly between 3 and 6 °C (Lowe and Davison 2006). However, in contrast to the hypothesis of the 'Thermal Specialisation Paradigm' (that these fish should have limited acclimatory ability), *P. borchgrevinki* demonstrated an ability to compensate for the temperature change, and decreased the oxygen consumption rate to equal that of cold acclimating fish. The fish demonstrated perfect compensation for temperature change according to the classification system of Precht et al. (1973). Major acclimation changes took place between day one and day nine when the steepest reduction in oxygen consumption rates was evident. However, some acclimatory adjustments were still occurring between day nine and day 25, as a trend for decreasing oxygen consumption rate continued over this period, but at a slower rate than the change over the first nine days. There was a slight increase in oxygen consumption rate on day 13, but this represents a period when air temperatures at Scott Base were unseasonably warm, and extra cold water changes were required to maintain water temperature at 4 ± 0.3 °C. This may have resulted in stress-related elevation in metabolic rate of these fish. It is clear that the decline in oxygen consumption rate did not result from the lack of feeding in these fish as cold acclimated fish did not demonstrate a decline in oxygen consumption rates while kept under the same non-feeding conditions. Perfect compensation in the rate of oxygen consumption suggests that these fish would not suffer the deleterious effects of elevated temperature associated with prolonged elevation of metabolic rate, including raised maintenance metabolic requirements and concomitant narrowing of the aerobic scope. It also suggests that the fish are not struggling to meet oxygen consumption requirements through time-limited anaerobic pathways.

Oxygen consumption following exhaustive exercise – time-course of acclimation

Measurement of the active metabolic rate of fish can be made using oxygen consumption measurements at the maximum sustainable swimming speed ($MO_{2\ max}$). Active metabolic rates vary depending on a species' lifestyle. Among temperate species, there can be a five-fold difference in the active metabolic rate of active and sluggish species (Johnston and Dunn 1987). Measurement of active metabolic rate also provides the information necessary for a calculation of absolute aerobic scope (active metabolic rate – resting metabolic rate) and factorial aerobic scope (active metabolic rate / resting metabolic rate) (Newell 1973; Johnston and Dunn 1987; Cech 1990). Swimming is energy-demanding, so the $MO_{2\ max}$ is usually significantly higher than the $MO_{2\ rest}$, often 10 to 15 times higher (Jobling 1993). Oxygen consumption rates rise to meet the increased requirements for aerobic ATP production during swimming. In these experiments, the maximum rate of oxygen consumption (and thus aerobic scope) could not be specifically measured, as the volume of water within the swimming tunnel was too great for swimming oxygen consumption measurements on a single fish. Consequently, fish were transferred to respirometers after reaching the point of exhaustive swimming, and oxygen consumption measurements were made very quickly following transfer. These measurements are therefore referred to as oxygen consumption post-exercise ($MO_{2\ post\text{-}exercise}$). Such measurements on *P. borchgrevinki* have been shown to be very close to the $MO_{2\ max}$ (Lowe and Davison 2006), so comparison with the $MO_{2\ max}$ data from other authors is possible, as is a very close approximation of aerobic scope.

In both warm acclimating and cold acclimating fish, exhaustive exercise caused an increase in the oxygen consumption rate. At the beginning of the acclimation period, the $MO_{2\ post\text{-}exercise}$ of cold acclimating fish was about three times greater than the $MO_{2\ rest}$ (factorial aerobic scope about 3.88). The factorial aerobic scope of cold acclimating fish was significantly higher than that of warm acclimating fish

(2.41) at the beginning of the acclimation period. Acutely increased temperature, between 3 and 6 ℃, has previously been found to cause a reduction in the aerobic scope of *P. borchgrevinki* (Lowe and Davison 2006). The reduction in the aerobic scope of warm acclimating fish occurred because at this early stage of the acclimation period, the resting oxygen consumption rate of these fish was high. This highlights the potential problem of the raised resting metabolic rate in warm acclimating fish - the elevated $MO_{2\ rest}$, without a change to the $MO_{2\ post\text{-}exercise}$, caused the narrowing of the aerobic scope in these fish, prior to the acclimation changes in resting MO_2. After these changes, at the end of the acclimation period, there was no significant difference between the aerobic scope of cold acclimated fish (3.54 ± 0.34) and warm acclimated fish (2.88 ± 0.32).

In both acclimation groups, the $MO_{2\ post\text{-}exercise}$ measured at the end of the acclimation period was lower than the $MO_{2\ post\text{-}exercise}$ measured at the start of the acclimation period (although this difference was not significant). This reduction in the $MO_{2\ post\text{-}exercise}$ may have occurred as a result of the lack of feeding, or because captive *P. borchgrevinki* tend to rest on the bottom of the aquarium tanks, in contrast to the constant swimming lifestyle of the wild fish, constituting a detraining effect. The $MO_{2\ post\text{-}exercise}$ obtained in these experiments (cold fish 125.0 mg O_2 kg^{-1} h^{-1}; warm fish 133.9 mg O_2 kg^{-1} h^{-1}) were lower than those measured by Lowe and Davison (2006) (221.5 mg O_2 kg^{-1} h^{-1}). Measurements were also slightly lower than those of Forster et al. (1987) (155 .6 mg O_2 kg^{-1} h^{-1}), although this can be partially accounted for because Forster et al. measured the $VO_{2\ max}$ (while the fish were swimming), rather than $MO_{2\ post\text{-}exercise}$ measured in these experiments (measured immediately after the fish had finished swimming).

There was no significant difference between the $MO_{2\ post\text{-}exercise}$ of warm and cold acclimating fish at the start of the acclimation period, or between these groups at the end of the acclimation period, indicating that the $MO_{2\ post\text{-}exercise}$ is less thermally sensitive than the resting oxygen consumption rate. A similar pattern of thermal insensitivity of exercising metabolic rate has been found in the Nile

tilapia, *Oreochromis nilotica* (McKenzie et al. 1996). However, during the middle of the acclimation period, there was a significant decline in the $MO_{2\ post\text{-}exercise}$ of warm acclimating *P. borchgrevinki*. This drop in the $MO_{2\ post\text{-}exercise}$ of warm-acclimating fish corresponded to the period when there were major changes occurring within the resting oxygen consumption system, so perhaps undergoing these changes involved a trade off in maximum oxygen consumption rate and thus aerobic scope.

Oxygen consumption after exhaustive exercise – end of the acclimation period

The relationship between temperature and maximum oxygen consumption rate is complex. Simply raising the temperature of cold acclimated fish to 4 °C did not result in an elevation of $MO_{2\ post\text{-}exercise}$, which might be expected to occur as a result of simple Q_{10} effects. Clearly this upper limit is governed by the interplay between numerous factors, not just temperature. Temperature is important however, as lowering the temperature of warm acclimated fish to -1 °C did result in a significantly reduced $MO_{2\ post\text{-}exercise}$ compared with the $MO_{2\ post\text{-}exercise}$ at 4 °C (Q_{10} = 2.9). This also demonstrates that warm fish had acclimated their aerobic performance to 4 °C. At the end of the acclimation period, the aerobic scope of warm acclimated fish was greatest at 4 °C, and was reduced at the natural environmental temperature of -1 °C. Fish had made acclimatory adjustments which enabled them to maintain their performance at the new temperature, thereby impairing their performance at -1 °C.

Oxygen consumption during recovery from exhaustive exercise

Following exhaustive exercise, the oxygen consumption rates of fish remain elevated for variable lengths of time. This raised metabolic rate results from the energy requirement for restoration of metabolic and cellular homeostasis after the changes caused by exercise, for example the restoration of ion and fluid

volume shifts (Franklin et al. 1996). In some species, the highest rate of oxygen consumption ($MO_{2\,max}$) can be recorded during recovery from exhaustive exercise. This is the case for example in Atlantic cod, *Gadus morhua* and Greenland cod, *G. ogas* (Bushnell et al. 1994). However, in *P. borchgrevinki*, anaerobic pathways are reduced and exhaustive swimming only results in a moderate accumulation of lactate (Davison et al. 1988) and a small oxygen debt, which is repaid rapidly. In this species, oxygen consumption levels usually recover within an hour following exhaustive exercise (Forster et al. 1987; Lowe and Davison 2006). Rapid recovery from exercise has also been reported in other species such as herring larvae, *Clupea harengus* (Franklin et al. 1996) and the Antarctic eelpout *P. brachycephalum* (Hardewig et al. 1998).

Rapid recovery of oxygen consumption rate following exhaustive exercise was evident in the results of these experiments. *P. borchgrevinki* showed an initial steep decline in oxygen consumption rate during the first 60 minutes following exhaustive exercise. In the following 140 minutes, a trend for decreasing oxygen consumption rate was still apparent, but was not as pronounced as in the first 60 minutes. After 200 minutes, oxygen consumption rates were very similar to rates before exercise. Neither the acclimation temperature, or the time spent at the acclimation temperature had any effect on this pattern. A thermal independence of recovery of oxygen consumption rate following exhaustive exercise has been previously reported in herring larvae, *C. harengus*, raised at 5 and 12 °C (Franklin et al. 1996). However, in the Nile tilapia, *O. nilotica*, the energetic costs associated with recovery from exhaustive exercise were increased at elevated environmental temperatures (McKenzie et al. 1996).

Resting ventilation frequency – time-course of acclimation

Oxygen for supply to the tissues must first be extracted from the medium, so changes in demand for oxygen at the tissues result in changes to the ventilation pattern. In fish, receptors monitor changes in arterial blood oxygen content and

regulate ventilation in response to these changes. Generally, requirements for increased oxygen supply are met by an increase in ventilatory stroke volume, and small changes in ventilation frequency. This provides energetic advantages because the costs of pumping the viscous water medium across the gills is great (Perry and Wood 1989). In these experiments, the ventilation frequency (frequency of opercular movements; Cech 1990) was measured during the four week acclimation period. Ventilation frequency has been shown to be thermally sensitive in *P. borchgrevinki.* Wilson et al. (2002) demonstrated an acute increase in maximum ventilation frequency of more than 250 % in *P. borchgrevinki* between -1 and 8 °C. Ventilation frequency was also thermally sensitive in the notothenioid fish *Lepidonotothen nudifrons* (Pörtner et al. 2000). However, Lowe and Davison (2006) found the ventilation frequency of resting *P. borchgrevinki* to be thermally independent between 0 and 6 °C. Opercular movement was reportedly deeper at 6 °C, but ventilation stroke volume was not measured. Changes in ventilation amplitude are also more common than changes in ventilation frequency in *P. borchgrevinki* in response to hypoxia (Wells 1987).

The ventilation frequency of cold acclimated *P. borchgrevinki* in this experiment was around 29 min^{-1}. This is lower than rates recorded for this species by Forster et al. (1987) of around 44 min^{-1}, but closer to those recorded by Wells (1987) of around 25 min^{-1}. It is slightly higher than results recorded by Wilson et al. (2002) of about 21 min^{-1}; and Lowe and Davison (2006) of about 19 min^{-1}. The stability of resting ventilation frequency of cold acclimated fish throughout the acclimation period suggests that there was no significant change to the ventilation frequency caused by lack of feeding or confinement stress. However, individual variation in ventilation frequency was large. This may occur as a result of differences in the response of individuals to confinement, or, because fish began the acclimation period in different digestive states, some having recently fed, others being post-absorptive. The individual variation in ventilation frequency was reduced by the end of the acclimation period in cold acclimated

fish, suggesting perhaps that all fish had reached a common, post-absorptive state. Ventilation frequency was thermally sensitive and showed an immediate increase on exposure to 4 °C. However, in a similar pattern to that described in $MO_{2\ rest}$, after day 10, the ventilation frequency of these fish began to fall, and on day 15, was not significantly different from the rate of cold acclimated fish. This pattern is intuitive, as around this period of acclimation, resting oxygen consumption rate had also fallen, making elevated ventilation frequency unnecessarily wasteful of energy. Compensatory changes acting on the oxygen consumption system, also clearly involved changes to the branchial exchange of oxygen.

Summary

These results agree with the findings of Seebacher et al. (2005), that the 'Thermal Specialisation Paradigm' needs to be re-evaluated. According to the paradigm, the Antarctic notothenioid fish *P. borchgrevinki*, inhabiting a thermally stable environment, should possess very limited ability to acclimate to changes in temperature. However, in contrast to this idea, this species exhibited an ability to regulate oxygen consumption in response to changes in external temperature, and demonstrated 'perfect compensation' as defined by Precht et al. (1973). Acclimatory changes were most evident in the rates of resting oxygen consumption and ventilation frequency. Acclimation changes acted to reverse the temperature induced rate elevation of these processes within a time-course of around seven to 10 days. The reduction in the resting metabolic rate of these fish is critical to maintenance of aerobic performance, and therefore survival, at elevated temperatures, because increased resting metabolic rate would otherwise result in a reduction in aerobic scope. Acclimatory changes to the rate of oxygen consumption ensure that oxygen supply to the tissues is preserved. In turn, this ensures sufficient energy provision for other essential life processes, such as a maintenance of sustained swimming ability, which is the subject of the next chapter.

Chapter Four

The effect of warm acclimation on the prolonged swimming ability of *Pagothenia borchgrevinki*

Introduction

In many species of fish and some other aquatic organisms, survival and Darwinian fitness are correlated with swimming performance. Swimming performance influences success in several essential fish activities, including escape from predators, prey capture, reproductive behaviour, larval selection of settlement habitats, adult habitat selection, and migration (Archer and Johnston 1989; Plaut 2001; Johnston and Temple 2002; Fisher et al. 2005). For *Pagothenia borchgrevinki*, swimming ability is particularly important for the capture of actively moving prey, and escape from predators such as Emperor penguins and Weddell seals (Davis et al. 1999; Ponganis et al. 2000; Franklin et al. 2003). Accordingly, the ability to maintain locomotor performance despite environmental temperature change is crucial if *P. borchgrevinki* is to cope with climatic warming.

During their daily activities, fish utilise several modes of swimming which are broadly categorised as burst swimming (or fast starts), sustained swimming and prolonged swimming (Beamish 1978). Burst swimming is maintained for less than 20 seconds and is fuelled by anaerobic pathways (Gibb and Dickson 2002; O'Steen and Bennett 2003). This type of swimming is mainly utilised during predator-prey interactions (Wakeling and Johnston 1998). Sustained swimming is swimming that is maintained for longer than 200 minutes, which does not end in fatigue (Beamish 1978). This type of swimming is fuelled by the aerobic

production of ATP (Gibb and Dickson 2002). Prolonged swimming is maintained for less than 200 minutes and usually ends in fatigue (Beamish 1978). ATP is provided firstly by aerobic pathways and then, closer to the point of fatigue, anaerobic pathways. Prolonged swimming speed is usually slightly higher than sustained swimming speed, and both are much lower than burst swimming speed (Deegan et al. 2005). Prolonged swimming ability is measured in the laboratory as critical swimming speed (Ucrit) by swimming fish in a swimming flume and incrementally increasing water velocity until fish become fatigued (Beamish 1978).

The effects of acute temperature change on swimming performance are species-specific and also vary according to the type of swimming performance investigated. Often, burst swimming performance is found to be relatively insensitive to temperature change, for example, in the Antarctic notothenioids *P. borchgrevinki, Trematomus bernacchii,* and *T. centronotus* (Wilson et al. 2001), and in 5 °C acclimated short-horn sculpin, *Myoxocephalus scorpius* (Beddow et al. 1995). However, the burst swimming performance of the sub-Antarctic notothenioid *Eleginops maclovinus* was found to be temperature dependent between 2 and 10 °C (Fernández et al. 2002), and the fast-start performance of rainbow trout (*Oncorhynchus mykiss*) was found to be temperature sensitive between 5 and 20 °C (Johnson and Bennett 1996). Sustained or prolonged swimming performance is commonly sensitive to temperature change, for example in Atlantic cod, *Gadus morhua* (Castonguay and Cyr 1998), chub mackerel, *Scomber japonicus* (Dickson et al. 2002), flannelmouth sucker, *Catostomus latipinnis* (Ward et al. 2002), rainbow trout, *O. mykiss* (Gamperl et al. 2002) and juvenile European sea bass, *Dicentrarchus labrax* (Claireaux et al. 2006). The difference in the effect of temperature on burst and prolonged (or sustained) swimming is presumed to result because prolonged swimming is much more complex than burst swimming, involving red muscle working aerobically with energy derived from the mitochondria. Therefore prolonged

swimming involves more biochemical steps which may be effected by temperature, and ultimately influence whole animal performance (Davison 2005).

The ability of fish to acclimate their swimming performance to chronic temperature change is also species-specific, and varies with the type of swimming performance (Johnston and Temple 2002; O'Steen and Bennett 2003). For example, in two cyprinid fishes, tinfoil barbs (*Puntius schwanenfeldii*) and river barbels (*Barbus barbus*), sustained swimming speeds were shown to acclimate strongly, while burst swimming speeds did not acclimate to a range of temperatures (O'Steen and Bennett 2003). Male mosquitofish (*Gambusia holbrooki*) (Hammill et al. 2004) and golden trout (*O. mykiss whitei*) (Myrick and Cech 2003) are also able to acclimate their sustained swimming performance, while female mosquitofish (*G. holbrooki*) can acclimate their burst swimming performance (Condon and Wilson 2006).

Most studies on swimming performance in Antarctic fish focus on *P. borchgrevinki* because of its willingness to swim in experimental flumes. In studies of acute temperature change, burst swimming performance of *P. borchgrevinki* has been shown to be thermally insensitive (Wilson et al. 2001). In contrast, sustained and prolonged swimming performance is highly temperature sensitive, with fish possessing a thermal performance breadth between 3 and 5 °C (Lowe 2004; Wilson et al. 2002). The effects of chronic temperature change also vary according to the mode of swimming performance. Acclimation studies have revealed that burst swimming ability does not acclimate after three weeks at 4 °C (Wilson et al. 2001) and the lack of acclimation ability was suggested to result from the species' evolution in the thermally stable Antarctic environment. However, a recent study of prolonged swimming performance (measured as critical swimming speed, Ucrit) demonstrated that *P. borchgrevinki* can acclimate its performance to 4 °C during a four week acclimation period. At the end of the acclimation period, there was no significant difference in the Ucrit of cold (-1 °C) or warm (4 °C) fish when these were swum at their appropriate acclimation

temperature (Seebacher et al. 2005). Following on from the research of Seebacher et al. (2005), the main aims of the current study were to confirm the ability of *P. borchgrevinki* to acclimate its prolonged swimming performance and to determine a time-course for the acclimation changes.

Materials and Methods

These experiments were conducted at the Scott Base Wet Laboratory facility in McMurdo Sound. *P. borchgrevinki* were collected in McMurdo Sound and held at the laboratory as described in chapter two (General Experimental Methods). Experimental fish were randomly selected. Eight fish (mass 106.8 ± 4.2 g, range 92 - 126 g; total length 210.3 ± 4.5 mm, range 192 - 230 mm); were acclimated to -1 °C for 28 days (cold fish), and eight fish (mass 93.3 ± 3.4 g, range 83 - 110 g; total length 188.1 ± 3.7 mm, range 175 - 205 mm) were transferred to 4 °C for 28 days of warm acclimation (warm fish, Group 1). There was no significant difference between the mass or length of either acclimation group. Note, these were the same fish used in the oxygen consumption experiments described in chapter three. Fish were not fed during the experimental period. While lack of feeding has been shown to impair the swimming performance of some species (for example Simpkins et al. 2004, rainbow trout *Oncorhynchus mykiss*), in a previous study, the swimming ability of unfed *P. borchgrevinki* acclimated for one month to -1 °C was not impaired compared with freshly caught fish (Seebacher et al. 2005). As initial measurements seemed to suggest that acclimation changes to swimming performance had taken place early in the acclimation period, a second group of eight fish (Group 2) was transferred to 4 °C for measurement of Ucrit in the first five days of the acclimation period (mass 79.7 ± 6.6 g, range 62.1 – 114.2 g; total length 209 ± 0.7 mm, range 185 - 244 mm).

Prolonged swimming ability – time-course of acclimation

Regular measurements of prolonged swimming ability (measured as critical swimming speed, Ucrit) were made throughout the acclimation period. Fish were swum individually in an 80 L Blazka-style swimming tunnel (Fig. 3.1), (Blazka et al. 1960). The cylindrical tunnel was made from clear perspex with a plastic mesh at each end, and water flow was created using a revolving impeller. Plaut (2001) highlighted the potential artefacts created by Blazka-type swimming tunnels, which may contribute to non-laminar water flow. These included the creation of a uniformly microturbulent environment, blocking effects caused by the position of the fish in the water current and the possibility that the fish may experience wake from its own movement. The design of the swimming tunnel used in these experiments sought to eliminate these artefacts where possible, and Plaut (2001) concluded that while laminar water flow could not be guaranteed with use of a Blazka-type swimming tunnel, the method was still ecologically relevant.

Before each swimming trial, the swimming tunnel was filled with fresh, fully aerated seawater at the appropriate acclimation temperature (-1 °C for cold acclimating fish and 4 °C for warm acclimating fish). The water temperature in the swimming tunnel was monitored throughout the experiment and did not vary by more than 0.3 °C. Fish were initially introduced to the swimming tunnel running at low velocity (14 cm s^{-1}) for a 60 minute settling period. The water velocity was then increased in an incremental fashion (6 cm s^{-1} every 10 minutes) until the fish became exhausted. Following standard protocols, exhaustion was considered to have occurred when a fish could no longer hold its place in the water column, and twice fell back against the rear mesh of the swimming tunnel (Kolok 1999; Hammill et al. 2004). Once a fish was exhausted, swimming time and swim tunnel velocity were recorded for calculation of Ucrit. It has previously been shown that repeated swimming does not produce a training effect in *P. borchgrevinki* (Seebacher et al. 2005).

Prolonged swimming ability at the reverse acclimation temperature

At the end of the 28 day acclimation period prolonged swimming ability of Group 1 (4 ℃) and -1 ℃ fish was measured at the normal acclimation temperature (-1 ℃ for cold fish and 4 ℃ for warm fish) and the reverse acclimation temperature (4 ℃ for cold acclimated fish and -1 ℃ for warm acclimated fish). Apart from the change in water temperature, the same procedure as described above was used for the measurement of Ucrit.

Data analysis and statistical methods

Ucrit was determined using the following equation (Brett 1964):

$$Ucrit = Uf + ([Tf/Ti]Ui)$$

Where:
Uf = the highest speed maintained for the entire time increment
Tf = the time taken to reach exhaustion in the final speed interval
Ti = the time interval length
Ui = the speed increment

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance was taken at the level of $P< 0.05$. All data are presented as the mean ± standard error, unless otherwise stated. Results within acclimation groups were compared using one-way analysis of variance (ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. Results between acclimation groups were compared using repeated measure two-way analysis of variance (ANOVA) (after a Bartlett's Test confirmed homogeneity of variance) and Bonferroni post-hoc

tests. Swimming ability at the acclimation temperature was compared with swimming ability at the reverse acclimation temperature using paired t-tests.

Results

Fish swimming behaviour within the swimming tunnel was very similar to that observed by previous authors (Montgomery and Macdonald 1984; Forster et al. 1987; Wilson et al. 2002). During the low speed familiarisation period fish tended to rest on the pectoral fins at the bottom of the swimming tunnel. As water velocity was increased to near 1 body length second $^{-1}$ (bls^{-1}), fish could no longer rest on the bottom, and maintained station in the tunnel using labriform locomotion powered by the pectoral fins. At higher water velocities, between 1.5 and 1.8 bls^{-1}, labriform locomotion became inadequate for holding position and fish switched to subcarangiform locomotion, involving contraction of the myotomal muscles. Subcarangiform locomotion could only be supported for brief periods and fatigue was reached shortly after this mode of locomotion was employed.

Prolonged swimming ability – time-course of acclimation

There was no significant change in the Ucrit of cold fish throughout the acclimation period ($P>0.05$), mean values were around 1.9 body lengths per second (bls^{-1}) (for example, day 6, 1.96 ± 0.11 bls^{-1}; day 26 1.88 ± 0.06 bls^{-1}). The Ucrit of warm fish (Group 1) was also stable throughout the acclimation period. Mean values of around 2 bls^{-1} were recorded and these were not significantly different from the Ucrit of cold fish ($P>0.05$), (for example, day 6, 2.10 ± 0.07 bls^{-1}; day 26 2.04 ± 0.11 bls^{-1}). Inter-individual variation in Ucrit was high in both cold and warm fish (Fig. 4.1).

Because there was no significant difference in the prolonged swimming ability of cold and warm acclimated fish when the first measurements were made on day six, Ucrit was measured in a second group of fish (Group 2) on day one, three and five of acclimation to 4 ℃ (Fig. 4.2). On day one of the acclimation period, the Ucrit of these fish (1.70 ± 0.09 bls^{-1}) was significantly lower than the Ucrit of warm fish (Group 1) on day six of the acclimation period ($P<0.05$), but not cold fish on day six. After three and five days of acclimation to 4 ℃, the Ucrit was not significantly different from that of Group 1 fish after six days acclimation to 4 or -1 ℃.

Prolonged swimming ability at the reverse acclimation temperature

Cold acclimated fish swum at the reverse acclimation temperature (4 ℃) showed significantly impaired Ucrit (1.56 ± 0.05 bls^{-1}) compared with results obtained at the normal acclimation temperature (1.88 ± 0.06 bls^{-1}, $P=0.009$) and also compared with warm acclimated fish swimming at 4 ℃ (Ucrit = 2.04 ± 0.11 bls^{-1}; $P<0.05$). When warm acclimated fish were swum at the reverse acclimation temperature (-1 ℃), their performance declined (Ucrit -1 ℃ = 1.71 ± 0.11) compared with swimming at 4 ℃ ($P=0.02$). Warm fish also swam slower at -1 ℃ than cold fish at this temperature, however this difference was not statistically significant.

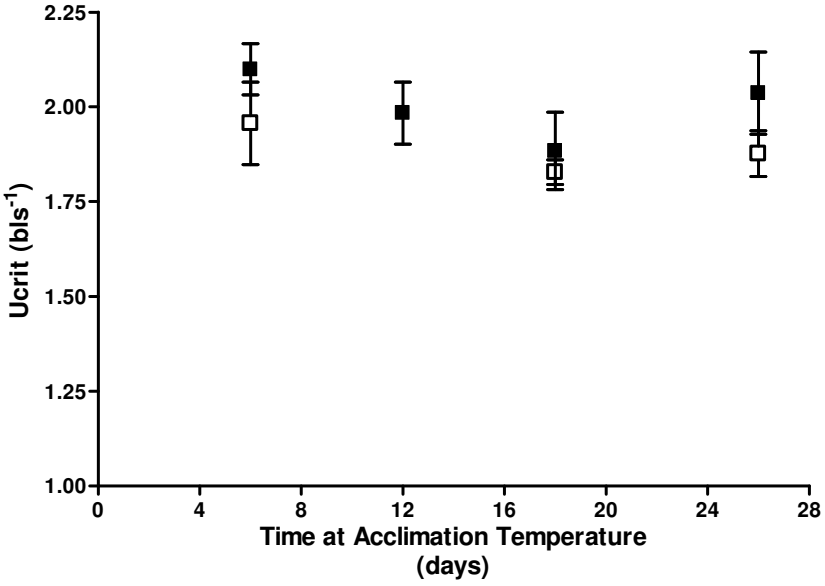


Fig. 4.1. Critical swimming speed (Ucrit) of *Pagothenia borchgrevinki* (Group 1) during 28 days of acclimation to -1 ℃ (□) or 4 ℃ (■). The Ucrit of each acclimation group did not vary significantly during the acclimation period. There was no significant difference between the Ucrit of cold and warm fish on any day measurements were made. Note, no cold fish measurements were made on day 12.

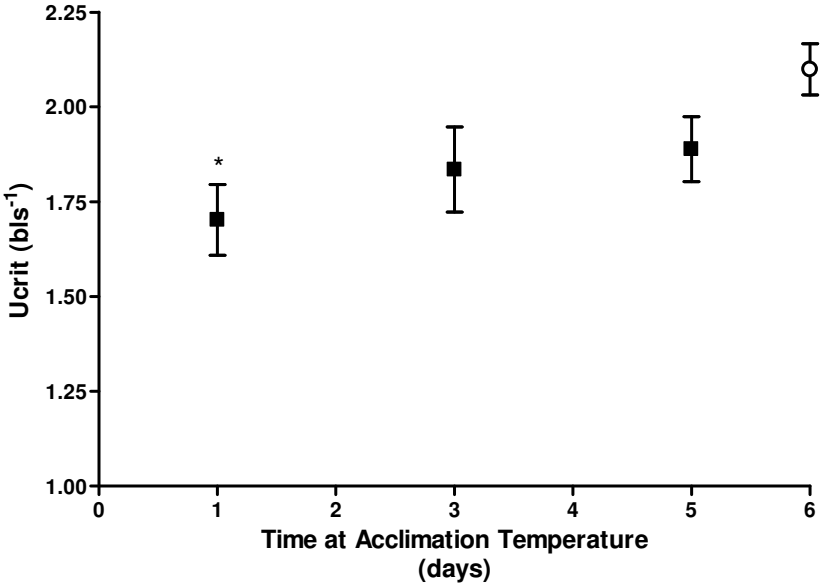


Fig. 4.2. Critical swimming speed (Ucrit) of *P. borchgrevinki* (Group 2), during the first five days of acclimation to 4 ℃. * Significantly different from the Ucrit of 4 ℃ acclimated fish on day 6 of the acclimation period (Fig. 4.1). The Ucrit of Group 1 fish on day six of the acclimation period has also been plotted for comparison (○).

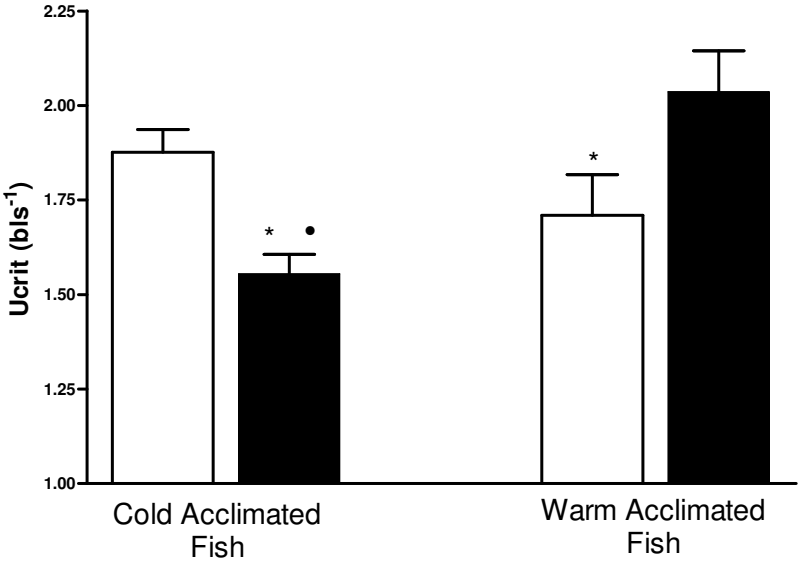


Fig. 4.3. Critical swimming speed (Ucrit) of cold and warm acclimated *P. borchgrevinki* (Group 1), swum at the normal acclimation temperature (cold fish -1 ℃, warm fish 4 ℃) and the reverse acclimation temperature (cold fish 4 ℃, warm fish -1 ℃). □ Swim at -1 ℃. ■ Swim at 4 ℃. * Significantly different from the Ucrit at the normal acclimation temperature. • Significantly different from the Ucrit of warm acclimated fish swum at 4 ℃.

Discussion

Most Antarctic notothenioids are sedentary, and even those species which have radiated to fill niches in the water column may be relatively inactive (Eastman 1993; Davison 2005). Therefore, most research on swimming in notothenioid fish has focused on *P. borchgrevinki,* being one of very few Antarctic species which is able and willing to swim in a swimming flume (Davison 2005). In its natural environment, this negatively buoyant species swims frequently to maintain position in the water column, but also spends some time resting on pieces of platelet ice (Andriashev 1970). Low temperature has a slowing effect on the rates of biological processes and this in turn may be expected to impair the locomotor performance of Antarctic fish. The extremely low body temperature of Antarctic fish would prevent most teleosts from swimming, but, while Antarctic species are not high speed champions, they are capable of locomotion at sub-zero temperatures, indicating that there has been some evolutionary adaptation of swimming performance (Archer and Johnston 1989; Bennett 1990; Franklin and Johnston 1997; Wilson et al. 2001; Franklin et al. 2003). It has been suggested that the relatively low swimming speeds achieved by *P. borchgrevinki* may result from the use of labriform locomotion, rather than being a direct result of low temperature (Tuckey and Davison 2004). The cost of prolonged locomotion in *P. borchgrevinki* is not high, estimates of around 0.0914 ml O_2 kg^{-1} h^{-1}, for a 50 g fish swimming at around 1 bls^{-1} have been reported (Forster et al. 1987). Locomotion costs are moderate due to the streamlined shape and low mass/length ratio of *P. borchgrevinki* (Franklin et al. 2003). It is also possible that the schooling behaviour of this fish may reduce the costs of locomotion for individuals (Beamish 1978), although this has never been tested specifically in this species.

