Zell- und systemphysiologische Untersuchungen der Temperaturtoleranz bei Fischen

Cellular and systemic investigations of the physiology of temperature tolerance in fish

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'This whole Ice Age thing is getting old.
You know what I could go for?
Global warming.'

Contents

Summary

In the light of climate change, scenarios of global warming and their implications for organisms and ecosystems, the physiological mechanisms that define thermal sensitivity and limit thermal tolerance have gained a wider interest. In an integrative approach, this thesis set out to address thermal tolerance in temperate, sub-Antarctic and Antarctic fish examining its functions, limits and mechanistic links between the organismic, cellular and molecular level.

At the organismic level, the role of oxygen in limiting thermal tolerance of the Antarctic eelpout *Pachycara brachycephalum* was investigated in *in vivo* nuclear magnetic resonance (NMR) experiments during gradual warming from 0 to 13°C. The effects of temperature on respiration, blood flow, energy metabolism, intracellular pH regulation, and tissue oxygenation were studied under normoxia and hyperoxia. Under normoxia, thermal tolerance was limited by the capacities of the circulatory system supplying oxygen to the tissues. Hyperoxia alleviates oxygen uptake and reduces costs of ventilation and circulation, which were mirrored in lower oxygen consumption rates than under normoxia, especially at higher temperatures. Yet additional oxygen could not shift or widen windows of thermal tolerance, probably due to further secondary limiting processes like thermally induced changes in membrane composition.

At a lower level of organismic complexity, thermal sensitivity of energy allocation to protein, DNA/RNA and ATP synthesis and ion regulation was studied in the cellular energy budgets of hepatocytes isolated from *P. brachycephalum* and sub- and high-Antarctic notothenioids. Organismic thermal limitations proved not to be reflected at the cellular level. Provided with sufficient oxygen and metabolic substrates cellular energy budgets remained stable over the investigated temperature range, widely surpassing the thermal tolerance windows of the whole organism. These findings corroborate the idea that capacity limitations of the organismic level are constricting thermal tolerance and support the recent concept of a systemic to molecular hierarchy, in which the most complex systemic level ultimately defines thermal tolerance.

At the molecular level, temperature sensitive expression of mitochondrial uncoupling proteins (UCP) was studied during warm and cold acclimation of *P. brachycephalum* and the temperate common eelpout *Zoarces viviparus*, respectively, to investigate the role of this protein in the adaptive plasticity of mitochondrial energy metabolism. Associated with a general mitochondrial proliferation during cold acclimation in *Z. viviparus*, protein and mRNA expression levels of UCP2 increased in liver and muscle tissue. During warm acclimation in *P. brachycephalum*, UCP2 expression was also increased but in contrast to otherwise relatively

stable mitochondrial capacities. Increased levels of UCP2 may be necessary to regulate high mitochondrial membrane potentials resulting from unchanged capacities in the warm, thus preventing formation of reactive oxygen species. These findings may be indicative of an alternative way of mitochondrial warm adaptation in Antarctic fish.

In conclusion, the data presented here demonstrate that thermal tolerance of the various levels of organisation in fish differ when studied on their own, but in a complex organism are in mutual control of each other, with the highest organisational level showing the highest thermal sensitivity. Within a narrow thermal window, slow warm acclimation of the individual appears possible even in stenothermal Antarctic fish, which in an integrated response of all levels of organisational complexity may shift towards an alternative eurythermal mode of life, thus increasing aerobic scope and windows of thermal tolerance.

Zusammenfassung

Physiologische Mechanismen, die die Temperaturtoleranz eines Organismus bestimmen, haben vor dem Hintergrund von Klimawandel, globaler Erwärmung und ihren Auswirkungen auf Organismen und Ökosysteme an Bedeutung gewonnen. In der vorliegenden Arbeit wurde daher in einem umfassenden Ansatz die Funktion von an der Temperaturtoleranz beteiligten Prozessen und deren Grenzen an borealen, subantarktischen und hochantarktischen Fischarten untersucht. Dabei wurde der Schwerpunkt auf die mechanistischen Verbindungen zwischen den organismischen, zellulären und molekularen Ebenen gelegt.

Auf der organismischen Ebene wurde die Rolle von Sauerstoff in der Limitierung der Temperaturtoleranz mit Hilfe von *in vivo* Kernspinresonanzexperimenten während einer schrittweisen Erwärmung von 0 auf 13°C an der antarktischen Aalmutter *Pachycara brachycephalum* untersucht. Temperatureffekte auf Respiration, Blutfluss, Energiestoffwechsel, intrazelluläre pH-Regulation und Gewebeoxygenierung wurden dabei unter normoxischen und hyperoxischen Bedingungen studiert. Unter Normoxie war die Temperaturtoleranz durch die Kapazität des Herz-Kreislauf-Systemes in der Sauerstoffversorgung limitiert. Hyperoxie erleichtert die Sauerstoffaufnahme und reduziert die Kosten von Ventilation und Herz-Kreislauf-System, was sich in einem verringerten Sauerstoffverbrauch vor allem unter erhöhten Temperaturen widerspiegelte. Zusätzlicher Sauerstoff konnte allerdings die Temperaturtoleranzfenster weder verschieben noch erweitern, was darauf hinweist, dass nachfolgende Prozesse wie z. B. temperaturinduzierte Veränderungen von Membraneigenschaften auf die Temperaturtoleranz wirken.

Auf zellulärer Ebene wurde der Effekt von Temperatur auf die Energieverteilung im zellulären Energiebudget anhand der zentralen Prozesse ATP-, Protein-, und RNA-Synthese sowie Ionenregulation in isolierten Leberzellen von *P. brachycephalum* und sub- und hochantarktischen Notothenoiden untersucht. Zelluläre Energiebudgets blieben über den gesamten untersuchten Temperaturbereich stabil, sofern die Zellen mit ausreichend Sauerstoff und Metaboliten versorgt wurden. Das Temperaturtoleranzfenster auf zellulärer Ebene war somit bei weitem größer als auf organismischer Ebene. Diese Befunde unterstützen die Theorien, dass Kapazitätslimitierungen auf systemischer Ebene die Temperaturtoleranz einschränken und eine Hierarchie von systemischer zu molekularer Ebene besteht.

Auf molekularer Ebene wurde die temperaturabhängige Expression mitochondrialer Entkopplerproteine (UCP) nach Akklimatisation in *P. brachycephalum* und der borealen

Aalmutter *Zoarces viviparus* untersucht, um Hinweise auf eine Beteiligung dieses Proteins an der Anpassungsfähigkeit des mitochondrialen Energiestoffwechsels zu finden. Im Einklang mit einer generellen mitochondrialen Proliferation in der Kälte konnte auch eine erhöhte mRNA- und Proteinexpression von UCP2 in Leber- und Muskelgewebe von *Z. viviparus* gefunden werden. Im Gegensatz dazu war bei der antarktischen Aalmutter die Expression bei gleichbleibender mitochondrialer Kapazität in der Wärme erhöht. Dieser erhöhte UCP Spiegel könnte zur Regulation eines hohen mitochondrialen Membranpotentiales nötig sein, das aus den unveränderten mitochondrialen Kapazitäten in der Wärme resultiert und somit der Bildung reaktiver Sauerstoffverbindungen entgegenwirkt. Diese Strategie deutet auf einen alternativen Weg mitochondrialer Wärmeanpassung in antarktischen Fischen hin.

Zusammenfassend kann gesagt werden, dass die Temperaturtoleranz der verschiedenen Organisationsebenen eines Organismus sich unterscheiden, wenn man sie separat betrachtet. Im Zusammenspiel des gesamten Organismus beeinflussen sie sich jedoch gegenseitig, werden aber letztendlich durch die höhere Sensitivität der höchsten Organisationsebene limitiert. In einem engeren Temperaturfenster erscheint auch eine längerfristige Wärmeakklimation auf Individuenebene in stenothermen antarktischen Fischen möglich. Unter moderaten Akklimationsbedingungen könnten sie alternativ zur Eurythermie über eine gemeinsame Reaktion aller Organisationsebenen aerobic scope und Temperaturtoleranzfenster vergrößern.

1 Introduction

During the last decade, the physiological mechanisms that define thermal sensitivity and limit thermal tolerance have gained wider interest in the context of climate change and its implications for organisms and ecosystems. The main focus of this thesis shall lie in the investigation of the mechanisms of thermal tolerance and their underlying energetic limitations of Antarctic fish, as the cold and stable Antarctic environment has led to adaptations making Antarctic fish species particular susceptible to thermal stress.

1.1 Concepts of thermal tolerance and functional entities

Ectothermal organisms cannot actively regulate their body temperature and are hence subject to temperature effects that influence and limit all physical and biochemical processes in their cells. Even simple unicellular ectotherms cannot adjust their metabolic performance to the whole range of temperatures found in the environment and more complex organisms are found to be even more thermally sensitive: the rise in complexity from unicellular eukaryotes to the metazoa has led to a gain in performance but also to an increase in metabolic rate and oxygen demand and hence to a greater thermal sensivity. Thus, the conventions of thermal tolerance are an issue of general importance to all ectothermal species, in particular to the more complex organisms.

Especially in the light of global warming, the significance of thermal tolerance becomes evident, as can be witnessed in thermally induced shift in zooplankton species (Southward et al., 1995) or the decline of cod stocks in the warming North Sea (O'Brien et al., 2000). Shelford (1931) was the first to develop a general theoretical model depicting consecutive stages of tolerance of ectothermal organisms towards abiotic factors, which in the following has been modified by several authors (Southward, 1958; Weatherley, 1970; Jones, 1971). It was finally refined with particular respect to the role of oxygen and decline of aerobic scope (the capacity of aerobic metabolic energy provision) in thermal tolerance (Pörtner, 2001). A number of recent studies have defined critical temperature thresholds for annelids (Sommer et al., 1997), sipunculids (Zielinski and Pörtner, 1996), crustaceans (Frederich and Pörtner, 2000) and fish (Van Dijk et al., 1999), which were associated with a drastic increase in oxygen demand and (where measured) declined aerobic scopes. Based on these insights, the current model relates to a thermally induced decline in aerobic scope as measure for thermal tolerance (for review, see Pörtner, 2001). Oxygen limitation sets in prior to functional failure and it appears that organismic thermal tolerance is defined by the capacity limitations of the most complex organisational level, namely the oxygen supply mediated by the circulatory (i.e.

cardio-vascular) system (Pörtner, 2002b; Lannig et al., 2004). Earlier authors have suggested that once the circulatory system's limits are exceeded or oxygen consumption of the distributive mechanisms themselves becomes overly high, oxygen supply may become increasingly hampered and consequently the organism's aerobic scope would decline (Weatherley, 1970; Jones, 1971). Thermal tolerance appears therefore closely connected to oxygen demand, and Pörtner and coworkers (Frederich and Pörtner, 2000) termed the temperatures above and below which aerobic scope declines as upper and lower *pejus* temperatures ($T_{p\,II}$ and $T_{p\,I}$; cf. figure 1). The *pejus* range, characterised by a declining aerobic scope, extends until the onset of anaerobic metabolism, which is marked by the critical temperatures $T_{c\,I}$ and $T_{c\,II}$, and beyond which survival is no longer possible (Zielinski and Pörtner, 1996; Sommer et al., 1997). In contrast to the long-term ecological tolerance range that is likely to be reflected by optimal aerobic scope between $T_{p\,I}$ and $T_{p\,II}$, physiological tolerance also extends into the *pejus* range, in which short-term survival is still possible but energy too limited to support high activity, growth and reproduction. Therefore, the threshold temperatures T_p between the optimum and *pejus* range presumably denote species-specific ecological and geographical distribution boundaries (Pörtner, 2001).

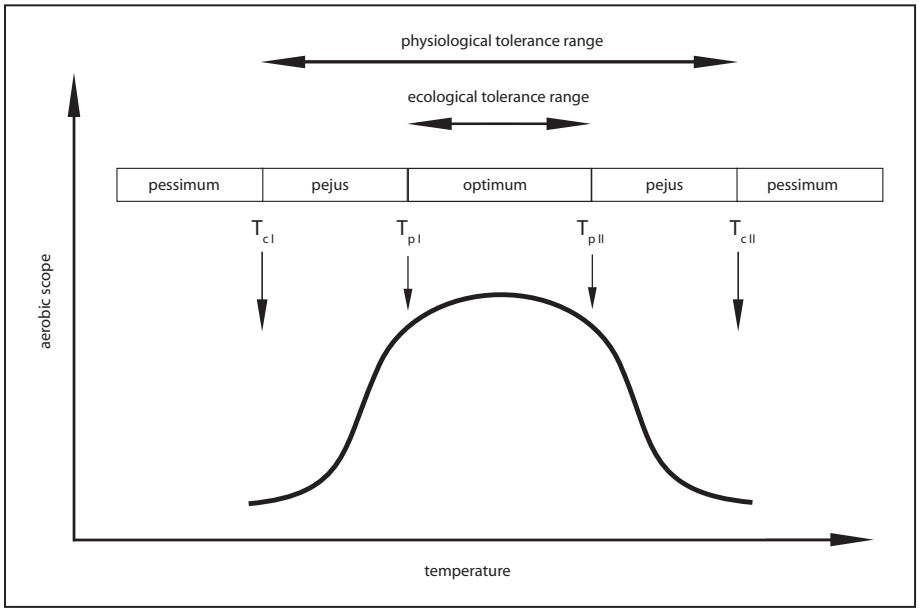


Figure 1: Model of oxygen limited thermal tolerance (after Frederich and Pörtner, 2000).

According to the theory of symmorphosis (Taylor and Weibel, 1981) and the concept of a systemic to molecular hierarchy of thermal tolerance (Pörtner, 2002b), an organism is fine-tuned to yield a functional entity, which is optimally adjusted to the energetic needs and supplies in a particular environment. Although in part adaptable to changing (seasonal)

environmental conditions, functional capacities of all systemic levels are thought not to be expressed in excess of the direct environmental needs, which are framed by the upper and lower *pejus* temperatures.

The environmental demands to metabolism may vary throughout the laditudinal cline and with them the size of the thermal tolerance windows. Cold stenotherm fish are observed to possess rather narrow thermal tolerance windows and are not able to support life functions at higher temperatures. In eurythermal temperate fish, 'envelopes' of thermal tolerance are wider but nonetheless mark the species-specific range of temperatures in which the organisms can survive (Brett and Groves, 1979). In Antarctic fish species, low and stable temperatures and high oxygen availability have led to adaptations, which are expressed by low metabolic rates associated with reduced capacities of oxygen supply, which makes these fish exceptionally sensitive to changing temperatures. These effects will be discussed in detail in the following chapters.

1.2 Inhabitation of the Southern Ocean

Radiation of the recent teleostei (bony fish) into the Southern Ocean began about 25 mio years ago in the early Miocene (Anderson, 1994; Arntz et al., 1994), when the polar Antarctic climate began to stabilise. The opening of the Drake Passage some 35 mio years ago had led to the forming of the circumpolar current and the Antarctic convergence and had isolated the water masses of the Southern Ocean from the surrounding seas, favouring the development of a stable cold-stenotherm Antarctic ecosystem, in which the constantly low water temperatures only range between –1.86°C and 1.0°C (Olbers et al., 1992).

1.3 Systemic adaptations to the cold

Ectothermal organisms consequently have had to adjust their life strategies to the environmental conditions of the Antarctic ecosytem. Like many species in the Arctic, most Antarctic fish species produce antifreeze proteins (AFPs) and glycoproteins (AFGPs), to protect their body fluids, which are hypoosmotic to sea water, from freezing (DeVries, 1971; Fletcher et al., 2001). These are peptides of various molecular masses (Schneppenheim and Theede, 1982; Schrag et al., 1987) that adsorb to forming ice crystals, thus they prevent further growth and cause thermal hysteresis.

Low environmental temperatures generally lead to increased viscosity, which has direct consequences for most vital processes, among others membrane fluidity, enzymatic function, blood circulation and gas diffusion. To maintain cell membrane fluidity, the content of unsaturated fatty acids and the ratio of phosphatidyl ethanolamine to phosphatidyl choline

(PE:PC) are frequently increased in the cold (Hazel, 1995), a process known as homeoviscous adaptation (Sinensky, 1974; Moran and Melani, 2001). Because of low metabolic rates and high oxygen solubility in the cold, Antarctic fish can afford to possess lower hematocrits than fish of lower latitudes to reduce blood viscosity (Egginton, 1997). In the case of the white-blooded Antarctic icefishes (*Channichthyidae*), red blood cells containing hemoglobin are even completely absent (Di Prisco, 2000). A resulting reduction in the oxygen carrying capacity of the blood is tolerable only because of increased physical solubility of oxygen in the blood and cytosol in the cold, and on the other hand, because of the passive and sluggish mode of life, which is also mirrored in a higher oxygen affinity of the remaining hemoglobin (Wells and Jokumsen, 1982; Sidell, 1998). Moreover, in comparison to fish that possess hemoglobin, icefish hold higher heart and blood volumes as well as increased mitochondrial densities (Sidell, 1991; O'Brien and Sidell, 2000; O'Brien et al., 2003). Under stress free conditions, even some of the Antarctic fish species that normally rely on respiratory pigments, can survive without them (Di Prisco, 2000). High viscosity at cold temperatures leads to decreased diffusion processes in the cytosol, affecting gas and metabolite transport to the mitochondria (Sidell, 1991). In combination with cold induced decreases in enzyme activities, this will ultimately result in a reduction of available energy and oxygen, consequently energy demand and metabolic rate would have to be lowered. To maintain physiological functions and prevent functional hypoxia, adjustments of metabolism to cold are therefore necessary, some of which involve mitochondrial proliferation.

1.4 Mitochondrial adaptation and stenothermality

Mitochondrial densities are found to be temperature dependent, cold adapted species display higher mitochondrial densities than species from temperate areas (Dunn et al., 1989) and mitochondrial proliferation in terms of number, size and cristae surface can be observed in the course of cold acclimation experiments (Johnston and Dunn, 1987; St-Pierre et al., 1998; Guderley and St-Pierre, 2002). High mitochondrial densities in the cold are advantageous as they enhance the oxidative capacities of an organism and shorten diffusion distances between the capillaries and mitochondria (Archer and Johnston, 1991). Additionally, frequently observed increased lipid contents ease diffusion, transport and storage of oxygen, which diffuses in lipids 4 to 5 times faster than in the cytosol (Sidell, 1991; 1998).

Yet, a drawback of high mitochondrial densities is a resulting elevated energy demand and, as a consequence, an elevated standard metabolic rate (SMR). Scholander et al. (1953) and Wohlschlag (1960) found remarkably higher metabolic rates in polar fish species at low temperatures, than expected from metabolic rates of tropical fish extrapolated to the same low

temperatures. Their observations led to the hypothesis of metabolic cold adaptation (MCA), which on the basis of recent findings has been controversially discussed, first of all by Holeton (1974) and Clarke (Clarke, 1983; 1991; 1993), and disproved for the high-Antarctic notothenioids (Clarke and Johnston, 1999). Today, it is widely believed, that MCA is only weakly expressed in Antarctic fish and that complete cold compensation is not reached (Hardewig et al., 1998).

This may in part be due to the fact that elevated metabolic rates, resulting from mitochondrial proliferation and increased energy consumption due to proton leakage rates over the inner mitochondrial membrane (which will be discussed in detail below) are compensated for (Pörtner, 2001). Compensation can be accomplished by modifications of membrane properties (Miranda and Hazel, 1996; Pörtner et al., 1998; Logue et al., 2000). Furthermore, mitochondrial enzymes of some cold-adapted fish display higher activation energies (Hardewig et al., 1999a; Pörtner et al., 1999a; Pörtner et al., 2000). Thus, metabolic rates at low temperatures can be kept on a level, which would be predicted by extrapolation of metabolic rates of temperate fish with lower mitochondrial densities. Still, the trade-off of this kind of cold adaptation can result in an increased temperature sensitivity, which becomes manifest in the stenothermality of these animals (Pörtner et al., 1999b). Once the enzymes' high activation energies are provided by elevated environmental temperatures, metabolic rate and thus metabolic energy consumption in these animals will rise substantially, hereby limiting the tolerable thermal range. Stenothermality hence can be considered a direct consequence of cold adaptation.

1.5 The cellular energy budget

Cells exposed to suboptimal conditions face stress in terms of distribution of metabolic resources, consequently the energy available for cellular maintenance and proliferation has to be carefully allocated to those metabolic processes, which are of eminent importance for cell survival. In other words, energy distribution in the cell has to follow some sort of hierarchy under stress conditions to secure the longest possible sustainment of basic cellular functions. Atkinson (1977) suggested that there is a hierarchy in ATP consuming processes, which in accordance with their functional importance show different sensitivities towards a reduction of the cellular energy load. He felt that 'there is a hierarchy of such processes in terms of their responses to the value of the energy charge. Energy-storing sequences, such as the syntheses of polysaccharides or fat, should be most sensitive to a decrease in energy charge. Biosynthesis of structural macromolecules should be next, and activities that are essential for maintenance of life should be able to function at lower values of

energy charge'. According to that notion, a situation of reduced energy (i.e. ATP) availability, which can be due to a shortage either in substrate or oxygen availability, first the metabolic processes related to growth and reproduction are down-regulated, then the processes of cellular maintenance, including ion pumps and exchangers that maintain ionic homeostasis (or enantiostasis, as it is rather called in ectotherms). It is yet questionable, whether in the intact cell these energy shifts occur as a reaction to a reduction in energy charge or to prevent a decrease in energy charge and it is an intriguing question as to how these shifts are in fact elicited.

1.6 Cellular homeostasis and ion regulation

As mentioned above, in the *pejus* range between T_p and T_c, first metabolic limitations become effective, not only influencing growth and reproduction (Pörtner et al., 2001) but possibly also cellular homeostasis, for example ion regulation (Van Dijk et al., 1999). Ion regulation and pH regulation in particular are very important in ectothermal organisms, which have to maintain intra- and extracellular buffering capacities over a wide range of temperatures. The imidazole moieties of the amino acid histidine play a central role in intracellular pH regulation, as they are the only functional groups with a pK within the physiological range (pK'= 6.92). According to the α-stat hypothesis by Reeves (1972), intracellular pH (pH_i) is regulated following the shift of imidazole pK with temperature (-0.015 to –0.020 pH • $°C^{-1}$). This prevents changes in imidazole dissociation status and thus conserves the ionisation status of proteins in all cellular compartments. First thought to completely rely on passive mechanisms, temperature dependent intracellular pH regulation was found to also involve active mechanisms, which were then included into the theory (Reeves, 1985; Cameron, 1989). The differential contributions of active and passive mechanisms appear to depend on the degree of eury- or stenothermality of an organism – the more eurythermal an organism, the more active processes are involved in pH regulation (Sartoris and Pörtner, 1997; Van Dijk et al., 1997), presumably to render the animal more flexible in its reaction towards changing temperatures (Pörtner et al., 1998; Sartoris et al., 2003a).

1.7 Proton leak

Adaptive flexibility towards temperature changes is not only of great importance in cellular homeostasis but also and especially so within the mitochondria. As has been laid out above, thermal tolerance is closely connected to oxygen demand and mitochondria constitute the primary cellular oxygen consumers (only 10% of cellular SMR can be attributed to non-

mitochondrial respiration) and therefore. In this light, it is interesting to notice that all mitochondria are characterised by a basal level of uncoupling of the oxidative phosphorylation, which further increases oxygen demand. This apparently wasteful process called proton leak might have a regulative function and contribute to mitochondrial adaptive flexibility, which shall be discussed in this chapter.

Proton leak appears to be largely insensitive to changes in cellular energy charge (Buttgereit and Brand, 1995) and is rather a function of membrane potential instead (Brand et al., 1999; Brand, 2000). Proton leak reactions and the ATP synthase compete for the same driving force, the mitochondrial electrochemical proton gradient, which is built up as electrons are passed down the respiratory chain and which constitutes the primary energy source for cellular ATP synthesis (cf. figure 2: (a)). Therefore, not all of the energy available in the electrochemical gradient is coupled to ATP synthesis. Some is consumed by leak reactions, in which protons pumped out of the matrix are able to pass back into the mitochondria through proton conductance pathways in the inner membrane, which circumvent the ATP synthase. These non-productive proton conductance pathways are physiologically important and comprise 15-25% of the standard metabolic rate (SMR) in isolated mammalian tissues and cells, 30% in rat hepatocytes, 50% in resting perfused rat muscle, 34% working perfused rat muscle, and 20-40% of basal metabolic rate in rats (Brand et al., 1994; Brand et al., 1999), and about 10% of mitochondrial respiration in isolated liver mitochondria of the notothenioid *Lepidonotothen nudifrons* (Hardewig et al., 1999a). Basal leak rates might be accomplished by proteins like the adenine nucleotide translocase (ANT), the transhydrogenase, the glutamate/aspartate antiporter and the dicarboxylate carrier (Skulachev, 1999; Wojtczak and Wiecedilckowski, 1999; Pörtner et al., 2000; Jackson, 2003). Additionally, there is some evidence for regulatory modulation of leak rates in resting and working perfused rat muscle, indicating that the contribution of proton leak declines at higher metabolic rates, when flux through the ATP synthase must increase (Rolfe and Brand, 1996; Rolfe et al., 1999).

Controlled dissipation of the electrochemical proton gradient has been first observed in the brown adipose tissue (BAT) of mammals. It is mediated by the first known uncoupling protein (UCP1) (Nicholls et al., 1978), homologues of which have more recently been found in ectotherms, amongst others in fish (Stuart et al., 1999; Liang et al., 2003). They all belong to the family of mitochondrial membrane transporter proteins (Walker, 1992) and provide a channel for protons to flow back into the matrix (figure 2).

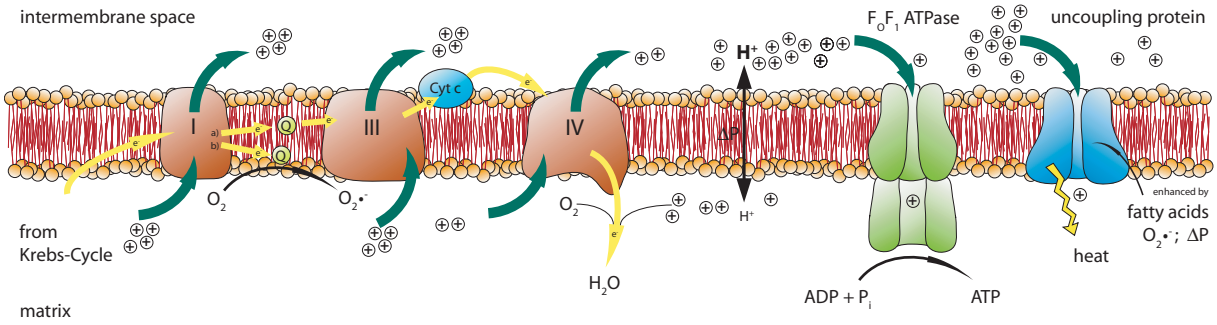


Figure 2: Schematic overview of oxidative phosphorylation and proposed UCP function. The oxidation of reducing equivalents generated during substrate oxidation in the Krebs-cycle or β-oxidation of fatty acids in the complex I, III and IV leads to the separation of protons and electrons. Protons are pumped out of the mitochondrial matrix into the intermembrane space, whilst electrons are passed down the complexes of the respiratory chain (a) or can be passed on molecular oxygen to form superoxide (b) (see text for further explanations). Membrane potential builds up over the inner mitochondrial membrane, which is primarily used to produce ATP by the F_OF_1-ATPase but which is also dissipated as heat by the basal proton leak and mediated by UCP.

The various roles of UCP homologues have been widely discussed, with particular respect to their implications for energy metabolism. While UCP1 is widely accepted as a mediator of proton leak in mammalian brown adipose tissue (Klingenberg and Echtay, 2001; Klingenberg et al., 2001), the functional significance of its homologues is still under dispute. UCP1 acts in thermogenesis in the brown adipose tissue, but the widespread occurrence of its homologues in many tissues and all four eukaryotic kingdoms (Laloi et al., 1997; Jarmuszkiewicz et al., 1999; Jarmuszkiewicz et al., 2000; Vianna et al., 2001) suggests a more central role for UCPs in metabolic regulation. Further speculations as to the function of UCP have been nourished by the fact that UCP (and proton leak) have been reported to be stimulated by various metabolites and proteins as ROS (Echtay et al., 2002), coenzyme Q (Klingenberg et al., 2001), retinoids (Rial et al., 1999) and fatty acids. The latter observation led to the protonophore theory (not depicted in figure 2), in which UCP transport the anionic form of fatty acids (FFA^-) out of the mitochondrial matrix, which diffuse back through the membrane in their protonated form as FFA-H (for further information, refer to Lowell, 1996; Ricquier and Bouillaud, 2000).

1.8 Functions for UCPs in ectotherms

UCP are unlikely to be involved in thermoregulation in fish and other water breathing ectotherms; due to the high thermal capacity of water any heat that is produced is instantly lost over the gills. In their habitats, fish can experience wide fluctuations of ambient water temperature throughout the year and they have to adjust their metabolic energy supply according to the thermally induced energy demand. Uncoupling protein homologues in ectotherms might thus be involved in metabolic processes related to thermal adaptation rather

than thermoregulation. In mammals and birds, UCP1, UCP2 and UCP3 show temperature sensitive expression and their levels increase upon cold exposure (Ricquier and Kader, 1976; Raimbault et al., 2001; Simonyan et al., 2001; Vianna et al., 2001) and it is conceivable that expression levels of ectothermal UCP are also dependent on temperature.

Skulachev (Skulachev, 1998) suggested a protective function for mammalian UCP2 in the prevention of reactive oxygen species (ROS) formation by controlled mild uncoupling, a theory also supported by other authors (Brand, 2000; Pecqueur et al., 2001; Richard et al., 2001). Mitochondrial ROS tend to form especially under conditions of high membrane potential or high protonmotive force, when respiration slows and electrons accumulate on ubiquinone (Q) (cf. figure 2: (b)), which increases the steady state concentrations of its reduced form, ubisemiquinone (QH•). Electrons leaking from ubisemiquinone could react with molecular oxygen to produce superoxide, which in turn produces other ROS. Mitigating proton motive force, uncoupling could lessen the reductive tension in the system and thus lower ROS production. Provided with the ability to control both ATP synthesis and ROS production via uncoupling by UCP, an organism would be able to more freely modulate its basal metabolic rate, making it more flexible towards changing environmental conditions and energetic demands (as has been described in Bishop and Brand, 2000). Consequently, by temperature sensitive control of expression and function of a putatively regulative protein like UCP (Medvedev et al., 2001; Pecqueur et al., 2001), animals would possess a means of thermal adaptation on the molecular level, helping it avoid modifying the suite of proteins of the respiratory chain.

1.9 Concept of this thesis

The objective of this thesis is to apply an integrative approach to the above-described mechanisms of thermal tolerance in temperate, sub-polar and polar fish, with special attention to mechanistic links between systemic, cellular and molecular levels. The thesis will center around three questions, which focus on the existence of thermally induced capacity limitations at various levels of organisational complexity and the connections among them.

1. Is thermal tolerance limited by oxygen availability at the whole organismic level?

This part of the thesis was designed to investigate the hypothesis of an oxygen limited thermal tolerance in fish (Pörtner, 2001). By use of flow-through respirometry, *in vivo* ^{31}P-NMR spectroscopy and MRI, the effects of temperature on energy metabolism, intracellular pH, blood-flow and tissue oxygenation were investigated under normoxia

and hyperoxia. The key question of this suite of experiments was whether additional oxygen could improve oxygen supply to mitochondria and thus shift or widen the windows of thermal tolerance in the Antarctic eelpout *Pachycara brachycephalum*.