Fish size influences swimming performance, with absolute Ucrit scaling positively with body size. However, when relative swimming speeds are considered (bls^{-1}),

small fish are capable of proportionately greater speeds than large fish (Forster et al. 1987; Kolok 1999; Elsworth et al. 2003). In juvenile fish ontogeny may alter the relationship between fish size and swimming ability, for example, in juvenile green sturgeon, *Acipenser medirostris*, Ucrit increases with size up to a total length of 266 mm, but above 266 mm Ucrit decreases. Decrease in Ucrit above 266 mm reflects underlying muscle changes that occur during development and act to favour burst swimming ability over sustained swimming ability (Allen et al. 2006). Ontogenetic effects on fish swimming speed have also been demonstrated in *Notothenia neglecta*, coral reef fish and fish from the family Cottidae (Archer and Johnston 1989; Temple and Johnston 1998; Green and Fisher 2004). There are also links between fish swimming performance and metabolic rate (Bennett 1990; Cano and Nicieza 2006; Claireaux et al. 2006) and morphology, phylogeny and ecotype (Jordan et al. 2005).

In the current study, cold acclimated fish reached swimming speeds of around 1.9 bls^{-1}, which was marginally lower than speeds reported by Forster et al. (1987) and Wilson et al. (2002), and slightly higher than those reported by Montgomery and Macdonald (1984). There was no significant difference in the Ucrit of cold fish at any time during the acclimation period, confirming that lack of feeding and repeated swimming did not affect swimming performance. Variation of Ucrit between individuals was high, even when relative swimming speeds (incorporating body size) were compared. Inter-individual variation in fish swimming ability is common and has been reported by other authors (for example Lee et al. 2003; Breen et al. 2004; Fisher et al. 2005). Such variation is not considered to result from experimental error, but has been shown to be repeatable and statistically valid (see Kolok 1999 for review).

At low swimming speeds, early in the swimming challenge, *P. borchgrevinki* swum using labriform locomotion, which is common among notothenioid fish. It is assumed that the ancestral notothenioid also employed this mode of locomotion, suggesting that its prevalence is a result of phylogeny rather than an

adaptation to cold Antarctic conditions. *P. borchgrevinki*, with its large fan-shaped pectoral fins, is well suited to labriform swimming; during low-speed swimming the fins are rotated in an oscillatory fashion, so that the fish rows itself through the water (Eastman 1993). To achieve greater speeds while still maintaining labriform locomotion, the pectoral fins are used in a flick-and-glide action (Davison 2005). Labriform swimming is powered by the red muscle fibres, which are small diameter fibres that contain many mitochondria, have a good blood supply and high levels of intracellular myoglobin, ensuring they remain well oxygenated during routine activity. The red muscle fibres of *P. borchgrevinki* are distributed almost exclusively in the pectoral fins, and receive blood from the hypobranchial arteries which run from the gills straight to the fins (Davsion 2005).

Labriform locomotion is suitable for swimming speeds up to about 1.5 to 1.8 bls^{-1}, above which fish switch to subcarangiform locomotion (Davison 2005) which involves undulation of the posterior half of the body (Eastman 1993). Switching to subcarangiform locomotion results in only a minor increase in swimming speed, but rapidly leads to fatigue (Davison 2005). Subcarangiform locomotion is powered by the myotomal trunk muscles which are dominated by white muscle fibres in adult red-blooded notothenioids (Eastman 1993; Davison 2005). White muscle fibres are large, have a poor blood supply and generate ATP through glycogen-fueled anaerobic metabolism (Davison 2005). Because *P. borchgrevinki* is only capable of sustaining a modest oxygen debt during exercise (Forster et al. 1987), the use of white muscle fibres, and hence the duration of subcarangiform locomotion is necessarily short lived.

Changes in environmental variables such as salinity, oxygen concentration, photoperiod and especially temperature influence the swimming performance of fish (Randall and Brauner 1991). For example, acute exposure to salt water reduces Ucrit in freshwater acclimatised salmon parr (*O. mykiss*) and Adriatic sturgeon (*A. naccarii*), and this is attributed to an increase in plasma sodium concentration and extracellular chloride concentration (Brauner et al. 1992;

McKenzie et al. 2001a). However, ontogeny may influence sensitivity to salinity, for example, young-of-the-year *A. naccarii* can compensate its swimming performance to moderate the effects of acutely changed salinity (McKenzie et al. 2001b). Adult European seabass, *Dicentrarchus labrax* can also make compensatory adjustments in swimming performance following salinity changes (Chatelier et al. 2005). In Atlantic cod, *G. morhua*, swimming performance declines in hypoxic conditions (Herbert and Steffensen 2005) and at temperatures below 10 °C the Ucrit of largemouth bass, *Micropterus salmoides* is sensitive to changes in photoperiod (Kolok 1991).

Acute temperature change affects the rates of most biological processes from a cellular to a whole animal level (O'Steen and Bennett 2003), and it is temperature that is arguably the most significant environmental factor to impact fish swimming performance (Beamish 1978). In European sea bass, *D. labrax*, sustained swimming ability is reduced at low temperatures, increases to a maximum at the optimum temperature and then decreases above the optimum temperature (Bennett 1990; Claireaux et al. 2006). This pattern is also evident in other species, although the optimum temperature and performance range differs, and optimum temperatures may also vary depending on the season (Taylor et al. 1996; Özilbigin and Wardle 2002). Loss of locomotor ability at low temperatures is often compensated for by increased bouts of antagonistic or evasive behaviours (Bennett 1990). Reduced swimming ability at high or low temperatures is thought to result from changes in the internal environment, rather than changes to the physical properties of water. The processes which are thought most to impact on changed locomotory performance are differences in molecular kinetics and the rates of biological processes that convert chemical energy into thrust (Randall and Brauner 1991; Fuiman and Batty 1997) as well as impaired oxygen delivery (Randall and Brauner 1991). However, the change in water viscosity associated with changed temperatures does have an impact on larval swimming ability in some species (see for example Fuiman and Batty

1997). Temperature has also been shown to affect the repeat swimming performance of rainbow trout, *O. mykiss* (Jain and Farrell 2003).

In contrast to burst swimming ability (Wilson et al. 2001), acute temperature increase has been shown to significantly impair the prolonged swimming performance of *P. borchgrevinki* (Wilson et al. 2002; Lowe 2004; Seebacher et al. 2005), with fish possessing a thermal performance breadth of between 3 °C (Lowe 2004) and 5 °C (Wilson et al. 2002). Wilson et al. (2002) reported a 17 % decline in Ucrit between -1 and 4 °C, and Lowe (2004) reported a 26 % decline in Ucrit between these temperatures. However, the results of the present study demonstrate that *P. borchgrevinki* is able to acclimate its swimming performance to this 5 °C increase in temperature. Acclimation changes occurred rapidly, with no significant difference in the swimming performance of cold and warm acclimated fish swum at their acclimation temperature on day six of the acclimation period. Major changes appear to have taken place between day one and day three, as the Ucrit of 4 °C fish on day one was significantly lower than the Ucrit of warm fish on day six, but there was no significant difference between the Ucrit of warm acclimated fish on day three and day six. Since swimming performance is significant for the survival of fish (Archer and Johnston 1989; Plaut 2001; Johnston and Temple 2002; Fisher et al. 2005), it makes sense that locomotory performance is a target for early acclimatory changes. However, in some studies, acclimatory changes in locomotory performance have been reported to take several weeks (for example Heap et al. 1986). Swimming fish at the reverse acclimation temperature at the end of the acclimation period confirmed that fish had acclimated to the changed conditions. When swum at the reverse acclimation temperature, fish swimming performance was impaired compared with performance at their normal acclimation temperature, and the performance of the other acclimation group.

The physiological mechanisms that underpin changes in locomotory ability vary between species but may include changes to the relative proportions of different

muscle types and/or their contractile properties, the number and/or surface area of mitochondria, and changes in myofibrillar ATPase activity (Hammill et al. 2004). For example, acclimation changes in goldfish, *Carassius auratus*, and killifish, *Fundulus heteroclitus* include changes to myofibrillar ATPase activity and twitch contraction speed (Johnson and Bennett 1995) and in brown trout, *Salmo trutta*, acclimation induces changes in red muscle fibres and capillaries (Day and Butler 2005). Also, seasonal acclimation to winter conditions results in greater total mitochondrial volume in trout, *O. mykiss* (Egginton et al. 2000).

Summary

Acutely exposing *P. borchgrevinki* to a 5 °C rise in temperature results in impaired locomotor performance. However, given a very short acclimation period, this species has demonstrated a remarkable ability to adjust its swimming performance in response to this change. Fish were able to acclimate their performance to 4 °C, and by acclimating to this new temperature, their performance was impaired at the natural environmental temperature of -1 °C. The ability to acclimate locomotory performance is ecologically significant as swimming ability is linked to essential daily activities including escape from predators and prey capture.

Chapter Five

The effect of warm acclimation on the cardiovascular system of *Pagothenia borchgrevinki*

Introduction

The anatomy of the notothenioid cardiovascular system is similar to that of other teleost fish. The heart has four chambers, which are arranged in series (sinus venosus, atrium, ventricle, bulbus arteriosus) and the heart index (heart weight expressed as percentage of body mass) is in the same range as those of other teleosts (Tota et al. 1991). However, there are a range of specialisations to the physiology of these systems (Davison et al. 1997). These include maintenance of a high cardiac output despite a low heart rate (achieved by a high stroke volume), a low resting haematocrit with the capacity to increase this substantially in times of oxygen demand, low vascular resistance, partly achieved by the low resting haematocrit, and high cholinergic tonus on the heart and spleen in resting conditions (Axelsson et al. 1992; Axelsson et al. 1994; Davison et al. 1997). For example, cholinergic tonus values of 55 % (Axelsson et al. 1992), 50 % (Axelsson et al. 1994) and 46 % (Franklin et al. 2001) have been reported for *Pagothenia borchgrevinki*. Cholinergic tone is even higher in *Trematomus bernacchii,* reaching 80 % at 0 ℃ (Franklin et al. 2001). Therefore, as discussed by Egginton et al. (2006) the major influence on notothenioid heart rate is the degree of vagal tone, with fish varying heart rate by removing the ‘brake’ rather than applying the ‘accelerator’. It is thought that such specialisations represent adaptations to the polar environment, however it is also possible that they may represent characteristics inherited from ancestral notothenioids (Egginton et al. 2006).

The cardiovascular systems of fish are very sensitive to changes in temperature (Driedzic and Gesser 1994; Farrell 1997; Taylor et al. 1996), with an acute rise in temperature usually causing an increase in cardiac output, which most often results from increases in heart rate (De Vera and Priede 1991). The aerobic swimming performance of fish is closely linked with both cardiovascular function and respiratory function, because these systems are responsible for supplying oxygen and metabolites to the working muscles (Franklin et al. 2007). In chapter four, acclimation of aerobic swimming performance in *P. borchgrevinki* at 4 °C was evident. It was expected that these acclimatory changes to swimming performance would be accompanied by changes to the cardiovascular system, which provides oxygen supply to the working muscles. Investigation of these acclimatory changes to the cardiovascular system was the subject of this chapter.

Recent work by Franklin et al. (2007) examined acclimatory changes in the cardiovascular system of *P. borchgrevinki* held for one month at 4 °C and then acutely exposed to temperatures between -1 and 8 °C. In cold acclimated controls (-1 °C for one month) factorial scope for cardiac output was greatest at -1 °C, and decreased as environmental temperature was increased between -1 and 8 °C. This occurred because increased temperature resulted in an increase in resting cardiac output, but no increase in maximal cardiac output. However, when fish were acclimated for one month to 4 °C, factorial scope for cardiac output was shown to be thermally independent between 4 and 8 °C. The aim of the current study was to investigate the thermal plasticity of the cardiovascular system of *P. borchgrevinki* in further detail, and to elucidate the mechanisms of thermal independence of cardiac output in this species. Specifically, the effects of acclimation on heart rate and ventilation rate, and the effect of acclimation on the thermal sensitivity of the cardiovascular system were determined. It is vital to have information on the cardiovascular system because its continued, unimpaired function is critical to an organism's survival (Driedzic and Gesser

1994). The cardiovascular system of *P. borchgrevinki* is known to be particularly sensitive to temperature change (Lowe et al. 2005), so clearly acclimation changes to the cardiovascular system are essential if this species is to survive predicted oceanic warming.

Materials and Methods

These experiments were conducted at the Scott Base Wet Laboratory facility in McMurdo Sound. *P. borchgrevinki* were collected in McMurdo Sound and held at the laboratory as described in chapter two (General Experimental Methods).

Fish were not fed during the experimental period as it has previously been shown that feeding can affect metabolism for up to two weeks (Boyce and Clarke 1997) and that metabolic rate and heart rate are correlated in notothenioids (Campbell et al. 2007).

Acclimation to 4 ºC

Twelve fish were randomly selected for acclimation to 4 ºC (warm acclimated fish). These fish were transferred to two 100 litre static tanks maintained at 4 ± 0.3 ºC. Thirty litres of water were replaced daily in each tank without significant alteration to the tank temperature. Six of the warm acclimated fish were held for 14 days at 4 ºC before experimental use (mass 76.0 ± 9.3 g, range 38 - 93 g; total length 205.5 ± 9.4 mm, range 165 - 227 mm), while the remaining six fish were acclimated for 28 days to 4 ºC (mass 48.6 ± 17.4 g, range 21 - 135 g; total length 168.2 ± 15.4 mm, range 138 - 243 mm). Six control animals (cold acclimated fish) were randomly selected and retained in the flow-through aquarium system (mass 81.0 ± 10.5 g, range 40 - 107 g; total length 201.5 ± 9.5 mm, range 160 - 220 mm). Cold acclimated fish were held for 28 days before

experimental use. There was no significant difference between the mass and length of any group.

Heart rate, ventilation rate and thermal sensitivity of the cardiovascular system

At the end of the acclimation period, fish were individually anaesthetised in 0.1 g l^{-1} MS222 (ethyl *m*-aminobenzoate methanesulphonate) dissolved in seawater. Each fish was then placed in a surgical sling where the gills were continuously irrigated with an aerated solution of 0.05 g l^{-1} MS222. Fish were fitted with two subcutaneous electrocardiogram (e.c.g.) electrode wires positioned ventrally, in close proximity to the location of the heart. Electrodes were secured using one ventral and one dorsal suture. After surgery, fish were placed into a 10 litre experimental tank at their acclimation temperature for a 24 hour recovery period. This period has previously been shown to be adequate for the recovery of cardiovascular variables to resting levels (Lowe et al. 2005). Water quality was ensured by maintaining a constant flow of -1 °C water through the experimental tank (for cold acclimated fish), or regular replacement of three litres of tank water with clean water, pre-heated to 4 °C (for warm acclimated fish). Following the 24 hour recovery period, electrodes were connected to a bioamplifier (ML 136, AD Instruments, Australia) linked to a computerised data acquisition system (AD Instruments, Powerlab). The signal was displayed on a laptop computer and digital filtering was used to separate the raw data into heart (e.c.g.) and ventilation (opercular muscle) signals.

Baseline measurements of resting heart rate and ventilation rate at the acclimation temperature were made over the first 60 minutes. Water temperature within the experimental tank was then changed with the addition of either cold sea water (-1 °C, on tap), or pre-warmed aerated sea water. Each fish was exposed to a series of temperatures; 30 minutes at -1, 2, 4, 6 and 8 °C, in that order, with each experimental temperature separated by a 60 minute recovery

period at the normal acclimation temperature. Lowe et al. (2005) demonstrated that *P. borchgrevinki* heart rate recovered from thermal disturbance during a 60 minute recovery period, and that there was no cumulative effect of exposure to high temperatures. Water was added gently to minimise disturbance to the fish. Stabilisation of tank water at each temperature was monitored and was always complete within two minutes. Measurements of heart and ventilation rate during analysis were made from the time that the new temperature had stabilised within the tank.

Data analysis and statistical methods

Mean heart rate and mean ventilation rate at each temperature for each fish was determined for every three minute block. Variation within acclimation groups was compared using one-way analysis of variance (ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. Variation between acclimation groups was compared using two-way analysis of variance (ANOVA), after a Bartlett's Test confirmed homogeneity of variance. Where a treatment effect was indicated Bonferroni post-hoc tests were carried out. Pearson's correlation analysis and linear regression were used to determine the relationship between heart rate and ventilation rate (cardio-respiratory synchrony) of fish at each temperature. The Q_{10} values for heart and ventilation rates were calculated using the Van't Hoff equation (Hoar 1975) and heart and ventilation rates at each test temperature were compared with rates at the acclimation temperature using one sample t-tests. Analyses were carried out using GraphPad Prism Version 4.00 software for Windows (GraphPad Software, San Diego, USA). Statistical significance was taken at the level of $P<0.05$ and data are presented as mean ± SEM, unless otherwise stated.

Results

Heart rate

Mean resting heart rate in cold acclimated fish was 24.1 ± 1.1 beats per minute (bpm) which was not significantly different from the 26.7 ± 2.3 bpm shown by fish acclimated to 4 ℃.

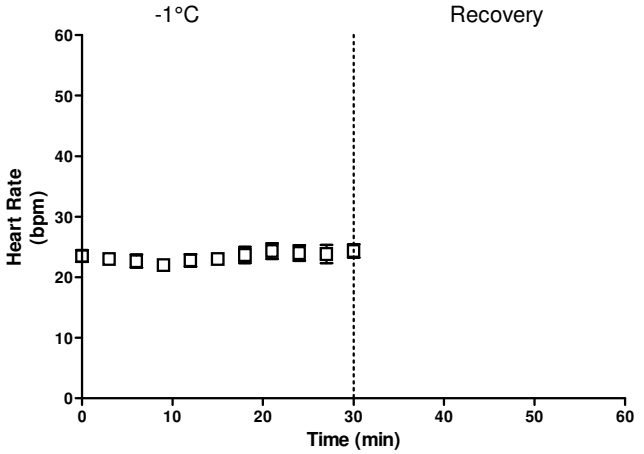
Figures 5.1 to 5.5 show graphs of heart rate in fish exposed to temperatures of -1, 2, 4, 6 and 8 ℃. Simply changing the water, but not the temperature, in the experimental tank had no effect on heart rate. On exposure to any warmer temperature, cold acclimated fish responded by increasing their heart rate (Fig. 5.2a to 5.5a). The increase was not instantaneous, but showed a rise for approximately the first 15 minutes of exposure, with a slight overshoot, falling back to a new steady rate for the last 10 minutes. This new rate was directly related to the exposure temperature, with a Q_{10} of 1.9 over the temperature range used (Fig. 5.6a). On re-exposure to -1 ℃, heart rate in these fish rapidly fell back towards initial values, reaching this within 10 minutes.

Fish that had been acclimated to 4 ℃ for 28 days showed a very different response to any acute change in temperature. Any increase in temperature elicited a rapid significant bradycardia, irrespective of the starting temperature, that lasted up to 10 minutes. Thus, a fish at its acclimation temperature of 4 ℃ taken to 6 or 8 ℃ showed an immediate and significant bradycardia (Fig. 5.4c, 5.5c), while the same animal taken down to a test temperature of -1 or 2 ℃ showed this bradycardia upon return to 4 ℃ (Fig. 5.1c, 5.2c). Any decrease in temperature in these fish tended to produce a small, usually not significant tachycardia (Fig. 5.1c, 5.2c) which was never observed in cold acclimated fish. Following either the tachycardia or bradycardia, heart rate always returned to the initial rate. Data from the last 10 minutes of exposure to any temperature showed that heart rate in these fish was insensitive to temperature (Fig. 5.6c).

Fish exposed to only 14 days at 4 °C looked very similar to fish exposed for 28 days. However, although the tachy- and bradycardia events were present, they were not as developed as seen in the longer exposure fish (Fig. 5.1b to 5.5b). Fourteen day acclimated fish did show a rise in heart rate at 8 °C (Fig. 5.5b) and a small, but not significant rise at 6 °C (Fig. 5.4b), although in neither case did they reach the values shown by the -1 °C acclimated fish (Fig. 5.4a, 5.5a).

Fig. 5.1. Heart rate of *Pagothenia borchgrevinki* during 30 minutes at -1 ℃ and 30 minutes recovery at the acclimation temperature. **a.** Fish acclimated for one month to -1 ℃ (no recovery data is presented for this group as -1 ℃ is the normal acclimation temperature of these fish). **b.** Fish acclimated for two weeks to 4 ℃. **c.** Fish acclimated for one month to 4 ℃. * Significantly different from the heart rate at 0 minutes.

a.



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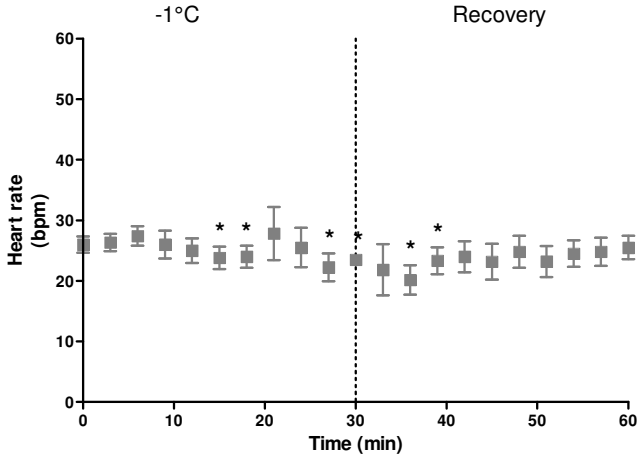

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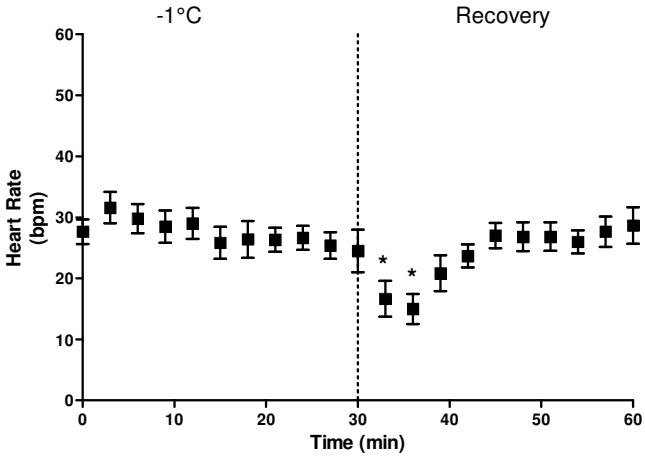
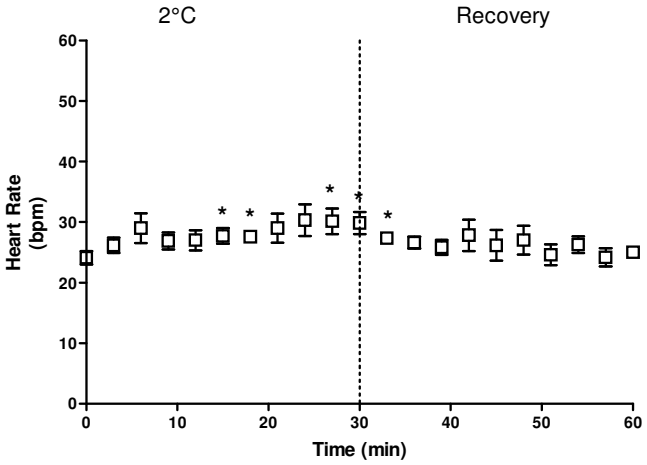


Fig. 5.2. Heart rate of *P. borchgrevinki* during 30 minutes at 2 ℃ and 30 minutes recovery at the acclimation temperature. **a.** Fish acclimated for one month to -1 ℃. **b.** Fish acclimated for two weeks to 4 ℃. **c.** Fish acclimated for one month to 4 ℃. * Significantly different from the heart rate at 0 minutes.

a.



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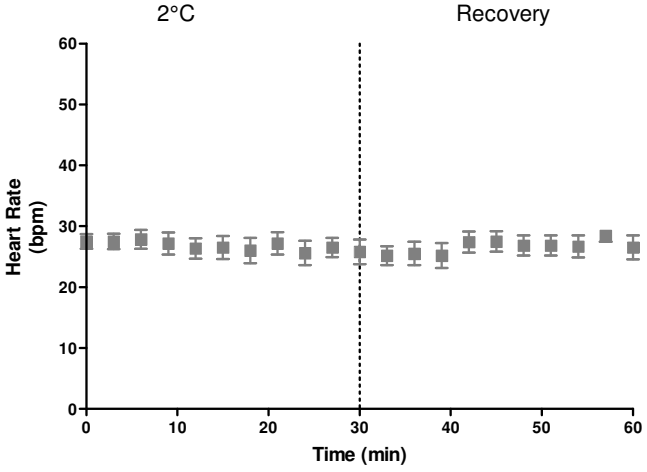

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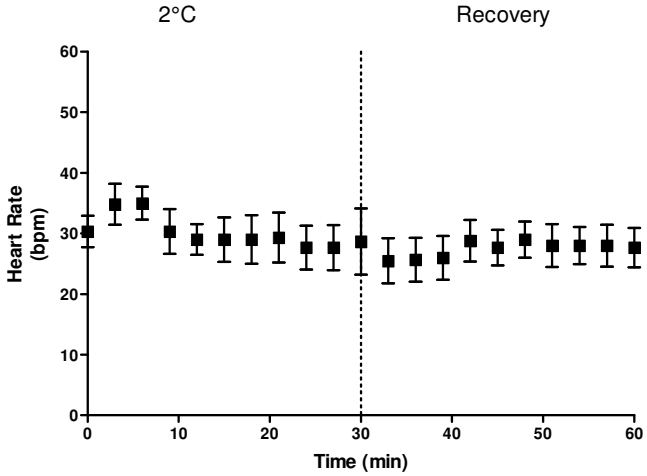
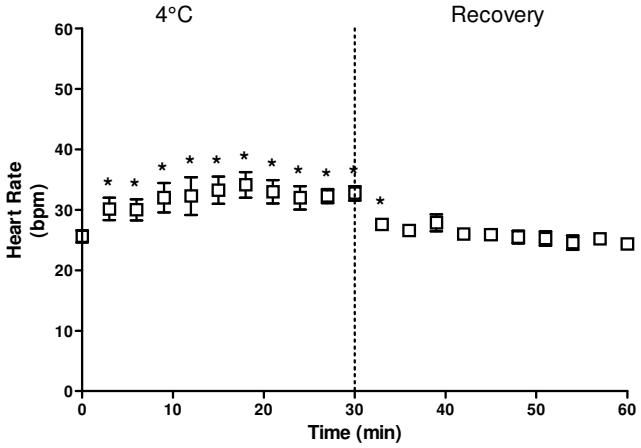


Fig. 5.3. Heart rate of *P. borchgrevinki* during 30 minutes at 4 ℃ and 30 minutes recovery at the acclimation temperature. **a.** Fish acclimated for one month to -1 ℃. **b.** Fish acclimated for two weeks to 4 ℃. **c.** Fish acclimated for one month to 4 ℃. No recovery data is presented for warm acclimated fish as 4 ℃ is the normal acclimation temperature of these groups.* Significantly different from the heart rate at 0 minutes.

a.



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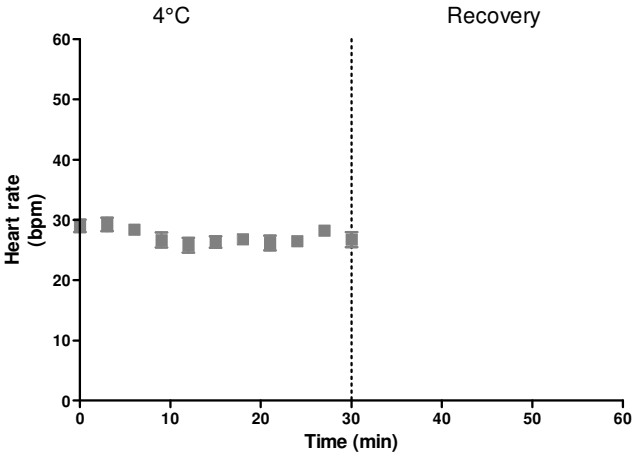

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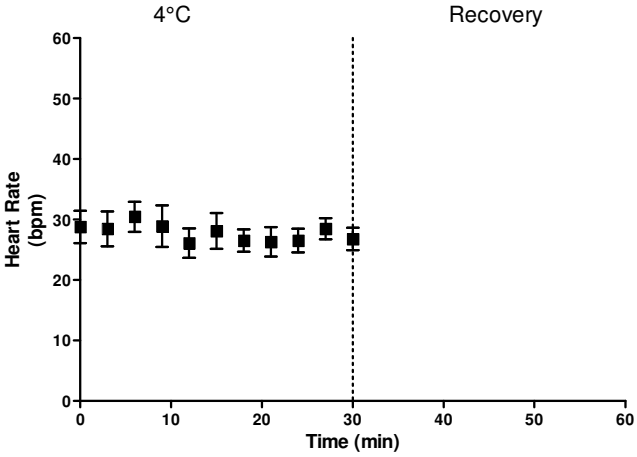
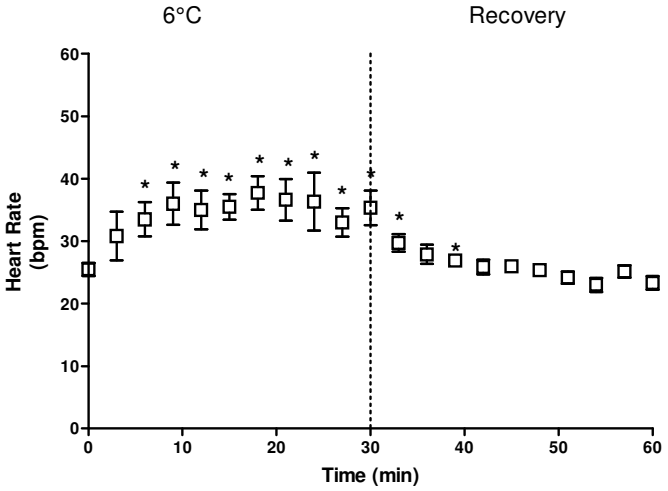


Fig. 5.4. Heart rate of *P. borchgrevinki* during 30 minutes at 6 ℃ and 30 minutes recovery at the acclimation temperature. **a.** Fish acclimated for one month to -1 ℃. **b.** Fish acclimated for two weeks to 4 ℃. **c.** Fish acclimated for one month to 4 ℃.* Significantly different from the heart rate at 0 minutes.

a.



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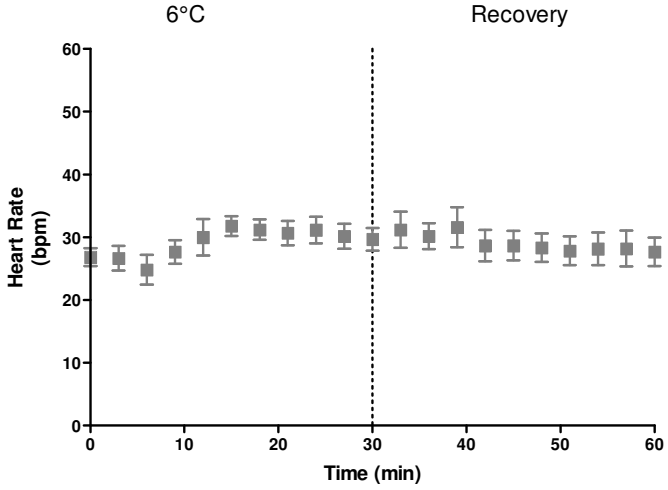

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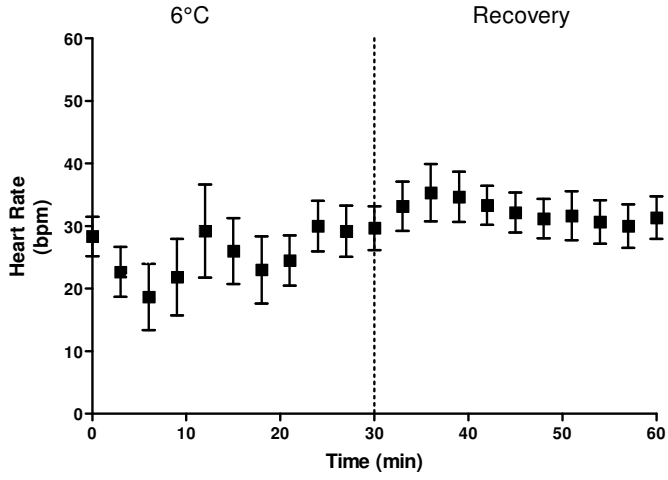
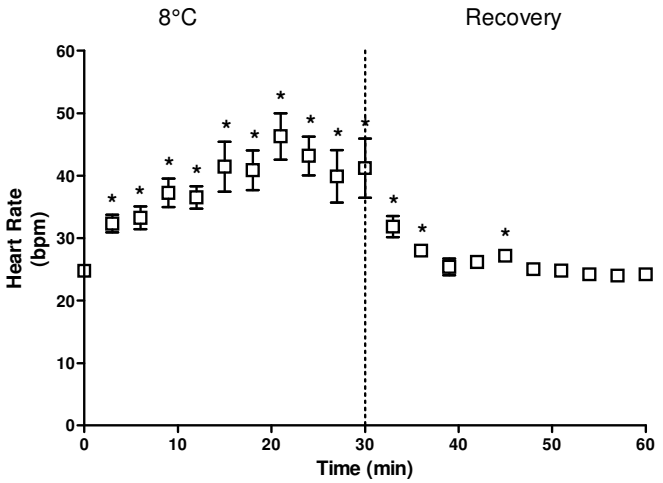


Fig. 5.5. Heart rate of *P. borchgrevinki* during 30 minutes at 8 ℃ and 30 minutes recovery at the acclimation temperature. **a.** Fish acclimated for one month to -1 ℃. **b.** Fish acclimated for two weeks to 4 ℃. **c.** Fish acclimated for one month to 4 ℃.* Significantly different from the heart rate at 0 minutes.

a.