2. Are potential organismic limitations reflected at the cellular level?

On a lower level of organismic complexity, experiments were designed to test Atkins' hypothesis of a hierarchy in energy consuming processes in the cell (Atkinson, 1977), with particular respect to thermally induced energetic constraints in cellular metabolism. Using specific inhibitors of some key metabolic processes of the cell, thermal tolerance and possible shifts in energy allocation due to energetic limitations were investigated in hepatocytes of high- and sub-Antarctic notothenioid fishes.

3. Is cellular energy metabolism able to adapt to thermal stress? A case study of temperature sensitive expression of the uncoupling protein 2, which is putatively involved in the regulation of proton leak. Proton leak comprises a substantial fraction of the cellular energy budget and may be of kinetic relevance to the elasticity of the mitochondrial energy metabolism (Brand, 2000). Members of the uncoupling protein family bear high similarities between each other and all include the identical signal sequences of the mitochondrial transporter family (Walker, 1992), suggesting a well-conserved and central function in metabolism. On the molecular level, this study aimed to characterise UCP2 and examine UCP2 expression in response to acclimation to borderline temperatures in the temperate and sub-Antarctic eelpouts *Zoarces viviparus* and *Pachycara brachycephalum*.

2 Methods

2.1 Animals

All fish species used in the experiments for publication I-III belonged to the order Perciformes. For publication I and II and the intra-familial comparison in publication III, the physiology of two closely related members of the family Zoarcidae (eelpouts), the Antarctic eelpout *Pachycara brachycephalum* (publication I-III) and the temperate common eelpout *Zoarces viviparus* (publication III) was investigated. The zoarcids comprise some 220 mostly benthic species and have originated in the Eocene about 50 million years ago in the Northern Pacific, from where they radiated from the Pacific abyssal into temperate and polar waters. To date, they are spread worldwide from deep-sea habitats into the shallow waters of boreal coasts. *Z. viviparus* (max. size about 50cm total length) lives in shallow waters from 0-40m in an area from the English Channel in the South into the Irish Sea, the North Sea and the Baltic and along the Norwegian coast into the Northeast Atlantic, the White Sea and the Barents Sea. It is ovoviviparous and feeds on gastropods, chironomids, crustaceans, eggs and fry of fishes. The bathydemersal *P. brachycephalum* occurs circum-Antarctic in deep waters from 200-1800m and feeds on mussels, gastropods, amphipods and polychaetes (Gon and Heemstra, 1990; Anderson, 1994). Like the majority of zoarcids, *P. brachycephalum* is oviparous.

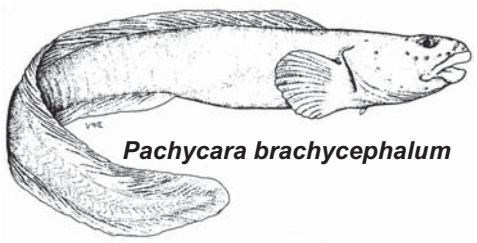
Eurythermal common eelpouts *Z. viviparus* from the Baltic Sea were caught during summer 2001 in the Kieler Förde. Fish were kept at 13 ‰ salinity, and were acclimated to 2.0 ± 0.5 °C (cold-acclimated) or 10.5 ± 0.5 °C (habitat temperature) for at least 2 months. Antarctic eelpouts (*P. brachycephalum*) were caught close to the Antarctic Peninsula during the cruise ANT XVIII of the German research vessel "POLARSTERN" in March 2000 near Deception Island using baited traps at a depth of 475 m and during cruise ANT XIX in April/May 2001 at a depth of 500 m close to King George Island. Water temperature was 0.4°C at a salinity of 34.5 ‰. Until the start of the experiments in June 2000, the fish were first kept in aquaria onboard RV POLARSTERN, then transferred to and kept at the Alfred Wegener Institute (Bremerhaven, Germany) in well-aerated sea-water of 0.0 ± 0.5 °C (habitat temperature) and 5.0 ± 0.5 °C (warm-acclimated) at 32-34 ‰ salinity for at least 2 months. All fish were kept under a 12:12-h light-dark cycle and were fed live shrimps *ad libitum* once a week. Feeding was terminated 7 days prior to experimentation to ensure that standard metabolic rate (SMR) was measured.

Fish used for the experiments in publication II were of the deepwater Antarctic family Artedidraconidae and the family Nototheniidae, which occur from the high latitudes of the Southern Hemisphere into coastal Antarctic regions and range between 15 and 30cm total

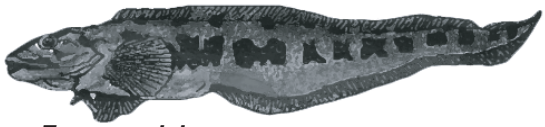
length. Both families belong to the sub-order Notothenioidei, which comprise most of the fish species described in the Southern Ocean (Gon and Heemstra, 1990). Members of the Nototheniidae are mostly benthic with some pelagic and cryopelagic exemptions, the absence of a swim bladder in this family is compensated for by lipids and low mineral content of the bones, leading to near neutral buoyancy. The sub-Antarctic benthopelagic species *Lepidonotothen larseni* occurs from 45°S-70°S in depths between 30 and 550m around the Antarctic Peninsula, the Scotia Arc and the sub-Antarctic Islands. It mainly feeds on krill, hyperiid amphipods and mysids. The high Antarctic species *Trematomus eulepidotus*, *T. pennellii* and *T. bernacchii* are all demersal and occur in a depth range from shallow waters (mainly *T. eulepidotus*) to about 700m between 60°S and 78°S from the Antarctic continental shelf to South Orkney (*T. eulepidotus*, *T. bernacchii*) and the Scotia Arc (*T. pennellii*). They feed on polychaetes, amphipods, gastropods, copepods and fish eggs. *T. lepidorhinus* is a bathydemersal nototheniid and can be found in depths of 200-800m on the inner slope of the Southern ocean and the Antarctic shelf except the Antarctic Peninsula in the high latitudes from 60°S-78°S. It feeds on amphipods, copepods, polychaetes and mysids.

The representative of the demersal Artedidraconidae, *Artedidraco orianae*, can be found in depths of 80-800m on the sublittoral and continental shelf of East Antarctica (Ross Sea, South Victoria Land, Weddell Sea) from 66°S-77°S. It feeds mainly on gammaridean amphipods, with substantial amounts of errant polychaetes and rarely also on isopods.

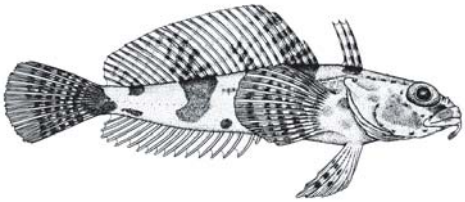
All Notothenioidei were caught in bottom trawls and semi pelagic trawls between November 2003 and January 2004 on cruise ANT XXI/2 of RV POLARSTERN. Fish of the sub-Antarctic nototheniid species *Lepidonotothen larseni* were caught off Bouvet Island (54°30,22 S; 003°14,37 E), the remaining species *Artedidraco orianae* (Artedidraconidae), and the trematomid nototheniids *Trematomus lepidorhinus*, *T. eulepidotus, T. bernacchii* and *T. pennellii* in the eastern Weddell Sea. Until experimentation, fish were maintained onboard the vessel in an air-conditioned container equipped with aquaria and aerated recirculated natural seawater at 0.5 ± 1.0°C for 2-3 weeks to ensure they were in good health. Fish were not fed prior to the experiments, which were all carried out in the laboratories onboard.



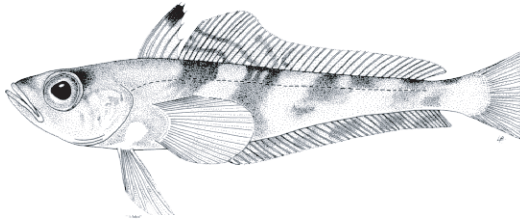
Pachycara brachycephalum



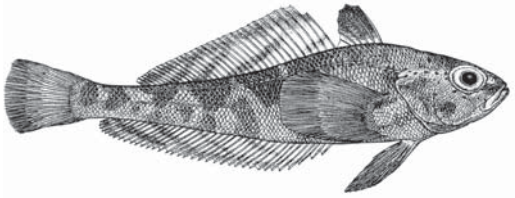
Zoarces viviparus



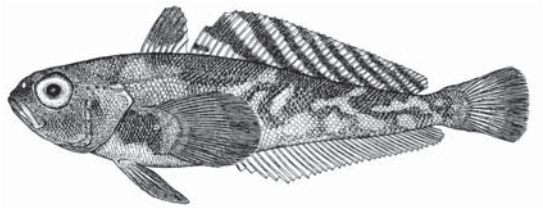
Artedidraco orianae



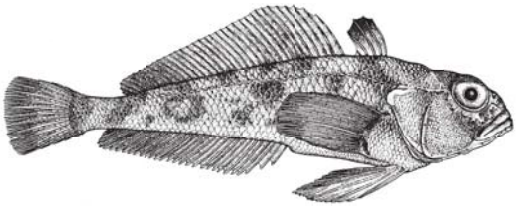
Lepidonotothen larseni



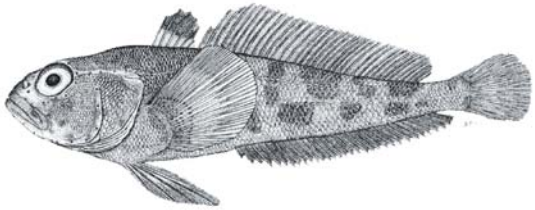
Trematomus lepidorhinus



Trematomus eulepidotus



Trematomus pennellii



Trematomus bernacchii

Figure 3: Fish species used in the experiments (Antarctic species taken from Gon & Heemstra (1990), picture of *Z. viviparus* drawn by J. Ulleweit)

2.2 Analyses by nuclear magnetic resonance techniques

Experiments were conducted using a 4.7 T magnet with a 40cm horizontal wide bore and actively shielded gradient coils. Inside the magnet, non-anaesthetized animals were placed in a cylindrical flow-through perspex chamber of approx. 300ml volume, in which they could move without restraint. The fish remained inside the magnet throughout the whole experiment (for up to 9 days). A 5 cm ^{1}H-^{31}P-^{13}C surface coil, directly placed under the animal chamber, was used for excitation and signal reception. To monitor temperature and oxygen

concentration of in- and outflowing water, fluoroptic temperature and oxygen sensors were installed directly upstream and downstream of the animal chamber inside the magnet. Seawater was supplied to the chamber hydrostatically out of a 50l thermostatted reservoir. Water flow could be controlled to ±1ml between 2 and 500ml*min^{-1}. Oxygen partial pressure (P_{O2}) in the reservoir was adjusted by a gas-mixing pump.

Two experimental series were carried out, one under normoxia (P_{O2}: 20,3 to 21,3kPa) and one under hyperoxia (P_{O2}: 45 kPa). Temperature in both series was increased between 0 and 15°C by 1°C*12 hrs^{-1}. Before experimentation, fish were left inside the experimental setup for at least 24 hours to recover from handling stress, as evidenced from control ^{31}P-NMR spectra. Respiration measurements were carried out during a three-hour period prior to each increase in temperature. Experiments under normoxia and hyperoxia were carried out alternately, in order to smoothen out potential effects of aquarium captivity on oxygen consumption (Saint-Paul, 1988). *In vivo* ^{31}P-NMR spectra (see publication I for details) were acquired continuously throughout the whole experiment to measure changes in intracellular pH (pH_i) represented by the position of the signal of inorganic phosphate (P_i), relative to phosphocreatine (PCr) as an internal standard. The spectra were corrected for temperature and intracellular ion concentrations of marine organisms according to Bock *et al.* (2001).

Alternating with spectroscopy, a flow weighted MR imaging method (see publication I) was applied to examine blood flow in the *Aorta dorsalis*. In the images obtained, blood vessels were picked manually and changes in the ratio of signal intensity over noise intensity were used to determine relative changes in blood flow. Signal intensities of regions of interest (ROI) in the fish were put in proportion to those of ROIs of the same position in a blank image.

To monitor oxygen supply to white muscle and liver, we applied a T_2* weighted gradient echo MR sequence for blood oxygenation level-dependent (BOLD, see publication I) contrast magnetic resonance imaging (Ogawa et al., 1990). In this method, the different magnetic properties of oxyhemoglobin (which is diamagnetic) and deoxyhemoglobin (paramagnetic) are used to account for changes within the ratio of oxy:deoxyhaemoglobin and thus overall blood oxygenation level.

2.3 Respiration

Whole animal respiration was measured simultaneously to the NMR experiments using fluoroptic sensors (optodes) connected to the water in- and outflow of the NMR animal chamber described below. For the measurements, the water flow through the animal chamber was reduced depending on animal size and temperature, such that the animals depleted oxygen

concentrations by 10 to 15%. Optodes were calibrated to the respective temperature and oxygen consumption was calculated as follows:

$$\dot{M}_{O_2} = \left(\frac{\Delta P_{O_2} \times \beta_{O_2} \times \dot{V}}{W} \right)$$

$\dot{M}_{O_2}$: oxygen consumption rate [µmol•g fw^{-1}•h^{-1}]

ΔP_{O2} : difference in partial pressure between in- and outflowing water [kPa]

β_{O2} : oxygen capacity of water [µmol•l^{-1}•kPa^{-1}]

$\dot{V}$: flow rate [l•h^{-1}]

W : animal weight [g]

In addition to the NMR experiments a parallel experimental series was run with five animals kept in a 50l tank under normoxic and hyperoxic conditions, respectively. Temperature treatment was identical to the one in the NMR experiments (see below). Respiration frequency was counted at each temperature and video recordings were stored on a VHS video system for later analysis of the gill opercular width. The product of ventilatory frequency and amplitude (i.e. opercular width) delivered a qualitative proxy for ventilatory effort.

Measurements of cellular respiration were carried out in two parallel setups consisting of Perspex respiration chambers that could be volume adjusted between 300-1500µl and temperature controlled by a thermostat. Respiration was measured using micro-optodes, connected to a laptop computer. 300µl of cell solution were spun down shortly and 200µl of the medium exchanged for fresh medium. The cells were then resuspended and put into the respiration chambers. The chambers were sealed airtight and a micro-optode was inserted through the lid. Blank respiration was recorded for 20min, then the optode was withdrawn and inhibitor stock solution was added to the suspension with a microlitre glass syringe. After reintroduction of the micro-optode, respiration was recorded for 40min. The cells were removed, the respiration chambers washed twice with distilled water and 70% ethanol and a new experiment run with fresh cells and a different inhibitor. Cell solutions were diluted to 1,5 • 10^7 cells • ml^{-1} and kept on ice on a shaking desk throughout the experiments. Respiration rates were calculated to nmol O2 • 10^6 cells^{-1} • min^{-1} and respiration in the presence of an inhibitor was calculated as a percent fraction of its respective blank respiration to account for

potential deterioration of cell quality over time. Cell viability was checked after the last run and always higher than 90%.

2.4 Cell isolation

Hepatocytes were isolated following a protocol modified after Mommsen *et al.* (Mommsen et al., 1994). Fish were anaesthetised (0,5g MS-222/l); the liver was carefully excised and transferred into a Petri dish on ice with 4ml/ g freshweight of solution 1 (see publication II for formulation). Fish were killed afterwards by a cut through the spine and removal of the heart. To remove blood, the liver was washed by perfusion of the *Vena cava hepatica* in vitro with ice-cold solution 1, until no more blood cells were visible in the drain. Then, the liver was perfused on ice via the *Vena cava* with 2ml /g fw. ice-cold collagenase solution and gently massaged for about 10 minutes. Peritoneal tissue was removed, the rest finely chopped and gently shaken on ice for about 60 minutes, until total disintegration of the tissue. The solution was then filtered through a 250µm mesh-size gaze. Hepatocytes were collected by gentle centrifugation and washed repeatedly by centrifugation in solution 1 + 1% BSA, until the lipid phase and all erythrocytes were removed. Cells were stored at 0°C on a shaking desk. Cell titres were assessed in a Fuchs-Rosenthal haemocytometer dish and viability of cells was determined by Trypan blue exclusion (>95%). Total protein content was measured according to Bradford (Bradford, 1976). Samples of cell solution were frozen in liquid nitrogen, stored at –80°C and broken up by ultra sound treatment before analysis.

2.5 Inhibitors

Cycloheximide was used to inactivate peptidyl transferase activity of the ribosomal 60S subunit (i.e. to inhibit protein synthesis; for concentrations used, see publication II). To estimate the energetic needs of the Na^+/K^+-ATPase, ouabain was used. Actinomycin D was administered to block RNA and DNA synthesis. To inhibit mitochondrial ATP synthesis (F_oF_1-ATPase), cells were incubated with oligomycin. In a set of preliminary experiments the minimum concentrations of inhibitors sufficient for maximum reduction of oxygen consumption were determined, since it has been shown that overdoses of inhibitors can lead to an overestimation of the particular metabolic process due to side effects and even to cell death (Wieser and Krumschnabel, 2001). Due to potential cross reactivity, inhibitors were never used in combination with each other.

2.6 Molecular Biology

2.6.1 Protein isolation, gel electrophoresis and western blot analysis

Membrane enrichments were prepared from about 100 mg of frozen tissue by disruption with a hand homogenizer using ice-cold homogenisation buffer (see publication III for formulation). Cellular debris was removed by low-speed centrifugation and the membranes were pelleted from the supernatant crude extract by final high-speed centrifugation. Membrane pellets were resuspended in a minimum volume of homogenisation buffer. Total protein was measured using the method of Bradford (Bradford, 1976) and a BSA standard.

Protein samples were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Laemmli, 1970). A prestained marker was used for the determination of molecular size. After electrophoresis, the proteins were transferred to nitrocellulose membranes; the obtained blots were then stained with Ponceau S to control for equal loading and successful transfer (Sambrook et al., 1989). After de-staining blots were blocked in a blocking buffer containing dry-milk (see publication III). A monoclonal rabbit anti-human UCP2 antibody was used for immunodetection and blots were incubated under agitation with the primary antiserum diluted in blocking buffer. Following a series of washes, blots were incubated with mouse anti-rabbit antibody conjugated to horseradish peroxidase. Antibody binding was visualized by chemiluminescence, detected and quantified with a cooled CCD-camera system. Normal rabbit serum was substituted for primary antibodies to assess non-specific immunoreactivity. Membrane preparations were used to determine the optimal concentration ratio for antigen over primary and secondary antibody. For quantification, a protein concentration was used in a range, where the signal changed linearly with antibody binding.

2.6.2 RNA-Isolation

Animals were anaesthetized (0,5g MS-222/l) before being killed. Samples of different tissues were quickly removed, placed in sterile tubes and frozen immediately in liquid nitrogen. Until used for RNA or protein isolation, the samples were stored at -80°C.

For the preparation of cDNA, mRNA was obtained from total RNA isolated from frozen tissue. The RNA was quantified spectrophotometrically in triplicate samples at 260nm. A_{260}/A_{280} ratios were always >1.9. Formaldehyde agarose gel electrophoresis according to Sambrook (1989) was used to verify the integrity of the RNA.

2.6.3 Characterisation of UCP2

Fragments of the UCP2 gene were isolated by means of reverse transcription followed by PCR (RT-PCR). Primers were designed using highly conserved regions of published sequences of the carp and zebra fish UCP2 gene (Stuart et al., 1999) as a reference. Reverse transcription was performed with Superscript RT and the reverse primer 2 (for all primer details, refer to table 1 in publication III) using mRNA as templates (see publication III for a detailed description). For the amplification of the resulting single strand cDNA, forward primer 1 was used in combination with the reverse primer 2 in a PCR reaction resulting in a 440-nucleotide fragment. The procedure was repeated with a second set of primers (primers 3/4) to yield a fragment of 550 nucleotides. Primers were designed on the basis of conserved regions of the published UCP2 sequence for *D. rerio*.

The cDNA was amplified with *Taq*-Polymerase, the obtained PCR fragments prepared for cloning and purified by gel electrophoresis. After cloning, plasmids were isolated from overnight cultures. To verify the presence and size of inserts, the isolated plasmids were analysed by restriction digestion with *Eco*RI. For each fragment, the DNA sequences of positive clones were determined for both strands and sequences were analysed by alignment. The full-length cDNA was determined by means of the RACE technique (rapid amplification of cDNA ends). The isolated cDNA fragments were used to design 3' RACE forward primers and 5' RACE reverse primers with sequences identical for both eelpout species (primers 5-9). Cloning, sequencing and assembly of the RACE fragments was performed following the same protocols as outlined above, yielding the full-length cDNA sequence of UCP2 for *P. brachycephalum* and *Z. viviparus*. The cDNA sequences have been submitted to Genbank and can be obtained under the following accession numbers: Genbank AY625190 (ZvUCP2); Genbank AY625191 (PbUCP2). Analyses of the deduced amino acid sequences of hydrophilicity after van Heijne and Kyte-Doolittle were carried out to locate putative transmembrane helices. Additionally, phylogenetic analysis was performed by the construction of a phylogenetic tree from the deduced amino acid sequences and a number of published sequences of UCP homologues (see publication III).

2.6.4 Construction of probes and sequence determination

For the construction of species-specific probes for *Z. viviparus* and *P. brachycephalum* cDNA clones for the UCP2 gene and β-actin were isolated using RT-PCR. Reverse transcription was performed following the protocol outlined above with the reverse primer 11, again using mRNA as templates. The cDNA was amplified as outlined above, using primer 10

and 11 in a PCR reaction resulting in a 137-nucleotide fragment. The primer pair was designed within a given region of 150 bp that was identical in both species.

A 215bp cDNA fragment of the β-actin gene from both organisms was isolated from an existing fragment of 377bp (cf. Lucassen et al., submitted) with essentially the same protocol using primer pair 12/13. All fragments were purified by gel electrophoresis and then cloned in *Escherichia coli*.

2.6.5 Quantification of protein specific mRNA

For RNA quantification, ribonuclease protection assays (RPA) were performed. Total RNA was hybridized simultaneously to antisense probes for UCP2 and β-actin, in case of liver RNA, or UCP2 and 18S-rRNA, for muscle RNA, respectively. Probes were synthesized by in vitro transcription with T7 or T3 RNA Polymerase with the plasmids containing the respective cDNA fragments (described above). For 18S-rRNA, a commercial plasmid containing a highly conserved 80bp fragment was used. All probes were labelled with α-^{32}P uridine 5´-triphoshate. To equalize protected fragment intensities, specific radioactivities were used for UCP2, β-actin and 18S-RNA; the probes were always prepared freshly and purified by PAGE under denaturing conditions (see publication III). The DNA templates were removed prior to electrophoresis by DNase I treatment.

After hybridisation, the RNA:RNA hybrids were treated with RNase and co-precipitated with yeast RNA. The RNA was dissolved in loading dye and separated by denaturing PAGE. After drying of the gel, radioactivity was detected and quantified with a phosphorous storage image system.

2.7 Statistical analysis

Data in publication I were examined for significant differences between normoxic and hyperoxic experimental series by a one-factorial analysis of covariance (ANCOVA) and a post-hoc Student-Newman-Keuls test. Within each experimental series, specific segments were compared by a paired sample contrasts analysis. Slopes were compared to one another using an f-test. Regressions and squared correlation coefficients were calculated using Sigma Plot 2000.

For publication II, statistical analyses of differences within cellular respiration rates and among and between inhibited proportions of total respiration were carried out. Differences between control and elevated respiration rates were determined by t-tests. To test for temperature sensitivity of the specific inhibited proportions of total respiration, data were

arcsin transformed and Spearman Rank correlations and one-way analyses of variance (ANOVA) were performed. Furthermore, differences between inhibitor sensitive respiration at control and elevated temperatures were determined by t-tests, which were also applied to test for differences of the total means (within the range of 0-15°C) of inhibitor sensitive respiration between the investigated species.

Statistical analyses of differences among treatments in publication III were performed by t-tests. All differences were considered significant if $P < 0.05$. If not stated otherwise, all data are presented as values ± standard error of the mean (SEM).

3 Publications

List of publications and declaration of my contribution towards them

Publication I

F C Mark, C Bock, H O Pörtner (2002). Oxygen limited thermal tolerance in Antarctic fish investigated by MRI and ^{31}P-MRS.

American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 283:R1254-R1262

The ideas for the experiments were developed by the second and third author and myself, the experiments conducted and analysed by myself in cooperation with the second author. The first draft of the manuscript was written by myself and revised together with the second and third author.

Publication II

F C Mark, T Hirse, H O Pörtner (2004). Thermal sensitivity of cellular energy budgets in Antarctic fish hepatocytes.

Polar Biology (submitted)

I developed the outline and design of the experiments in cooperation with the third author. Supported by the second author, I carried out the experiments on board RV POLARSTERN. I analysed the data and wrote the manuscript, which was revised together with the third author.

Publication III

F C Mark, M Lucassen, H O Pörtner (2004). Are mitochondrial uncoupling proteins involved in thermal acclimation in temperate and polar fish?

Physiological Genomics (submitted)

Together with the second and third author, I planned the concept and outline of this study. I carried out the experiments and data analysis and wrote the manuscript, which was revised in cooperation with the second and third author.

Further publications:

Publication IV

H O Pörtner, F C Mark, C Bock (2004). Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques.

Respiratory Physiology & Neurobiology 141:243-260

All authors contributed equally to the concept and realisation of this review article.

PUBLICATION I

Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and ^{31}P-MRS

F C Mark, C Bock & H O Pörtner

2002

American Journal of Physiology

283:R1254-R1262

Am J Physiol Regul Integr Comp Physiol 283: R1254–R1262, 2002.
First published August 8, 2002; 10.1152/ajpregu.00167.2002.

Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and ^{31}P-MRS

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Mark, F. C., C. Bock, and H. O. Pörtner. Oxygen limited thermal tolerance in Antarctic fish investigated by MRI and ^{31}P-MRS. *Am J Physiol Regul Integr Comp Physiol* 283: R1254–R1262, 2002. First published August 8, 2002; 10.1152/ajpregu.00167.2002.—The hypothesis of an oxygen-limited thermal tolerance was tested in the Antarctic teleost *Pachycara brachycephalum*. With the use of flow-through respirometry, in vivo ^{31}P-NMR spectroscopy, and MRI, we studied energy metabolism, intracellular pH (pH_i), blood flow, and oxygenation between 0 and 13°C under normoxia (P_{O_2}: 20.3 to 21.3 kPa) and hyperoxia (P_{O_2}: 45 kPa). Hyperoxia reduced the metabolic increment and the rise in arterial blood flow observed under normoxia. The normoxic increase of blood flow leveled off beyond 7°C, indicating a cardiovascular capacity limitation. Ventilatory effort displayed an exponential rise in both groups. In the liver, blood oxygenation increased, whereas in white muscle it remained unaltered (normoxia) or declined (hyperoxia). In both groups, the slope of pH_i changes followed the alpha-stat pattern below 6°C, whereas it decreased above. In conclusion, aerobic scope declines around 6°C under normoxia, marking the pejus temperature. By reducing circulatory costs, hyperoxia improves aerobic scope but is unable to shift the breakpoint in pH regulation or lethal limits. Hyperoxia appears beneficial at sublethal temperatures, but no longer beyond when cellular or molecular functions become disturbed.

aerobic scope; heat stress; thermal tolerance limits; magnetic resonance imaging; magnetic resonance spectroscopy

FISH AND INVERTEBRATES endemic to the Antarctic Ocean live in a physically very stable and well-defined environment. Very low temperatures between −1.9 and +1°C and excellent oxygen availability at low metabolic rates have led to physiological features that reflect adaptation to the permanent cold. To reduce blood viscosity, most Antarctic fish hold only low numbers (7) or are completely devoid [*Channichthyidae* (6)] of red blood cells. High levels of lipid and mitochondrial numbers result in improved oxygen diffusion and shorter cytosolic diffusion distances (42, 43). As a consequence of the high degree of cold temperature specialization, Antarctic fish are greatly restricted in their biogeographic distribution and are strongly confined to their environment, indicated by a low tolerance to heat (44). Stenothermality therefore appears to be the direct consequence of being highly adapted to the extreme environmental conditions of the Southern Ocean (34). However, the physiological mechanisms limiting thermal tolerance are still under dispute and several models of temperature tolerance have been introduced (47, 52).

On the basis of Shelford's law of tolerance (41), the recent work of Zielinski and Pörtner (57), Sommer et al. (45), van Dijk et al. (50), and Frederich and Pörtner (11) led to the concept of an oxygen-limited thermal tolerance. As most clearly visible in the spider crab *Maja squinado* (11), limits of thermal tolerance during both heating and cooling are indicated by a set of low and high pejus temperatures (T_p). T_ps denote the beginning of decreased oxygen supply to an organism resulting in a drop in its aerobic scope and hence a reduction of scopes for activity, and possibly for growth and reproduction. In the pejus range between T_p and the critical temperature T_c, animals still can survive, but only under the above mentioned restrictions until T_c is reached, characterized by the onset of anaerobic metabolism (for review, see Ref. 29). In ecological terms, T_p is therefore of great importance, as it may be found close to the temperature limits of biogeographical distribution.

It is hence conceivable that thermal tolerance limits relate to the loss of balance between O_2 demand and supply. On the warm side, for instance, high mitochondrial densities as found in Antarctic species may result in greater energy losses due to proton leak (15, 33, 34), which, with rising temperature, would soon lead to a situation in which oxygen demand surpassed oxygen availability. Limited oxygen availability to tissues might be the first manifestation of thermal intolerance and lead to lower optimum temperatures (35) before heat-induced damage at lower levels of complexity, i.e., organ or cellular functions, contributes to heat death of an animal (29, 30).

As a contribution to an understanding of the physiological basis of temperature-dependent biogeography in the light of global warming, we tested the hypothesis that oxygen limitation is the first line in a hierarchy of thermal tolerance limits in Antarctic fish (29). The key

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question is whether additional oxygen has a significant impact on thermal tolerance and how such an effect may become visible. In the context of earlier findings of T_cs in temperate and Antarctic zoarcids, *Zoarces viviparus* and *Pachycara brachycephalum* (50), we chose the Antarctic eelpout *Pachycara brachycephalum* as an experimental animal. Members of the fish family Zoarcidae are cosmopolitan and thus constitute good model organisms for a comparison of Antarctic fish to closely related species from temperate waters.

MATERIAL AND METHODS

Animals. Antarctic eelpouts (*Pachycara brachycephalum*) were caught in March 2000 near Deception Island (Antarctica) using baited traps at a depth of 475 m. Water temperature was 0.4°C at a salinity of 34.5‰. Fish were 24–30 cm in size and weighed 36–74 g. Until the start of the experiments in June 2000, the fish were kept in aquaria onboard RV Polarstern and at the Alfred Wegener Institute (Bremerhaven) at ambient temperatures of 0 ± 0.5°C and a salinity of 32.5‰. Fish were fed fresh shrimp ad libitum fortnightly and starved 8 days before experimentation to ensure that standard metabolic rate (SMR) was measured. Experiments were carried out between June and November 2000.

Experimental protocol. Experiments were conducted using a 4.7-T magnet with a 40-cm horizontal wide bore and actively shielded gradient coils (Bruker Biospec 47/40 DBX System). Inside the magnet, nonanesthetized animals were placed in a cylindrical flow-through Perspex chamber (Rietzel) of ~300 ml vol (15-cm long, 7-cm wide, and 6 cm in height), in which they could move without restraint (Fig. 1). The fish remained inside the magnet throughout the whole experiment (for up to 9 days). A 5 cm ^{1}H-^{31}P-^{13}C surface coil, directly placed under the animal chamber, was used for excitation and signal reception. To monitor temperature and oxygen concentration of in- and outflowing water, fluoroptic temperature (Polytec) and oxygen sensors (Comte) were installed directly upstream and downstream of the animal chamber inside the magnet. Seawater was supplied to the chamber hydrostatically out of a 50-liter reservoir, the temperature of which could be controlled to ±0.1°C by means of cryostats (Lauda). Water flow could be controlled to ±1 ml between 2 and 500 ml/min. P_{O_2} in the reservoir was adjusted by a gas-mixing pump (Wösthoff).