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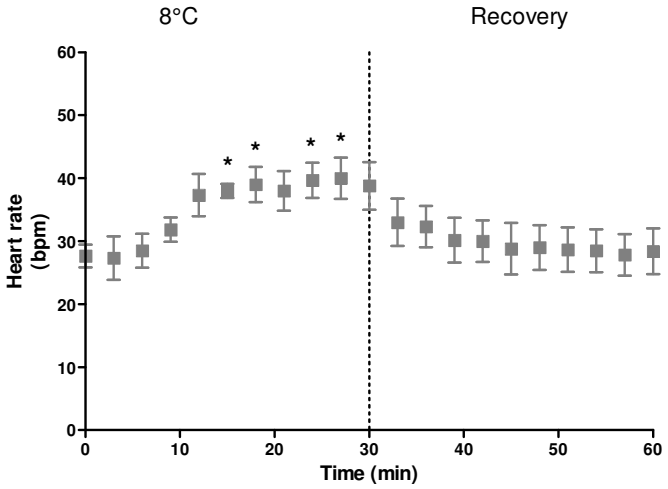

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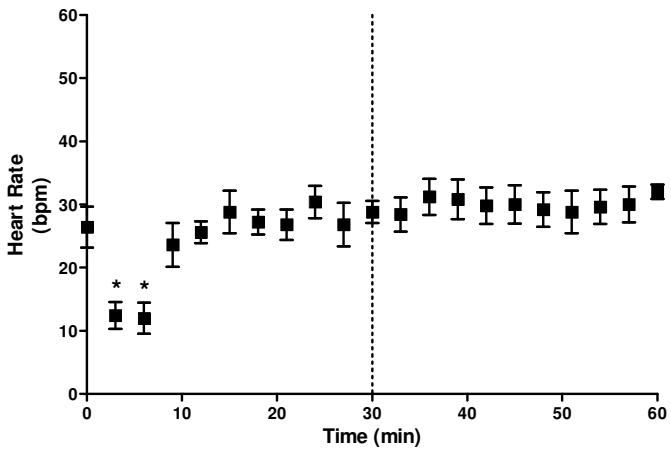
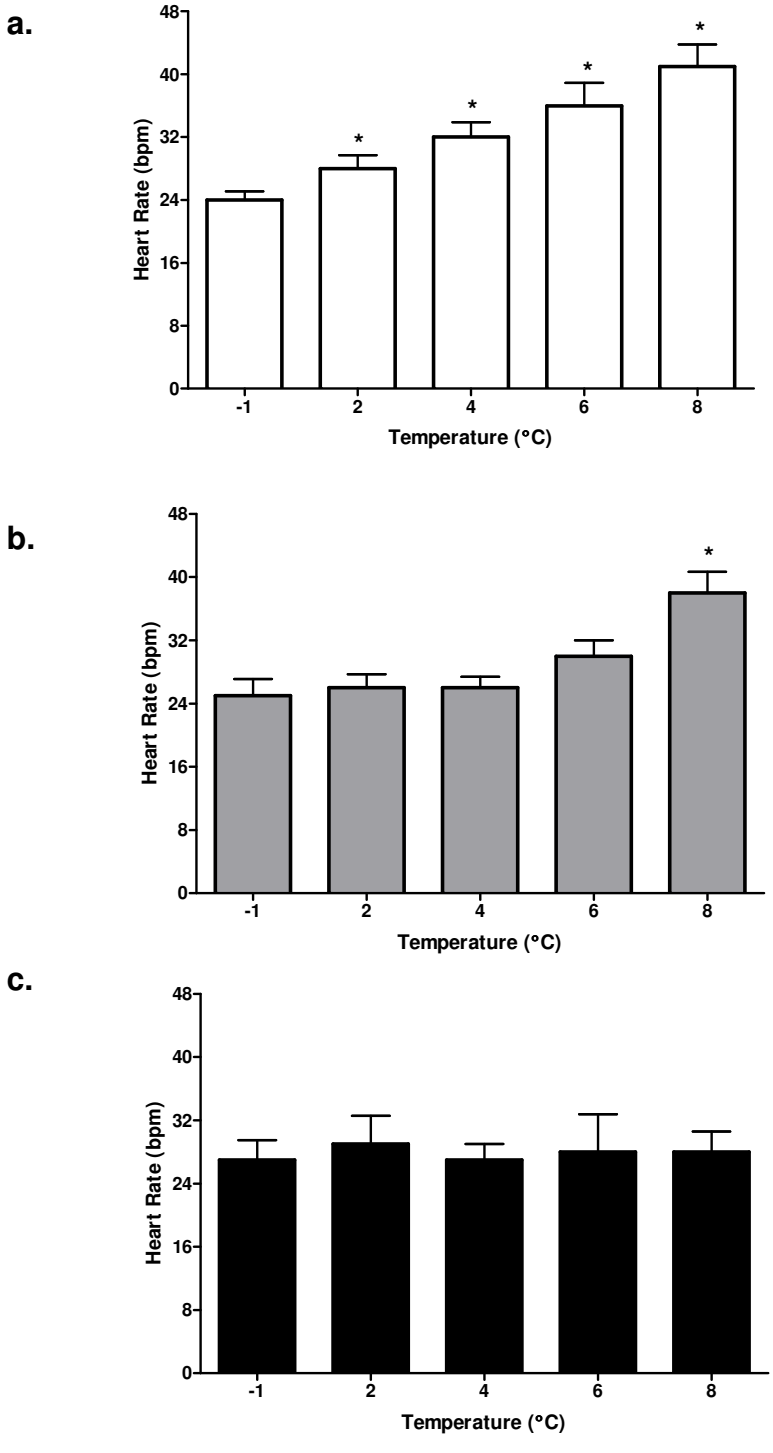


Fig.5.6. The effect of acute exposure to temperatures between -1 and 8 ℃ on the resting heart rate of *P. borchgrevinki*. **a**. Cold acclimated fish (28 days). **b**. Warm acclimated fish (14 days). **c**. Warm acclimated fish (28 days). * Significantly different from the heart rate at the normal acclimation temperature (-1 ℃ for cold acclimated fish; 4 ℃ for warm acclimated fish).



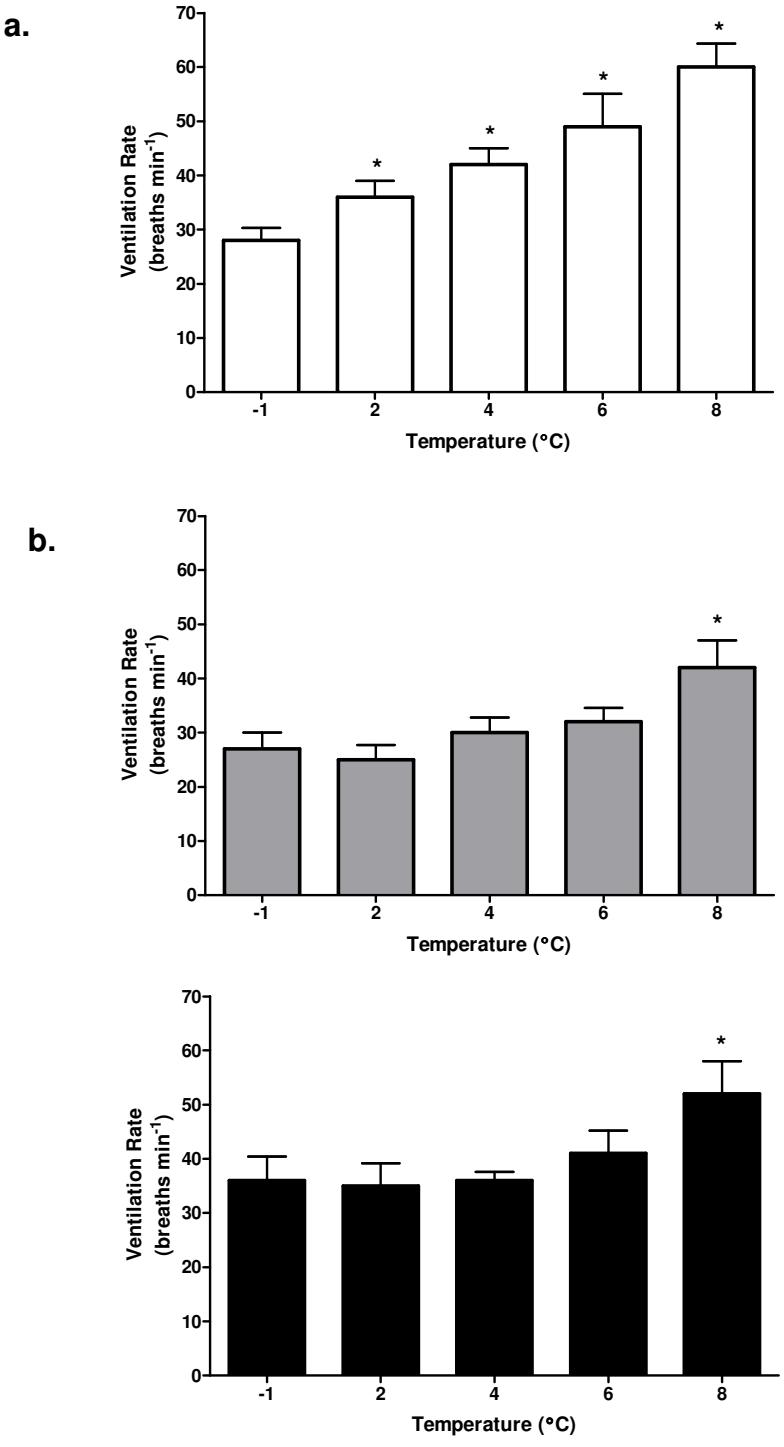
Ventilation rate

Resting ventilation rate in cold acclimated fish at their acclimation temperature (-1 °C) was 28.1 ± 2.3 breaths per minute. Ventilation rate was thermally sensitive between -1 and 8 °C (Q_{10} = 2.4) (Fig. 5.7a).

The resting ventilation rate of warm acclimated fish (28 days) at their acclimation temperature (36.0 ± 1.6 breaths per minute) was significantly higher than the ventilation rate of cold acclimated fish at -1 °C ($P<0.05$), but lower than the rate of these cold acclimated fish at 4 °C (42.0 ± 3.0 breaths per minute). Ventilation rate was thermally insensitive between -1 and 6 °C, but very sensitive between 6 and 8 °C (Q_{10} = 6.34) (Fig. 5.7c).

Fish acclimated for 14 days to 4 °C had a similar ventilation rate (30.5 ± 2.8 breaths per minute) to cold acclimated fish, and this was significantly lower than the rate for warm acclimated fish (28 days) ($P<0.01$). Ventilation rate was thermally insensitive between -1 and 6 °C in these fish, but thermally sensitive between 6 and 8 °C (Q_{10} = 6.56) (Fig. 5.7b).

Fig.5.7. The effect of acute exposure to temperatures between -1 and 8 ℃ on the resting ventilation rate of *P. borchgrevinki*. **a**. Cold acclimated fish (28 days). **b**. Warm acclimated fish (14 days). **c**. Warm acclimated fish (28 days). * Significantly different from the ventilation rate at the normal acclimation temperature (-1 ℃ for cold acclimated fish; 4 ℃ for warm acclimated fish).



Cardio-respiratory coupling

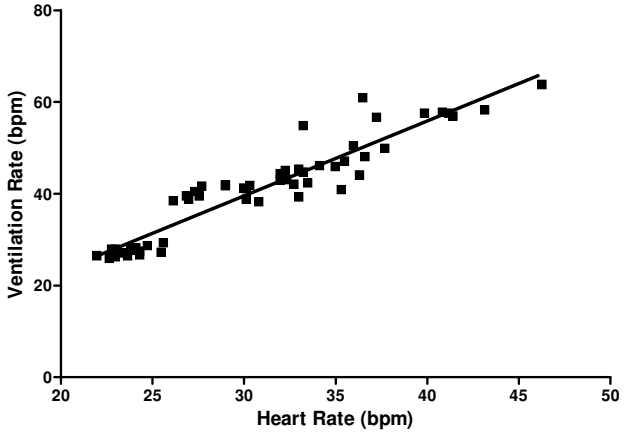
In cold acclimated fish there was a linear relationship between heart rate and ventilation rate at all experimental temperatures. Data were pooled to produce Fig. 5.8. In resting fish at -1 ºC data were clustered around a point at 24 bpm and there was essentially a 1:1 ventilation/heart rate ratio. As temperature increased, both heart rate and ventilation increased, with ventilation increasing at a faster rate than heart rate, and thus the ventilation/heart rate ratio moving away from the ideal of 1:1. At a heart rate of 45 bpm the ratio was about 1.4:1. However, the data shown in Fig. 5.8a tend to mask a tendency for these two variables to match each other for periods of time.

After only 14 days of acclimation there was a greater spread of data points, though with a tendency for them to cluster around a heart rate of 25 – 30 bpm, and a ventilation/heart rate ratio of approximately 1:1. At the highest temperature (8 ºC) both ventilation and heart rate increased, though the ratio remained at around 1:1 (Fig. 5.8b).

Following acclimation to 4 ºC for one month, both heart rate and ventilation rate were independent of temperature change up to 6 ºC and so most of the data points were clustered around a point (approximately 25 bpm heart rate, 35 bpm ventilation) with a few outliers (Fig. 5.8c), giving a ventilation/heart rate ratio of around 1.4:1. At 8 ºC, heart rate did not change, but ventilation increased dramatically so again, data points were clustered around a single point with a ratio of around 2:1. It was possible to plot linear regressions, though correlation at the lower temperatures was not good (r^2 = 0.205, P = 0.002). At 8 ºC, the slope was 1.03 (r^2 = 0.584, P = 0.006).

Fig.5.8. The relationship between heart rate and ventilation rate of *P. borchgrevinki* during acute exposure to -1, 2, 4, 6 and 8 °C. **a**: Cold acclimated (28 days, pooled data for all temperatures) (r^2 = 0.88). **b**: Warm-acclimated fish (14 days, pooled data for all temperatures) (r^2 = 0.79). **c**: Warm-acclimated fish (28 days). Pooled data for -1 to 6 °C (r^2 = 0.21). 8 °C r^2 = 0.58.

a.



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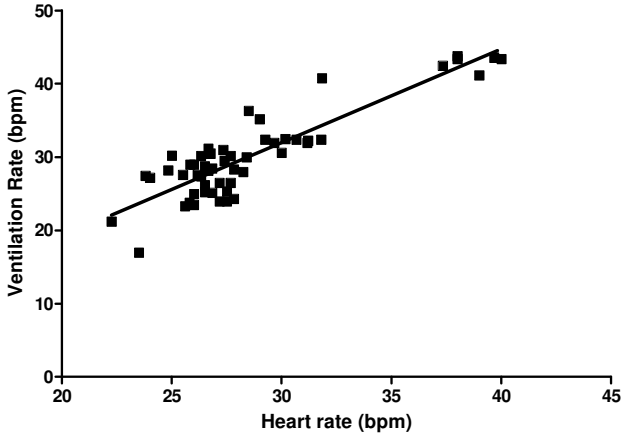

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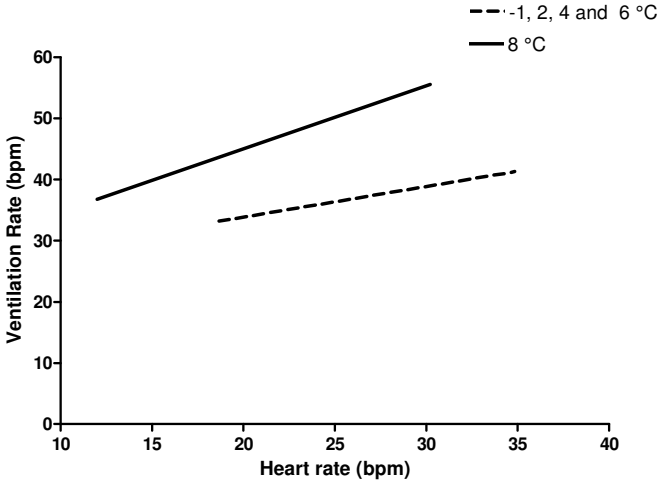

Discussion

Heart rate

Temperature is one of the most important determinants of heart rate in many species of fish, with an increase in temperature causing an increase in rate (Driedzic and Gesser 1994). However, there are seemingly conflicting data on the effect of temperature on the heart rate of *P. borchgrevinki*. Two studies have reported the heart rate of these fish to be reasonably insensitive to the effects of acute increases in temperature (Forster et al. 1998; Franklin et al. 2001), and this has been linked to increased inhibitory cholinergic control of heart rate at increased temperatures, as fish treated with atropine and a beta blocker show a normal Q_{10} response to increased temperature (Franklin et al. 2001; Lowe et al 2005). In a study on the related notothenioid fish, *Trematomus bernacchii,* no change in heart rate was found until a temperature of approximately 2.5 ℃ was reached, but above this temperature, heart rate rose rapidly (Axelsson et al. 1992). In contrast, Lowe et al. (2005) and the current study (cold acclimated fish) found the heart rate of *P. borchgrevinki* to be thermally sensitive, increasing with a Q_{10} of between 2.0 – 3.3. The present study provides a potential explanation for these differences. In the earlier studies, the time period involved was relatively short, as little as 10 minutes in the Forster et al (1998) work, while in the Axelsson et al (1992) study, heart rate started to increase at 2.5˚C, or about 20 minutes after the start of exposure to increased temperatures. In the present work, temperature was changed quickly (two minutes) but heart rate did not immediately respond, taking up to 15 minutes to stabilise, often with a small overshoot (Fig. 5.1a - 5.5a). It would appear that in these Antarctic fish where heart rate is controlled largely by neural cholinergic inhibition, there is initial cholinergic control of the heart rate during exposure to increased temperatures, but this control is modified as exposure time is extended.

The mean resting heart rate recorded for cold acclimated *P. borchgrevinki* was about 24 bpm, which compares well with the 20 bpm reported for this species by Axelsson et al. (1994). However, lower values of 18 bpm and 16 bpm were recorded by Franklin et al. (2001), and Lowe et al. (2005) respectively. On re-exposure of the cold acclimated fish to -1 °C, the reduction in heart rate back to these initial levels was rapid, and was complete in less than 10 minutes. This rapid fall in rate suggests that the change in heart rate simply occurred as a normal Q_{10} response as the body, and thus the blood, cooled. If a 5 % blood volume is assumed, and circulation time in a 100 g *P. borchgrevinki* is approximately 2.25 minutes (working on cardiac output values of 22 ml min^{-1} kg^{-1}; Franklin et al., 2007), then significant blood cooling would be expected to occur well within the 10 minutes noted for heart rate to return to normal levels.

In contrast to acute exposure to 4 °C, fish held for 14 and 28 days at this temperature were able to acclimate their heart rate and compensate for the change in temperature. The resting heart rate of warm acclimated fish at 4 °C was not significantly different from the heart rate of cold acclimated fish at -1 °C. Acclimation to 4 °C for 28 days changed the thermal sensitivity of the heart rate (Fig 5.6c) while acclimation for a shorter time period (14 days) showed that the changes were only partially completed by this time (Fig. 5.6b). Between -1 and 8 °C, heart rate was thermally insensitive in these warm acclimated fish (28 days). The rapid periods of tachy- and bradycardia in these fish are notable as they show that the fish are able to sense small changes in temperature and use neural, rather than hormonal mechanisms to control heart rate. Heart rate control was presumably achieved by variation in cholinergic and, to a lesser extent, adrenergic control, as these systems have previously been identified as important for heart rate control in this species (Axelsson et al. 1992; Axelsson et al. 1994; Davison et al. 1997; Franklin et al. 2001; Lowe et al. 2005), while circulating catecholamines do not seem to be important (Forster et al. 1998).

Maintenance of a stable and low heart rate despite an increased environmental temperature could be considered detrimental, as increased temperature generally results in increased oxygen requirements for ectothermic animals. This demand must be supplied by increased oxygen delivery from the cardio-respiratory system, which may be facilitated by an increased cardiac output. Antarctic fish alter cardiac output mainly via heart rate rather than stroke volume (Axelsson et al. 1992; Franklin et al. 2007), so an unchanged heart rate indicates minor changes to cardiac output. Certainly, during warm acclimation in this species, resting VO_2 shows an initial rise (as expected) but then falls back such that cold and warm acclimated animals have the same VO_2 (see chapter three). This would explain the low heart rate in warm acclimated fish at 4°C, but does not explain why these warm fish display a need to keep heart rate constant. It is possible that the increased demand for oxygen at elevated temperatures is partly compensated for by an increase in the oxygen carrying capacity of the blood. This may occur through an increase in the number of circulating red blood cells (increased haematocrit, Hct). Acute increases in temperature have previously been shown to cause an increase in Hct in cold acclimated *P. borchgrevinki* (Franklin et al. 2001) and this was caused by contraction of the spleen resulting in red blood cell release into the circulation. Interestingly, Nilsson et al. (1996) demonstrated that the spleen of *P. borchgrevinki* is under cholinergic control, and that increased cholinergic tone on the spleen results in its contraction and a concomitant increase in Hct. It is possible that cholinergic control on the spleen is increased at elevated temperatures at the same time that cholinergic control of the heart is increased. Further research in this area would be interesting.

Ventilation rate and cardio-respiratory synchrony

Resting ventilation rates of cold acclimated fish at -1 °C were similar to those reported by other authors (Wells 1987; Wilson et al. 2002), although somewhat lower than Forster et al. (1987). The ventilation rate of these fish was thermally sensitive between -1 and 8 °C (Q_{10} = 2.4), and this is in agreement with the

findings of Wilson et al. (2002). The length of acclimation time at 4 ℃ was important in determining ventilation rate. After a 14 day acclimation period, the ventilation rate at 4 ℃ was not significantly different to that of cold acclimated fish at -1 ℃. However, after 28 days at 4 ℃, the ventilation rate was significantly elevated compared with control fish. The elevated ventilation rates obtained in the current study conflict with data obtained in previous experiments, which found no significant difference in the ventilation rates of cold and warm acclimated fish after a 28 day acclimation period (see chapter three). It seems therefore, that the acclimatory response of ventilation in this species may vary between individuals. Warm-acclimation also changed the thermal sensitivity of ventilation rate. In both warm acclimation groups, ventilation rate was independent of temperature between -1 and 6 ℃, but rose dramatically between 6 and 8 ℃ (warm acclimated 14 days Q_{10} 6 – 8 ℃ = 6.56; warm acclimated 28 days Q_{10} 6 – 8 ℃ = 6.34). Changes in the thermal sensitivity of heart rate and ventilation rate during warm-acclimation contribute to changes in cardio-respiratory synchrony.

In fish there is a close matching of the respiratory and cardiac rates, which is termed cardio-respiratory synchrony. Cardio-respiratory synchrony may facilitate efficient oxygen uptake by synchronising blood and water flow at the gills (Egginton et al. 2006). The phenomenon has been demonstrated in several fish species (for example De Vera and Priede 1991; Taylor 1992) including four species of Southern Ocean notothenioids (Campbell et al. 2005). Cardio-respiratory synchrony was evident in cold acclimated *P. borchgrevinki* in the current study and was close to the hypothesised optimal synchrony of 1:1. However, in warm acclimated fish (28 days), there was tight control of heart rate during acute exposure to varying environmental temperatures, and cardio-respiratory synchrony was not evident. Deviation from cardio-respiratory synchrony was also evident in warm acclimated fish (14 days) where heart rate was tightly controlled between -1 and 6 ℃. At 6℃ and above, heart rate control was less efficient and cardio-respiratory-synchrony was evident.

Summary

In summary, acclimatory changes to the cardiovascular system allowed maintenance of normal heart rates of *P. borchgrevinki* at 4 °C. The maintenance of normal heart rates following acclimation to 4 °C is made possible because acclimatory changes involve a reduction in the demand for oxygen at the tissues (chapter three), thus, there is no requirement for increased oxygen delivery to the tissues after acclimation to 4 °C. Acclimation to 4 °C also affected the thermal sensitivity of the cardiovascular system, with warm acclimated fish demonstrating thermal independence of heart rate between 4 and 8 °C which was brought about via bouts of bradycardia at raised temperatures. Maintenance of cardiovascular function at raised temperatures is ecologically important because the cardiovascular system supplies oxygen and metabolites to the tissues, which in turn facilitates aerobic activity.

Chapter Six

The effect of warm acclimation on the haematological properties and enzyme activities of *Pagothenia borchgrevinki*

Introduction

Acclimation changes that maintain whole animal performance at altered temperatures are determined by modifications at the biochemical and molecular level (Cossins and Bowler 1987; Johnson and Bennett 1995; Hochachka and Somero 2002), and these usually include modifications of haematological parameters and enzymatic activities. Haematological parameters of fish are sensitive to stress, and for many species, one of the most perturbing stressors is a change in temperature. The haematological responses of fish to changed temperature are species specific, with differences between acute and prolonged temperature change (Ryan 1995). Likewise, the activities of metabolic enzymes may be altered by temperature, the effects of which are species-specific and vary between acute and prolonged exposure. Metabolic enzymes are enzymes which are involved in the production of adenosine triphospahte (ATP), therefore, their function is critical for the maintenance of cellular activities, most of which derive their energy from ATP hydrolysis (Hochachka and Somero 2002).

Haematology

Previous research has suggested that serum ion concentrations rise in marine fishes and fall in freshwater fishes in response to lowered environmental temperature (O'Grady and DeVries 1982). However, this view was challenged in a review by Burton (1986). Burton suggested that, although there was a definite rise in plasma osmolality in species inhabiting waters close to or below 0 °C, there was no general tendency for

other marine species to demonstrate increased osmolality with decreased temperatures. Likewise, Burton challenged the idea that freshwater species demonstrate a fall in plasma osmolality in response to decreased temperature, criticising the lack of supporting experimental evidence (Burton 1986). However, it is evident that marine fish which inhabit polar waters, have raised plasma osmolalities compared with temperate marine species. Raised plasma osmolality contributes to freezing resistance and is thought to result from increased concentrations of organic compounds in some species, and/or increased concentrations of plasma sodium and chloride in other species (O'Grady and DeVries 1982; Burton 1986; Gonzalez-Cabrera et al. 1995). Acute and prolonged increases in temperature have previously been shown to have an effect on the regulation of plasma ion concentrations in nototheniid fish (Gonzalez-Cabrera et al. 1995; Forster et al. 1998; Lowe and Davison 2005) and these findings will be considered in the context of results from the current study.

Within the Nototheniidae, there is a relationship between lifestyle and both blood oxygen carrying capacity and haemoglobin oxygen affinity. Typically, all nototheniids possess haemoglobin with a low affinity for oxygen compared with temperate species with similar lifestyles (di Prisco 2000). However, among the nototheniids themselves, more active species possess haemoglobin with a lower affinity for oxygen than sedentary species, and this enables the transfer of oxygen to the tissues at relatively high oxygen concentrations. Typically, active nototheniids also possess greater oxygen carrying capacities than do sedentary species. For example, the inactive nototheniid *Trematomus loennbergi* has a higher affinity for oxygen and a lower blood oxygen carrying capacity than the active *P. borchgrevinki*, which has low blood oxygen affinity, a sigmoidal dissociation curve and a high carrying capacity (Montgomery and Wells 1993). Maintenance of sufficient oxygen supply to the tissues is essential for continued cellular function at changed temperatures, therefore, the effects of warm acclimation on the oxygen-carrying capacity of *P. borchgrevinki* were investigated.

Normal plasma glucose concentrations of *P. borchgrevinki* are higher than most temperate or tropical species, and this is probably related to their active lifestyles (Lowe

and Davison 2005). There are currently no published values on the effect of long-term temperature change on the plasma glucose concentration of *P. borchgrevinki*, but acutely raised temperature has been shown to induce a slow rise (around 24 hours) in plasma glucose concentration in this species (Lowe and Davison 2005). It is difficult to determine whether this slow rise in plasma glucose concentration is a component of the thermal stress response, because stress-related changes in plasma glucose concentration usually occur quickly in fish (Barton 1997; Pankhurst 2004). For example, raised plasma glucose concentrations were evident 10 minutes after a stressor in golden perch (*Macquaria ambigua*) (Carragher and Rees 1994) and 30 minutes after a stressor in Eurasian perch (*Perca fluviatilis*) (Jentoft et al. 2005). As indicated by Lowe and Davison (2005), the slow rise of glucose concentration may impair its usefulness as an indicator of acute stress in *P. borchgrevinki,* but it was unknown what effect long-term exposure to elevated temperature may have on blood glucose concentration.

Enzyme activity

Metabolic enzymes are enzymes which are involved in the production of adenosine triphospahte (ATP), therefore, their function is critical for the maintenance of cellular activities, most of which derive their energy from ATP hydrolysis (Hochachka and Somero 2002). There are three main pathways for the synthesis of ATP. Aerobic ATP synthesis fuels long-term bouts of work and involves the complete oxidation of substrates. This process requires oxygen as the terminal proton and electron acceptor and utilises substrates such as glucose, glycogen, fatty acids or amino acids. A second pathway, fermentation, partially catabolises substrates in the absence of oxygen, and produces anaerobic end-products. In animals, the most common form of fermentation is anaerobic glycolysis, which utilises glycogen or glucose as a substrate and produces lactate as an end-product. In fish, the lactate is stored in the white muscle during burst work and is converted back to glycogen during recovery. Anaerobic glycolysis produces ATP rapidly, but unlike aerobic ATP production, it can not be maintained long-term. In vertebrate tissues containing creatine phosphate, a third pathway, phosphagen mobilisation, may be utilised. This pathway is also oxygen independent and is

catalysed by only one enzyme - creatine phosphokinase (Hochachka and Somero 2002). Because it is catalysed by only one enzyme, this ATP-producing pathway is the quickest and can supply immediate needs, but the supply of creatine phosphate within the cell is limited and is exhausted quickly (Dunn and Johnston 1986).

Changes in temperature are likely to impact the catalytic cycles of enzymes (Marshall 1997; Marshall et al. 2000). The general catalytic cycle of an enzyme can be divided into three main stages. The first stage involves recognition and binding of the substrate, and this is mediated by a number of amino acids located within the enzyme. In the second stage changes in the active site induce conformational stresses, which ultimately result in the formation of a new product, which is released from the enzyme in the third stage (Marshall 1997). Weak bonding interactions are involved at each stage, and these are sensitive to the effects of temperature. Fish in thermally variable environments must be able to compensate for changes in temperature in order to maintain fully functional catalytic cycles of enzymes. Compensation may occur on a daily or seasonal basis in thermally fluctuating environments, but it is also assumed to have occurred over evolutionary time scales to enable fish to adapt to the cold conditions of the Antarctic.

At low temperatures, the rates of enzyme catalysed reactions are reduced (Lucassen et al. 2003). This results from temperature-induced changes to enzyme structure and interactions with ligands, which ultimately affect the formation of the enzyme-substrate complex (di Prisco 2000). However, in cold adapted species, changes at the biochemical level (cold adaptation) are thought to offset these temperature-related effects and maintain aerobic performance (Johnston and Harrison 1985; Dunn 1988; Storelli et al. 1998 and see Guderley 2004 for review). Cold adaptation involves a proliferation of mitochondria (seen as an increase in mitochondrial volume density) and an associated increase in the number of mitochondrial enzymes (Dunn et al. 1989; Johnston et al. 1994; Johnston et al. 1998 and see Somero 2002 and Davison 2005 for review). Increased numbers of metabolic enzymes make it possible to increase the catalytic activity (kcat), and this partially or completely compensates for the rate-

depressing effects of low temperature (di Prisco 2000; Somero 2004). There is also some evidence that temperature adaptation involves minor amino acid changes which affect enzyme flexibility. Work on Pacific damselfishes (*Chromis spp.*) demonstrated that a single amino acid substitution in a functionally significant hinge region could change the kinetic characteristics of an A_4-LDH molecule from a temperate to a tropical ortholog (Johns and Somero 2004). These authors considered it likely that in many species, certain sites within enzymes (which influence the flexibility of the moving portions of the enzyme) were more likely to be involved in temperature adaptation, and they termed these areas 'hot spots' of adaptation. Further research is required to determine the rate of occurrence of these types of changes in other species. In most studies of nototheniid fish, only enzymes involved in aerobic metabolism show evidence of cold adaptation (Dunn and Johnston 1986; Torres and Somero 1988; Crockett and Sidell 1990; Torres and Somero 1998), although high activity of LDH from brain tissue has been demonstrated by Kawall et al. (2002), a finding which the authors considered indicative of cold adaptation. High LDH activity in myotomal muscle tissues from *P. borchgrevinki* has also been demonstrated by Tuckey and Davison (2004). There is some evidence that glycolytic capacity is cold adapted in benthic zoarcids (Zakhartsev et al. 2004 and see Somero 2002 for review). There is very little information available on the effect of raised temperature on metabolic enzyme activity in Antarctic notothenioid fish (but see Seebacher 2005). However, given that acclimation changes in *P. borchgrevinki* involve changes to the metabolic rate (chapter three), it was hypothesised that there would be concomitant changes in metabolic enzyme activity in fish acclimated for one month to 4 °C.

The aim of this chapter was to examine the effect of prolonged exposure to 4 °C on the haematology and metabolic enzyme activity of *Pagothenia borchgrevinki*. Measurements of plasma osmolality, sodium and chloride concentration as well as total haemoglobin concentration ([Hb]), haematocrit (Hct, the percentage of blood volume that is composed of erythrocytes) and mean corpuscular haemoglobin content (MCHC, an index of erythrocyte volume) were made in warm (4 °C for one month) and cold (-1

°C for one month) acclimated fish and were compared with values for fish 72 hours post-capture. Plasma glucose concentration was also measured in these fish.

The activity and thermal denaturation profiles of two metabolic enzymes from *P. borchgrevinki* were measured after one month of acclimation to either cold (-1 °C) or warm (4 °C) conditions and also in fish 72 hours after capture ('fresh' fish) and after one week at 4 °C. Cytochrome C oxidase (CCO) activity was measured as an indicator of aerobic ATP production and mitochondrial acclimation (Cai and Adelman 1990; Tschantz et al. 2002; Lucassen et al. 2003) and lactate dehydrogenase (LDH) activity was measured as an indicator of anaerobic ATP production (Torres and Somero 1998; Tschantz et al. 2002).

Materials and Methods

Haematology

Specimens of *P. borchgrevinki* were collected in McMurdo Sound and held in acclimation conditions as described in chapter two (General Experimental Methods). Eight warm acclimated fish (mass 93.3 ± 3.4 g, range 83 - 110 g; total length 188.1 ± 3.7 mm, range 175 - 205 mm) were held for one month at 4 °C and eight cold acclimated fish (mass 106.8 ± 4.2 g, range 92 - 126 g; total length 210.3 ± 4.5 mm, range 192 - 230 mm) were held at -1 °C for one month. There was no significant difference between the mass or the length of the two fish groups. Fish were not fed during the acclimation period. Control values were obtained from a third group of fish ('fresh' fish, n=8) which were held in the cold water (-1 °C) flow-through aquarium for 72 hours post-capture before sampling (mass 64.4 ± 3.6 g, range 53 - 82 g; total length 196.9 ± 0.4 mm, range 185 - 214 mm). Seventy two hours is considered adequate for the return of haematological parameters to resting conditions after the stress of capture and confinement (Ryan 1995). Due to the limited availability of animals, the mass of these fish was significantly lower than the mass of both cold and warm acclimated fish

($P<0.05$). Blood and plasma samples were obtained from fish following the procedure detailed in chapter two. It has been suggested that the use of chronically implanted cannulae is the most reliable method for the collection of blood samples from resting fish, and that data collected from acutely sampled fish may be in error (Wells et al. 1990; Macdonald and Wells 1991). However, it was not possible to use cannulae in these experiments, as experimental fish were also involved in swimming trials and measurements of resting oxygen consumption in the week prior to removal of blood samples. Inserting cannulae would have impaired the results for these experiments. Time did not permit for cannuale to be inserted after the experiments and still allow for an adequate period of recovery from surgery before sampling. Therefore, acute sampling methods were employed, and during this procedure handling, air exposure and disturbance were minimised to prevent undue stress to the fish, and sampling was undertaken rapidly to minimise disturbance of haematological variables. It is considered unlikely that this method caused more stress to fish than would result from the loss of blood and surgery required for cannulae insertion.

The osmolality of 8 µl plasma aliquots was measured using a Wescor 5100 C vapour pressure osmometer. The osmometer was calibrated prior to sampling using standard solutions. Plasma sodium concentration was measured using a Sherwood flame photometer and a calibration curve plotted using standard solutions. Chloride concentration of 10 µl plasma aliquots was determined using a Radiometer CMT10 chloride meter. The chloride meter was calibrated prior to use with standard solutions and the supporting electrolyte was changed after every 10 samples.

Total haemoglobin concentration ([Hb]) was determined from a 5 µl sample of whole blood using the cyanmethaemoglobin method (Sigma diagnostics kit 525). This method is based on the oxidation of haemoglobin and its derivatives to methaemoglobin. In the presence of alkaline potassium ferricyanide, methaemoglobin reacts with potassium cyanide and forms cyanmethaemoglobin. Absorbance of cyanmethaemoglobin was measured at 540 nm using a Unicam 8625 UV/VIS spectrometer. The colour intensity of cyanmethaemoglobin at 540 nm is proportional to the total haemoglobin

concentration. Total haemoglobin concentration could therefore be determined using a calibration curve drawn from a range of dilutions of cyanmethaemoglobin standard solutions. Haematocrit (Hct) was determined at Scott Base as part of the sampling procedure described in chapter two. Mean corpuscular haemoglobin content (MCHC) was calculated as total haemoglobin concentration / fractional haematocrit.

Plasma glucose concentration was determined using the glucose oxidase / peroxidase enzymatic method (Sigma diagnostics kit 510). This method is based on the coupled enzymatic reactions:

$$C_6H_{12}O_6 + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine (colourless)} \xrightarrow{\text{Peroxidase}} \text{Oxidised o-Dianisidine (brown)}$$

The intensity of the brown colour was measured at 450 nm using a Unicam 8625 UV/VIS spectrometer and this intensity is proportional to the original concentration of glucose in the plasma. Glucose concentrations were determined using the following calculation:

$$\text{Plasma glucose (mmol L}^{-1}\text{)} = \frac{A(\text{test}) / A(\text{standard}) \times 100}{18}$$

Where:

A = absorbance at 450nm

Test = experimental sample

Standard = a 5.56 mmol L^{-1} standard solution

Enzyme activity

Tissues for analysis of enzyme activity were collected from the same three groups of fresh, cold acclimated and warm acclimated fish used in the haematology experiments. A fourth group of fish (*n*=8), held for one week at 4 °C, were also used in the analysis of enzyme activity (mass 79.7 ± 6.6 g, range 62 – 114 g; total length 209.1 ± 0.7 mm, range 185 - 244 mm). The mass of these fish was significantly lower than the mass of cold acclimated fish ($P<0.05$). Tissues were collected and stored as described in chapter two. White muscle was removed from a section of tail fillet, and red muscle was dissected from the region adjacent to the base of the pectoral fins, the whole heart was removed, followed by the liver, which was cleaned carefully to eliminate endoparasites. In Christchurch tissue homogenates of 10 % weight / volume were prepared in an extraction medium containing phosphate buffer solution (25 ml 150 mM NaCl and 20 ml 10 mM NaH_2PO_3 made up to 500 ml with water; pH 7). Homogenates for use in LDH analysis were centrifuged at a low speed (2000 rpm) for two minutes to remove tissue clumps. Homogenates for use in CCO analysis were not centrifuged. Prepared homogenates were then used for analysis of enzyme activity and thermal denaturation. Each assay was initially optimised by varying the homogenate concentration to achieve a linear change in absorbance over time. For both CCO and LDH, activity was assayed at 4 °C at pH 7. Assay temperature was controlled by circulating temperature controlled water through the cuvette holder from an external recirculating water bath. Enzyme samples were kept frozen until immediately prior to use. Absorbance was measured using a Shimadzu UV1601 PC spectrometer.

Cytochrome C oxidase (CCO) activity of *P. borchgrevinki* tissue was measured using a colorimetric assay (Sigma kit CYTOC-OX1). This method is based on the oxidation of ferrocytochrome C to ferricytochrome C by cytochrome C oxidase. This is a biphasic reaction, with an initial burst of activity in the first 60 seconds followed by a slower reaction rate. The initial burst of activity is measured in this assay. As cytochrome C is oxidised, a decrease in absorption at 550 nm is observed. The decrease in absorption

was measured and used in the following equation to calculate the activity of CCO in the tissue samples:

$$\text{Cytochrome C oxidase activity (units/ml)} = \frac{\Delta A \times \text{dil} \times 1.1}{(\text{vol. enzyme}) \times 21.84}$$

Where:
ΔA = change in absorption over one minute
dil = dilution factor of sample
1.1 = reaction volume (ml)
vol. of enzyme = sample volume (ml)
21.84 = the difference in extinction coefficients ($\Delta\varepsilon^{mM}$) between reduced and oxidised cytochrome C at 550 nm
Unit definition: one unit will oxidise 1 µmole of ferrocytochrome C per minute at pH 7 at 25 ℃.

Lactate dehydrogenase (LDH) activity was assayed using a technique that monitors the reduction of pyruvate to lactate in the presence of LDH using NADH as the indicator. Tissue samples were first diluted in 1.74 ml of phosphate buffer solution (25 ml 150 mM NaCl and 20 ml 10 mM NaH_2PO_3 made up to 500 ml with water; pH 7), then tissue-specific volumes of 3 mg/ml NADH and 0.8 mg/ml sodium pyruvate were added to the cuvette to initiate the reaction. Reaction volumes that produced a linear rate of pyruvate reduction were determined for each tissue and used in the experimental assays. The change in absorbance was measured at 340 nm for five minutes in all tissues except the heart, for which absorbance was measured over three minutes. The mean decrease in absorbance over one minute was used in the following calculation to determine LDH activity:

$$\text{LDH activity } (\mu\text{mol min}^{-1}\text{ L}^{-1}) = \frac{\Delta A \times V \times 1000}{\varepsilon \times d \times \Delta t \times v}$$

Where:

ΔA = change in absorbance

V = total volume (ml)

ε = extinction coefficient of NADH

d = distance of light path (cm)

t = time (minutes)

v = sample volume (ml)

Values were then calculated and expressed as μmol min^{-1} g tissue^{-1}

To determine the effect of thermal denaturation on enzyme activity the same assays for CCO and LDH outlined above were used. However, prior to the initiation of the assay reaction, enzyme samples were floated in eppendorf tubes in an insulated flask at 50 ℃. Activity was then assayed at five, 10 15 and 20 minutes after the beginning of incubation at 50 ℃ and values compared with the activity of the sample before incubation at 50 ℃ (0 minute activity). Thermal denaturation of CCO was assayed using extractions from red muscle tissues and thermal denaturation of LDH was measured using extractions from white muscle tissues.

Data analysis and statistical methods

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance was taken at the level of $P<0.05$. All data are presented as the mean ± standard error, unless otherwise stated. Results for all haematological parameters and enzyme activity for different tissues within an acclimation group were compared using one-way analysis of variance

(ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. For comparison of enzyme activity between different tissues and between acclimation groups, two-way analysis of variance (ANOVA) was used, after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. The effect of acclimation on thermal denaturation of enzymes was compared using repeated measure two-way analysis of variance (ANOVA) and post-hoc Bonferroni analyses. Change of enzyme activity during thermal denaturation was measured using repeated measure one-way analysis of variance (ANOVA) and post-hoc Bonferroni analyses.

Results

Haematology

Acclimation to both -1 ℃ and 4 ℃ caused a significant reduction in the plasma osmolality of *P. borchgrevinki* compared with fresh fish (fresh fish 592.4 ± 3.5 mmol kg^{-1}; cold acclimated fish 539.1 ± 2.4 mmol kg^{-1}; warm acclimated fish 529.0 ± 17 mmol kg^{-1}; $P<0.05$) (Fig. 6.1). The decline in plasma osmolality was brought about by a reduction in plasma sodium concentration (Fig. 6.2), and plasma chloride concentration (Fig. 6.3), which were significantly reduced in both cold and warm acclimated fish compared with fresh fish.

A significant increase in [Hb] was evident in warm acclimated fish compared with fresh fish ($P<0.05$), but this was not apparent in cold acclimated fish (Fig. 6.4). [Hb] in fresh fish was 18.53 ± 1.94 g L^{-1} and this rose to 28.47 ± 2.28 g L^{-1} in warm acclimated fish. The Hct of warm acclimated fish (25.25 ± 1.97 %) was elevated in comparison with cold acclimated fish (18.29 ± 1.44 %; $P<0.05$) (Fig. 6.5). Fresh fish Hct (23.71 ± 1.87 %) was not significantly different from warm acclimated fish, but was elevated compared

with cold acclimated fish (P<0.05). Acclimation to both cold and warm conditions caused a significant increase in MCHC (Fig. 6.6) and a decrease in plasma glucose concentration (Fig. 6.7) compared with fresh fish.

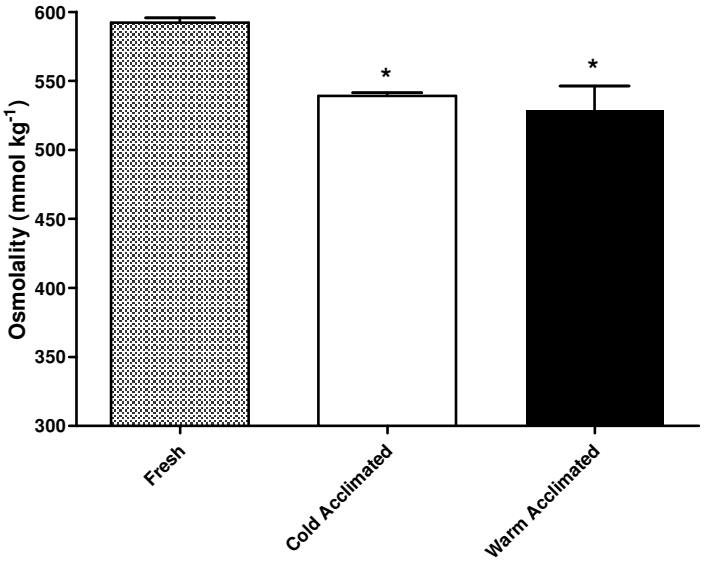

Fig. 6.1. The effect of acclimation on the plasma osmolality of *Pagothenia borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 ℃, warm acclimated fish after four weeks at 4 ℃). * Significantly different from the plasma osmolality of fresh fish. There was no significant difference between the plasma osmolality of cold acclimated and warm acclimated fish.

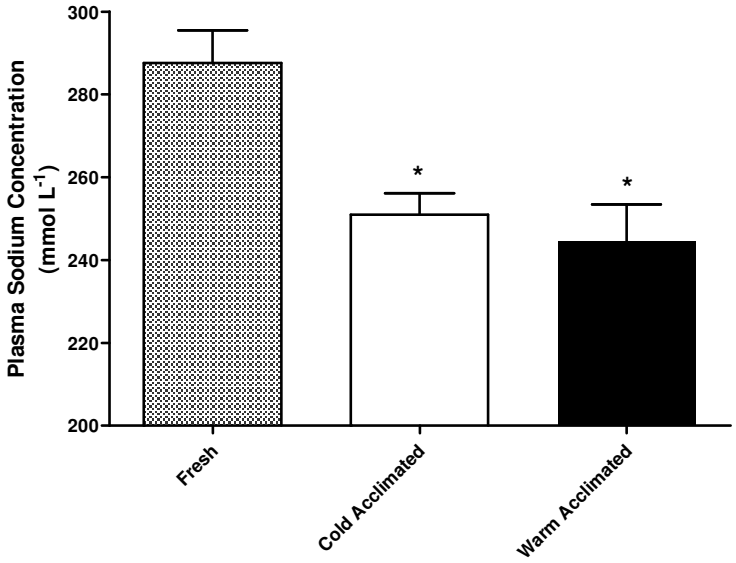

Fig. 6.2. The effect of acclimation on the plasma sodium concentration of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 ℃, warm acclimated fish after four weeks at 4 ℃). * Significantly different from the plasma sodium concentration of fresh fish. There was no significant difference between the plasma sodium concentration of cold acclimated and warm acclimated fish.

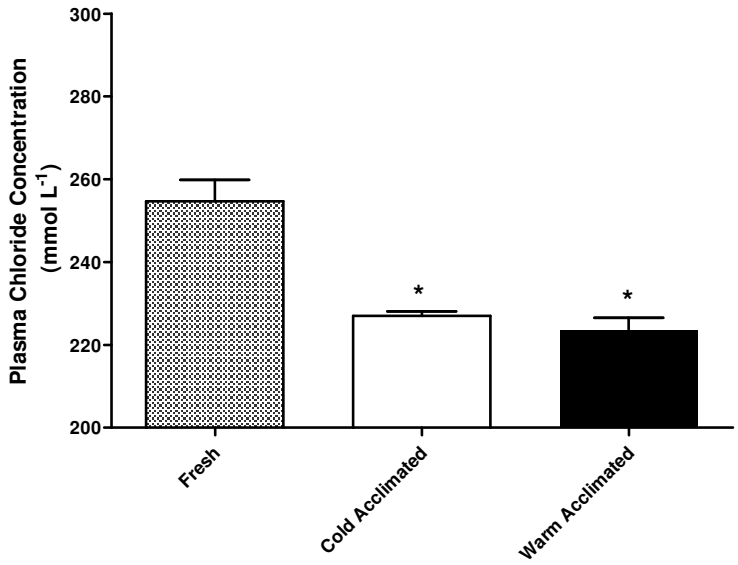

Fig. 6.3. The effect of acclimation on the plasma chloride concentration of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 ℃, warm acclimated fish after four weeks at 4 ℃). ***** Significantly different from the plasma chloride concentration of fresh fish. There was no significant difference between the plasma chloride concentration of cold acclimated and warm acclimated fish.

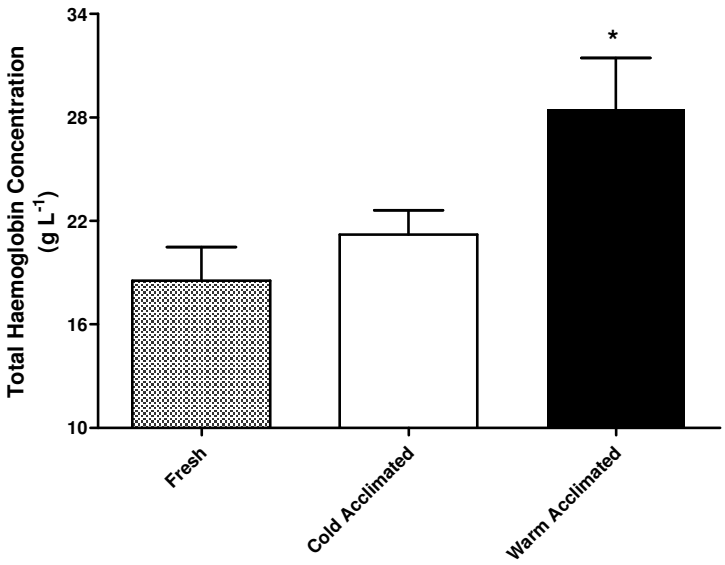

Fig. 6.4. The effect of acclimation on the total haemoglobin concentration ([Hb]) of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 ℃, warm acclimated fish after four weeks at 4 ℃). * Significantly different from the [Hb] of fresh fish. While the [Hb] of warm acclimated fish was elevated compared with cold acclimated fish, this difference was not statistically significant.

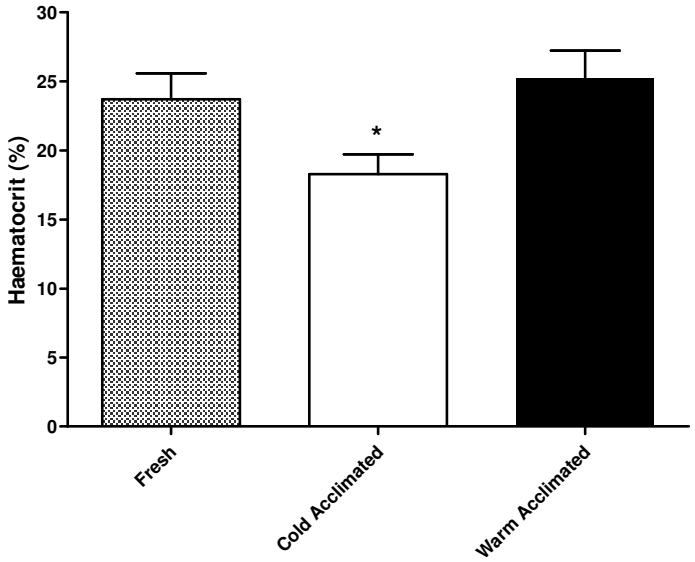

Fig. 6.5. The effect of acclimation on the haematocrit (Hct) of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 °C, warm acclimated fish after four weeks at 4 °C). * Significantly different from the Hct of fresh fish and warm acclimated fish. There was no significant difference between the Hct of fresh fish and warm acclimated fish.

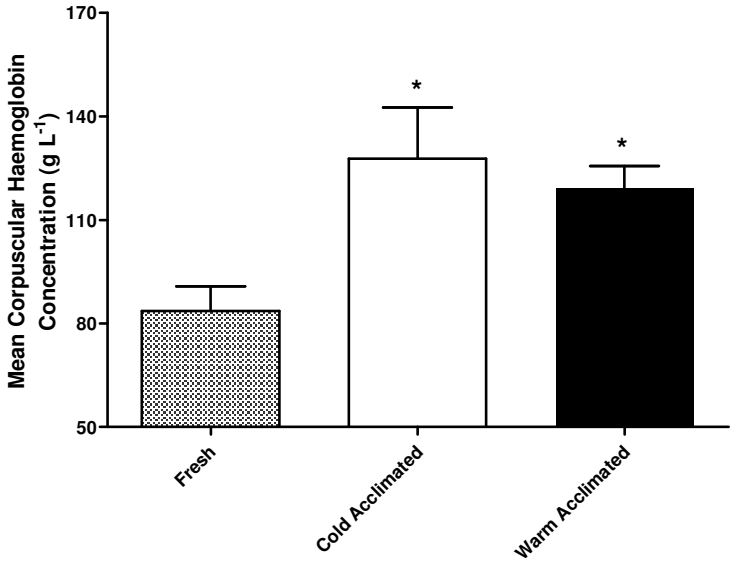

Fig. 6.6. The effect of acclimation on the mean corpuscular haemoglobin concentration (MCHC) of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 °C, warm acclimated fish after four weeks at 4 °C). * Significantly different from the MCHC of fresh fish. There was no significant difference between the MCHC of cold acclimated and warm acclimated fish.

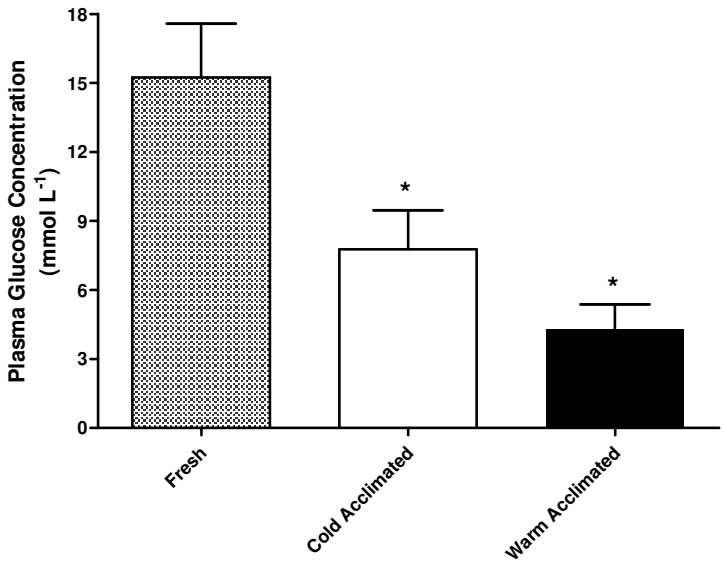

Fig. 6.7. The effect of acclimation on the plasma glucose concentration of *P. borchgrevinki* (fresh fish sampled 72 hours post-capture, cold acclimated fish sampled after four weeks at -1 °C, warm acclimated fish after four weeks at 4 °C). * Significantly different from the plasma glucose concentration of fresh fish. There was no significant difference between the plasma glucose concentration of cold and warm acclimated fish.

Enzyme activity

Inter-individual variation in CCO activity was high in all acclimation groups. In all groups except 4 °C (one month), CCO activity was statistically similar in all tissues. Warm acclimated fish (4 °C one month) demonstrated significantly increased CCO activity in the red muscle compared to both the activity of CCO in the white muscle, liver and heart, and compared to the red muscle CCO activity of the other acclimation groups (Fig. 6.8).

In all acclimation groups LDH activity was greatest in the heart followed by the white muscle. Activity was very limited in the red muscle and liver. Acclimation for one month to cold and warm conditions significantly reduced the LDH activity of the white muscle

compared with fresh fish and fish held for one week at 4 ℃ ($P<0.05$). Cold acclimated fish (-1 ℃ one month) had heart LDH activity that was significantly elevated compared with fresh fish (Fig. 6.9).

There was no significant effect of acclimation on the thermal denaturation of red muscle CCO at 50 ℃. Enzyme activity after five minutes of incubation at 50 ℃ showed a high level of inter-individual variation, some tissue preparations exhibiting increased activity while the activity of others decreased. After a 10 minute incubation period, enzyme activity of all acclimation groups was reduced and after 15 minutes activity had ceased in all but a few tissue preparations (Fig. 6.10). LDH proved to be more thermally stable than CCO, with all acclimation groups demonstrating activity after 15 minutes at 50 ℃, and all groups except warm acclimated fish demonstrating some activity after 20 minutes at this temperature. Overall, acclimation had no significant effect on the pattern of denaturation of LDH (Fig. 6.11), although after five minutes warm acclimated fish (4 ℃ one month) showed reduced LDH activity compared with fish held for one week at 4 ℃ and also fresh fish. After 10 minutes, the LDH activity of 4 ℃ (one week) fish was also elevated compared with cold and warm (4 ℃ one month) fish.

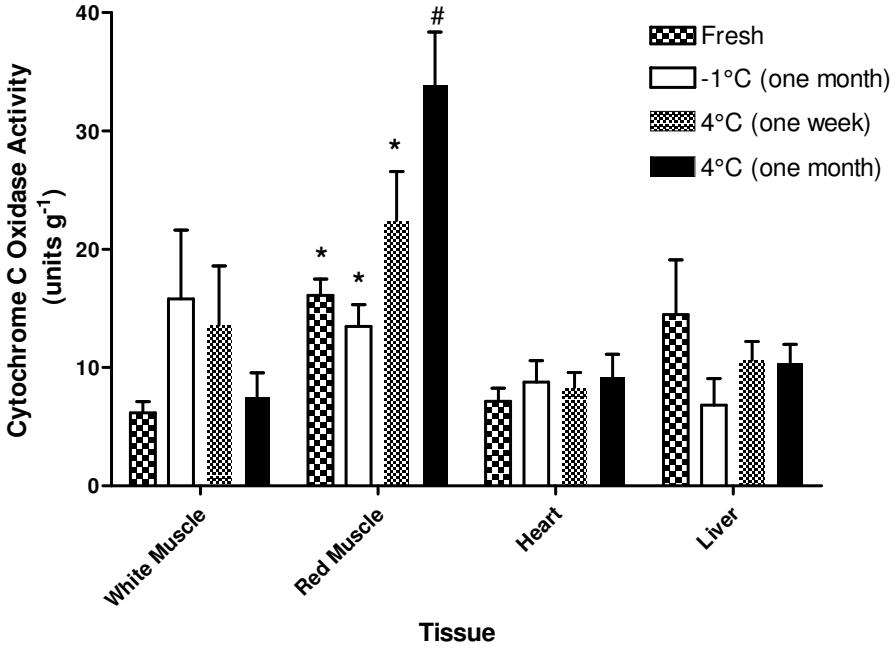

Fig. 6.8. Cytochrome C Oxidase (CCO) activity in white and red muscle and liver and heart of *P. borchgrevinki* held in a range of acclimation conditions. ***** Significantly different from the red muscle CCO activity of warm acclimated fish (4 °C one month). **#** Significantly different from the CCO activity in the white muscle, liver and heart of warm acclimated fish (4 °C one month). Unit definition: one unit will oxidise 1 µmole of ferrocytochrome C per minute at pH 7 at 25 °C. Note, while the CCO activity in the white muscle of fresh fish is lower than the other three acclimation groups, the high level of inter-individual variation in the results meant that this difference was not statistically significant.

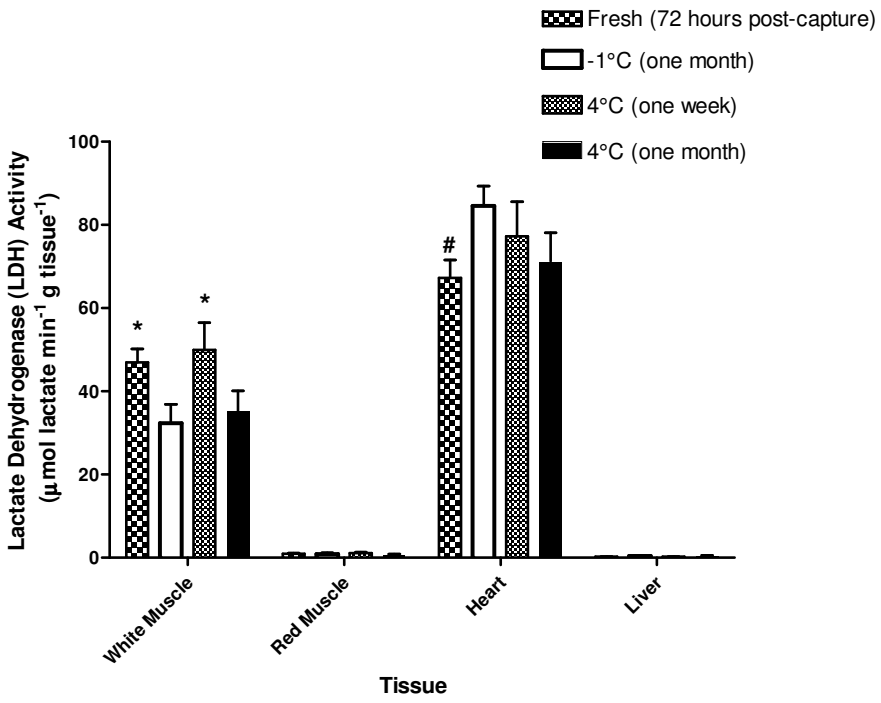

Fig. 6.9. Lactate dehydrogenase (LDH) activity in white and red muscle and liver and heart of *P. borchgrevinki* from different acclimation conditions. * Significantly different from the white muscle LDH activity of cold acclimated fish (-1 °C one month) and warm acclimated fish (4 °C one month). # Significantly different from the heart LDH activity of cold acclimated fish.

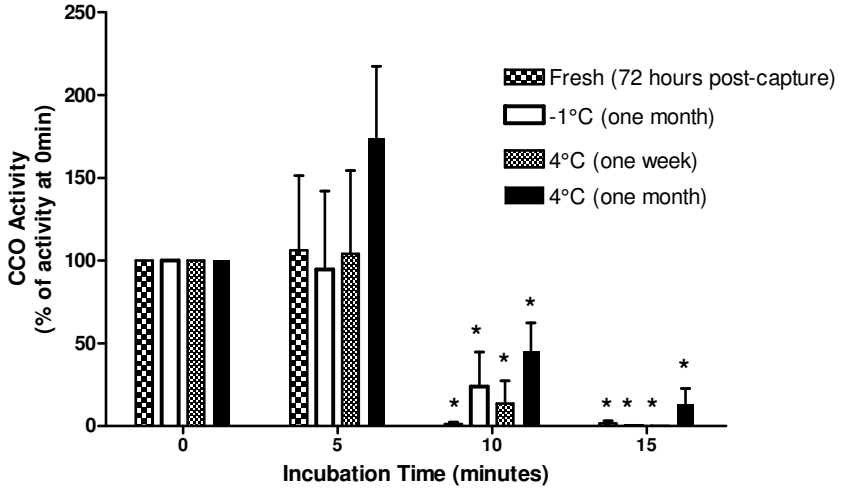

Fig. 6.10. *P. borchgrevinki* red muscle cytochrome C oxidase (CCO) activity following incubation at 50 °C. * Significantly different from the enzyme activity of the same acclimation group at 0 minutes. Neither acclimation to cold (-1 °C) nor warm (4 °C) conditions significantly affected the pattern of thermal denaturation of CCO.