Two experimental series were carried out, one under normoxia (P_{O_2}: 20.3–21.3 kPa) and one under hyperoxia (P_{O_2}: 45 kPa). Temperature in both series was increased between 0 and 15°C by 1°C/12 h. Before experimentation, fish were left inside the experimental setup for at least 24 h to recover from handling stress, as evidenced from control ^{31}P-NMR spectra. Respiration measurements were carried out during a 3-h period before each increase in temperature. Here, the water flow through the animal chamber was reduced from 300 to 3 ml/min (depending on animal size and temperature), such that the animals depleted oxygen concentrations by 10–15%. Experiments under normoxia and hyperoxia were carried out alternately to smooth out potential effects of aquarium captivity on oxygen consumption (M_{O_2}) (39). In vivo ^{31}P-NMR spectra [sweep width: 5,000 Hz; flip angle: 45° (pulse shape: bp 32; pulse length 100 μs); repetition time (TR): 1.0 s; 600 scans; duration: 10 min; size: 4 kilobytes] were acquired continuously throughout the whole experiment to measure pH_i and its changes represented by the position of the signal of P_i, relative to phosphocreatine (PCr) as an internal standard. The spectra were corrected for temperature and intracellular ion concentrations of marine organisms according to Ref. 4.

Alternating with spectroscopy, a flow-weighted MR imaging method (Fig. 1) was applied to examine blood flow in the Aorta dorsalis [similar to Ref. 3, using the following parameters: matrix, 128 × 128; field of view, 4 × 4 cm; 5 slices at 2 mm each; sweep width, 50,000 Hz; flip angle, 45° (using a hermite pulse of 2,000 μs); TR, 100 ms; echo time (TE), 10 ms; acquisition time, 1 min; 2 averages]. In the images obtained, blood vessels were picked manually and changes in the ratio of signal intensity over noise intensity were used to determine relative changes in blood flow. To correct for movements of the fish inside the chamber, the position of the animal in relation to the excitation profile of the surface coil was taken

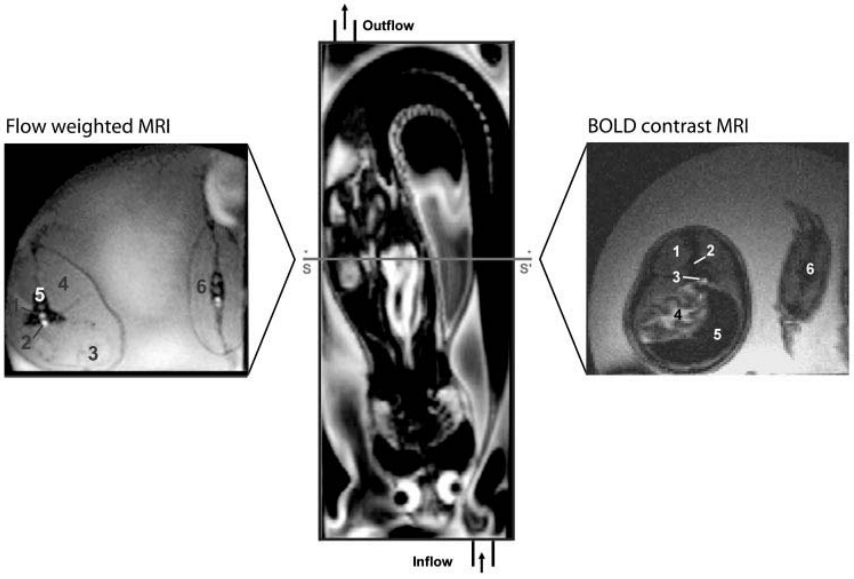

Fig. 1. Schematic view of a specimen of *P. brachycephalum* inside the experimental chamber (adapted from Ref. 4). *Left*: a typical flow-weighted MR image is depicted, its orientation indicated by the line (S-S′) crossing the animal's trunk region (1, aorta dorsalis; 2, vena cava posterior; 3, stomach; 4, dorsal muscle; 5, spine; 6, tail). *Right*: a T_2* weighted MR image [blood oxygenation level dependent (BOLD)] of the same anatomic position (1, dorsal white muscle; 2, spine; 3, blood vessels; 4, stomach; 5, liver; 6, tail).

into account. For better comparability of the data obtained from different fish, baseline corrections were applied to individual data. Signal intensities of regions of interest (ROI) in the fish were put in proportion to those of ROIs of the same position in a blank image.

To monitor oxygen supply to white muscle and liver, we applied a T_2* weighted gradient echo MR sequence for blood oxygenation level-dependent (BOLD) contrast MRI (27) [matrix, 128 × 128; field of view, 4 × 4 cm; 5 slices at 2 mm each; sweep width, 50,000 Hz; flip angle, 11° (pulse shape, sinc3; pulse length 2,000 μs); TR, 100 s; TE, 40 ms; acquisition time, 4 min; 4 repetitions; 2 averages]. In this method, the different magnetic properties of oxyhemoglobin (which is diamagnetic) and deoxyhemoglobin (paramagnetic) are used to account for changes within the ratio of oxy:deoxyhemoglobin and thus overall blood oxygenation level (Fig. 1).

In addition to the NMR experiments, a parallel experimental series was run with five animals kept in a 50-liter tank under normoxic and hyperoxic conditions, respectively. Temperature treatment was identical to the one described above. Respiration frequency was counted at each temperature and animals were filmed using a VHS video system for later analysis of the gill opercular width, carried out using the public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/). The product of ventilatory frequency and amplitude (i.e., opercular width) delivered a qualitative proxy for ventilatory effort.

Statistics. Data were examined for significant differences between normoxic and hyperoxic experimental series by a one-factorial analysis of covariance (ANCOVA) and a post hoc Student-Newman-Keuls test (Super ANOVA, Abacus Concepts); the level of significance was $P < 0.05$. Within each experimental series, specific segments were compared by a paired sample contrasts analysis (Super ANOVA). Slopes were compared with one another using an f-test. Again, a $P < 0.05$ was considered significant. Regressions and squared correlation coefficients were calculated using Sigma Plot 2000 (SPSS). All values are presented as means ± SE.

RESULTS

As evidenced from control ^{31}P-NMR spectra, handling stress elicited by the introduction of the fish into the setup resulted in a slight reduction of PCr/P_i ratios from which the fish recuperated within 1–2 h. For the remaining time of the control period and throughout the whole of the experiment, there was no detectable change in the levels of high-energy phosphates (data not shown), which is commonly accepted as a sign of animal well being (4, 26). As could be seen from MR imaging, fish remained calm and only rarely moved inside the animal containers (data not shown), similar to the behavior the fish show in our aquariums, where they tend to hide in narrow plastic tubes.

$\dot{M}O_2$ under control conditions (normoxia, 0–1°C) equivalent to standard metabolic rate (SMR) was in accordance with published data for Antarctic eelpouts (50, 53, 55) and did not differ significantly from hyperoxic control $\dot{M}O_2$. With rising temperature, $\dot{M}O_2$ of *Pachycara brachycephalum* followed a typical exponential function under normoxia (Fig. 2*B*). However, exposure to hyperoxia and warmer temperatures resulted in a more linear increase in $\dot{M}O_2$, reflecting a strong reduction of the exponential increment observed under normoxic conditions. The two patterns of $\dot{M}O_2$ differed significantly above 8°C, from where the need for oxygen under normoxia increasingly exceeded the level of $\dot{M}O_2$ under hyperoxia. The Q_{10} between 2 and 12°C was 3.40 ± 0.55 and 2.63 ± 0.48 for normoxia and hyperoxia, respectively (means ± SE).

These findings were also reflected in the blood flow through the main dorsal blood vessel (Aorta dorsalis) of the fish (Fig. 2*C*). Although blood flow generally seemed to increase with rising temperature under both normoxic and hyperoxic conditions, it was only under normoxia that it rose steadily up to 6°C and reached levels significantly higher than under control conditions (as indicated by the asterisks in Fig. 2*C*). During warming above 7°C, no further increase in blood flow occurred. In contrast, blood flow under hyperoxia did not increase significantly, but remained fairly constant regardless of the temperature applied.

In both groups, the increase in ventilatory frequency was virtually identical over the range of temperatures, with a tendency toward a slightly lesser increment above 8°C under hyperoxia (data not shown). The same observation holds for ventilatory amplitude above 5°C. Below 5°C, opercular movement was too feeble under hyperoxia to be accurately measured (<1 mm), resulting in a significant difference between hyperoxia and normoxia below 5°C (data not shown). Ventilatory effort (Fig. 2*A*) hence showed an exponential incline with rising temperature slightly lower under hyperoxia (with a statistically significant difference in relation to normoxia only for 3 and 4°C, however).

BOLD contrast in white muscle (Fig. 3*A*), depicting blood oxygenation levels, did not change significantly with increasing temperature under normoxia, although there was a slight trend of decreasing oxygenation at higher temperatures. In the hyperoxic series, BOLD contrast showed a pronounced decrease between 5 and 6°C, with tissue oxygenation levels being significantly lower between 6 and 13°C than between 0 and 5°C. In the liver, however, tissue oxygenation levels displayed a nonsignificant trend to increase with temperature in both experimental series. This trend was somewhat more pronounced under hyperoxia (Fig. 3*B*).

White muscle pH_i under normoxia at 0°C was 7.41 ± 0.02, whereas pH_i values in the hyperoxic group were somewhat higher at low temperatures (Fig. 4). We did not observe significant differences in temperature-dependent pH_i changes between hyperoxia and normoxia. In both groups, pH_i regulation followed a pattern close to the one predicted by the alpha-stat hypothesis, however, only below 6°C. Whereas the hypothesis predicts that rising temperature should cause an acidification of −0.017 pH units/°C (36, 37), we found a slope of ΔpH/°C of −0.012 units (R^2 0.89) under normoxia and −0.015 units/°C (R^2 0.98) under hyperoxia, respectively. Above 6°C, pH regulation followed a significantly different pattern with a ΔpH of −0.004 units/°C (R^2 0.51) for the normoxic and −0.007 units/°C (R^2 0.75) for the hyperoxic series. In general, the decrease of pH_i with rising temperature appeared slightly

larger under hyperoxia than under normoxia; however, the differences in slope were not significant.

All fish died around 13°C, independent of the oxygen concentration. There was no obvious difference between hyperoxia and normoxia, possibly also due to the greater influence of interindividual variability on thermal tolerance compared with oxygen concentration. Shortly before death (~30 min), there was a pronounced drop in white muscle pH_i. This was consistently observed in all the animals included in the study.

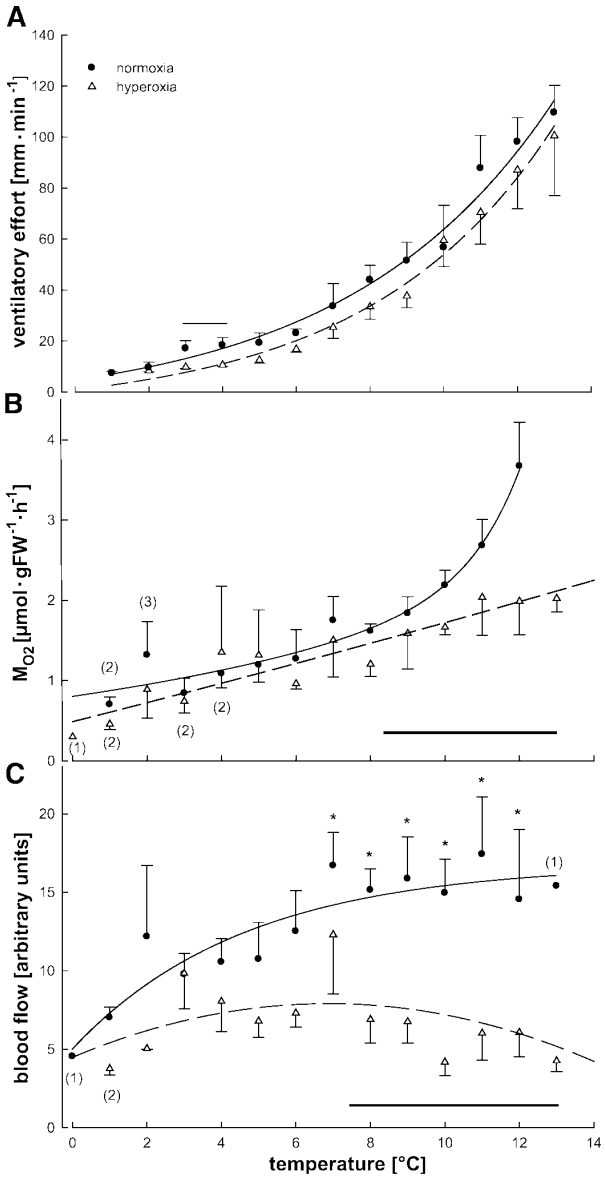

Fig. 2. Ventilatory effort (*A*), oxygen consumption (*B*; M_{O_2}), and arterial blood flow in the *Aorta dorsalis* (*C*) of *P. brachycephalum* under normoxia and hyperoxia with rising temperature. *A*: ventilatory effort as the product of ventilatory frequency and amplitude. Effort increased exponentially with rising temperature in both groups. As indicated by the horizontal line, it was significantly lower under hyperoxia between 3 and 4°C (n = 4 or 5). Normoxia: $f = (-6.94 \pm 7.64)+(11.69 \pm 4.68)\cdot\exp(0.18 \pm 0.03\cdot x)$; $R^2 = 0.98$. Hyperoxia: $f = (-7.40 \pm 5.89)+(8.29 \pm 3.09)\cdot\exp(0.20 \pm 0.03\cdot x)$; $R^2 = 0.99$. *B*: as indicated by the horizontal line, M_{O_2} above 8°C was significantly different between normoxia and hyperoxia. Under normoxia, M_{O_2} showed a large exponential increment, which could not be detected under hyperoxia (n = 3–7 for the normoxic and n = 3–6 for the hyperoxic series, unless indicated otherwise). Normoxia: $f = (0.80 \pm 0.13)\cdot\exp(0.08 \pm 0.04\cdot x)+ (0.0002 \pm 0.0014)\cdot\exp(0.74 \pm 0.48\cdot x)$; $R^2 = 0.96$. Hyperoxia: $f = 0.47+(0.13\cdot x)$; $R^2 = 0.99$. *C*: arterial blood flow, as derived from flow-weighted MR images. Under normoxia, blood flow increased during warming to 7°C, and it remained constant and significantly elevated above that temperature (depicted by *). Blood flow under hyperoxia remained fairly constant. The black line indicates the temperature area between 8 and 13°C, in which blood flow differed significantly between both experimental series (n = 3–6 for the normoxic and n = 4–6 for the hyperoxic series, unless indicated otherwise). Line fits indicate an overall trend within the data sets.

DISCUSSION

Oxygen and the cardiovascular and ventilatory systems. Fanta et al. (10) showed that ventilation frequencies of Antarctic fish (*Notothenia* sp., *Trematomus* sp.) decrease under hyperoxia, an effect that has been reported for various marine and freshwater fish species (2, 14). This stands in opposition to our observations in *Pachycara brachycephalum*, where ventilation frequency did not differ between normoxia and hyperoxia. Instead, ventilation amplitude was reduced under hyperoxia, although significantly only at slightly elevated habitat temperatures between 3 and 4°C. Even though ventilation frequency might be lowered in some species and the P_{O_2} difference between blood and water rises, it is commonly found that arterial P_{O_2} rises in proportion to the P_{O_2} of the medium under hyperoxia due to increased oxygen availability (46, 48, 56). O_2 can passively enter the blood via the gills and the skin; even under normoxia, up to 35% of the total amount of oxygen consumed at rest in the Antarctic eelpout *Rhigophila dearborni* can be attributed to cutaneous uptake (53). Hyperoxia thus alleviates the workload required for sufficient oxygen supply to tissues and at the same time increases the scope for active oxygen uptake and, in consequence, aerobic scope.