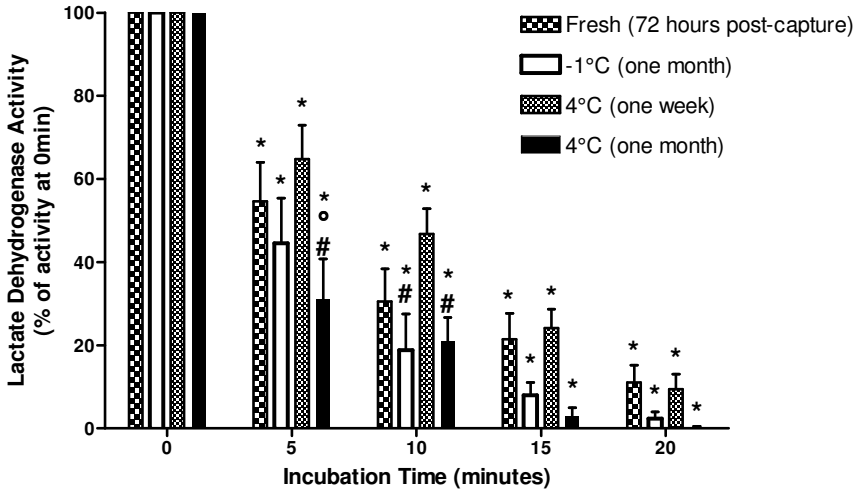

Fig. 6.11. *P. borchgrevinki* white muscle lactate dehydrogenase (LDH) activity during incubation at 50 °C. * Significantly different from the enzyme activity of the same acclimation group at 0 minutes. # Significantly different from the enzyme activity of warm acclimated (one week) fish at the same time interval. ° Significantly different from the enzyme activity of fresh fish at the same time interval. Acclimation did not significantly affect the pattern of thermal denaturation of LDH in any acclimation group.

Discussion

Haematology

The serum osmolality of Antarctic fish is typically higher than temperate and tropical marine species. Increased serum osmolality mainly results from increased concentrations of sodium and chloride which is actively regulated by a change in the set points of ion pumps (O'Grady and DeVries 1982; Gonzalez-Cabrera et al. 1995). High serum osmolality was evident in the results of the current study. Fresh fish had osmolalities of around 592 mmol kg^{-1}, which is more than double the values of Atlantic sturgeon (*Acipenser oxyrinchus*) and shortnose sturgeon (*A. brevirostrum*) (Baker et al. 2005). This value compares well with previous measurements of *P. borchgrevinki* plasma osmolality (O'Grady and DeVries 1982; Lowe and Davison 2005) and plasma

sodium and chloride concentrations of fresh fish also compare well with previous findings (O'Grady and DeVries 1982; Franklin et al. 1993; Forster et al. 1998). It has previously been shown that acute temperature elevation, to 6 °C, causes a delayed increase in plasma osmolality in *P. borchgrevinki*, but no change is evident during acute exposure to 3 °C. The increase in plasma osmolality at 6 °C is transient and is thought to result from a temporary perturbation of osmotic control resulting from the temperature-induced influx of ions (Lowe and Davison 2005). Osmotic disruption does not occur immediately, but is delayed (Forster et al. 1998; Lowe and Davison 2005), for example Forster et al. (1998) found that 10 minutes of exposure to 10 °C had no effect on the plasma osmolality of *P. borchgrevinki*.

Lowe and Davison (2005) tested the effect of prolonged exposure to 4 °C on osmoregulation in *P. borchgrevinki* and reported warm-induced hypo-osmoregulation in this species, and this has also been described in the Antarctic nototheniids *Trematomus bernacchii* and *T. newnesi* (Gonzalez-Cabrera et al. 1995). In the study on *T. bernacchii* and *T. newnesi,* Gonzalez-Cabrera et al. (1995) attributed the observed hypo-osmoregulation to the compensation of Na^+ / K^+-ATPase activity in the gills and kidneys of these fish, leading to active removal of Na^+ and Cl^-. However, in the current study hypo-osmoregulation was found to occur as a result of acclimation, regardless of whether this was to cold or warm conditions. Both warm and cold acclimated fish demonstrated a reduction in plasma osmolality of between 60 and 70 mmol kg^{-1} compared with fresh fish, and both plasma sodium and plasma chloride concentrations were reduced in acclimated fish compared with fresh fish. It is not clear why acclimation should cause this reduction in plasma osmolality, although a recent study by Polakof et al. (2006) has highlighted an interaction between osmoregulatory ability and nutritional status in gilthead sea bream (*Sparus auratus*). In the sea bream, Na^+, K^+-ATPase activity at low salinities was impaired in unfed fish compared with fed fish. Impaired Na^+, K^+-ATPase activity resulted in reduced plasma osmolality of unfed fish compared with fed fish at low salinities. Therefore, it is possible that the no feeding protocol of the current study contributed to the observed decline in plasma osmolality of *P. borchgrevinki.* However, there is a key difference in the impact of the non-feeding

protocol on the fish used by Polakof et al. (2006) and those in the current study. The sea bream used in the study by Polakof et al. (2006) were starved for 14 days prior to measurements of plasma osmolality and this period was sufficient to cause a significant decline in body mass and a disruption of metabolic status in this species. In the current study, *P. borchgrevinki* were unfed for 28 days before measurements of plasma osmolality were made, but at the end of this period there was no significant change in either the metabolic status (see chapter three) or the mass / condition index (see chapter seven) of acclimated fish. These results suggest that the no feeding protocol was not of sufficient length to disrupt fish condition or major physiological function, including osmoregulation. Other factors were probably responsible for the observed hypo-osmoregulation, but further research is required to elucidate these.

The haemoglobin concentration of *P. borchgrevinki* is generally higher than other nototheniid fish and this has been correlated with the active lifestyle of this species (Wells et al. 1980; Macdonald and Wells 1991). *P. borchgrevinki* has five functionally distinct haemoglobins, between 70 – 80 % of which are Hb 1. The multiplicity of haemoglobins and the low heats of oxygenation compared with temperate fish indicate that the haemoglobin system of this active species is very specialised (di Prisco 2000). In the current study, fresh fish and cold acclimated fish had [Hb] of between 18 and 21 g L^{-1}, which compare favourably with earlier studies (Wells et al. 1984; Wells et al. 1989; Ryan 1995), although these are slightly lower than the values reported by Wells et al. (1980) and Lowe and Davison (2005). Warm acclimation caused the [Hb] to rise to about 28 g L^{-1}. Previous studies have demonstrated an increase in [Hb] in this species in response to severe agitational stress (Wells et al. 1984), acute temperature increase (Ryan 1995), prolonged temperature increase (Lowe and Davison 2005, but note changes were not statistically significant due to high levels of inter-individual variation), hypoxia (Wells et al. 1989) and as an immediate but short term response to confinement (Ryan 1995). In the current study elevated [Hb] was associated with increased Hct in warm acclimated fish.

The resting Hcts of teleost fish are species-specific (Baker et al. 2005) and are thought to reflect the lifestyle of a species more closely than its latitudinal distribution (Wells et al. 1980). In Antarctic fish, there is a general trend for a reduction in Hct to offset the effects of increased blood viscosity which result at low temperatures (Wells et al. 1980). The presence of erythrocytes has a major effect on blood viscosity (Wells et al. 1990; Montgomery and Wells 1993; Egginton 1996), with a 20% Hct having about the same effect on blood viscosity as a 20 °C drop in temperature (Macdonald and Wells 1991). Increased blood viscosity would impair perfusion of the vascular system, and consequently oxygen delivery to the tissues, unless blood pressure was raised or vascular resistance lowered (Eginton1996). Reduced Hct clearly reduces blood oxygen carrying capacity, but this is sustainable in Antarctic fish because of their low metabolic rates and the increased oxygen solubility in water and plasma at low temperatures (Egginton 1996; di Prisco 2000). In fact, experimental reduction of *T. bernacchii* Hct to less than 1% does not impair its performance during periods of enforced exercise (di Prisco 2000), and this species can also survive periods of carbon monoxide exposure, which disables the haemoglobin (di Prisco et al. 1992). High blood viscosity at polar temperatures is also compensated for by low vascular resistance, resulting from the specialisation of vascular geometry, which is typical of Antarctic notothenioid fish (Axelsson et al. 1992). In the Channichthyidae, compensation for increased viscosity is taken to the ultimate extreme with the loss of the red blood cells and haemoglobin as the oxygen carrier. This removal of haemoglobin as an oxygen carrier means that the blood viscosities of these fish are very low (Montgomery and Wells 1993) and at -1.8 °C are similar to those found in the New Zealand blue cod (*Parapercis colias*) at 15 °C (Macdonald and Wells1991). Some authors have suggested that the red-blooded nototheniids are following along the same evolutionary pathway as the channichthyids, and will also lose their haemoglobin in the course of evolutionary time (Wells et al. 1990).

While the resting Hct of *P. borchgrevinki* is low compared to temperate fishes, this species has the ability to increase Hct in response to a range of stressors. These stressors include confinement (Ryan 1995), acute thermal stress (Franklin et al. 1991;

Egginton 1996; Forster et al. 1998; Lowe and Davison 2005), hypoxia (Wells et al. 1989), handling (Wells et al. 1984) and exercise (Davison et al. 1988; Franklin et al. 1993). In all of these studies, the increase in Hct was linked to contraction of the spleen (which serves as a store of erythrocytes) and the consequent release of erythrocytes into the circulation. This mechanism was confirmed in *P. borchgrevinki* by Wells et al. (1989) who showed that increased oxygen carrying capacity during hypoxia was correlated with decreased spleen mass. Further evidence came from Franklin et al. (1993) who demonstrated that spleen-ligated *P. borchgrevinki* experienced no change in Hct and reached fatigue more quickly during exercise than control fish. Decrease in splenic mass and increased oxygen carrying capacity during hypoxia and high temperature was also reported by Davison et al. (1994). Increased Hct provides the necessary extra oxygen carrying capacity required by this active species in times of stress. The response has been demonstrated in other teleost species (for example Yamamoto 1987; Yamamoto 1988; Yamamoto and Itazawa 1989; Wells and Weber 1990), although in *P. borchgrevinki* cholinergic innervation of the spleen capsule is important (Nilsson et al. 1996), in contrast to other teleost species where adrenergic nerves (releasing epinephrine) have a key role in controlling the spleen (Kita and Itazawa 1990; Nilsson 1994).

Warm acclimation caused a significant increase in the Hct of *P. borchgrevinki* compared with cold acclimated fish, a result which was not found by Lowe and Davison (2005). It could be suggested that this increase in Hct is an indication that warm acclimated fish are stressed. However, the results of other experiments on this same group of fish imply that this is unlikely. Resting oxygen consumption and ventilation rates were not elevated at the end of the acclimation period (chapter three), sustained swimming ability was not impaired compared with cold acclimated fish (chapter four), and plasma glucose concentrations, which may rise in response to stress in fish (Burton 1997; Begg and Pankhurst 2004), were not elevated compared with control fish (this chapter). It seems more likely that the increase in Hct was advantageous for these fish. At 4 °C the concentration of oxygen in the water is reduced, and it could therefore be necessary for the fish to increase Hct to improve the oxygen carrying capacity of the blood. It is

interesting to note here that while an increase in Hct provides extra oxygen carrying capacity, it also increases blood viscosity, and consequently causes an increase in the workload of the heart. Presumably at 4 °C, the benefits of improved oxygen carrying capacity outweigh the detrimental effects of more viscous blood, which may be lessened at this elevated temperature. The Hct of fresh fish was elevated compared with cold acclimated fish and is also high in comparison with results found by other authors for the same species at a similar time period post-capture (Franklin et al. 1993; Ryan 1995; Lowe and Davison 2005). This is unlikely to be a result of the sampling procedure, as the results for cold acclimated fish (using the same sampling method as for fresh fish) compare well with these previous studies. However, it is possible that the 72 hour post-capture period was not sufficient to allow the recovery of fish Hct from the stress of capture, handling and confinement, although this has previously been described as sufficient time for recovery from these stressors (Ryan 1995; Lowe and Davison 2005).

Acclimation to both cold and warm conditions caused an increase in MCHC compared with fresh fish, which may be considered an indication of cellular shrinking. However, the MCHC values obtained for fresh fish are much lower than those reported by previous authors (Wells et al. 1989; Franklin et al. 1993; Ryan 1995; Lowe and Davison 2005), and are more likely to indicate that fresh fish were stressed (particularly when Hct results are considered), and this was associated with cellular swelling (therefore decreased MCHC). Other authors have also reported cell swelling occurring in acutely sampled fish (Wells et al. 1990). Cell swelling occurs when the Na^+ / H^+ exchanger on the red blood cell membrane is activated in response to circulating catecholamines, causing an influx of Na^+ and Cl^- into the cells (Forster et al. 1998). The MCHC values obtained for cold and warm acclimated fish are closer (although still somewhat lower) to those reported by previous authors for fish in an unstressed resting state (Wells et al. 1989; Franklin et al. 1993; Lowe and Davison 2005), so it is considered that rather than the values for these acclimation groups being elevated, the values obtained for fresh fish were low. Because there is no significant difference between the MCHC of warm and cold acclimated fish, this suggests that the increase in Hct and [Hb] demonstrated

in warm acclimated fish did not result from cell swelling, but simply increased numbers of circulating red blood cells.

Elevated blood glucose concentration (hyperglycaemia) is generally accepted as an indication of stress in fish (Barton 1997; Begg and Pankhurst 2004) and acute thermal stress has been shown to lead to a delayed hyperglycaemia in *P. borchgrevinki* (Lowe and Davison 2005). However after long-term acclimation to 4 ℃ (five to six weeks) this hyperglycaemia was no longer apparent (Lowe and Davison 2005). The results of the current study are consistent with these earlier findings, with warm acclimated fish showing no significant difference in plasma glucose concentration compared with cold acclimated fish. Both groups of fish demonstrated a decrease in plasma glucose concentration compared with fresh fish and this probably results from the non-feeding protocol for acclimating fish. However, as no change in condition index was apparent between fresh fish and acclimated fish (both cold and warm), it is considered that fish were not detrimentally affected by this decreased plasma glucose concentration. Plasma glucose measurements obtained in the current study are in close agreement with those from previous studies (Lowe and Davison 2005).

Enzyme activity

Most cellular activities rely on the hydrolysis of ATP for energy, the production of which is catalysed by a number of metabolic enzymes. Maintaining the activity of these enzymes at changed temperatures is therefore critical to the continued function of an organism. Enzyme function requires a balance between molecular stability and structural flexibility. Molecular stability is necessary to provide the appropriate geometry for ligand binding and to prevent denaturation, while flexibility is important to allow catalysis at an appropriate rate to meet metabolic demands (Fields 2001). The balance between flexibility and stability is temperature dependent (Cossins and Bowler 1987; Dunn 1988; Marshall 1997; di Prisco 2000; Fields 2001; Lucassen et al. 2003), at cold temperatures enzyme movement is slowed and this may mean that catalysis no longer occurs at a metabolically useful rate. However, at warm temperatures enzyme flexibility

may be so increased that structural stability is compromised and substrate binding will not occur (Fields 2001). Temperature change may also affect enzyme activity by causing changes to cellular pH, the energy of activation (Ɛa, a measure of the energy required to form an activated complex), and the diffusion rates of substrate and product (Marshall 1997). At extreme cold temperatures, there is a danger of cold denaturation of enzymes, which is thought to result from interactions between non-polar groups in proteins and water, leading to protein unfolding (Marshall 1997). Hot denaturation of enzymes is also possible at extreme high temperatures (Pörtner 2002).

Compensation for changes in temperature must act to overcome these perturbations to enzyme function and maintain a balance between structural stability and flexibility. Temperature compensation in enzymes may be achieved by one or a combination of three possible mechanisms: first, a change in the cellular concentration of enzymes (quantitative strategy), brought about by varying the relative rates of synthesis and degradation; second, a change in the types of enzyme isoforms present (qualitative strategy), achieved by on/off synthesis of different enzyme isoforms or by maintaining a set of isoforms and varying the concentration of each; and third the modification of the kinetics of pre-existing enzymes (modulative strategy), for example, changed activation energy (Cossins and Bowler 1987; Vetter and Buchholz 1998). For example seasonal, thermal acclimation of the LDH of Norwegian coastal cod (*Gadus morhua*) involves changes in LDH concentration and adjustment of thermodynamic and kinetic properties, but not the expression of alternative isoforms (Zakhartsev et al. 2004). However, in the goldfish, *Carassius auratus,* acclimation to -1 or 26 ℃ leads to changes in the structure of myofibrillar ATPase, which is apparent because of the changed temperature of thermal denaturation of these enzymes (Johnston et al. 1975).

Acclimation affects the enzymes of different species variably (Johnston and Dunn 1987). For example, Tschantz et al. (2002) examined cold acclimation in five species of closely related fish from the family Centrarchidae (the ‘sunfishes’). These fish demonstrated species-specific responses to cold temperatures, with no obvious pattern related to phylogeny. Many organisms also show seasonal variation in enzyme activity

(Guderley 2004), for example, the eastern red spotted newt (*Notophthalmus viridescens viridescens*) showed seasonal differences in CCO activity, but interestingly, there was no significant difference in LDH activity between seasons (Berner and Bessay 2006). CCO activity of the cod *G. morhua* also demonstrates seasonal variability (Pelletier et al. 1993), as does the activity of CCO in some tissues of the channel catfish (*Ictalurus punctatus*) (Seddon and Prosser 1997). Seasonal differences in acclimation responses are thought to relate to the acclimatisation state of the animals when they are collected, which is related to a range of environmental factors. In the current study, fish were all collected early in the Antarctic summer season, so seasonal factors were not responsible for the observed differences in enzyme activity. Acclimation effects are also tissue-specific (Johnston and Dunn 1987), for example, in the carp *Cyprinus carpio*, temperature compensation of CCO activity was greater in tissues with high aerobic metabolic rate (heart and liver) than in white muscle (Cai and Adelman 1990).

The two metabolic enzymes investigated in this study were cytochrome C oxidase (CCO) and lactate dehydrogenase (LDH). CCO is a protein complex that spans the inner mitochondrial membrane and is the terminal member of the electron transport chain. It is responsible for the transport of electrons across the membrane coupled with the synthesis of ATP (Robinson and Capaldi 1977). CCO activity is a good indicator of aerobic capacity. Evolutionary adaptation to low temperature is usually associated with an increase in mitochondrial numbers (Egginton et al. 2002) and a concomitant increase in the tissue-specific activity of CCO (Hardewig et al. 1999b). In the current study, warm acclimation of *P. borchgrevinki* resulted in a rise in the activity of red muscle CCO compared with fresh fish, cold acclimated fish and fish held for one week at 4 °C. An increase in red muscle CCO activity in response to acclimation is in agreement with previous studies on enzyme acclimation, and particularly the work of Seebacher et al. (2005) who demonstrated an increase in CCO activity in *P. borchgrevinki* acclimated to 4 °C. These authors noted an increase of about 20 units g^{-1} as a result of warm acclimation, and a similar increase was noted in the current study. Thermal compensation of enzyme activity has been hypothesised not to occur in organisms inhabiting thermostable environments. Some experimental evidence supports this

hypothesis, for example the whitespotted bamboo shark (*Chiloscyllium plagiosum*) showed no temperature compensation in citrate synthase or LDH activity after prolonged exposure to a 5 ℃ increase in temperature (Tullis and Baillie 2005). However, *P. borchgrevinki* is clearly a remarkable exception to this theory, inhabiting one of the world's most thermostable environments, yet still possessing the ability to compensate enzyme activity for changed temperatures.

LDH is the terminal enzyme of anaerobic glycolysis in vertebrate tissues, and is involved in catalysis of the interconversion of pyruvate and L-Lactate with the concurrent conversion of NADH and NAD^+ (Kawall et al. 2002). The reaction involves an initial binding of adenine dinucleotide cofactor, then binding of substrate, catalysis and the release of product and adenine dinucleotide (Marshall et al. 2000). LDH is abundant in the cytosol of fish white muscle cells and is a good indicator of anaerobic metabolic capacity (Torres and Somero 1988; Sharpe et al. 2001). The somatic tissues of the haemoglobinless channichthyids have particularly high concentrations of glycolytic enzymes including LDH (Bacila et al. 1989). LDH activity in *P. borchgrevinki* white muscle was around 30 to 45 μmol min^{-1} g^{-1} wet weight tissue, and interestingly, was much higher in heart tissue. The activities of LDH obtained in the current study for fresh *P. borchgrevinki* white muscle tissues compare well with previous results for the labriform swimmer *Notothenia neglecta* (also an Antarctic notothenioid) (Dunn and Johnston 1986,), but are lower than those recorded by Tuckey and Davison (2004) for *P. borchgrevinki* white muscle. In contrast to the findings of Seebacher et al. (2005), warm acclimation did not increase LDH activity in any of the four tissues analysed in the current study. In fact, both cold and warm acclimation caused a reduction in white muscle LDH activity compared with fresh fish. It is possible that this reduction in LDH activity resulted from the effect of confinement. In the aquarium system, fish were not exposed to predators and did not come into contact with prey items, and this reduced the need for burst swimming movements. Perhaps as a consequence, the activity of enzymes associated with anaerobic energy metabolism, such as LDH, were down-regulated. It would be interesting to test this assumption using other anaerobic

enzymes from acclimated *P. borchgrevinki.* In contrast, fish used in the study of Seebacher et al. (2005) were swum repeatedly during the acclimation period.

Both CCO and LDH exhibited heat denaturation temperatures over 40 °C greater than the normal environmental temperatures encountered by *P. borchgrevinki*. Such a finding is not unusual among Antarctic fish (Marshall 1997; Giardina et al. 1998; Marshall et al. 2000), however the disruption of enzyme function (physiological denaturation) probably occurs at a much lower temperature, and Antarctic fish enzymes are considered thermally unstable above about 5 °C (Somero et al. 1996; Somero 1998). Warm acclimation did not result in a change in the thermal denaturation pattern of CCO, so it is possible that the increased activity of this enzyme was the result of a proliferation of enzyme concentration, rather than the expression of different enzyme isoforms.

Summary

Acclimation changes in whole animal performance are underpinned by changes at the molecular and biochemical level. In *P. borchgrevinki* several haematological and enzymatic changes contribute to the maintenance of metabolic capacity observed after a 5 °C rise in temperature. These include a significant increase in total haemoglobin concentration and haematocrit; and increased activity of red muscle CCO. Decreased plasma glucose concentration and plasma osmolality and increased MCHC occur as a result of acclimation, regardless of whether this is to cold or warm conditions.

Chapter Seven*

The effect of warm acclimation on aspects of *Pagothenia borchgrevinki* behaviour and ecology:

thermal preference, survival of x-cell fish and long-term acclimation

Introduction

Previous chapters have demonstrated that *Pagothenia borchgrevinki* possesses the necessary physiological mechanisms to acclimate to 4 °C. This chapter considers how acclimation affects certain aspects of the behaviour and ecology of this species. These questions are significant in terms of assessing the effects of Southern Ocean warming on wild populations of *P. borchgrevinki*. Three main questions are considered: does acclimation to 4 °C alter the thermal preference of *P. borchgrevinki*?; can individuals with X-cell disease acclimate to 4 °C?; and can acclimation changes evident over one month be sustained during long-term exposure to 4 °C?

Thermal preference

Fish responses to temperature can be divided into tolerance, resistance and preference, and these have been defined by Jobling (1981). The tolerance zone is the range of temperatures where 50 % of the population could theoretically survive indefinitely. Within the tolerance zone, fish usually spend most of their time at, or close to a certain temperature, which is the preferred temperature. Outside of the tolerance zone are those temperatures where survival time is dependent on exposure time, and this is the zone of resistance. Two methods have been widely employed in laboratory investigations of preferred temperature (Jobling 1981). In the 'gravitation method', fish are placed into a thermal gradient for a length of time, during which they swim to the preferred temperature (Jobling 1981; Konecki 1995). In the second method, thermal preferenda are determined for groups of fish acclimated to a range of temperatures. The thermal preferenda are then plotted, and a line is drawn through the data points.

*An abbreviated version of this chapter (excluding the thermal preference data) is in press at the Journal of Fish Biology

Where the plotted line intersects with the 'line of equality', the acclimation temperature equals the preferred temperature of the species (Jobling 1981).

Many species that colonise heterogeneous thermal environments will actively seek zones of preferred temperatures. This behaviour is considered to be a homeostatic mechanism which facilitates thermoregulation, as fish regulate their body temperature by selecting warmer or cooler waters (Baras 1995; van Dijk et al. 2002; Clutterham et al. 2004). The zones of preferred temperature are species-specific, so water temperature is important in influencing the natural distribution of fish. For example, niche segregation of Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*) has been attributed to differences in thermal preference (Larsson 2005). Thermally motivated niche segregation has also been observed in shallow mangrove ponds, where warmer waters are colonised by *Cyprinodon artifrons*, while cooler areas are preferred by *Floridichthys carpio* and *Gambusia yucatana* (Heath et al. 1993). Laboratory studies which investigate the thermal preferences of fish using thermal gradients usually produce results that are consistent with the preferred temperatures of wild animals (Heath et al. 1993; Lafrance et al. 2005).

Previous studies have demonstrated a link between acclimation temperature and thermal preference in fish (Cincotta et al. 1984; Tsuchida 1995; Rodriguez and Ramirez 1997). For example, in six species of North American freshwater fish (*Notemigonus crysoleucas, Notropis analostanus, Rhinichthys atratulus, Catostomus commersoni, Lepomis gibbosus* and *Micropterus salmoides*), preferred temperature increased as acclimation temperature increased (Cincotta et al. 1984). However, in some species thermal preference temperatures are independent of acclimation temperature. For example, in the marbled rockfish (*Sebastiscus marmoratus*), acclimation temperature affects critical thermal maxima and critical thermal minima, but not final preferred temperature (Kita et al. 1996). In larval sea lamprey, *Petromyzon marinus*, summer preferred temperature is affected by acclimation temperature, but winter preferred temperature is not (Holmes and Lin 1994). Macdonald et al. (1987) briefly discussed unpublished data on the thermal preference of cold acclimated *P. borchgrevinki,* which

suggested that these fish actively seek the cold end of a thermal gradient (-1.8 to 5.4 ℃). The aim of the current study was to determine whether acclimation to 4 ℃ alters the thermal preference of *P. borchgrevinki.* If fish exposed to elevated temperatures demonstrate changes in thermal preference, then this could potentially affect the natural distribution of the species during the course of climatic change.

X-cell disease

X-cell disease is well documented in marine teleost fish (see for example Mellergaard and Nielsen 1996; Mellergaard and Lang 1999; Miwa et al. 2004), but still little is known about its epidemiology. Infected fish are characterised by the proliferation of large spherical cells, which are usually apparent in the epidermal tissues such as the skin, gills and pseudobranch (Davison 1998), though lesions occasionally appear internally (Kent et al. 1988). X-cell disease is most commonly reported in the gill tissues of Antarctic fish, where the large number of x-cells cause a characteristic whitened appearance and swelling of the gill filaments (Franklin and Davison 1988; Davison 1998). X-cells have a distinct cellular structure. Cells are large and are surrounded by an extracellular coat. The cell nucleus is large and spherical and contains conspicuous nucleoli. Mitochondria are clearly different from those of surrounding tissues and the cytoplasm contains variable numbers of electron-dense granules (Davison 1998). In *P. borchgrevinki* infection of the gills with x-cell disease reduces lamellar blood flow and consequently impairs aerobic performance (Davison and Franklin 2003), as well as the ability to tolerate hypoxia (Davison et al. 1990). In situations requiring increased oxygen delivery to the tissues, such as during exercise, fish must recruit more lamellae to facilitate oxygen delivery, and this is when the disease becomes most limiting (Davison et al. 1990). During the initial stages of acclimation to 4 ℃, *P. borchgrevinki* experiences a substantial increase in the demand for oxygen at the tissues, and must therefore increase its rate of oxygen uptake, and consequently recruit more lamellae. While this can be achieved in healthy individuals (chapter three), it was hypothesised that individuals infected with x-cell disease would be limited in their ability to survive during these initial stages of acclimation. This hypothesis was tested in the current

study. Wild populations of *P. borchgrevinki* often include significant numbers of x-cell individuals (Davison 1998), if these individuals are unable to survive the challenges of increasing ocean temperatures, then this may seriously impact the abundance of wild populations.

Long-term acclimation

Most studies of thermal acclimation in fish involve an acclimation period of around four to six weeks, and this was the case in the research described in previous chapters. However, a recent study has suggested that some moderate stressors, which appear to have a modest impact over a short time period, may become harmful over a longer time period (Michaelidis et al. 2007). Consequently an assessment of *P. borchgrevinki* condition and aerobic performance was undertaken during a six month acclimation period, to determine whether the patterns established during one month at 4 °C would be maintained over this longer interval. Longer acclimation studies of this nature provide a more complete picture of the possible impacts of oceanic temperature change on marine species than results from short term acclimation studies alone.

Fish condition was determined using Fulton's Condition Index, which incorporates fish mass and length data and provides an indication of the relative fitness of individual fish within a population. Previous studies have demonstrated that fish exposed to long-term thermal stress experience a reduction in condition index (for example Kocovsky and Carline 2001; Muhlia-Almazan et al. 2003). A similar decline in the condition of *P. borchgrevinki* during warm acclimation may indicate that this species experiences stress at 4 °C. Alternatively, within the optimal temperature range for growth, elevated temperature may lead to an increase in food intake and a concomitant increase in growth rate (although this is dependent on the availability of food and the scale of the thermally mediated elevation in resting metabolic rate) (Jobling 1993). If fish are feeding at maximal rates, ingested energy that is not used to fuel metabolism, or lost as waste products is available for growth (Cossins and Bowler 1987; Weatherly and Gill 1987; Jobling 1993). If *P. borchgrevinki* demonstrated an increase in growth rate during

warm acclimation then this would be reflected in increases in mass and length compared with cold acclimated individuals. Limited data are available on the growth rates of Antarctic fish, so it was unclear whether observable differences in growth rates would be apparent during the six month acclimation period. However a previous study has reported a growth rate of between 13.8 and 27.9 mm per year for *P. borchgrevinki* (La Mesa and Vacchi 2001).

Resting oxygen consumption rate ($MO_{2\ rest}$) and prolonged swimming ability were measured at regular intervals during the six month acclimation period. The significance of these physiological variables was discussed in chapter three and chapter four, so this will only be briefly summarised here. Oxygen is required by metabolising tissues to produce ATP, which fuels most cellular processes. Acute exposure to raised temperatures increases the rate of many cellular processes, and consequently the demand for ATP is elevated. In turn, this causes an increase in the oxygen consumption rates of many organisms (Jobling 1993). Prolonged elevation of resting metabolic rate has several deleterious consequences, including a reduction in the aerobic scope for activity (if there is no associated increase in maximum oxygen consumption rate), diversion of energy away from essential processes such as growth and reproduction and extra stress on the heart, as heart rate and cardiac output usually increase to maintain oxygen delivery to the tissues (Jensen 1993). During a one month acclimation period at 4 °C, *P. borchgrevinki* was able to correct the thermally induced elevation of resting metabolic rate and displayed an $MO_{2\ rest}$ similar to that of cold acclimated animals (chapter three). In the current chapter the aim was to determine if this compensation was a short lived and unsustainable response, or, if it could be maintained long-term.

Swimming performance often influences the success of fish in their daily activities, including escape from predators, prey capture, reproductive behaviour, larval selection of settlement habitats, adult habitat selection, and migration (Archer and Johnston 1989; Plaut 2001; Johnston and Temple 2002; Fisher et al. 2005). Therefore, the maintenance of locomotor performance following temperature change is critical to the

survival of many species. Acute temperature effects on swimming performance are species-specific and also vary according to the type of swimming performance investigated. Burst swimming performance is usually insensitive to temperature change, whereas sustained or prolonged swimming performance is commonly temperature sensitive (Beddow et al. 1995; Castonguay and Cyr 1998; Wilson et al. 2001; Claireaux et al. 2006). As described in chapter four, *P. borchgrevinki* can maintain prolonged swimming performance at 4 °C during a one month acclimation period. In the experiments described in this chapter, the aim was to determine whether prolonged swimming ability could be maintained during a six month acclimation period at 4 °C.

Methods

Thermal preference

Experiments on thermal preference were conducted in the temperature controlled facilities at Canterbury University. Fish were caught in McMurdo Sound and shipped to New Zealand, as described in chapter two (General Experimental Methods). All fish were initially held in the cold aquarium facility (0.0 ± 0.3 °C) for two months prior to experimental use. Four experimental fish were then removed and held for one month in the 4 °C facility (4 ± 0.3 °C), described in chapter two (mass 58.2 ± 12.3 g, range 39 – 93 g; total length 187.8 ± 12.2 mm, range 164 - 220 mm). Results were compared with four control fish identified and separated from non-control fish in the cold aquarium (mass 55.8 ± 9.1 g, range 34 - 78 g; total length 193.3 ± 10.4 mm, range 165 - 215 mm). There was no significant difference between the mass or length of the two fish groups.

A horizontal thermal gradient was created in a black trough made from industrial plastic (2.5 x 0.6 x 0.3 m, length x width x height). The trough was placed in the temperature

controlled cold room, air temperature -1.5 ± 0.5 ℃ (the size of the trough was dictated by the limited space available within this room). When filled with water, and in the absence of heating mechanisms, the water temperature of seawater within the trough was 0 ± 0.3 ℃. To create a thermal gradient, two heat exchangers, linked to two temperature-controlled water baths, were placed in the base of the plastic trough. The base of the trough was covered with very fine nylon mesh to preserve a uniform appearance along the length of the gradient, including the area where the heat exchangers were positioned. By carefully controlling the amount of heat produced by the heat exchangers, in combination with the cooling effect of the cold aquarium air, a horizontal thermal gradient of 0 to 4.5 ℃ was created within the trough. Thermal zones 0.5 x 0.5 x 0.3 m (length x width x height) were clearly labelled on the outside of the trough using bright yellow tape. These zones corresponded to temperatures of 0 – 0.5 ℃, 0.6 – 1.5 ℃, 1.6 – 2.5 ℃, 2.6 – 3.5 ℃ and 3.6 - 4.5 ℃. In the interest of clarity in the presentation of results, these zones will be referred to as 0, 1, 2, 3 and 4 ℃ respectively. Space constraints made it impossible to create a longer gradient incorporating a wider range of temperatures. A second constraint associated with the length of the gradient was the tendency for temperatures within the gradient to begin to equalise after a period of around 2.5 hours. Many attempts were made to try and correct this problem, however, none of these adjustments produced a significant alteration in the length of time a gradient could be maintained. Fluorescent lighting above the tunnel was even and continuous. In preliminary trials, six cold acclimated fish were placed into the trough, without a thermal gradient, for 90 minutes. This was important to confirm that tank dynamics did not influence position selection. The fish used in these preliminary trials were not the control fish used in later experiments within the thermal gradient.

The thermal preference of each fish was measured individually in the thermal gradient. Once a thermal gradient was established, each fish was placed into the trough and left undisturbed for 90 minutes. Fish position throughout the 90 minutes was recorded using a wall-mounted Sony video camera recorder (Handycam Vision CCD TRV218E). Each fish was exposed to the thermal gradient on two separate occasions, three weeks

apart. On the first occasion fish were initially placed into the cold (0 ℃) zone of the thermal gradient, while on the second occasion fish were placed into the warm (4 ℃) zone of the gradient. Thermal preference experiments were always conducted in the morning, between 9.00 and 11.30 am to reduce the potential influence of circadian rhythms on temperature selection. The first 30 minutes fish spent within the gradient were considered a familiarisation period, and only data from the following 60 minutes were used for analysis. Data were analysed by recording the position of each fish every two minutes and then calculating the mean number of minutes each acclimation group spent at each temperature. The position of each fish at the end of the 90 minute period was also recorded.

X-cell disease

X-cell diseased *P. borchgrevinki* were routinely captured while fishing for healthy fish, using the technique described in chapter two. At the Scott Base wet laboratory, individuals with x-cell disease were separated from non x-cell individuals and housed in a separate tank within the flow-through aquarium system for 72 hours before experimental use. Individuals with between 60 and 80 % of the gills infected with x-cell disease were then separated for use in these experiments. As described by Davison (1998), x-cell disease spreads from distal to proximal ends of the gill filaments, and all gill filaments are fairly uniformly infected, so an evaluation of percent infection was straightforward. Seven control fish were held in the cold flow-through aquarium (-1.0 ± 0.3 ℃) (mass 39.9 ± 5.1 g, range 27 – 56 g; total length 153.7 ± 3.4 mm, range 140 - 170 mm; condition factor 1.1), and seven fish were placed into two warm acclimation tanks as described in chapter two (mass 33.0 ± 3.3 g, range 24 – 47 g; total length 147.7 ± 5.1 mm, range 120 - 160 mm; condition factor 1.0). There was no significant difference between the weight or the length of the two acclimation groups. Fish were not fed during the acclimation period. Fish survival time at -1 ℃ and 4 ℃ was recorded. This was compared with the survival time of fish used in chapter three and chapter four, (for resting oxygen consumption and prolonged swimming measurements), which were acclimated for one month to either -1 or 4 ℃. The initial

intention was to measure $MO_{2\ rest}$ in both cold and warm acclimated x-cell groups at regular intervals during the acclimation period. However, most warm x-cell individuals died before day 10, so measurements of $MO_{2\ rest}$ were made only on day five and day 12 of the acclimation period (only two warm fish survived to day 12). Oxygen consumption was measured using closed-box respirometry techniques at the acclimation temperature of each group (-1 °C for cold fish; 4 °C for warm fish), as described in chapter three, and briefly outlined here. A single fish was introduced to a respirometer (volume 1 410 – 1 500 ml) 24 hours before oxygen consumption measurements were made. Each respirometer was immersed in an aquarium tank at the acclimation temperature of the fish it contained. A continuous flow of seawater into the respirometer, from the surrounding tank, was maintained using an aquarium pump.

Immediately prior to measurement the aquarium pump was withdrawn from the respirometer. All air bubbles were removed and the respirometer was sealed using a tight fitting respirometer bung. The fish depleted the oxygen in the respirometer for 70 minutes, during which time the oxygen tension of a 1 ml water sample from inside the chamber was measured every 10 minutes. The water sample was injected into the measuring unit of a Strathkelvin oxygen microcell (MC100) which contained an IL 1302 oxygen electrode. The electrode was attached to a Strathkelvin oxygen meter (model 781). Readings were taken two minutes after the injection of the sample to allow the meter time to stabilise. The oxygen meter was calibrated before each experiment using air-saturated seawater at the same acclimation temperature as the experimental fish. The zero level was checked using a solution of 3.81 g L^{-1} sodium borate to which crystalline sodium sulphite was added and partially dissolved. Measurements were ended if the oxygen tension in the respirometer fell below 100 mm Hg before the end of the 70 minutes. Oxygen consumption rates were then determined using the following equation:

$$\frac{\Delta PO_2 \times C \times V \times 31.999}{t \times M}$$

Where:

ΔPO_2 = change in oxygen partial pressure over the measurement period (mm Hg)

C = oxygen capacitance of seawater at a given temperature (μmol L^{-1} mm Hg^{-1})

V = volume of water in the respirometer (L)

31.999 = molecular weight of oxygen

t = duration of measurement (h)

M = mass of fish (g)

The oxygen capacitance value used was 2.31 μmol L^{-1} mm Hg^{-1}.

Long-term acclimation

These experiments were conducted in the temperature controlled facilities at Canterbury University. Fish were collected in McMurdo Sound, transported to New Zealand and housed in the aquarium facilities at Canterbury University, as described in chapter two. Fish were held in the cold aquarium for two months prior to experimental work. Four control fish were randomly selected, weighed, measured and labelled (using coloured suture thread inserted through the dorsal fin) (mass 58.3 ± 8.7, range 37 – 80 g; total length 193.3 ± 10.6 mm, range 164 - 215 mm). These fish were then held in a separate tank within the cold aquarium facilities (0.0 ± 0.3 ℃). Four fish were randomly selected and transferred to the 4 ℃ aquarium (as described in chapter two) (mass 61.2 ± 12.5, range 43 – 96; total length 189.3 ± 11.9 mm, range 167 - 221 mm). These fish were also weighed, measured and labelled with suture thread, at the start of the acclimation period. There was no significant difference between the weight or length of the two acclimation groups. Measurements of $MO_{2\ rest}$ were made on both groups of

fish at regular intervals between day 31 and 158 of the acclimation period. Measurement of oxygen consumption followed the same method described in chapter three, and for x-cell fish (this chapter). Fish were fed immediately after measurement of oxygen consumption, and were not fed again until after the next oxygen consumption measurement was made. In this way, a period of at least 15 days elapsed between feeding and the measurement of oxygen consumption, which minimised the influence of the specific dynamic action (SDA) on oxygen consumption readings.

Prolonged swimming ability was measured in both groups of fish between day 43 and day 169. The method used for measurement of prolonged swimming ability was the same as described in chapter four, and is briefly summarised here. Fish were swum individually in a cylindrical, 80 L Blazka-style swimming tunnel (Fig. 3.1), (Blazka et al. 1960). Before each swimming trial, the swimming tunnel was filled with fully aerated seawater at the appropriate acclimation temperature (-1 °C for cold acclimated fish and 4 °C for warm acclimated fish). The water temperature in the swimming tunnel was monitored throughout the experiment and did not vary by more than 0.3 °C. Fish were initially introduced to the swimming tunnel running at low velocity (14 cm s^{-1}) for a 60 minute settling period. The water velocity was then increased in an incremental fashion (6 cm s^{-1} every 10 minutes) until the fish became exhausted. Following standard protocols, exhaustion was considered to have occurred when a fish could no longer hold its place in the water column, and fell back against the rear mesh of the swimming tunnel (Kolok 1999; Hammill et al. 2004). Once a fish was exhausted, swimming time and swim tunnel velocity were recorded for calculation of Ucrit using the following equation (Brett 1964):

$$Ucrit = Uf + ([Tf/Ti]Ui)$$

Where:

Uf = the highest speed maintained for the entire time increment

Tf = the time taken to reach exhaustion at the final speed interval

Ti = the time interval length

Ui = the speed increment

At the end of the six month acclimation period, fish were weighed and measured and mass and length data were used for the calculation of Fulton's Condition Index:

$$\frac{100 \times \text{mass (g)}}{\text{length}^3 \text{ (cm)}}$$

For comparison, Fulton's Condition Index was also determined for fish used in experiments in Antarctica. This included six fresh fish (72 hours after capture) and fish used in the one month resting oxygen consumption and one month prolonged swimming ability experiments (chapter three and four), and this information is presented along with the results for six month acclimated fish.

Data analysis and statistical methods

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance was taken at the level of $P<0.05$. All data are presented as the mean ± standard error, unless otherwise stated. Weight and length data were compared using unpaired t-tests, and condition index

comparisons were made using one-way analysis of variance (ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. Results for temperature preference, six month resting oxygen consumption rate and prolonged swimming ability were compared using two way analysis of variance (ANOVA), after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. The resting oxygen consumption rate and survival time of x-cell fish was compared using one-way analysis of variance (ANOVA) and post-hoc Bonferroni analyses.

Results

Thermal preference

There was no clear pattern in the final temperatures selected by either cold or warm acclimated *P. borchgrevinki* (Fig. 7.1). Individuals varied in their final selection, with all temperature zones except 2 °C selected on three occasions as the final preference, and 2 °C selected on four occasions. The initial starting temperature had no impact on final temperature preference, although no fish starting in the gradient at 0 °C selected this as their final preferred temperature. Likewise, no fish starting at 4 °C selected this as their final preferred temperature.

When fish were initially placed at the 0 °C end of the thermal gradient, warm acclimated fish demonstrated a very clear preference for spending most time at 0 °C, and cold acclimated fish spent significantly more time swimming than at 1 °C (Fig. 7.2). However, when fish were initially placed into the 4 °C end of the gradient, these patterns were no longer evident, and no particular temperature was favoured by either acclimation group (Fig. 7.3). High levels of inter-individual variation in selected

temperatures were particularly evident when fish were initially placed at the 4 ℃ end of the gradient.

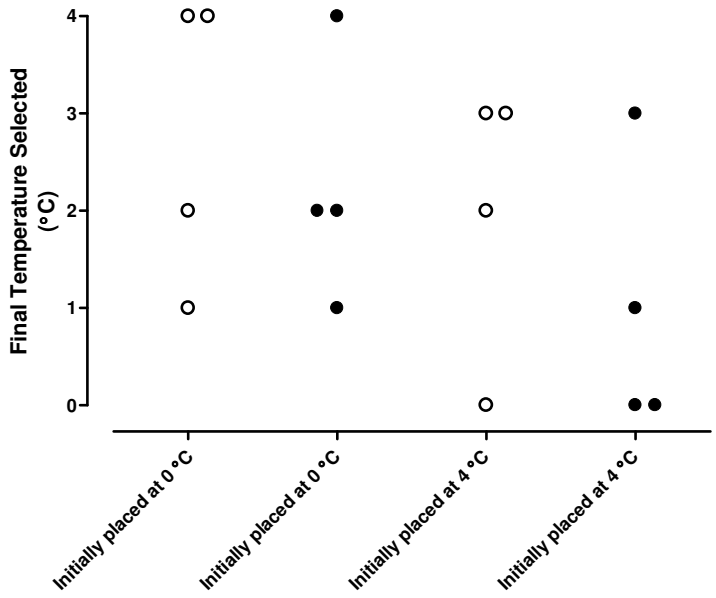

Fig. 7.1. Final temperature selected by individual *Pagothenia borchgrevinki* in a horizontal temperature gradient. ○ Cold acclimated fish (-1 ℃ one month). ● Warm acclimated fish (4 ℃ one month). There was no significant difference between the final temperature selected by either acclimation group, or between the final temperature selected when fish were initially placed at 0 or 4 ℃.

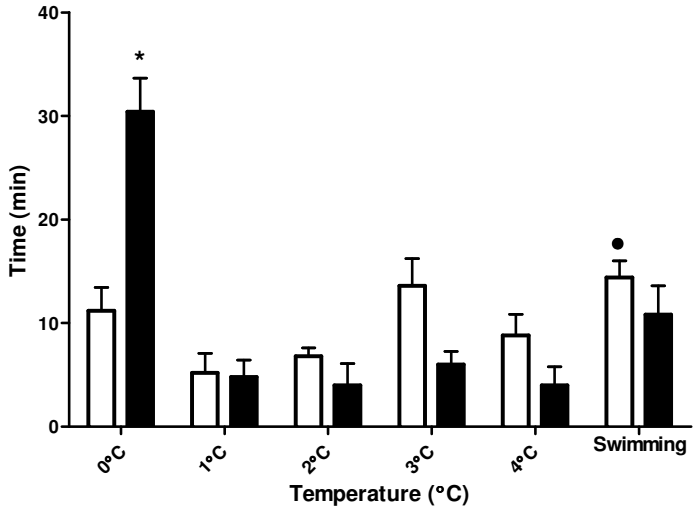

Fig. 7.2. Mean number of minutes spent by cold acclimated (-1 ℃, one month; □) and warm acclimated (4 ℃, one month; ■) *P. borchgrevinki* at different temperatures in a thermal gradient during a 60 minute period. Fish were initially placed at the 0 ℃ end of the gradient. * Significantly different from the amount of time warm acclimated fish spent at any other temperature, or swimming. ● Significantly different from the amount of time cold acclimated fish spent at 1 ℃.

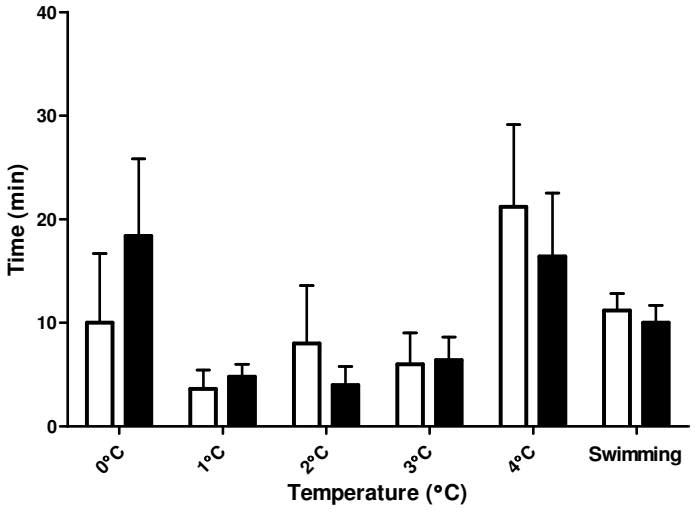

Fig. 7.3. Mean number of minutes spent by cold acclimated (-1 ℃, one month; □) and warm acclimated (4 ℃, one month; ■) *P. borchgrevinki* at different temperatures in a thermal gradient during a 60 minute period. Fish were initially placed at the 4 ℃ end of the gradient. There was no significant difference in the amount of time either group spent at any temperature. No temperature was significantly favoured by individuals within either acclimation group.

X-cell disease

X-cell diseased *P. borchgrevinki* kept at 4 ℃ for five days did not have significantly greater resting oxygen consumption rates ($MO_{2\ rest}$) (50.8 ± 8.9 mg O_2 kg^{-1} h^{-1}) than x-cell fish kept for five days at -1 ℃ (41.2 ± 5.0 mg O_2 kg^{-1} h^{-1}) (Fig. 7.4). The rates of diseased cold fish are higher than those recorded for healthy fish at the same time in the acclimation period. However, diseased fish at 4 ℃ had lower oxygen consumption rates than healthy fish held at this temperature (see chapter three). Only two individuals survived for 12 days at 4 ℃, and the mean $MO_{2\ rest}$ of these two fish was not significantly different from the $MO_{2\ rest}$ of fish at -1 ℃ for 12 days.

All healthy fish (fish used in the oxygen consumption and critical swimming experiments, chapter three and four) survived for the whole of the 28 day acclimation period, in both cold and warm acclimation conditions (Fig. 7.5). Fish with x-cell disease held at -1 ℃ survived for an average of 27 ± 0.3 days, very close to the length of the whole acclimation period. However, x-cell fish exposed to 4 ℃ demonstrated very poor rates of survival. The mean survival time of these fish was only 8.5 ± 2.0 days, which was significantly lower than the survival time of any other group, and the earliest deaths occurred after 3 days at 4 ℃. No individual survived for the whole acclimation period, and the last two individuals died after 17 days at 4 ℃.

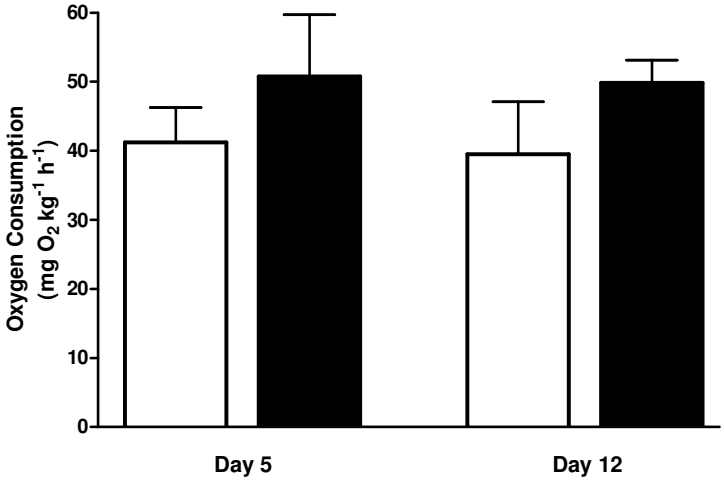

Fig. 7.4. Resting oxygen consumption rate ($MO_{2\ rest}$) of *P. borchgrevinki* with x-cell disease during exposure to cold (-1 ℃, □) and warm (4 ℃, ■) conditions. There was no significant difference in the $MO_{2\ rest}$ of either group on day five or on day 12. The day 12 results of 4 ℃ fish are from two individuals only, as other warm acclimating individuals did not survive to this time.

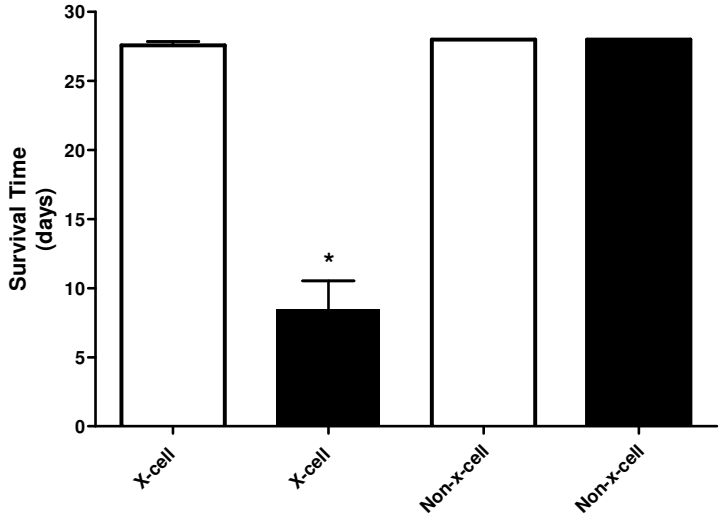

Fig. 7.5. Mean survival time of *P. borchgrevinki* during acclimation to cold (-1 ℃, □) and warm (4℃, ■) conditions. Data for individuals with and without x-cell disease are presented. ***** Survival time that is significantly lower than all other groups. Note, a survival time of 28 days represents the end of the acclimation period. No standard errors are presented for non-x-cell fish as all of these individuals survived for the whole of the acclimation period.

Long-term acclimation

There was no significant change in the mass or length of either cold or warm fish at the end of the six month acclimation period compared with these values at the start of the acclimation period (Fig. 7.6). After six months of acclimation to 4 °C, warm fish mass and length, and warm fish condition index were not significantly different from the mass and length (Fig. 7.6), or condition index (Fig. 7.7) of cold acclimated fish. The condition index of warm, but not cold, acclimated fish (six months) was significantly reduced compared with those of one month cold and warm acclimated fish (fish used in the oxygen consumption and critical swimming experiments, chapter three and four), and also freshly caught fish. Neither acclimation to cold nor to warm conditions for one month significantly altered the condition index compared with fresh caught fish.

The $MO_{2\ rest}$ of fish acclimated for six months to both -1 °C and 4 °C were much more variable than those observed during the one month acclimation period (chapter three). The $MO_{2\ rest}$ of cold acclimated fish was not significantly different from the $MO_{2\ rest}$ recorded for fish acclimated for one month to -1 °C on any day during the six month acclimation period. Similarly, the $MO_{2\ rest}$ recorded for fish during the six month warm acclimation period was not significantly different from the results obtained for fish acclimated for one month to 4 °C. There was no significant difference between the $MO_{2\ rest}$ of cold and warm acclimated fish on any day during the six month acclimation period (Fig. 7.8).

The prolonged swimming ability (Ucrit) of both cold and warm acclimated fish during the six month acclimation period was significantly lower than the Ucrit of fish acclimated for one month to similar conditions (chapter four). For both cold and warm acclimated fish, there was a trend towards decreasing Ucrit throughout the six month acclimation period (for example cold fish Ucrit day 43: 1.41 ± 0.02 bl s^{-1}; day 168: 1.25 ± 0.14 bl s^{-1}), however, because of the high degree of inter-individual variation in Ucrit this trend was not statistically significant. The Ucrit of warm acclimated fish was not significantly different from the Ucrit of cold acclimated fish on any day during the acclimation period.

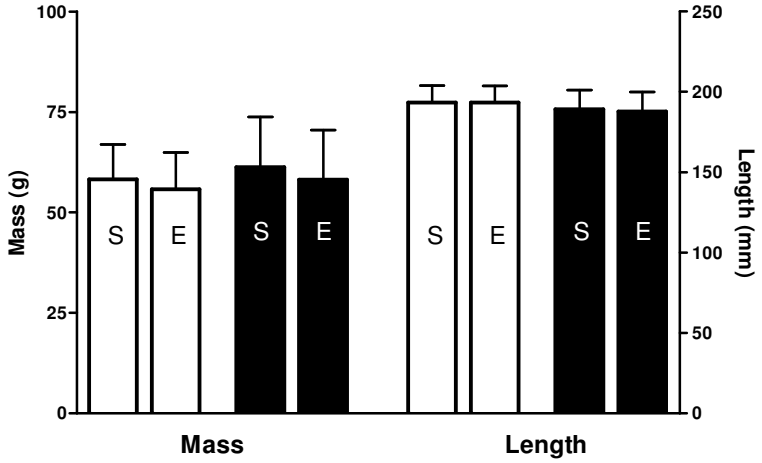

Fig. 7.6. Mass and length of *P. borchgrevinki* at the start (S), and the end (E) of a six month acclimation period. □ Cold acclimated fish (-1 ℃). ■ Warm acclimated fish (4 ℃). There was no significant difference between the mass or length of the two acclimation groups at the start, or at the end of the acclimation period.

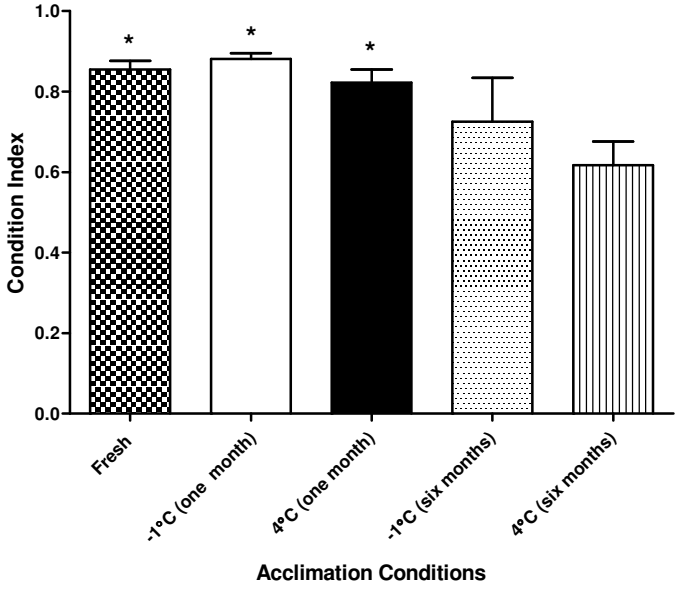

Fig. 7.7. Condition index of *P. borchgrevinki*. Data collected at the end of the acclimation period are presented for 'fresh' (72 hours post-capture) and acclimated (to -1 °C or 4 °C for one or six months) fish. There was no significant difference between the condition index of fish acclimated for six months to cold or warm conditions. There was no significant difference between the condition index of fresh fish and fish acclimated for one month to -1 or 4 °C. * Significantly different from the condition index of warm acclimated fish (six months), but not cold acclimated fish (six months).

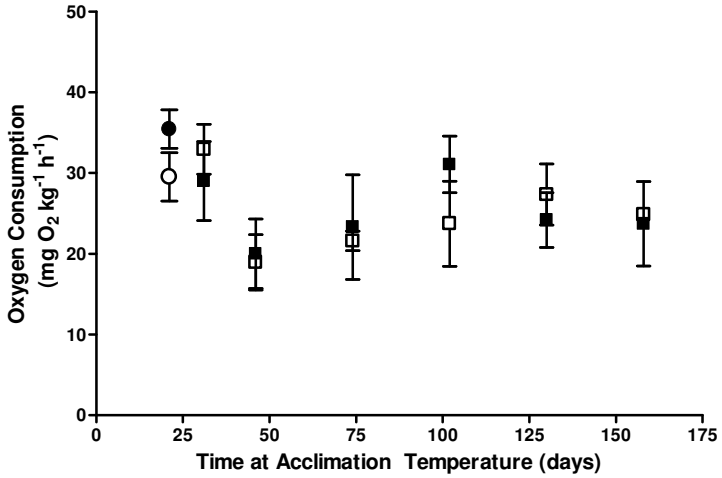

Fig. 7.8. Resting oxygen consumption rate ($MO_{2\ rest}$) of *P. borchgrevinki* acclimated for six months to cold conditions (-1 °C, □) and warm conditions (4 °C, ■). There was no significant difference between the rates of either group on any day during the acclimation period. The resting oxygen consumption rate of a separate group of *P. borchgrevinki* acclimated for 21 days to the same temperatures are included for comparison (○ cold acclimated fish; ● warm acclimated fish). There was no significant difference between the resting oxygen consumption rates of fish acclimated for one month or six months to these temperatures.

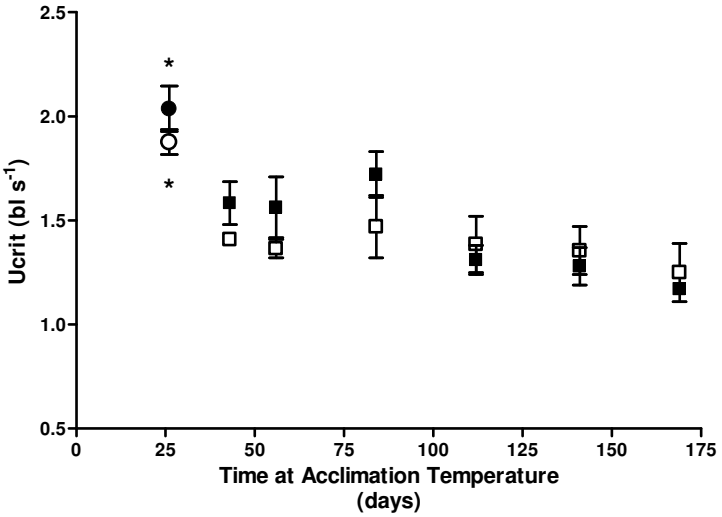

Fig. 7.9. Critical swimming speed (Ucrit) of *P. borchgrevinki* during six months of acclimation to cold (-1 ℃, □) and warm (4 ℃, ■) conditions. There was no significant difference between the Ucrit of either acclimation group on any day during the acclimation period. The Ucrit of a separate group of *P. borchgrevinki* acclimated for 26 days to the same temperatures are included for comparison (○ cold acclimated fish; ● warm acclimated fish). * Significantly greater than the Ucrit of fish during the six month acclimation period.

Discussion

Thermal preference

Research has demonstrated that many species of fish seek out zones of preferred temperatures, a behaviour which is thought to benefit thermoregulation (Weatherley and Gill 1987). The preferred temperature is species-specific, and is linked to the optimum temperature for growth of a species, which is the temperature at which maximally fed fish have the greatest growth rates (Jobling 1981). For example, in laboratory settings brown trout, *Salmo trutta*, consistently prefer to settle at the optimum temperature for growth (Larsson 2005) and roach, *Rutilus rutilus* select temperatures at the high end of their optimum temperature range for growth (van Dijk e al. 2002). Preferred

temperatures are also often linked with the upper lethal temperature of a particular species. For example, Tsuchida (1995) found a highly positive correlation between the final temperature preferendum and the upper lethal temperature of 14 coastal fish species, and this was also apparent in marbled rockfish, *S. marmoratus*, although the physiological mechanisms responsible for this relationship have not been elucidated (Kita et al. 1996). The preferred temperature has also been linked with the temperature at which scope for activity, and thus the ability to do work, is greatest, for example in larval sea lamprey *Petromyzon marinus* (Holmes and Lin 1994).

Within a species, certain environmental factors may also influence the preferred temperature, including salinity, feeding, size and ontogeny, and oxygen concentration. For example, the preferred temperature of the Mayan cichlid, *Cichlasoma urophthalmus*, is influenced by salinity. The final preferred temperature of this species was 39.2 ℃ at 15 ‰ salinity, but only 26.9 ℃ in 30 ‰ salinity (Stauffer and Boltz 1994). In roach, *R. rutilus*, temperature preference is influenced by feeding. Fed fish have a preferred temperature of about 27 ℃, while starved fish have a circadian pattern of temperature preference. At night, starved fish migrate into cooler waters, while in the daytime, they seek warmer water. This behaviour is considered a trade-off between the energetic advantages of cooler water (reduced metabolic rate) and the increased possibility of finding food in warmer waters during daytime hours (van Dijk et al. 2002). In the Atlantic cod, *Gadus morhua*, temperature preference changes ontogenetically with smaller, younger fish selecting higher temperatures than larger, older fish (Lafrance et al. 2005). Oxygen availability also affects temperature selection in the Atlantic cod *G. morhua.* When oxygen availability is low, these fish select cooler temperatures than when oxygen supply is plentiful. Selecting lower temperatures is considered to benefit fish as they undergo a reduction in routine oxygen consumption and a leftward shift in the oxyhaemoglobin dissociation curve (Schurmann and Steffensen 1992).