Because oxygen solubility is elevated at low temperatures, icefish (*Channichthyidae*) resort to O_2 transport in solely physical solution and can afford to abandon the use of respiratory pigments like hemoglobin (6). Sluggish benthic zoarcids and nototheniids that still rely on hemoglobin only do so at very low hematocrit levels between 10 and 18% [*P. brachycephalum*: 13%, personal observation; *R. dearborni*: 10.5 ± 3.0% (53); Nototheniids: 10–18% (7)], thus reducing blood viscosity, which again lowers the costs of blood circulation. At low temperatures, physically dissolved oxygen can constitute up to 30% of the total amount of blood oxygen and much of the improved O_2 supply

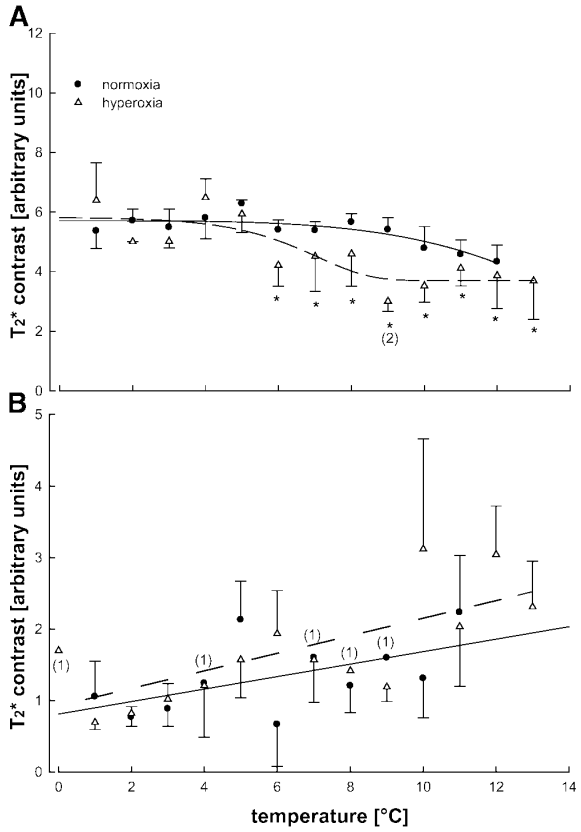

Fig. 3. White muscle (*A*) and liver (*B*) tissue oxygenation under normoxia and hyperoxia with rising temperature, as derived from BOLD contrast of T_2^* weighted MR images. *A*: under normoxia, white muscle tissue oxygenation levels remained constant with rising temperature, whereas in the hyperoxic series oxygenation levels between 6 and 13°C were significantly lower than below 6°C (*) (n = 2 or 3 for the normoxic and n = 2–5 for the hyperoxic series). Line fits indicate an overall trend within the data sets. *B*: in both experimental series there was a trend in liver tissue oxygenation levels to increase with rising temperature. This trend appeared to be more pronounced under hyperoxia, although individual oscillations were large (n = 2 for the normoxic and n = 3–5 for the hyperoxic series, unless indicated otherwise). Normoxia: $f = 0.81 + 0.09 \cdot x$; $R^2 = 0.32$. Hyperoxia: $f = 0.85 + 0.13 \cdot x$; $R^2 = 0.52$.

under hyperoxia occurs by enhancing the levels of physically dissolved oxygen.

Good oxygen availability and a stable, cold-stenothermal environment support low energy turnover lifestyles in Antarctic fish, not least via the reduction of the energy cost of cardiovascular and ventilatory work. If the capacity of ventilation and circulation is adjusted accordingly low, these fish become stenothermal, meaning that a temperature-induced rise in metabolic oxygen requirements cannot adequately be met by oxygen delivery through ventilation and the cardiovascular system. A decline in aerobic scope would therefore be the first consequence of thermal stress elicited by environmental warming.

Extending from earlier considerations by Jones (19), a decline in whole animal aerobic scope likely marks the temperature at which oxygen delivery capacities fall back behind the rising energy demand of cardiovascular and other aerobic tissues such as liver. Ventilatory and circulatory organs might therefore be among the first to be affected by progressive oxygen limitations, which in consequence lead to a vicious circle of an ever-increasing oxygen deficiency (11). While ventilation and blood circulation are sped up to augment oxygen supply, ventilatory and especially circulatory musculature consume most of the delivered oxygen themselves, and thus only exacerbate the deficit by further increasing M_{O_2}. Evidently, the cost of circulation explains much of the exponential rise in M_{O_2} observed under normoxia (Figs. 2 and 3), which is frequently found in fish respiration experiments (1, 50, 55). This is indirectly supported by observations by van Ginneken et al. (51), who found M_{O_2} to increase under hypoxia in tilapia (*Oreochromis mossambicus*). Starting from a fractional cost of 30% of SMR for ventilation and circulation in a resting fish (18), an increasing part of the SMR will have to be accredited to ventilation and especially circulation at high temperatures and thereby contribute to the loss in aerobic scope.

The increase and subsequent plateau in blood flow with rising temperature under normoxia indicate a cardiovascular capacity limitation above 7°C, resulting in a mismatch in oxygen delivery and demand. This leads to a drop in aerobic scope, suggesting the 7°C threshold to be a T_p (11), when blood flow becomes limited by the insufficient capacity of the heart to overcome frictional resistance within the vascular system. As under this situation of rising thermal stress fish cannot further upregulate hemoglobin oxygenation

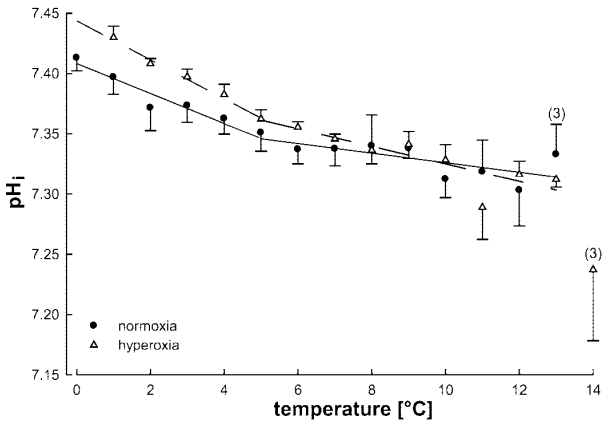

Fig. 4. White muscle intracellular pH (pH_i) values derived from in vivo ^{31}P-NMR spectra of the Antarctic eelpout *P. brachycephalum*. At temperatures below 6°C, pH_i regulation in normoxic and hyperoxic animals followed an alpha-stat pattern with a ΔpH of -0.012 units/°C ($R^2 = 0.89$) for the normoxic and -0.015 units/°C ($R^2 = 0.98$) for the hyperoxic experimental series, respectively. Beyond 6°C, ΔpH was -0.004 units/°C (R^2 = 0,51) for the normoxic and -0.007 units/°C ($R^2 = 0.75$) for the hyperoxic series, indicating a different pattern of pH regulation. In both cases, the increment of the function below 6°C was significantly different from the slope above 6°C (n = 5–7 for the normoxic and n = 4 or 5 for the hyperoxic series, unless indicated otherwise).

or blood P_{O_2} levels, oxygen extraction (i.e., ΔP_{O_2} between arterial and venous P_{O_2}s) is increased to meet the rising requirements for oxygen. Venous P_{O_2} drops, reflecting a worsening of oxygen supply to the heart in fish, as pointed out by Pörtner et al. (31) for the cod, *Gadus morhua*. Finally, a T_c is reached, which by definition (57) marks the onset of anaerobic metabolism and complete loss of aerobic scope (see Ref. 29). Our data set, however, which focused on an evaluation of T_ps, is not suitable to identify a distinct T_c. Van Dijk et al. (50) chose succinate in liver tissue as a reliable indicator of T_c in their experiments on *P. brachycephalum*, which were carried out onboard RV Polarstern shortly after animal capture. The authors found T_c to be situated around 9°C, which was the same temperature at which M_{O_2} was maximal. At this temperature, the animals lost balance and died. In our study, these processes likely occurred ~13°C. This indicates that stress levels in the animals might have been higher in van Dijk's experiments than in ours, owing to the nature of experimental conditions onboard the research vessel.

The data obtained under hyperoxia indicate that additional oxygen can lower cardiovascular costs and thereby overall M_{O_2}. This perception is strongly supported by the blood flow data (Fig. 2, *B* and *C*). While under normoxia, blood flow gradually increased until it reached a steady level above 7°C; it remained fairly constant under hyperoxia after a small increment between 1 and 4°C. In consequence, circulation did not breach the line above which it became counterproductive, i.e., consuming more oxygen than it could deliver. It can therefore be expected that under hyperoxia, M_{O_2} would display a more prolonged exponential phase and should support survival at higher temperatures than under normoxia. These findings are in accordance with a suggested hierarchy of thermal tolerance, where reduction in aerobic scope is the "first line of sensitivity" affected by thermal stress, giving way to the next set of limiting factors (29, 30). These factors are thus far unexplained in the eelpout but lead to death at ~13°C. Reduced O_2 demand and blood flow under hyperoxia at elevated temperatures suggest an enhanced functional reserve to the animal. This reflects an enhanced aerobic scope or upward shift in T_p, which must be considered significant. Yet a beneficial effect of O_2 on T_c is not clear, likely because T_c and, furthermore, molecular limits, coincide.

Tissue oxygenation. For further support of the above conclusions we monitored tissue oxygenation changes in white muscle and liver by applying BOLD imaging. However, various physiological and physical effects can differentially influence BOLD contrast. Only with adequate consideration of these effects will interpretation of these data become possible. Physiologically, BOLD contrast reflects the ratio of oxy- and deoxyhemoglobin, which depends on P_{O_2} and temperature, as well as Bohr and Root effects. The latter can be excluded in sedentary and benthic Antarctic fish (54), whereas the former would contribute to a drop in BOLD contrast during a potential heat-induced extracellular acidosis. This appears unlikely to explain the drop in BOLD contrast during hyperoxia compared with normoxia, as such a metabolic acidosis would be more severe during normoxia. A change in hematocrit can influence signal baseline as well (24) and evoke a rise in BOLD contrast regardless of the tissue, in line with a rise in hemoglobin-borne oxygen. Antarctic notothenioid teleosts are theoretically able to enhance hematocrit via release of sequestered erythrocytes from the spleen (9); it is unknown whether this occurs in zoarcids. Again, this predicted pattern contradicts the drop in BOLD contrast observed in white muscle, especially during hyperoxia vs. normoxia and thus appears unlikely. Finally, tissue perfusion rates are positively correlated with BOLD contrast (21) and the ratio of metabolic rate over blood flow (MR_{O_2}/BF) is negatively correlated to R_2^* ($1/T_2^*$), as recently shown for brain by Hyder et al. (17). Thus a rise in tissue perfusion would lead to a rise in BOLD contrast, similar to a rise in hemoglobin oxygenation. On the physical side, in relying on T_2^* weighted MR imaging, BOLD contrast is strongly influenced by T_2, local inhomogeneities of the magnetic field (22), dissolved molecular oxygen (20, 28), and other paramagnetic ions and molecules, all of which elicit a decrease in BOLD contrast. Especially liver tissue is known for its low T_1 and T_2 values due to its dense matrix and high concentrations of paramagnetic ions (25), and BOLD contrast changes may thus appear more pronounced in this tissue compared with other tissues with the same change in oxygenation.

Nonetheless, Lebon et al. (23) and Semple et al. (40) have shown for both human muscle and liver tissue that changes in T_2^* weighted MR images can be attributed to changes in blood oxygenation levels. Overall, application of BOLD contrast techniques should constitute a helpful in vivo tool to at least qualify if not quantify tissue oxygenation changes.

In the present study, field homogeneity was good (Fig. 1) and no differences between BOLD contrast under normoxia and hyperoxia were observed at control temperatures. Environmental hyperoxia is hence not likely to directly influence tissue oxygenation levels, as a consequence of reduced blood flow and ventilation rate (18). Despite increased O_2 demand during normoxic warming, blood oxygen levels in muscle tissue (Fig. 3*A*) remained constant throughout the experiments. Maintenance of aerobic scope at increased SMR would require a rise in blood P_{O_2} to maintain the balance between demand and supply. A moderate drop in P_{O_2} may in fact occur, concealed by the maintenance of T_2^*, owing to the flow dependence of BOLD contrast. As a corollary, the maintenance or fall of blood P_{O_2} in the light of increased organismic and cellular oxygen demand and blood flow indicates less aerobic flexibility.

Interestingly, we found a significant decline in muscle oxygenation above 5°C under hyperoxia, starting from O_2 levels similar to those under normoxia. The reduction in blood oxygenation evidently was not compensated for by an increase in blood flow. Even if a significant effect of blood flow on BOLD contrast oc-

curs, these interpretations hold. The lower levels of blood flow under hyperoxia (Fig. 2*C*) likely cause the decrease in blood oxygenation in the muscle tissue and reflect the lower whole animal O_2 demand.

When measuring white muscle tissue P_{O_2} invasively with microelectrodes, Fanta et al. (10) found decreasing muscle oxygenation under hyperoxia in one notothenioid species. However, even within the same genus, this was not a general response to elevated oxygen levels. It thus needs to be emphasized that blood oxygenation in white muscle, which is metabolically relatively inactive at rest, does not reflect oxygen supply to central aerobic organs such as the liver. Here (Fig. 3*B*) we found that tissue oxygenation was inclined to increase with rising temperature, a trend slightly more prominent under hyperoxia than under normoxia. It is conceivable that visceral blood oxygenation levels increase during warming to meet rising metabolic oxygen demands (8), this possibly being a trade-off at the expense of reduced blood supply to the less active muscular tissue (18). At unchanged levels of blood flow, this scheme might be more pronounced under hyperoxia. Moreover, as a consequence of the relatively low signal intensity in liver tissue (see above), any changes in BOLD contrast will appear more dramatic than, for instance, in muscle tissue. In conclusion, our data imply that oxygenation levels can be increased or remain unchanged in more vital organs such as liver when oxygen supply to muscle is reduced.

pH_i regulation. Initial values of white muscle pH_i under normoxia at 0°C (7.41 ± 0.02) were very similar to the 7.43 ± 0.06 obtained by Van Dijk et al. (50) using the homogenate method (32) and to the values found by Bock et al. (4) by NMR measurements in the same species at the same temperature. Hyperoxia-induced reduction of ventilatory effort at low ambient temperatures can evoke a respiratory acidosis, due to the accumulation of CO_2 in the blood (14). This can be compensated for within 2–3 days by a subsequent active uptake of HCO_3^- via the gill HCO_3^-/Cl^- exchanger (5, 16). Resulting elevated bicarbonate levels in the blood affect the mechanisms of pH_i regulation, again most likely HCO_3^-/Cl^- exchangers and HCO_3^-/Na^+ cotransporters (12), thereby explaining the higher initial pH_i values under hyperoxia.

Evidently, the decline in aerobic scope suggested to occur beyond 5–6°C parallels a shift in the pattern of temperature-dependent pH_i regulation, indicated by the distinct break around 5°C. The fact that this pattern remains more or less unchanged under hyperoxia leads to the conclusion that it matches the normoxic pejus threshold but is not influenced by oxygen availability. A possible cause for this shift in pH regulation might be in the thermal sensitivity of ion channels or a change in the relationship between membrane permeability and compensatory ion exchange. Thermal inactivation of ion transport (e.g., Na^+/H^+, Cl^-/HCO_3^- exchanger, H^+-ATPase) is very likely not yet involved, owing to the steady-state nature of temperature-dependent pH_i values reached. The slightly steeper slopes under hyperoxia may relate to the elevated blood bicarbonate levels but do not significantly shift the break temperature.