Macdonald et al. (1987) discussed the results of an unpublished study on the thermal preference of *P. borchgrevinki,* conducted by Macdonald, Montgomery and Paulin at Scott Base. In these experiments fish were exposed to a temperature gradient between

-1.8 and 5.4 ℃, and were shown to consistently select the cold portion of this gradient. Temperature selection was a gradual process, with fish making frequent exploratory swims throughout the gradient before settling at the colder extreme. This behaviour was attributed to thermal stimulation of non-specific peripheral transducers, such as the lateral line, or to central nervous system stimulation by oxygen deficit (created by increased metabolic rate or acceleration of motor activity), as no specific thermal receptors were located.

In the current study, the aim was to determine whether acclimation to 4 ℃ altered this pattern of thermal preference. However, neither cold nor warm acclimated fish in the current study showed a clear pattern of final temperature selection, or any tendency to spend more time in a particular temperature zone within the gradient. While warm acclimated fish did spend a significant portion of time at 0 ℃ if they were initially placed into this zone, this trend was not apparent if fish were initially placed into the 4 ℃ zone of the gradient. A lack of significant results in this study may suggest that *P. borchgrevinki* does not actively select preferred temperatures. Alternatively the result may be the consequence of one (or both) of two problems relating to the design of the horizontal gradient. Space restrictions within the cold aquarium meant that the length of the thermal gradient was limited. Consequently, the range of temperatures that were available within the gradient was also limited, to between 0 and 4.5 ℃, which is less than the -1.8 to 5.4 ℃ range reported by Macdonald et al. (1987). *P. borchgrevinki* may not have actively selected preferred temperatures within this limited thermal range because the temperatures available in the gradient fall within the thermal tolerance zone of this species (Somero and DeVries 1967). The length of the trough also limited the amount of time it was possible to maintain a clearly defined gradient, before horizontal mixing caused water temperature within the gradient to become homogeneous. A suitable gradient could be maintained for around 2.5 hours, so it was decided that a 90 minute experimental period should be used. Many temperature preference experiments, working within warmer gradients, use a longer time period (for example 36 hours used by Lafrance et al. 2005 with *G. morhua*), and it may be that the variable nature of selected temperatures illustrates that individuals were still exploring the

thermal gradient at the end of the 90 minute period, and had not settled at the final temperature recorded, but were merely resting there temporarily. Repeated attempts were made to correct this problem with the horizontal gradient, but these met with limited success. However if a more efficient thermal gradient could be established the results of a repeated study could prove interesting, as personal observations on the behaviour of warm acclimated *P. borchgrevinki* suggested that these animals became restless on re-exposure to -1 °C. The cardiovascular data presented in chapter five supports the hypothesis that warm acclimated fish become agitated on re-exposure to -1 °C. Initial exposure to -1 °C results in a transient, probably stress-related tachycardia in these fish (see chapter five). Critical swimming data also suggest that the function of warm acclimated fish is impaired at -1 °C. At the end of a one month acclimation period, warm acclimated fish swum at -1 °C demonstrate impaired swimming performance compared with their performance at 4 °C and compared with the performance of cold acclimated fish at -1 °C (see chapter four). As the preferred temperature is related to the temperature at which scope for activity is optimal (Holmes and Lin 1994), it seems reasonable to suggest that warm acclimated fish should select a temperature close to 4 °C, the optimal temperature for swimming performance.

X-cell disease

Fish with x-cell disease were not able to acclimate to 4 °C, and had a mean survival time of about eight days at this temperature. Death of x-cell fish at raised temperatures has serious implications for populations of Antarctic fish, in the light of oceanic warming. X-cell disease appears to be widespread among teleost fish in the Ross Sea (Bucke and Everson 1992; Montgomery and Wells 1993; Davison 1998) and the infection rates among populations of *P. borchgrevinki* are high (Davison 1998) with reports of 15 % (Davison and Franklin 2003), 16 % (Franklin and Davison 1988), and up to 44 % (Davison 1998) infection rates in some schools. Infection rates among *P. borchgrevinki* may be high due to increased transmission of the disease resulting from the schooling lifestyle of the species (Davison 1998). Loss of up to 44 % of some populations of *P.*

borchgrevinki may cause significant impacts on population dynamics and food web interactions in the Ross Sea.

The cause of x-cell disease is currently unknown. Miwa et al. (2004) used gene amplification techniques to examine ribosomal RNA from x-cell lesions of the flathead flounder *Hippoglossoides dubius*. These authors suggested that the disease was caused by an unknown protozoan parasite. However, Evans et al. (2000) suggested that pollutants may be significant in the aetiology of the disease in *Trematomus bernacchii*, because the incidence of the disease was higher at a polluted site (Winter Quarters Bay) than a pristine site (Backdoor Bay), in the Ross Sea region. There has also been some suggestion that the x-cell may be a virally transformed cancer cell (Alpers et al. 1977; Desser and Khan 1982). In contrast to its aetiology, the physical effects of the disease have been well documented. In *P. borchgrevinki* the disease has so far only been reported to attack the gill tissue, where the production of x-cells causes swelling of the gill filaments, loss of functional lamellae and a consequent reduction in the surface area available for gas exchange (Franklin and Davison 1988; Davison 1998). X-cell diseased *P. borchgrevinki* have high haematocrits, which may provide a greater blood O_2 carrying capacity, and partly compensate for the loss of gill function (Davison et al. 1990). Fish with x-cell disease also commonly have low condition indexes (Franklin and Davison 1988; Mellergaard and Nielsen 1996; Davison 1998) and this may be associated with increased stress due to the disease and/or a direct effect of the disease (Davison 1998). Research has demonstrated that the low condition indexes are not associated with an inability to catch prey (Mans 2000).

It seems likely that the impairment of oxygen delivery, caused by x-cell infection of *P. borchgrevinki* gills, may affect the ability of x-cell fish to survive and acclimate to 4 °C. In healthy fish, $MO_{2\ rest}$ is elevated during the first five days of exposure to 4 °C, and then later declines (chapter three). Oxygen consumption initially increases to supply the extra demand for oxygen, caused by temperature induced rate elevation of cellular processes. However, in x-cell fish the initial increase in $MO_{2\ rest}$ was not evident. After five days at 4 °C, warm fish oxygen consumption was not significantly different from the

rate of fish at -1 °C. It is probable that the impairment of gill function that is caused by x-cell disease prevents these fish from increasing oxygen uptake at the gills, and in the absence of sufficient oxygen supply, essential cellular processes may be impeded, leading eventually to death of these animals. The hypothesis that diseased fish are unable to increase oxygen uptake during times of heightened oxygen requirement is supported by the work of Davison et al. (1990), who demonstrated that the $VO_{2\ max}$ of infected fish is significantly reduced compared with healthy fish.

Long-term acclimation

Water temperature significantly affects fish growth. In a recent study climatically induced changes in ocean temperature were shown to have affected the growth rates of eight long-lived fish species in the southwest Pacific. In this study otoliths were used to examine changes in annual growth rates during the last century. It was found that increased ocean surface temperatures were correlated with increased growth rates of fish living at depths above 250 m. However, in fish species living at depths greater than 1000 m reductions in growth rates were evident, and this was correlated with cooling at these depths (Thresher et al. 2007). In another example, Cyr et al. (1998) acclimated male Atlantic cod (*G. morhua*) to 2.4 °C and between 6 and 10 °C for 10 months, and found a significant reduction in the body weight and length of cold acclimated fish. Depressed somatic growth in cold acclimated fish occurred because these individuals ate less than warm acclimated fish, even though both groups were provided with equal rations. In the current study, acclimation to 4 °C did not significantly affect the mass or length of *P. borchgrevinki* compared with cold acclimated fish. However, drawing conclusions on the effect of acclimation on the growth rate of this species would be unsound, as there was no significant change in the mass or the length of any fish over the six month acclimation period. Therefore, valid data on the growth rates of the species, and associated acclimation effects would need to be gathered over a longer time period.

Acclimation to 4 ℃ for either one month, or six months did not significantly alter the condition index of warm fish compared with fish kept at -1 ℃ for similar acclimation periods. Long-term exposure to thermally stressful conditions has previously been shown to cause a reduction in condition index of fish (for example Kocovsky and Carline 2001; Muhlia-Almazan et al. 2003). Therefore, the lack of change in the condition index of warm fish may be taken as further evidence that acclimated *P. borchgrevinki* are not experiencing stress at 4 ℃. The condition indexes of both cold and warm acclimated *P. borchgrevinki* (six months) were reduced in comparison with fish held for 72 hours, and fish held for one month in the facilities at Scott Base (although the difference was only statistically significant in warm acclimated fish). This finding indicates that fish transported to New Zealand, held for long periods in captivity, and removed from their normal food source experience a slight decline in condition in comparison with fish used in Antarctica. However time and logistical constraints make it currently impossible for long-term studies, such as described in this chapter, to be undertaken at the Scott Base facilities. Use of the condition index has been criticised by some authors because it does not account for periods of irregular growth, or growth that changes the relationship between mass and length (Cossins and Bowler 1987). Condition index may vary seasonally and/or spatially (Fenaughty 2006). Also, changes in the mass of an individual may reflect a range of changes in its body composition, and may not accurately reflect growth in terms of the energy gain of an individual. For example, deposition of 1 g of lipid leads to a weight gain of 1 g, while deposition of 1 g of protein leads to the deposition of about 4 g of water resulting in a 5 g weight gain (Jobling 1993). However, in situations such as the current study, where the comparison is between groups collected at the same location, in the same season and kept under the same feeding regime, use of the condition index is justified (Weatherley and Gill 1987), and favourable because it is straightforward to perform and non-lethal.

The $MO_{2\ rest}$ of fish acclimated for six months to cold and warm conditions did not change significantly from the rates recorded for fish acclimated for one month to these conditions. Clearly, warm acclimated fish can maintain the changes established during the one month acclimation period long-term, these are not short-term compensatory

mechanisms. Oxygen consumption rates of six month fish displayed more inter-individual variation than one month fish. This may result from the impact of long-distance transport and long-term laboratory confinement. Alternatively, it may be suggested that this variability resulted from the tail-end of the specific dynamic action of feeding (SDA, see chapter three). During the one month acclimation period a no-feeding protocol was followed, but this was clearly not possible during a six month acclimation period. A compromise had to be reached between maintaining fish condition and collecting oxygen consumption data that was free from the influence of SDA, so fish were fed immediately after each measurement of oxygen consumption, ensuring the largest possible gap between feeding and the next measurement. In this way, the shortest interval between feeding and oxygen consumption measurements was 15 days, which should be sufficient to avoid the effects of SDA (Wells 1987; Davison et al. 1990; Johnston and Battram 1993; Boyce and Clarke 1997; Boyce et al. 2000). For example, Johnston and Battram (1993) measured the SDA of the Antarctic notothenioid fish *Notothenia neglecta*, and found this to last around 208 hours in this species. Interestingly, these authors also found that the length and magnitude of the SDA was independent of acclimation temperature.

The prolonged swimming ability of warm acclimated fish during the six month acclimation period was not significantly different from that of cold acclimated fish. This illustrates that the acclimatory changes which act to maintain swimming ability at elevated temperatures are not short-lived compensatory mechanisms, but sustainable long-term changes. However, the prolonged swimming ability of six month acclimated fish, both cold and warm, was significantly reduced compared with fish acclimated for one month to the same conditions. This probably occurred because fish were held for a long period in static tanks, whereas wild fish are constantly swimming. Captive fish may experience a reduction in swimming ability resulting from their sedentary lifestyle within the aquarium tanks, and although this departure from natural conditions is not ideal, studies of this nature would not be possible except in a laboratory setting.

Summary

There was no obvious pattern in the temperature preference of *P. borchgrevinki* acclimated to cold conditions. Therefore, it was not clear what effect acclimation to 4 ℃ has (if any) on the temperature preference of this species. Further studies with a modified thermal gradient would be beneficial. *P. borchgrevinki* with x-cell disease were not able to acclimate to 4 ℃, unlike healthy members of this species. This finding is concerning given that high proportions of *P. borchgrevinki* populations are infected with the disease. Finally, long-term exposure to 4 ℃ was sustainable for *P. borchgrevinki.* There was no change in the condition index of this species compared with cold acclimated individuals, and $MO_{2\ rest}$ and Ucrit were not significantly different from those of cold acclimated individuals at any time during the acclimation period.

Chapter Eight

The acclimatory ability of three Antarctic trematomids: *Trematomus bernacchii*, *T. hansoni* and *T. pennellii*

Introduction

Previous chapters have demonstrated that the Antarctic notothenid fish *Pagothenia borchgrevinki* can acclimate to 4 ℃. However, this species, with its active, cryopelagic lifestyle, is unusual among the notothenids which are mainly sluggish bottom-dwellers (Gon and Heemstra 1990), so it is possible that the close relatives of *P. borchgrevinki* may not share its acclimatory ability. Therefore, the effects of long-term exposure to 4 ℃ were investigated in three different Antarctic species, from the genus *Trematomus* (*T. bernacchii*, *T. hansoni* and *T. pennellii*), the benthic lifestyles of which are more typical among the notothenids (see chapter one). Previous studies have held *T. bernacchii* for several weeks at 4 ℃, though most of these studies provide no information on fish survival rate at this temperature (Somero et al. 1968; Gonzalez-Cabrera et al. 1995; Evans et al. 1999; Hofmann et al. 2000; Guynn et al. 2002; Ream et al. 2003; Brauer et al. 2005; Petzel 2005; Podrabsky and Somero 2006). In studies which present survival data survival rates generally seem very low, for example Weinstein and Somero (1998) studied mitochondrial function in *T. bernacchii* kept at 4 ℃ for two weeks. At the end of this period 15 cold acclimated individuals were sampled (acclimated to -1.86 ℃), compared with only five warm

acclimated individuals (acclimated to 4 ℃). Carpenter and Hofmann (2000) also noted the death of four out of seven individuals after 21 days of exposure to 4 ℃. True acclimation changes by definition are those which act to maintain the ability of an organism to carry out essential biological processes such as predator escape, reproduction and prey capture (Hammill et al. 2004). However, the lack of information on survival rates of fish at 4 ℃ makes it impossible to determine whether true acclimation changes take place during the experimental periods employed, or if instead individuals which survive until the end of this time are merely tolerating exposure to the new temperature.

Most previous acclimation studies on *T. bernacchii* have investigated the impact of long-term exposure to 4 ℃ on plasma osmolality. Like other notothenioids, *T. bernacchii* have high serum osmolalitites of around 580 mOsm kg^{-1} (Morrison et al. 2006). Maintaining a high serum osmolality is advantageous for Antarctic fish because it depresses the freezing point of the blood (Somero and DeVries 1967), and also decreases the energy requirement for upholding an ionic gradient between the blood and the seawater (Prosser et al. 1970). However, acclimation to 4 ℃ has been shown to reduce the plasma osmolality of *T. bernacchii* (Gonzalez-Cabrera et al. 1995; Evans et al. 1999; Guynn et al. 2002; Morrison et al. 2006). The magnitude of the reported change in osmolality varies, for example an 11 % decrease in serum osmolality was reported by Petzel (2005), while Morrison et al. (2006) noted a 25 % decrease in serum osmolality during an acclimation period of the same length. Changes in osmolality occur during the first seven days of acclimation and are reversible when fish are returned to -1.5 ℃ (Gonzalez-Cabrera et al. 1995). Most of the reduction in plasma osmolality results from a decrease in plasma sodium and plasma chloride concentrations (Gonzalez-Cabrera et al. 1995; Petzel 2005). Changes in plasma osmolality are

accompanied by an increase in the activity of gill Na^+,K^+-ATPase (Gonzalez-Cabrera et al. 1995; Guynn et al. 2002; Morrison et al. 2006). Na^+,K^+-ATPase is an integral membrane protein which uses energy from ATP to transport Na^+ out of the cell and K^+ into the cell (Jorgensen and Andersen 1988), creating an electrochemical gradient that is important in maintaining the osmotic balance and resting membrane potential of cells (Blanco and Mercer 1998). The Na^+,K^+-ATPase also provides the potential energy for coupled transport of Na^+ and Cl^-, the mechanism which is used by marine teleosts to excrete excess Na^+ and Cl^- (Karnaky 1986). Increased Na^+,K^+-ATPase activity has been attributed to changes in its conformation, and in particular the number and type of α subunits present (Brauer et al. 2005). According to Morrison et al. (2006), warm acclimation causes the number of α1 and α2 subunits to increase, while the number of α3 subunits decrease, and this results in increased activity of the Na^+,K^+-ATPase. Warm acclimation also increases the drinking rates of *T. bernacchii*, and this is thought to be important in replacing water lost osmotically to seawater, as a result of the increased osmotic gradient between blood and seawater (Petzel 2005). Both the underlying stimulus for changes in osmolality, and the ecological significance of these changes are currently unclear.

There is less information available on the ability of *T. hansoni* and *T. pennellii* to acclimate to temperature change, although, a recent study demonstrated that acclimation of *T. pennellii* to 4 °C for eight weeks results in increased thermal tolerance, measured as survival time at 14 °C. Interestingly, *T. pennellii* was also significantly more tolerant of acute exposure to 14 °C than other notothenioid species, including *P. borchgrevinki*, *T. bernacchii* and *T. hansoni* (Podrabsky and Somero 2006).

The aim of the current work was to determine whether the three study species (*T. bernacchii*, *T. hansoni* and *T. pennellii*) possess the same ability to acclimate to 4 °C during a one month acclimation period as is displayed by the closely related *P. borchgrevinki*. Mean survival time and resting oxygen consumption rate at 4 °C were measured in all three species. In addition, measurements of plasma osmolality, plasma sodium and plasma chloride concentration as well as several blood parameters (glucose concentration, total haemoglobin concentration, haematocrit and mean corpuscular haemoglobin content) were measured in *T. bernacchii*.

Materials and Methods

These experiments were conducted at the Scott Base Wet Laboratory facility. All three species were collected in McMurdo Sound, and then separated into warm and cold acclimation groups as described in chapter two. For *T. bernacchii* six individuals were initially placed into a -1.0 ± 0.3 °C tank (mass 107.8 ± 27.2 g, range 31.4 – 267.4 g; total length 190.3 ± 12.6 mm, range 135 - 258 mm), and six were placed into a 4.0 ± 0.3 °C (mass 72.9 ± 12.8 g, range 36.4 – 134.3 g; total length 174.6 ± 9.2 mm, range 140 - 201 mm). *T. hansoni* and *T. pennellii* were not as readily available as *T. bernacchii*, so sample sizes were smaller for these species. For *T. hansoni* three cold fish (mass 97.1 ± 7.5 g, range 87.0 – 111.8 g; total length 224.3 ± 15 mm, range 198 - 250 mm), and three warm fish (mass 123.3 ± 9.3 g, range 109.6 – 140.9 g; total length 227.7 ± 4.3 mm, range 220 - 235 mm) were used in experiments. For *T. pennellii* three cold fish (mass 76.4 ± 12.9 g, range 51.2 – 93.7 g; total length 184.7 ± 10.2 mm, range 165 - 199 mm) and six warm fish (mass 46.7 ± 9.2 g, range 36.2 – 64.9 g; total length

148.3 ± 7.3 mm, range 135 - 160 mm) were used in experiments. For *T. bernacchii* and *T. hansoni*, there was no significant difference between the mass or the length of cold and warm fish. The length of cold *T. pennellii* was significantly greater than the length of warm fish, but there was no significant difference in the mass of these two groups.

The survival time of both cold and warm fish was recorded, and tanks were checked regularly, several times a day for the presence of dead or dying fish. Survival time was measured in complete days (24 hours) only, and death was considered to have occurred when fish lost equilibrium, could not be stimulated into movement and ceased to ventilate for a period of 15 minutes. Cessation of ventilation usually followed shortly after loss of equilibrium.

Measurements of resting oxygen consumption rates were made using the closed-box respirometry techniques described in chapter three. Measurements were consistently made in the morning to avoid possible complications caused by diel cycling of oxygen consumption, although Wells (1987) found no evidence for diel cycling in nine species of fish from McMurdo Sound. The initial intention was to measure oxygen consumption rate at regular intervals throughout the acclimation period in both cold and warm fish. However, limited survival of 4 °C fish made this impossible, so data were only collected for as long as warm individuals were available.

Blood samples were collected from *T. bernacchii* using the technique described in chapter two, and were transported to New Zealand for analysis. Cold acclimated fish were sampled after 28 days at -1 °C (n=5 samples as one fish died on day 26 of the acclimation period). On day six of the acclimation period,

only three of the six initial 4 ℃ fish had survived and all of these remaining fish began to show signs of loss of equilibrium. These fish were watched closely and blood samples were taken immediately after their death on day six. The osmolality of 8 µl plasma aliquots was measured using a Wescor 5100 C vapour pressure osmometer. The osmometer was calibrated prior to sampling using standard solutions. Plasma sodium concentration was measured using a Sherwood flame photometer and a calibration curve plotted using standard solutions. Chloride concentration of 10 µl plasma aliquots was determined using a Radiometer CMT10 chloride meter. The chloride meter was calibrated prior to use with standard solutions and the supporting electrolyte was changed after every ten samples. Total haemoglobin concentration ([Hb]) was determined from a 5 µl sample of whole blood using the cyanmethaemoglobin method (Sigma diagnostics kit 525), described in chapter six. Haematocrit (Hct) was determined at Scott Base as part of the sampling procedure described in chapter two. Mean corpuscular haemoglobin content (MCHC) was calculated as total haemoglobin concentration / fractional haematocrit.

Plasma glucose concentration was determined using the glucose oxidase / peroxidase enzymatic method (Sigma diagnostics kit 510), described in chapter six.

Data analysis and statistical methods

Statistical analysis was carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance was taken at the level of $P< 0.05$. All data are presented as the mean ± standard error. Fish mass and length were compared using unpaired t-tests. Survival

rates and oxygen consumption data were compared using one-way analysis of variance (ANOVA) after establishing homogeneity of variance using Bartlett's Test. Where a treatment effect was indicated, post-hoc Bonferroni analyses were carried out. Osmolality and haematology data was compared using unpaired t-tests.

Results

Survival at 4 °C

In all three *Trematomus* species, survival time at 4 °C was significantly shorter than at -1 °C. All cold acclimated *T. hansoni* and *T. pennellii*, and all but one *T. bernacchii* survived for the whole 28 day acclimation period. However, the mean survival time of fish at 4 °C was only 5 ± 0.5 days for *T. bernacchii* and 14 days for *T. hansoni* (14 ± 5) and *T. pennellii* (14 ± 3.6). The first fish deaths occurred after one day in *T. pennellii*, three days in *T. bernacchii* and eight days in *T. hansoni*. No warm *T. bernacchii* or *T. hansoni* survived for the whole of the acclimation period, and only one *T. pennellii* survived for the whole 28 days (Fig. 8.1).

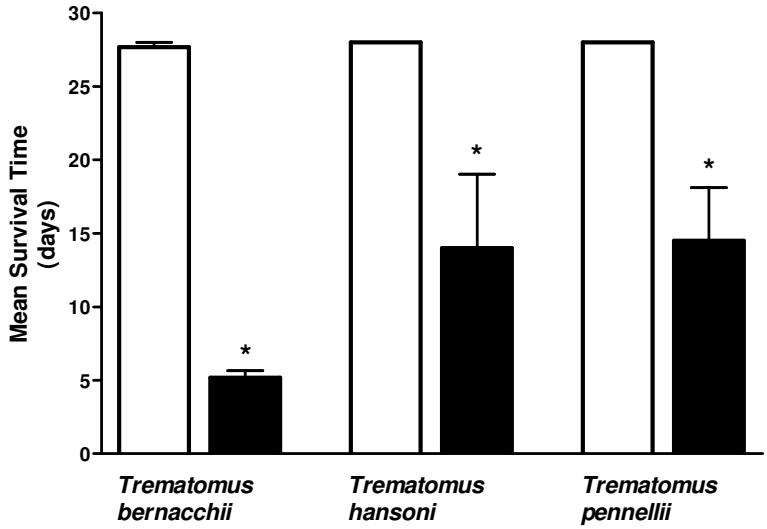

Fig. 8.1. Mean survival time of three *Trematomus* species (*bernacchii*, *hansoni* and *pennellii*) held in cold (-1 ℃; □) and warm (4 ℃; ■) conditions. * Significantly different from the mean survival time of the same species in cold conditions. No error bars are shown for cold acclimated *T. hansoni* or *T. pennellii* as all fish survived for the whole of the 28 day acclimation period.

Oxygen Consumption

All fish remained very still within the respirometers and adopted a tripod stance using the pelvic and anal fins, as described by Wells (1987), and similar to what is observed in the natural environment. *T. bernacchii* resting oxygen consumption rate ($MO_{2\ rest}$) was measured on day one and day five of the acclimation period. Cold acclimated fish on day one had an $MO_{2\ rest}$ of 45.8 ± 6.9 mg O_2 kg^{-1} h^{-1}, and this was not statistically different from the rate of the same fish on day five of the acclimation period (49.9 ± 8.8 mg O_2 kg^{-1} h^{-1}). Fish exposed to 4 ℃ had an MO_{2rest} of 35.3 ± 7.3 mg O_2 kg^{-1} h^{-1} on day one (24 hours

after exposure to 4 ℃), and this was not significantly different from the rate of cold acclimated fish on day one of the acclimation period. By day five only five 4 ℃ fish had survived, and two of these fish died approximately three hours after oxygen consumption measurements were made. Consequently, there was a high level of inter-individual variation in the results (and no statistical significance), however $MO_{2\ rest}$ had risen to 65.7 ± 16.3 mg O_2 kg^{-1} h^{-1} by day five in these fish (Fig. 8.2).

Oxygen consumption was measured on day two, ten and 16 of the acclimation period for *T. hansoni*. Cold acclimated fish had an $MO_{2\ rest}$ of between 35 to 42 mg O_2 kg^{-1} h^{-1} on these days. On day two of the acclimation period, the $MO_{2\ rest}$ of fish at 4 ℃ (33.4 ± 4.5 mg O_2 kg^{-1} h^{-1}) was not significantly different from that of cold acclimated fish. By day ten, only one 4 ℃ *T. hansoni* had survived, so data for $MO_{2\ rest}$ on day ten and day 16 were obtained from this individual alone. The $MO_{2\ rest}$ of this fish was similar to the $MO_{2\ rest}$ of cold acclimated fish on day ten and on day 16 (Fig. 8.3), although the statistical significance of these results is difficult to determine, due to the limited sample size of the 4 ℃ fish.

The $MO_{2\ rest}$ of *T. pennellii* was measured on day two, 16 and 24 of the acclimation period. Rates of cold acclimated fish were very stable for the whole of the acclimation period, at close to 31 mg O_2 kg^{-1} h^{-1}. The rate of 4 ℃ fish on day two (65.5 ± 10 mg O_2 kg^{-1} h^{-1}) was more than double the rate of cold acclimated fish on day two. By day 16 (n=4), warm acclimated fish demonstrated a reduction in $MO_{2\ rest}$, to 47.6 ± 8.3 mg O_2 kg^{-1} h^{-1}, which was not significantly different from the rate of cold acclimated fish at this time in the acclimation period. However, by day 24 only one 4 ℃ *T. pennellii* had survived, and interestingly, the $MO_{2\ rest}$ of this fish (which survived until beyond day 28 of the

acclimation period) was very similar to that recorded for cold acclimated fish on day 24 (4℃ fish 31.5 ± 0.0 mg O_2 kg^{-1} h^{-1}; -1 ℃ fish 31.3 ± 2.3 mg O_2 kg^{-1} h^{-1}) (Fig. 8.4).

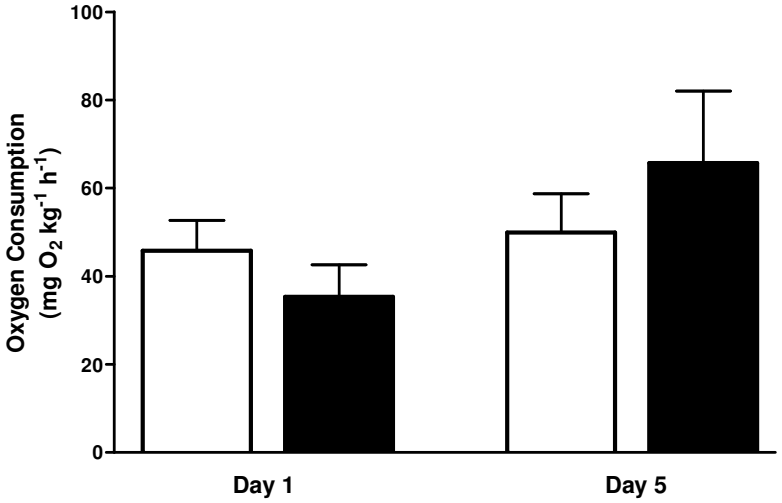

Fig. 8.2. Oxygen consumption at rest ($MO_{2\,rest}$) of *T. bernacchii* after one and five days at -1℃ (□) and 4 ℃ (■). $MO_{2\,rest}$ was not significantly different in either group on day one, or on day five.

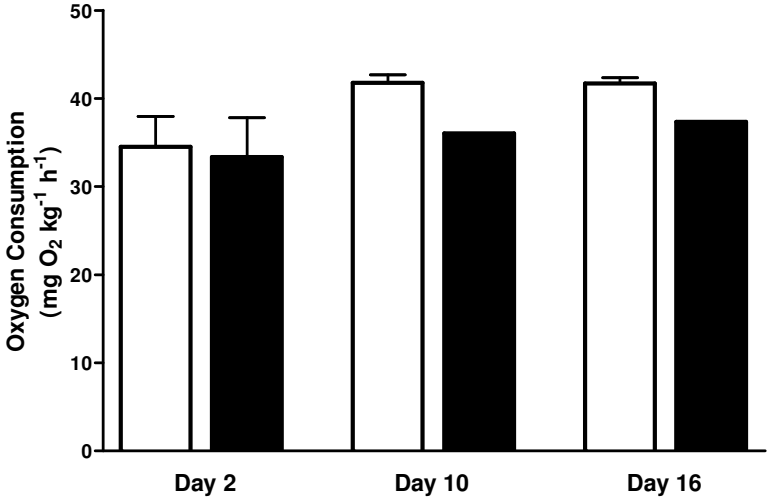

Fig. 8.3. Oxygen consumption at rest ($MO_{2\,rest}$) of *T. hansoni* after two, ten and 16 days at

-1 °C (□) and 4 °C (■). $MO_{2\ rest}$ was not significantly different in either group on any day. Note that the results for $MO_{2\ rest}$ of fish at 4 °C on day ten and 16 are from one fish only, as only one fish survived more than ten days at this temperature.

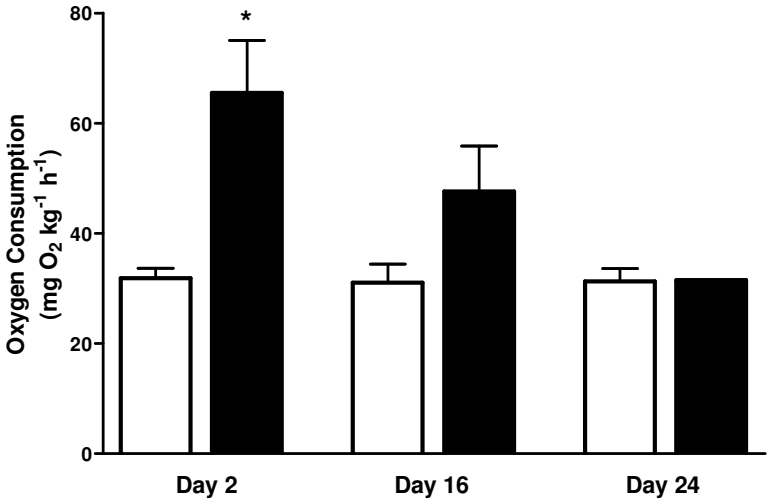

Fig. 8.4. Oxygen consumption at rest ($MO_{2\ rest}$) of *T. pennellii* after two, 16 and 24 days at -1 °C (□) and 4 °C (■). * Significantly different from the $MO_{2\ rest}$ of day two *T. pennellii* at -1 °C. Note that the results for $MO_{2\ rest}$ of fish at 4 °C on day 24 are from one fish only, as only one fish survived more than 16 days at this temperature.

Osmolality and Blood Parameters

The plasma osmolality of cold acclimated *T. bernacchii* was 537.5 ± 6.1 mmol l^{-1}, which was not significantly different from the plasma osmolality of fish held for six days at 4 °C (535.3 ± 6.6 mmol l^{-1}). This is reflected in the plasma sodium and plasma chloride concentrations, which are not significantly different in cold acclimated fish compared with 4 °C fish (Fig. 8.5).

The plasma glucose concentration of fish held at 4 ℃ for six days was over double that found in cold acclimated *T. bernacchii* (-1 ℃, 5.1 ± 0.4 mmol l^{-1}; 4 ℃ 14.1 ± 0.5 mmol l^{-1}) (Fig. 8.6). However, there were no other significant changes in the blood parameters of 4 ℃ fish compared with cold acclimated fish (Fig. 8.7, total haemoglobin concentration; Fig. 8.8, haematocrit; Fig 8.9, mean corpuscular haemoglobin content).