As pointed out by Sommer et al. (45), alpha-stat regulation of pH_i in a marine invertebrate was restricted to a temperature window between the T_c limits. The data obtained here for *P. brachycephalum* indicate that already the normoxic T_p correlates with a shift in pH regulation. This is also consistent with the data provided by van Dijk et al. (50), who found a deviation from alpha-stat pH_i regulation between 3 and 6°C in *P. brachycephalum* but located T_c close to 9°C (see above). Overall, the parallel events in oxygen metabolism and acid-base regulation confirm previous applications of the symmorphosis concept (49) to the limits of thermal tolerance, i.e., that the functional properties and capacities of several physiological systems are set to be optimal between the highs and lows of ambient temperatures and may thus show limitations or changes at similar levels of ambient temperatures (30; see Ref. 38 for endotherms).

In conclusion, under normoxia, a putative reduction of the aerobic scope, which coincides with a break in pH regulation around 5°C, can be made out between 6 and 7°C and is reflected in limited capacity of the circulatory system to enhance arterial blood flow. Our findings suggest that improved oxygen availability diminishes the effects of thermal stress by reducing the energy costs associated with oxygen distribution in the organism. High ambient oxygen levels will also help when oxygen demand is on the verge of exceeding oxygen availability as it is set by the functional capacity of the cardiocirculatory system. Although hyperoxia likely improves aerobic scope during thermal stress and may thereby widen the tolerance window delimited by the T_ps, the temperature dependence of pH regulation remains largely unaffected, likely due to fixed thermal properties of membranes or ion exchange mechanisms. This indicates that once the oxygen limitation of thermal tolerance has been alleviated, as shown by the uniform pattern of arterial blood flow under hyperoxia, further restrictive mechanisms at cellular or molecular levels may become effective. In general, our findings confirm that in vertebrates several processes are responsible for setting thermal tolerance limits, all of which seem tightly intertwined. Further work is necessary to elucidate the factors that restrict temperature tolerance once oxygen limitation is suspended; these may be located on a lower functional, i.e., cellular level (30). Overall, the capacity of Antarctic fish to adapt to climate-induced temperature changes appears very small. Oxygen-limited windows of thermal tolerance are narrow in this group and reflect its high sensitivity to current and, possibly, future scenarios of warming in Antarctic waters (13).

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PUBLICATION II

Thermal sensitivity of cellular energy budgets in Antarctic fish hepatocytes

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Thermal sensitivity of cellular energy budgets in Antarctic fish hepatocytes

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Abstract

Oxygen demand elicited by the main cellular energy consumers was examined in isolated hepatocytes of sub-Antarctic and high-Antarctic notothenioid and zoarcid (*Pachycara brachycephalum*) fish with respect to the role of cellular metabolism in co-defining thermal tolerance. The relative proportions of energy allocated to protein and RNA/DNA synthesis, ion regulation and ATP synthesis were quantified between 0 and 15°C by analysis of inhibitor sensitive cellular respiration. In all investigated species, protein synthesis constituted 25-37%, RNA synthesis 24-35%, Na^+/K^+-ATPase 40-45% and mitochondrial ATP synthesis 57-65% of total respiration. The sub-Antarctic nototheniid *Lepidonotothen larseni* displayed lower cellular protein synthesis rates but somewhat higher active ion regulation activities than its high-Antarctic confamilials, as is typical for more eurythermal species. Assumed thermal optima were mirrored in minimized overall cellular energy demand. Onset of thermal stress indicated by elevated energy turnover became visible between 3 and 0°C as well as beyond 6°C in the sub-Antarctic *L. larseni* and *P. brachycephalum*; whereas the high-Antarctic species displayed progressively rising respiration rates during warming with a cellular energetic minimum at 0°C. Sub-Antarctic fish showed signs of cold-eurythermy and appear to live close to their lower limit of thermal tolerance, while high-Antarctic notothenioids show high degrees of energetic efficiency at 0°C. All cellular preparations maintained energy budgets over a wide thermal range, supporting the recent concept that thermal limits are set by oxygen and associated energy limitations at the whole organism level.

Introduction

Fish of the perciform suborder Notothenioidei comprise most of the fish species living in the Southern Ocean (Gon and Heemstra, 1990). They began to radiate into Antarctic waters in the early Tertiary, gradually adapting to the progressive cooling, which set in after the opening of the Drake passage and the formation of the circumpolar current some 25 mio years ago (Eastman, 1993; Arntz et al., 1994). The further South into high-Antarctic waters some of these species diversified, the more they specialized on the permanent cold through unique adaptations at molecular, cellular and systemic levels (Kock, 1992; Detrich, 1997; Pörtner et al., 2005). Eventually, this even led to the loss of respiratory proteins in the white blooded Channichthyidae (Di Prisco, 2000). The downside of this high degree of cold-specialisation was the development of cold-stenothermy, mirrored by increased mitochondrial densities at uncompensated capacities (Johnston et al., 1998; D'Amico et al., 2002; Lucassen et al., 2003) combined with reductions in hematocrit (Egginton, 1997b) and cardiovascular output (Egginton, 1997a). Specialisation forced most of these species to lead a sluggish lifestyle. Extreme stenothermy also made them very susceptible to stress induced by warming, with upper thermal limits around 6°C in *Trematomus bernacchii*, *T. hansoni* and *T. borchgrevinki* (Somero and DeVries, 1967).

The first line of thermal limitation in animals is mirrored in the onset of functional hypoxia at the organismic level, followed by a hierarchical sequence of systemic to molecular stress events (Pörtner, 2002). Some of these principles have also been confirmed in Antarctic fish. For example, oxygen demand in increasing hyperthermia soon exceeds the capacity of oxygen supply and finally drives the animal into anaerobic metabolism (e.g. van Dijk et al. 1999). Findings obtained in a recent study of sub-Antarctic eelpouts (Mark et al., 2002) were in line with the suggested hierarchy in the processes defining thermal limits and elaborated a key role of the circulatory system in setting thermal tolerance, firstly by ensuring oxygen supply, secondly by contributing to oxygen demand through enhanced circulatory work at high temperatures. Oxygen demand is also generated at the cellular level. However, the potential changes in cellular processes and their oxygen demand upon cooling or warming and the resulting contributions of the various cellular processes to whole animal thermal intolerance have not yet been addressed.

Therefore, the present study sets out to investigate the potential role of cellular processes in thermal limitation. As cells exposed to suboptimal conditions may face a shift in the distribution of metabolic resources, analysis of the energy available to various processes of cellular maintenance and proliferation may provide a sensitive measure of environmental and/or thermal stress. Atkinson (1977) suggested that there is a hierarchy in ATP-consuming

processes, which in accordance with their functional importance show different sensitivities towards a reduction of the cellular energy load. This study aims to investigate temperature dependent energy allocation to the most important metabolic processes in hepatocytes of the highly thermally sensitive notothenioids. Energy allocation to protein synthesis, RNA/DNA synthesis, ion regulation (Na^+/K^+-ATPase), and ATP synthesis was examined with respect to thermally induced shifts and preferences in cellular energy allocation and the potential existence of threshold temperatures that might contribute to thermal tolerance limits of the whole organism.

Materials and methods

Animals

All fish used for the analysis of the energy budgets belonged to the perciform suborder of Notothenioidei and were caught in bottom trawls and semi-pelagic trawls between November 2003 and January 2004 on cruise ANT XXI/2 of the German research vessel Polarstern. Fish of the sub-Antarctic nototheniid species *Lepidonotothen larseni* (17.1±2.0cm; 34.3±13.5g) were collected off Bouvet Island (54°30,22 S; 003°14,37 E), high-Antarctic notothenioid, *Artedidraco orianae* (Artedidraconidae; 14.8±1.3cm; 30.0±6.5g), and the trematomid nototheniids *Trematomus lepidorhinus* (15.1±4.8cm; 45.6±31.8g), *T. eulepidotus* (21.1±2.7cm; 100.0±50.9g), *T. bernacchii* (23.3cm; 131.0g) and *T. pennellii* (21.7±3.2cm; 143.1±62.3g) were collected in the eastern Weddell Sea. Until experimentation, fish were maintained onboard the vessel in an air-conditioned container equipped with aquaria and aerated recirculated natural seawater at 0.5 ± 1.0°C for 2-3 weeks. Fish that did not demonstrate good health over this period were not used in the experiments. Fish were not fed prior to the experiments, which were all carried out in the laboratories onboard.

A second suite of experiments to record cellular respiration rates of hepatocytes of the sub-Antarctic eelpout *Pachycara brachycephalum* was conducted previously in the thermal range of 0 to 21°C. These experiments were carried out at the Alfred Wegener Institute between December 2001 and March 2002. Fish used in these experiments were caught at a depth of 500m close to King George Island (Antarctic Peninsula) during the cruise ANT XIX of Polarstern in April/May, 2001. Fish were transferred to the institute and kept in well-aerated water of 0.0 ± 0.5 °C at 32-34 ‰ salinity until experimentation.

Preparation of cellular isolates

Hepatocytes were isolated following a protocol modified after Mommsen et al. (1994). Fish were anaesthetised in MS-222 (3-Amino-benzoic-methanosulfonate, 0,5g/l); the liver was carefully excised and transferred into a Petri dish on ice with 4ml/g fresh weight of solution 1 (Hank's Medium without magnesium (to prevent blood clotting), containing: glucose: 5,6mM; KCl: 5mM; $NaHCO_3$: 4mM; Na_2HPO_4: 0,3mM; NaCl: 240mM; KH_2PO_4: 0,4mM; HEPES: 10mM; pH 7,4). Fish were killed afterwards by a cut through the spine and removal of the heart. Blood was removed from the liver by perfusion of the *Vena cava hepatica* with ice-cold solution 1, until no more blood cells were visible in the drain. Then, the liver was perfused on ice via the *Vena cava* with 2ml /g fw. ice-cold collagenase solution (solution 3: solution 1 + 1% BSA + 750U*ml^{-1} collagenase type IV) and gently massaged for about 10 minutes. Peritoneal tissue was removed, the rest finely chopped and gently shaken on ice for about 60 minutes,

until total disintegration of the tissue. The solution was then filtered through a 250µm mesh-size gaze. Hepatocytes were collected by gentle centrifugation (4 min at 60g) and washed repeatedly by centrifugation (2 min at 60g) in solution 2 (solution 1 + 1% BSA), until the lipid phase and all erythrocytes were removed. Cells were stored in solution 4 (solution 2 + 5mM glucose and 2mM $MgSO_4$) at 0°C on a shaking desk. Cell titres were assessed in a Fuchs-Rosenthal haemocytometer dish and viability of cells was determined by Trypan blue exclusion (>95%). Total protein content was measured according to Bradford (1976). Samples of cellular suspensions were frozen in liquid nitrogen, stored at –80°C and broken up by ultrasound treatment before analysis (4min at 0°C).

Respiration and inhibitors

Cells from about 5-10g liver tissue were freshly isolated each day. When necessary, several animals of the smaller species were pooled to collect enough liver fresh mass. Cellular suspensions were diluted to $15*10^6$ cells * ml^{-1} and kept under constant shaking on ice in solution 4 throughout the experiments. Measurements were carried out in duplicates in two parallel setups consisting of Perspex respiration chambers (Ranks Brothers, Cambridge, UK) that could be volume adjusted between 300-1500µl and temperature controlled by a thermostat (Lauda, Königshofen, Germany). Respiration was measured using micro-optodes and the TX system of PreSens (PreSens GmbH, Regensburg, Germany), connected to a laptop computer (Compaq Armada 500) via a MacLab system running the Chart 5.0 software (ADInstruments, Caste Hill, Australia).

300µl of the cell suspension were spun down briefly (1min, 60g, 0°C) and 200µl of the supernatant exchanged with fresh ice-cold solution 4. The cells were then resuspended and transferred into the respiration chambers. The chambers were sealed airtight and a micro-optode was inserted through the lid. Blank respiration was recorded for 20min, then the optode was withdrawn and inhibitor stock solution was added to the suspension with a microlitre glass syringe (Hamilton, Bonaduz, Switzerland). After reintroduction of the micro-optode, respiration was recorded for 40min. The cells were removed and respiration chambers washed twice with distilled water and 70% ethanol. A new experiment was run with fresh cells and a different inhibitor. Respiration rates were calculated and cellular respiration in the presence of an inhibitor was quantified in relation to its respective control rate to account for potential deterioration of cell quality over time. Cell viability was checked after the last run and was always found to be >90%.

The following inhibitors were used:

Cycloheximide was used at a concentration of 100μM to inactivate peptidyl transferase activity of the ribosomal 60S subunit (i.e. to inhibit protein synthesis, (Wieser and Krumschnabel, 2001; Langenbuch and Pörtner, 2003)). To estimate the energetic needs of Na^+/K^+-ATPase, ouabain was used at a concentration of 6,67mM (Pannevis and Houlihan, 1992; Krumschnabel et al., 1994a). Actinomycin D was administered at a concentration of 100nM to block RNA and DNA synthesis (Smith and Houlihan, 1995). To inhibit mitochondrial ATP production (F_oF_1-ATPase), cells were incubated with 10μg/ml oligomycin (Gamper and Savina, 2000).

All inhibitors were dissolved in DMSO, preliminary experiments had shown that DMSO addition of <5% of the total assay volume did not affect respiration rates. In a preliminary set of experiments we determined the minimum concentrations of inhibitors sufficient for maximum reduction of oxygen consumption, since it has been shown that overdoses of inhibitors can lead to overestimates of the particular metabolic process due to side effects and even to cell death (Wieser and Krumschnabel, 2001). Due to possible cross reactivity, inhibitors were never applied in combination.

Statistical analysis

Statistical analyses of differences between total cellular respiration rates and differences between inhibited fractions of total respiration were carried out using Prism 4.0a and InStat 3.0b (GraphPad Software, Inc.). Differences between control and elevated respiration rates in the warm were determined by t-tests and considered significant if $P < 0.05$.

To test for the temperature sensitivity of each cellular process identified by its specific inhibitor, data were arcsin transformed and analysed through Spearman Rank correlations and one-way analyses of variance (ANOVA). Furthermore, differences between inhibitor sensitive respiration at control and elevated temperatures were determined by t-tests, which were also applied to test for differences of the total means (within the thermal range of 0-15°C) of inhibitor sensitive respiration between investigated species.

Again, differences were considered significant if $P < 0.05$. If not stated otherwise, all data are presented as values ± standard error of the mean (SEM).

Results

Respiration

Oxygen consumption rates of isolated hepatocytes between 0 and 15°C were between 0.1 and 0.7 nmol O_2 * 10^6 $cells^{-1}$ * min^{-1} in all examined species (figure 1 & 2). In contrast to the high-Antarctic species, the sub-Antarctic notothenioid *L. larseni* showed the lowest rates of cellular oxygen consumption between 3 and 6°C (figure 1A). The sub-Antarctic *P. brachycephalum* displayed a similar pattern, lowest rates of oxygen consumption were around 3°C and rose upon warming as well as cooling (figure 2). These species were the only to show an increase in oxygen consumption upon cooling from 3 to 0°C. In the high-Antarctic species (figure 1B-E), lowest rates were found at 0°C, cellular rates were significantly higher at all temperatures than at 0°C. The increases in oxygen consumption followed variable patterns in each species: In *T. eulepidotus* hepatocytes (figure 1B) there was a steady increase in respiration rates with temperature with a tendency to level off between 12 and 15°C. Cells obtained from *T. pennellii* showed a more moderate increase up to 9°C, followed by a steep increment to rates of oxygen consumption similar to those of *T. eulepidotus* above 9°C (figure 1C). Oxygen demand of *T. lepidorhinus* hepatocytes rose during warming to 6°C (figure 1D) and remained constant thereafter. In *T. bernacchii* cellular respiration also rose and levelled off beyond 6°C (figure 1E), while in *A. orianae* hepatocytes oxygen consumption peaked at 12°C (figure 1F). Yet, in these two species the picture is incomplete due to insufficient availability of tissue samples.