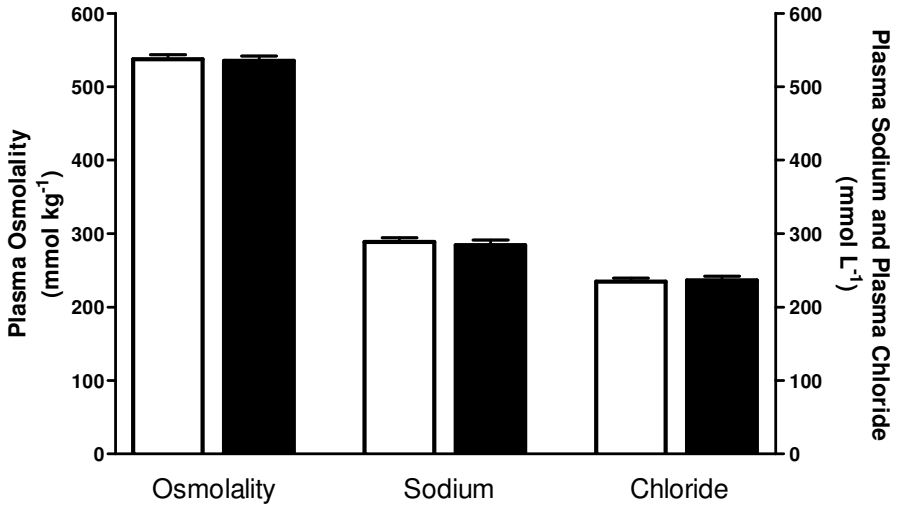

Fig. 8.5. Plasma osmolality, sodium and chloride concentration of *T. bernacchii.* □ Fish at -1 ℃ (28 days). ■ Fish at 4 ℃ (6 days).

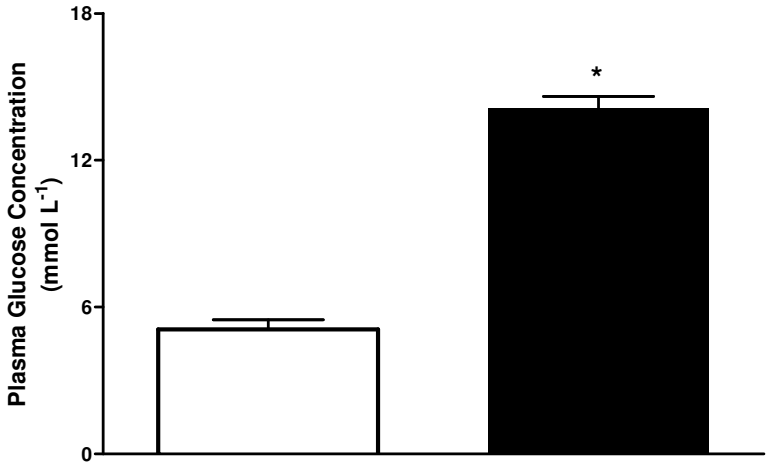

Fig. 8.6. Plasma glucose concentration of *T. bernacchii* held at -1 ℃ (□; 28 days) and 4 ℃ (■; 6 days). * Significantly different from the plasma glucose concentration of fish held at -1 ℃.

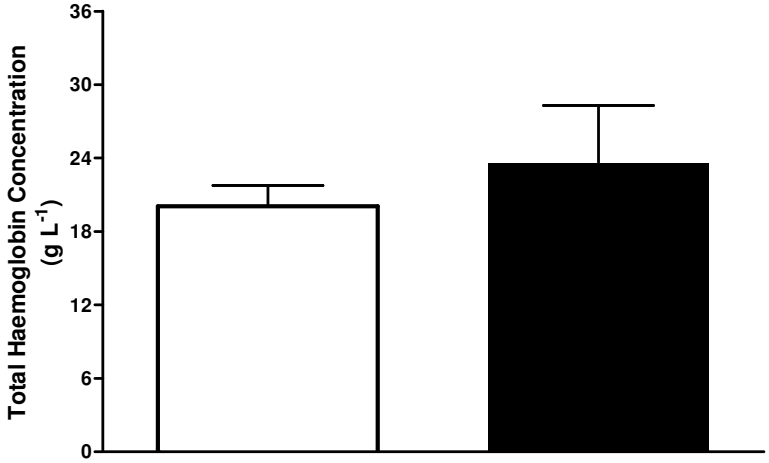

Fig. 8.7. Total haemoglobin concentration of *T. bernacchii* held at -1 ℃ (□; 28 days) and 4 ℃ (■; 6 days). There was no significant difference between the total haemoglobin concentration of -1 ℃ fish and 4 ℃ fish.

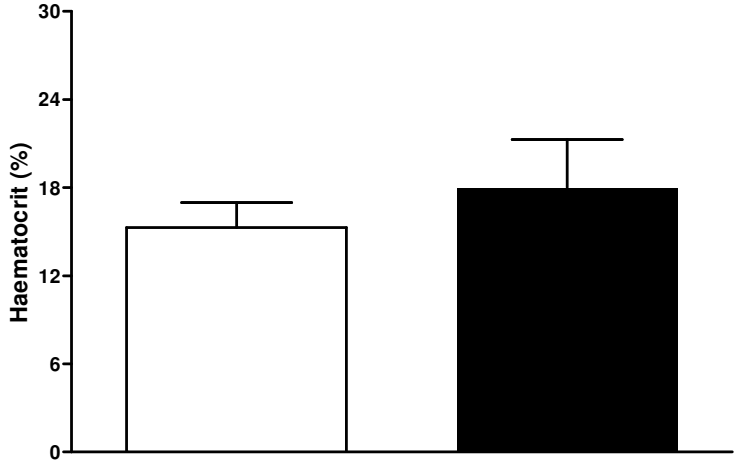

Fig. 8.8. Haematocrit of *T. bernacchii* held at -1 ℃ (□; 28 days) and 4 ℃ (■; 6 days). There was no significant difference between the haematocrit of -1 ℃ fish and 4 ℃ fish.

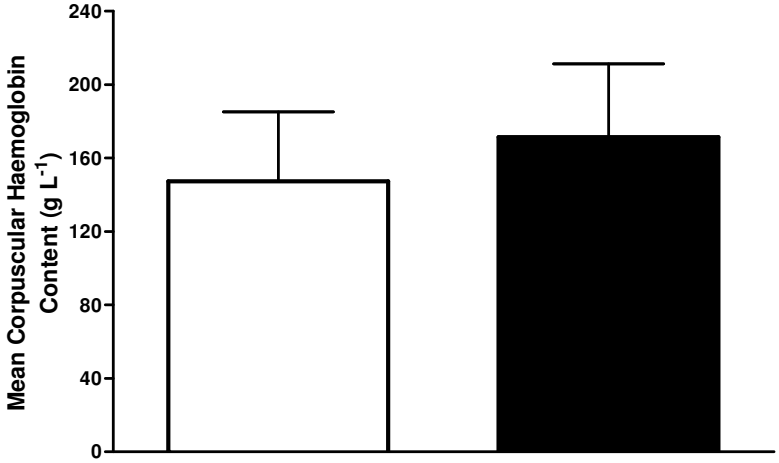

Fig. 8.9. Mean corpuscular haemoglobin content (MCHC) of *T. bernacchii* held at -1 ℃ (□) and 4 ℃ (■). There was no significant difference between the total haemoglobin concentration of -1 ℃ fish and 4 ℃ fish.

Discussion

The limited survival of fish at 4 ℃ clearly demonstrates that *T. bernacchii*, *T. hansoni* and *T. pennellii* are not capable of acclimating to this temperature. Only one fish survived for the whole 28 day acclimation period at 4 ℃, with most surviving for less than ten days. Fish death was preceded by fish becoming disorientated in the water column, floating upside down or on the side as well as an inability to sustain activity, even when provoked, which are similar characteristics to those reported by Podrabsky and Somero (2006) for death of *T. bernacchii* and *T. pennellii* during acute exposure to 14 ℃. The thermal tolerance zone of fish has been defined as the range of temperatures where 50 % of the population could theoretically survive indefinitely (Jobling 1981). With death of 14 out of 15 fish placed at 4 ℃, this temperature can not be considered to fall within the thermal tolerance zone of any of these three species, and this agrees well with early studies on the upper lethal temperature of notothenioids by Wohlschlag (1964) and Somero and DeVries (1967).

The physiological processes which lead to thermal death in fish have not been firmly established. In invertebrates, critical temperatures are characterised by hypoxia, followed by the initiation of anaerobic metabolism (Pörtner et al. 1998). For example, at low critical temperatures lugworm (*Arenicola marina*) ventilation is slowed and oxygen supply to the tissues is reduced, resulting in hypoxia and eventually death (Sommers et al. 1997). This also occurs at low temperatures in the sipunculid worm *Sipunculus nudus* (Zielinski and Pörtner 1996). Research on thermal limits in the temperate eelpout *Zoarces viviparous* and the Antarctic eelpout *Pachycara brachycephalum* suggests that impairment of oxygen supply

may also be the ultimate cause of heat death in fish (van Dijk et al. 1999). In both of these species, anaerobic end products accumulate in the tissues as the upper critical temperature is approached. While this build up could be attributed to mitochondrial malfunction, this is unlikely as eelpout mitochondria have been shown to function at temperatures well above the critical temperatures of these species (Weinstein and Somero 1998; Hardewig et al. 1999a). Van Dijk et al. (1999) therefore concluded that the accumulation of anaerobic by-products was most likely a result of insufficient oxygen supply at elevated temperatures, which may result from failure of respiration, or poor blood circulation or a combination of these factors. Similarly, Mark et al. (2002) attributed heat death of Antarctic fish to the failure of ventilatory and circulatory organs to meet increased metabolic demands for oxygen at elevated temperatures. At raised temperatures, fish may be caught in what the authors termed a 'vicious circle of ever increasing oxygen deficiency', where respiration and circulation systems speed up to increase oxygen supply to the tissues, which in turn further elevates the requirement for oxygen delivery.

It seems likely that oxygen deficiency may be the ultimate cause of death at 4 °C in the three species studied. The resting oxygen consumption rate of -1 °C *T. bernacchii* was between 45 and 49 mg O_2 kg^{-1} h^{-1}, which compares well with the results of Wells (1987). Exposure of *T. bernacchii* to 4 °C for one day did not result in a significant increase in resting oxygen consumption rate, even though this elevated temperature should result in increased metabolic costs of maintenance (Cossins and Bowler 1987). While some species show a thermal independence of oxygen consumption in the mid-part of their temperature range, this is unlikely to be the case here, as 4 °C is very close to the upper thermal limit of this species (Somero and DeVries 1967; Guynn et al. 2002). There was

an increase in $MO_{2\ rest}$ on day five, though this change was not statistically significant due to the small sample size, caused by fish deaths, and high levels of inter-individual variability. It is possible that *T. bernacchii* was not able to raise its rate of oxygen consumption sufficiently to meet the increased requirements for oxygen at 4 °C. A similar pattern was evident in *T. hansoni*, where there was no significant increase in oxygen consumption rate after two days at 4 °C, compared with fish held at -1 °C for two days. If oxygen supply becomes limiting at 4 °C in these species, then a build-up of anaerobic by-products would become evident prior to death, measurement of these by-products would be an interesting path for further study.

In *T. pennellii*, exposure to 4 °C resulted in a different pattern of oxygen consumption than that observed in the other two species. After two days at 4 °C, $MO_{2\ rest}$ was elevated in 4 °C fish compared with -1 °C fish. However, after 16 days at this temperature, the $MO_{2\ rest}$ of 4 °C fish had fallen and was much closer to that of -1 °C fish. This pattern is similar to that described for *P. borchgrevinki* in chapter three and seemed to suggest that fish were beginning to acclimate to 4 °C, though clearly the acclimation changes either took place too slowly, or were not adequate, as after day 16 only one of the initial six fish placed at 4 °C had survived. This one surviving fish successfully completed the acclimation period and after 24 days at 4 °C had an $MO_{2\ rest}$ that was almost exactly the same as fish acclimated to -1 °C. Perhaps, in this species at least, a small number of individuals possess genetic information which may provide them with a selective advantage at raised temperatures, an interesting concept when considering the possible impacts of oceanic warming for this species.

Failure of *T. bernacchii* to acclimate to 4 °C seems to contradict the results of several earlier studies, in which *T. bernacchii* was reportedly held for several weeks at 4 °C. However in these studies, the number of individuals initially placed at 4 °C compared with those remaining at the end of the prescribed period are seldom reported (Evans et al. 1999; Hofmann et al. 2000; Guynn et al. 2002; Ream et al. 2003; Brauer et al. 2005; Petzel 2005; Podrabsky and Somero 2006), and where this information is presented, survival rates are low (Gonzalez-Cabrera et al. 1995; Weinstein and Somero 1998; Carpenter and Hofmann 2000). Results from biochemical studies on this species also suggest that while a limited number of individuals may survive long-term exposure to 4 °C, truly adaptive acclimation changes do not occur during this time. For example work on *T. bernacchii* liver mitochondria demonstrated what the authors termed the high level of cold adaptation and stenothermy of this species (Weinstein and Somero 1998). *T. bernacchii* have the lowest mitochondrial Arrhenius Break Temperatures (ABTs) ever reported. The ABT is the temperature at which discontinuities in Arrhenius plots occur, thus indicating significant disruption of mitochondrial function. Holding *T. bernacchii* at 4 °C for two weeks does not significantly alter the ABT, revealing a failure of mitochondrial respiration to acclimate to 4 °C (Weinstein and Somero 1998). However, it is important to note here, that the work of other authors (particularly Johnston et al. 1994; Johnston et al. 1998 and Johnston 2003), suggests that changes in mitochondrial abundance, rather than mitochondrial function, are important in thermal adaptation. For example, Antarctic notothenioid fish show no up-regulation of mitochondrial oxygen consumption relative to temperate and tropical species, but there is an increase in the mitochondrial content of slow muscle fibres (Johnston 2003). Hence, it is possible that acclimation of *T. bernacchii* mitochondria to 4 °C may be brought about by a change in mitochondrial content, rather than a

change in the ABT. The keratocytes of *T. bernacchii* also demonstrate an inability to acclimate to 4 ℃. Keratocytes are motile cells which assist wound healing in fish by migrating across wounded areas and preventing infection. The motility of these cells is temperature-dependent, with a general tendency for keratocyte speed to increase as temperature increases. However, there is an optimal speed for keratocyte movement which varies between species. Work by Ream et al. (2003) demonstrated that *T. bernacchii* could not compensate mean keratocyte movement following long-term exposure to 4 ℃.

The work of Carpenter and Hofmann (2000) on heat shock proteins provides further evidence that *T. bernacchii* is not truly able to acclimate to 4 ℃, but rather experiences thermal stress at this temperature. Heat shock proteins (Hsp) are important molecular chaperones that assist with protein folding and biosynthesis, but do not make up part of the final protein product. There are many types of Hsps, some are constitutively expressed when an animal is in a resting state, while others are expressed in response to stress. Generally, Hsps are expressed in response to heat stress when the temperature rises 5 to 10 ℃ above the optimal temperature for growth of an organism. These heat induced chaperones bind to thermally denatured proteins until normal body temperature is restored, thus allowing the denatured proteins to refold into their functional states (Hofmann et al. 2000). After 21 days at 4 ℃, the endogenous levels of one of these heat shock proteins (*hsp70*) is significantly elevated in *T. bernacchii*, indicating that these animals experience thermal stress at this temperature. However, despite the elevation in *hsp70*, *T. bernacchii* do not seem to gain sufficient protection from thermal injury, with over 50 % mortality of 4 ℃ fish reported in this study (Carpenter and Hofmann 2000). In contrast to long term exposure to elevated temperatures, acute thermal shock does not lead to an

elevation in *hsp70*, a finding that has been attributed to the constitutive expression of the *hsp70* gene in *T. bernacchii* (Buckley et al. 2004).

The poor survival rates of the three species in the current study meant that collection of haematology data was very difficult. Ideally, *T. bernacchii* blood samples would have been collected from both cold and warm acclimated fish at the end of the 28 day acclimation period. However, early death of 4°C fish made this impossible, so that blood samples were collected from only three fish following six days of exposure to 4 °C. The plasma osmolality and sodium and chloride concentrations measured in cold acclimated fish are slightly lower than recorded in some previous studies (Davison et al. 1994; Davison et al. 1995; Guynn et al. 2002). After six days at 4 °C, no significant difference in the plasma osmolality of *T. bernacchii* was evident compared with fish at -1 °C. It is likely that the six day exposure time was not sufficient to observe the hypo-osmolality described by other authors for *T. bernacchii* at 4 °C (Gonzalez-Cabrera et al. 1995; Evans et al. 1999; Guynn et al. 2002; Brauer et al. 2005; Petzel 2005; Morrison et al. 2006). Losses of sodium and chloride that contribute to decreased plasma osmolality are reported to occur within the first 14 days of exposure to 4 °C, with the first significant difference in plasma osmolality noted after seven days at this temperature (Gonzalez-Cabrera et al. 1995).

After six days at 4 °C, the plasma glucose concentration of *T. bernacchii* was significantly elevated compared with cold acclimated individuals. It could be suggested that the short acclimation time of 4 °C fish may have resulted in elevation of plasma glucose levels. Cold acclimated fish were sampled after 28 days, while warm fish had only spent six days in the 4 °C aquarium tanks and may still have been suffering from the stress of capture and confinement.

However Lowe and Davison (2005) measured the plasma glucose concentration of *T. bernacchii* after 72 hours in a -1 °C flow-through aquarium, which was considered rested baseline data for this species. These authors reported a plasma glucose concentration of about 2 mmol l^{-1}, which is significantly lower than values obtained in the current study for fish held at 4 °C for six days, indicating that the observed elevation in plasma glucose was probably a result of the increased temperature rather than the effect of confinement and handling stress. The increase in plasma glucose concentration at 4 °C recorded in the current study is considerably larger than the increase elicited by acute exposure to either 3 or 6 °C (Lowe and Davison 2005), although these authors commented that glucose concentrations were continuing to rise after 24 hours at 6 °C, and may not have reached potential peak values. Hyperglycaemia is a common response of fish to stress (Kindle and Whitmore 1986; Barton and Schreck 1987; Vijayan and Moon 1992; Carragher and Rees 1994; Staurnes et al. 1994; van Dijk et al. 1999; Elofsson et al. 2000; Begg and Pankhurst 2004; Bracewell et al. 2004), with stressed temperate water teleosts reaching plasma glucose concentrations of between 10-18 mmol l^{-1} (Schwalme and Mackay 1985; Braley and Anderson 1992; Vijayan and Moon 1994; Wells and Pankhurst 1999). In most teleosts, the response is relatively rapid (occurring within about 15 to 30 minutes) (Begg and Pankhurst 2004; Bracewell et al. 2004) and this acts to provide the fish with energy to fuel the 'fight or flight reaction' (Pottinger et al. 2000). As described by Lowe and Davison (2005), the plasma glucose response of *T. bernacchii* is slow and varies between individuals, but is still a sensitive indicator of stress in this species. Therefore, elevation in plasma glucose concentrations in *T. bernacchii* exposed to 4 °C for six days suggests that these fish experience stress associated with this temperature increase.

The values recorded for cold acclimated *T. bernacchii* total haemoglobin concentration, haematocrit and mean cell haemoglobin concentration compare well with results from previous studies (Davison et al. 1994; Davison et al. 1995; Lowe and Davison 2005). After six days at 4 ℃, there was no significant difference in any of these variables compared with cold acclimated fish. These parameters also remained unchanged during acute thermal stress (24 hour exposure to 3 or 6 ℃) (Lowe and Davison), which suggests that mechanisms for increasing blood oxygen carrying capacity, such as increased haematocrit, are not utilised by *T. bernacchii* during periods of thermal stress.

Summary

The results of the current study demonstrate that three sedentary, benthic notothenioids (*T. bernacchii*, *T. hansoni* and *T. pennellii*) are not able to acclimate to a 5 ℃ increase in temperature. Survival time at this temperature was limited in all three species, with only one fish surviving for the whole 28 day acclimation period. It is possible that the ultimate cause of death was the inability of these species to supply oxygen at an adequate rate to meet the increased metabolic demands of fish at 4 ℃. Perhaps, as has been suggested by Coppes Petricorena (2007) these species no longer possess the genetic material required for acclimation, and hence survival in warmer waters? These are worrying results in terms of the ability of these species to survive ocean temperature increases resulting from global climate change.

Chapter Nine

General Conclusion

Antarctic marine organisms have long been considered thermally intolerant, a trait apparently arising from their specialisation to the thermally stable conditions of the Antarctic. The foundations of this concept were established in the work of Levins (1968), and then extended by Huey and Hertz (1984). Levins (1968) developed the 'Principle of Allocation' which stated that organisms possessing phenotypes suited for variable environments have reduced ability to perform in a stable environment. Consequently, in fluctuating environments, selection is thought to favour phenotypic plasticity, whereas stable environments are thought to favour selection for specialised phenotypes. Huey and Hertz (1984) later applied this theory to temperature adaptations of organisms, developing the 'Jack-of-all-temperatures is a master of none' hypothesis. According to this hypothesis, organisms inhabiting thermally variable environments have greater acclimatory ability than those inhabiting thermally stable environments, but as a consequence, they trade off their ability to perform optimally within a narrow temperature range. Some experimental evidence seems to support this hypothesis, for example La Terza et al. 2001 (Antarctic ciliate *Euplotes focardii*); Tullis and Baillie 2005 (whitespotted bamboo shark *Chiloscyllium plagiosum*); Schaefer and Ryan 2006 (zebrafish *Danio rerio*). However, there are some exceptions, for example cold-seep mussels, *Bathymodiolus childressi*, collected from a stenothermal pool (6.5 – 7.2 °C) have high thermal tolerances and can survive for long periods at temperatures 20 °C above their normal environmental temperatures (Berger and Young 2006). The results of the current study clearly demonstrate that the Antarctic fish *Pagothenia borchgrevinki* is another exception to the hypothesis that thermal specialisation necessarily results in a trade-off in thermal flexibility. These fish successfully acclimated to a 5 °C increase in temperature, demonstrating a range of important and interesting physiological adjustments.

One of the most significant acclimatory adjustments demonstrated by *P. borchgrevinki* was the change in resting oxygen consumption rate ($MO_{2\ rest}$). Initial exposure to 4 °C resulted in the expected Q_{10} elevation of $MO_{2\ rest}$, however during the first nine days of the acclimation period metabolic changes occurred, which resulted in a reduction of $MO_{2\ rest}$. At the end of the one month acclimation period perfect compensation of $MO_{2\ rest}$ (Precht 1973) was observed. Oxygen consumption following exhaustive exercise ($MO_{2\ post\text{-}exercise}$) was not as thermally sensitive as $MO_{2\ rest}$, so there was no significant difference between the $MO_{2\ post\text{-}exercise}$ of either cold or warm acclimated fish at any time during the one month acclimation period. Likewise, the oxygen consumption during recovery from exhaustive exercise was not significantly different in the two groups. Thermally mediated elevation of $MO_{2\ rest}$, but not $MO_{2\ post\text{-}exercise}$ caused an initial reduction in the aerobic scope of 4 °C *P. borchgrevinki*, therefore, the down-regulation of $MO_{2\ rest}$ was critically important for the maintenance of aerobic scope in these fish. Ventilation frequency was thermally sensitive in *P. borchgrevinki*, but demonstrated a pattern of thermal acclimation that was similar to that of $MO_{2\ rest}$, showing initial thermally induced elevation, but returning to initial frequencies over a time-course of about ten days. This time-course fits well with the nine day time-course recorded for changes in resting oxygen consumption.

Acclimatory adjustments were also evident in the prolonged swimming ability of *P. borchgrevinki*. Swimming ability seemed to acclimate rapidly to 4 °C. Results from earlier studies demonstrate that acute exposure of *P. borchgrevinki* to 4 °C causes impaired swimming performance (for example Seebacher et al. 2005). However, the first measurements of swimming ability in the current study were made after six days at 4 °C, and at this time, there was no significant difference between the prolonged swimming ability of fish at -1 or 4 °C. When swimming performance was measured in a second group of 4 °C fish on day one, three and five of the acclimation period, there was evidence of improvement in swimming ability during the first three days at this temperature. Rapid acclimation of swimming ability is particularly important for fish survival, as swimming ability impacts such essential activities as prey capture and predator escape.

Oxygen is supplied to the tissues by the cardiovascular system. Supply must meet, but not exceed the metabolic demands of the tissues. Since acclimation of *P. borchgrevinki*

involved perfect compensation of metabolic rate, and the maintenance of aerobic swimming performance at 4 °C, it was predictable that acclimatory changes were evident in the cardiovascular system. Acclimation involved changes to the cardiovascular system of *P. borchgrevinki*. These changes allowed maintenance of heart rates at 4 °C that were very similar to those recorded at -1 °C. Acclimation to 4 °C also affected the thermal sensitivity of the cardiovascular system, with warm acclimated fish demonstrating thermal independence of heart rate between 4 and 8 °C which was brought about via bouts of bradycardia during acute exposure to raised temperatures and tachycardia during acute exposure to low temperatures.

The acclimation changes evident in oxygen consumption, swimming performance and cardiovascular function were underpinned by adjustments at the biochemical level. Warm acclimation resulted in increased haematocrit (Hct) and increased total haemoglobin concentration ([Hb]). It is possible that these changes were beneficial for *P. borchgrevinki* because at 4 °C problems associated with increased blood viscosity may be reduced compared with fish at -1 °C. At cold temperatures, fluids, such as the blood, tend to become more viscous. Increased blood viscosity presents challenges for the cardiovascular system, including increased energetic requirements for circulation. Antarctic fish counter the increase in blood viscosity through a reduction in the number of circulating erythrocytes (Wells et al. 1990; Montgomery and Wells 1993; Egginton 1996). If the problems associated with increased blood viscosity at -1 °C are ameliorated at 4 °C, *P. borchgrevinki* could gain additional oxygen carrying capacity by increasing Hct and [Hb]. Increased oxygen carrying capacity would, in turn provide important benefits for other aspects of fish performance, such as improved prolonged swimming ability. It would be interesting to investigate the changes in blood viscosity associated with acclimation to 4 °C to determine whether this hypothesis is supported by experimental data. Acclimatory adjustments at the biochemical level also included changes in the activity of the enzyme cytochrome C oxidase (CCO). CCO is the terminal member of the electron transport chain, and provides an indication of aerobic metabolic capacity. Red muscle CCO activity was significantly elevated in warm acclimated fish (one month) compared with the CCO activity of freshly captured fish, cold acclimated fish (one month), and fish held for one week at 4 °C. Acclimation changes were not evident in the activity of the anaerobic metabolic enzyme lactate dehydrogenase.

Importantly, six month acclimation studies confirmed that the changes established during a one month acclimation period were not short-term compensatory measures, but were sustainable long-term. Throughout a six month acclimation period, there was no significant difference in either the resting oxygen consumption, or the prolonged swimming performance of cold or warm acclimated fish. This sets *P. borchgrevinki* apart from some other teleost species which possess short-term coping mechanisms to survive temporary exposure to stressful temperatures, but do not acclimate to these changes in the strictly adaptive sense of the term. For example, in its natural environment, the cold stenothermal freshwater gadid, *Lota lota* is able to tolerate temperatures up to 25 °C, which is well above its thermal preferendum (Hardewig et al. 2004). Such tolerance is brought about through down-regulation of metabolic rate, mediated by a reduction in the activities of the metabolic enzymes. However, this down-regulation of metabolic rate is accompanied by a reduction in feeding rates, and is a temporary measure, which aids short-term survival during seasonal extremes (Hardewig et al. 2004).

While overwhelming evidence suggests that *P. borchgrevinki* is an exception to the 'Thermal Specialisation Paradigm', it seems that this species may be unique among the notothenioids in its ability to acclimate. When the acclimation ability of three different Antarctic notothenioids (*Trematomus bernacchii, T. hansoni and T. pennellii*) was tested, all three demonstrated poor survival rates and an inability to acclimate to 4 °C. Mean survival times at 4 °C were 5.2, 14.5 and 14.0 days for *Trematomus bernacchii, T. hansoni and T. pennellii* respectively, which fall well short of the 100 % survival rate of *P. borchgrevinki* at 4 °C. These three species are sedentary and benthic which contrasts with the active, cryopelagic lifestyle of *P. borchgrevinki*, but is a more typical lifestyle among the notothenioids. Therefore, it is likely that this lack of acclimation ability may be widespread among Antarctic notothenioids.

The disparity in the acclimation abilities of the four species examined during this study raises interesting questions regarding which aspects of *P. borchgrevinki* physiology predispose this species for success in acclimation? While there may be several answers to this question, it seems that one of the most important of these is the ability of *P. borchgrevinki* to respond to hypoxia. Acute exposure of notothenioid fish to

elevated temperatures results in an increase in the rates of cellular processes, which in turn, results in increased tissue demand for oxygen. Therefore, it is very likely that during initial exposure to 4 °C, the tissues of Antarctic fish are exposed to hypoxic conditions. Research conducted by Fanta et al. (1989) suggested that the behavioural and physiological responses of notothenioids to hypoxia are species-specific. The responses of *T. bernacchii* and *T. hansoni* to environmental hypoxia involve an immediate economy in respiration and possibly a switch to anaerobic metabolism (Fanta et al. 1989). Unlike the majority of teleost fish (and elasmobranchs) (Farrell 2007), *T. bernacchii* does not demonstrate hypoxic bradycardia, possibly because the heart is already under strong inhibitory cholinergic control (Axelsson et al. 1992). The benefits of hypoxia-induced bradycardia were reviewed by Farrell (2007). It seems that one advantage may be that bradycardia enables fish hearts to beat more forcefully and to increase diastolic filling time, thereby assisting in the maintenance of cardiac stroke volume. This offsets potential decreases in contractility caused when decreasing PVO_2 (partial pressure of oxygen in the venous blood) causes the myocardium to become hypoxic (Farrell 2007). The absence of hypoxia-induced bradycardia in *T. bernacchii* may therefore impair the ability of this species to cope with conditions of hypoxia. In contrast, *P. borchgrevinki* possesses several physiological mechanisms which promote survival in hypoxic conditions. Previous research has demonstrated that *P. borchgrevinki* increases Hct, [Hb] and blood oxygen affinity in response to environmental hypoxia, in turn this results in approximately a 40 % increase in blood oxygen carrying capacity (Wells et al. 1989). Such haematological changes are commonly observed in aquatic vertebrates that are exposed to environmental hypoxia (Frey et al. 1998; Rutjes et al. 2007). Interestingly, the haematological effects demonstrated by hypoxic *P. borchgrevinki* are similar to the effects observed during exposure to elevated temperatures (Davison et al. 1994). Further investigation of hypoxia tolerance in the four study species would yield interesting information regarding the relationship between this ability and the capacity for thermal acclimation.

The conclusion of this research, that three out of four of the study species are unable to acclimate to 4 °C, is particularly disturbing given current trends of global climate change. While assessment of temperature change in the Antarctic is complicated (Phillpot 1985; Stearns et al. 1993; King and Harangozo 1998), data from occupied Antarctic stations between 1949 and 1996 illustrates on average a warming trend in air

temperature of 1.2 ℃ (Jacka and Budd 1998), and up to 3 ℃ on the western Antarctic Peninsula (King and Harangozo 1998). Warming has also occurred in the Southern Ocean. For example, between the 1950s and 1980s, Southern Ocean mid-depth temperatures (700 – 1100 m) have risen by 0.17 ℃ (Gille 2002) and summer surface temperatures in the ocean adjacent to the western Antarctic Peninsula have risen by more than 1℃ since 1951 (Meredith and King 2005). Warming is predicted to disrupt Southern Ocean circulation patterns (Hirst 1999; Bi et al. 2001) and in some models, the thermohaline circulation is predicted to cease altogether, resulting in a warming of the entire ocean by about 7 ℃ over several hundred years (Bi et al. 2001). Clearly the loss of viable populations of *T. bernacchii, T. hansoni* and *T. pennellii* seems likely if these predictions come to pass, a fate which may also be shared by sub-tidal Antarctic organisms (see Clarke et al. 2007 for review). Additionally, while tank acclimation experiments have demonstrated that *P. borchgrevinki* possess the physiological 'machinery' required for acclimation to elevated temperatures, the fate of this species also depends upon a whole range of integrated environmental responses to warming, which may include for example, the effects warming may have on the sea ice habitat, or on community interactions and food web links. Far less data is available concerning Antarctic organisms at this broader level, and much further research is required (Clarke et al. 2007). The impacts of oceanic warming have already been documented in the north-west Mediterranean Sea where mass-mortality of cold water invertebrates has been observed (Chevaldonné and Lejeusne 2003). Prevention of similar mass mortalities in the Antarctic requires continued research combined with immediate action to reduce global warming pollution.

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