Energy budgets

Within the cellular energy budgets, the largest oxygen consumers of the cell (RNA, protein and ATP synthesis, ion regulation) more or less uniformly claimed the same fraction of available oxygen over the entire investigated temperature range (figure 1). In all investigated species, mean cycloheximide sensitive respiration constituted 25-37%, mean actinomycine sensitive respiration 24-35%, mean ouabain sensitive respiration 40-45% and mean oligomycine sensitive respiration accounted for 57-65% (cf. table 1). By measuring the oligomycin sensitive fraction of total respiration, ATP-synthesis was accounted for. Oligomycin sensitive respiration is a cue for mitochondrial efficiency and degree of coupling, measured by the P/O ratio (moles of ATP produced per moles of O_2 consumed). Subtracting oligomycin sensitive respiration from total respiration yields an indirect measure of processes like proton leak and non-mitochondrial respiration, which on average accounted for 35-43% in the hepatocytes of the species investigated here.

In *Trematomus eulepidotus* we found a significant decrease in cycloheximide sensitive respiration with increasing temperature (figure 1B).

In parallel to an increase in cellular respiration, the energy budget of the sub-Antarctic species *Lepidonotothen larseni* (figure 1A) displayed significant reductions in cycloheximide, actinomycin D and ouabain sensitive respiration while oligomycine sensitive respiration was elevated between 3 and 0°C (67%, cf. table 1).

Differences in functional rates

Mean inhibitor sensitive fractions over the thermal range of 0 to 15°C showed further differences between sub- and high-Antarctic fish species (figure 3): cycloheximide sensitive respiration was significantly lower in hepatocytes of the sub-Antarctic *L. larseni* than in the high Antarctic trematomid species *T. eulepidotus*, *T. pennellii* and *T. bernacchii* (it was also lower in *T. lepidorhinus*, but not significantly so). In contrast, the ouabain sensitive fraction of hepatocyte total respiration displayed a trend towards higher levels than in the high Antarctic trematomids in the range of 0-15°C.

Discussion

The aim of this study was to elaborate temperature dependent energy allocation to key metabolic processes in the cell. It was based on the question whether warm temperatures would elicit disturbances in cellular energy allocation that might contribute to whole organism thermal intolerance, especially so in the thermally most sensitive high-Antarctic notothenioids.

Cellular respiration

Hepatocyte oxygen consumption was very similar in all investigated species, ranging from 0.1 nmol O_2* min^{-1}*10^6 $cells^{-1}$ at low temperatures to a maximum of about 0.7 nmol O_2* min^{-1}*10^6 $cells^{-1}$ in *T. pennellii* at 15°C (figure 1 & 2). This is in line with respiration data recorded in hepatocytes from a variety of fish species (Antarctic *Lepidonotothen kempi* (Langenbuch and Pörtner, 2003); goldfish: (Krumschnabel et al., 1994b); temperate zoarcids (*Z. viviparus*): Mark (unpubl.)). Moreover, hepatocytes of goldfish and trout measured at 20°C and 15°C, respectively, show respiration rates (0.4 and 0.6 nmol O_2* min^{-1}*10^6 $cells^{-1}$, respectively (Krumschnabel et al., 2001)) similar to those of the high Antarctic trematomids at 15°C. At the cellular level this observation confirms the absence of metabolic cold adaptation in these notothenioid species, as has been argued by Clarke & Johnston (1999) based on a literature survey of oxygen consumption data from whole animals.

Within the range of experimental temperatures, it becomes evident that cellular respiration does not necessarily increase upon warming. Metabolic processes are not always exponentially linked to temperature, Q_{10} may fluctuate and can adopt higher values towards temperature extremes (Haschemeyer and Mathews, 1982) or warming may only then result in elevated respiration rates. Within the cellular oxygen consumption data presented in this study, we found clear evidence of species-specific patterns of cellular respiration, which will likely influence whole organism thermal sensitivity. Hepatocytes of the sub-Antarctic species *L. larseni* appeared to have the lowest energetic requirements between 3 and 6°C, below and above which cellular oxygen consumption rose. This indicates a rise in metabolic energy turnover in the liver in the cold, pointing to an energetic optimum (i.e. a temperature range of minimal energetic costs) for the organism at temperatures significantly higher than 0°C (figure 1A). In fact, specimens of *L. larseni* were caught off Bouvet Island (54°30,22 S; 003°14,37 E), which is located within the oscillations of the Southern boundary of the Antarctic Polar front. Thus, water temperatures are influenced by both the cold waters of the Southern Ocean and the warmer waters of the South Atlantic and are bound to vary both annually and seasonally. Water temperatures at the time of the catch ranged around 0.6°C. This relatively warm

temperature contrasts values of –1.0 to -1.8°C found in the Weddell Sea, where the high-Antarctic notothenioids were caught.

Observations of an energetic optimum are corroborated by the cellular oxygen consumption data recorded for the Antarctic eelpout *Pachycara brachycephalum* (figure 2), which showed a similar pattern with lowest oxygen consumption at 3°C. In sub-Antarctic conditions around the Antarctic peninsula, this species is frequently found, yet in the high Antarctic, *P. brachycephalum* only occurs in warmer sub-Antarctic deep-water layers at temperatures above 0°C (R. Knust, AWI, pers. comm.). Accordingly, the eelpout as well as *L. larseni* probably live close to their lower ecophysiological limits in sub- or deep Antarctic waters. Consequently, they may be more cold eurythermal than the high-Antarctic cold stenotherms.

Different patterns of thermal responses could also be observed within the five remaining high Antarctic notothenioid species of *Trematomus* and *Artedidraco* (figure 1B-F): In *T. eulepidotus* there was a steady increase in cellular respiration with temperature with a tendency to level off between 12 and 15°C, possibly indicating the onset of a limitation of hepatic metabolic capacity just above 12°C. In contrast, hepatocytes of *T. pennellii* showed a dramatic 2- to 3-fold increase in respiration rate towards the warm end of the investigated thermal range. This drastic increment reflects an over-proportional rise in metabolic costs, which is still covered by the capacity of cellular energy production, indicated by unchanged energy budgets. *T. lepidorhinus* neither showed a steady rise in cellular oxygen consumption, nor an abrupt increment. In the light of unchanged energy budgets, this species may be the least thermally sensitive. For *T. bernacchii* and *A. orianae*, data are insufficient but indicate limited metabolic capacities at higher temperatures (figure 1E-F). These patterns may reflect different and specific thermal sensitivities of the different species and calls for the respective investigations at the whole animal level.

Cellular energy budgets

Methodological considerations

Interpretation of inhibitor data is often problematic and error prone and therefore any such energy budgets will have to be interpreted with adequate precaution. The choice of the medium used to measure cellular respiration is of eminent importance as is the concentration of the inhibitor. This has convincingly been shown in the work of Wieser and Krumschnabel (2001), who demonstrated for trout and goldfish hepatocytes that cellular respiration increased up to five-fold and the relative fraction of protein synthesis rose considerably in free amino acid enriched Leibovitz (L-15) medium as compared to Hank's balanced salt solution. The authors also provided evidence for cycloheximide to inhibit far more metabolic processes than

just protein synthesis when applied in overly high concentrations. Generally, by directly or indirectly affecting further metabolic processes, inhibitors will always act somewhat unspecific and the actual share of a particular metabolic process is overestimated. For example, we treated RNA/DNA synthesis and protein synthesis as discrete processes, which they are not: once mRNA synthesis is inhibited, protein synthesis will also decrease to some extent, due to a lack of new transcripts. Moreover, we did not account for Ca^{2+}-ATPase or proton leak, the first of which comprises up to 10% of total cellular respiration in rat thymocytes (Buttgereit and Brand, 1995) and the latter about 10% of maximum mitochondrial respiration in isolated liver mitochondria of the notothenioid *Lepidonotothen nudifrons* (Hardewig et al., 1999), respectively.

Still, cycloheximide, actinomycin D and ouabain sensitive respiration can add up to more than 100% of total cellular respiration, which is indicative of secondary inhibition of dependent processes. As a consequence, cellular energy budgets compiled by the use of inhibitors cannot claim to represent absolute contributions of the examined metabolic processes but can provide an idea of the fraction of energy allocated to a specific process and how energy allocation may change qualitatively with a change in experimental conditions like temperature.

Bearing all of this in mind and following a strict protocol, it is still possible to analyse thermally induced changes in the inhibitor sensitive fractions of total cellular respiration and gain valuable insights into cellular energy metabolism. In fact, the overall stability of the energy budgets regardless of temperature and despite large changes in cellular oxygen consumption provides indirect support for the validity of our measurements and the data analyses carried out.

Variability in energy budgets

Mean values of the data collected for the four inhibitors (cf. table 1) were in line with inhibitor sensitive fractions of respiration observed in fish cells by other authors: in the six species examined the mean cycloheximide sensitive respiration was 25-37% (Krumschnabel et al., 1994a; Krumschnabel et al., 1997; Smith et al., 2001; Wieser and Krumschnabel, 2001; Langenbuch and Pörtner, 2003), mean actinomycin D sensitive respiration 24-35% (rat cells: Buttgereit and Brand, 1995; fish cells: Smith and Houlihan, 1995; Casey et al., 2002), mean ouabain sensitive respiration 40-45% (Krumschnabel et al., 1994a; Krumschnabel et al., 1994b; Krumschnabel et al., 2001), and mean oligomycin sensitive respiration 57-64% (Gamper and Savina, 2000).

The variability patterns observed in cellular respiration were only in part reflected in the energy budgets, which proved to be rather stable and temperature insensitive.

In the sub-Antarctic species *L. larseni*, cycloheximide sensitive respiration appeared reduced below 6°C, indicating lower levels of protein synthesis in the cold in this species. At 3°C actinomycin D sensitive respiration appeared reduced, which may have consequences for RNA/DNA synthesis. Ouabain sensitive respiration is significantly reduced at 0°C when compared to 6°C and higher and indicates a potential shift in ion regulation processes. Alternatively, an unexplained ATP consuming process rose and caused the respective reduction in the share of protein and RNA synthesis or ion exchange in the energy budget.
According to the theory of a hierarchy of ATP-utilizing processes first brought forward by Atkinson (1977) and corroborated by Buttgereit & Brand (1995) and Wieser & Krumschnabel (2001), protein synthesis is the process most sensitive to a change in ATP supply and will decrease first when a cell's energy charge is reduced, followed by macromolecule (RNA/DNA) synthesis and then by ion regulation. In our cells, protein synthesis may be decreased due to increased energy demand by other, unidentified processes. This conclusion is supported by a rise in oligomycine sensitive respiration (i.e. ATP synthesis rate) and an overall increase in cellular respiration at 0 and 3°C to avoid a drop in energy charge. Judging from the stable cellular energetic conditions observed by NMR spectroscopy in alive specimens of *P. brachycephalum* by Mark et al. (2002) during warming, there is most likely no drop in energy charge in the cells of all species used in this study, especially as the isolated cells are not constricted by systemic limitations.

In the high-Antarctic notothenioids, cellular energy budgets displayed different characteristics: in *T. eulepidotus* hepatocytes (figure 1B), we found a significant decrease in cycloheximide-sensitive respiration with rising temperature, possibly indicating the onset of a cellular metabolic capacity limitation. This limitation may lead to the predominant use of cellular ATP supply by baseline cellular functions, i.e. ion exchange. In the other species, no significant changes in cellular energy metabolism could be observed. It is astounding to find so few signs for a shift in ATP-consuming processes in the cells, even though they have been warmed to temperatures far beyond the survival range of the whole organisms (Somero and DeVries, 1967). Thus, although showing signs of energetic optima at rather low temperatures, the restrictive mechanisms limiting whole organism thermal tolerance cannot be operative at the cellular level in these Antarctic and sub-Antarctic notothenioids.

When comparing total cycloheximide and ouabain sensitive respiration among the nototheniids in the range from 0 to 15°C (figure 3), cycloheximide sensitive respiration was significantly higher in most of the high-Antarctic notothenioids than in the sub-Antarctic species, while ouabain sensitive respiration was significantly lower. This bias in energy allocation indicates that the stenothermal high Antarctic notothenioids presumably possess

greater protein synthesis capacities than the eurythermal sub-Antarctic species but lower capacities of active ion regulation.

Storch and coworkers (Storch et al., submitted) observed higher protein synthesis capacities in the cold eurythermal Antarctic eelpout *P. brachycephalum* as compared to the warm eurythermal common eelpout *Zoarces viviparus*, and other authors found evidence for cold compensated protein synthesis in high-Antarctic fish (Smith and Haschemeyer, 1980) and sea urchin embryos (Marsh et al., 2001). Furthermore, several studies investigating pH and ion regulation in eurythermal and stenothermal fish (Pörtner and Sartoris, 1999; Bock et al., 2001; Sartoris et al., 2003a) found the greater part of pH regulation in eurythermal fish to be dependent on active processes like regulation via Na^+/K^+-ATPase, while in stenothermal fish less costly passive processes prevailed. Our data for protein synthesis rates in the sub-Antarctic nototheniid *L. larseni* on the one hand and for Na^+/K^+-ATPase rates on the other are consistent with these findings for eurythermal fish and mark this species down as a more eurythermal species in comparison to the stenothermal high-Antarctic species.

Conclusions

Provided with sufficient energy and oxygen, cells can survive and maintain metabolic functions within a far wider thermal window than the more complex organisms from which they originate (cf. Somero and DeVries, 1967). Apart from small changes in energy allocation in cycloheximide and oligomycin sensitive respiration in the sub-Antarctic notothenioid species *L. larseni*, there were no distinct shifts in energy allocation over the investigated thermal range, nor did we identify any threshold temperatures beyond which abrupt changes in energy budget occurred. Energetic limitations to thermal tolerance must therefore be set at the organismic level. Here they occur through a mismatch in oxygen supply and demand, provoking a progressive reduction in aerobic scope (Pörtner et al., 2004). Accordingly, our present findings indirectly support the concept of oxygen limited thermal tolerance (Pörtner, 2001, 2002). In an earlier study in the sub-Antarctic eelpout *P. brachycephalum* (Mark et al., 2002), we found evidence for a limitation in functional capacity of the cardio-vascular system, which has subsequently also been observed in cod (Sartoris et al., 2003b; Lannig et al., 2004) and rainbow trout (Farrell and Clutterham, 2003), in line with earlier findings by Heath (1973). The cod studies by Lannig, Sartoris and coworkers revealed the primary limiting role of the cardio-vascular system: Over a wide thermal range arterial oxygen partial pressure remained constant, while venous oxygen partial pressure decreased steadily from a maximum at the animals' optimal temperature towards both the cold and warm ends of the thermal range. Under progressively increasing thermal stress, cardio-vascular capacities are not sufficient to

provide full aerobic scope and tissue oxygen extraction leaves venous blood increasingly oxygen depleted, eventually culminating in cellular oxygen limitation. Moreover, many fish species lack coronary circulation and rely on the venous oxygen reserve for myocardial oxygen supply (Farrell, 1993), thus only exacerbating the dilemma.

These considerations are consistent with the concept of a hierarchy of systemic to molecular processes contributing to thermal limitation in a complex organism (cf. Pörtner, 2002; Pörtner et al., 2004). The most sensitive process with respect to thermal tolerance is supposed to be set at the highest levels of organisational complexity. In fish, cardio-vascular more than ventilatory performance appears to be the bottleneck of thermal tolerance, although the various levels of organisation have evolved into a complex organism, in which the capacities of systemic to molecular processes closely match. Taylor and Weibel (1981) developed the concept of symmorphosis, which states that the functional capacities of individual components are designed to suit the higher unit, i.e. the organism. Furthermore, the concept indicates that an organism's functional capacities are never expressed in excess of its direct environmental needs. At the cellular level, one might thus find a wider thermal tolerance once the constraints at the higher levels of organisational complexity are no longer operative. The optimum of maximum cellular energy efficiency is still set to within the thermal range of the whole organism. Once organismic oxygen supply to cells by the cardio-vascular system declines, cellular hierarchies of energy allocation become effective and lead to reductions in the scopes for growth and reproduction, then in cellular maintenance metabolism (DNA synthesis and ion homeostasis) and ultimately to cell death.

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Figure 1

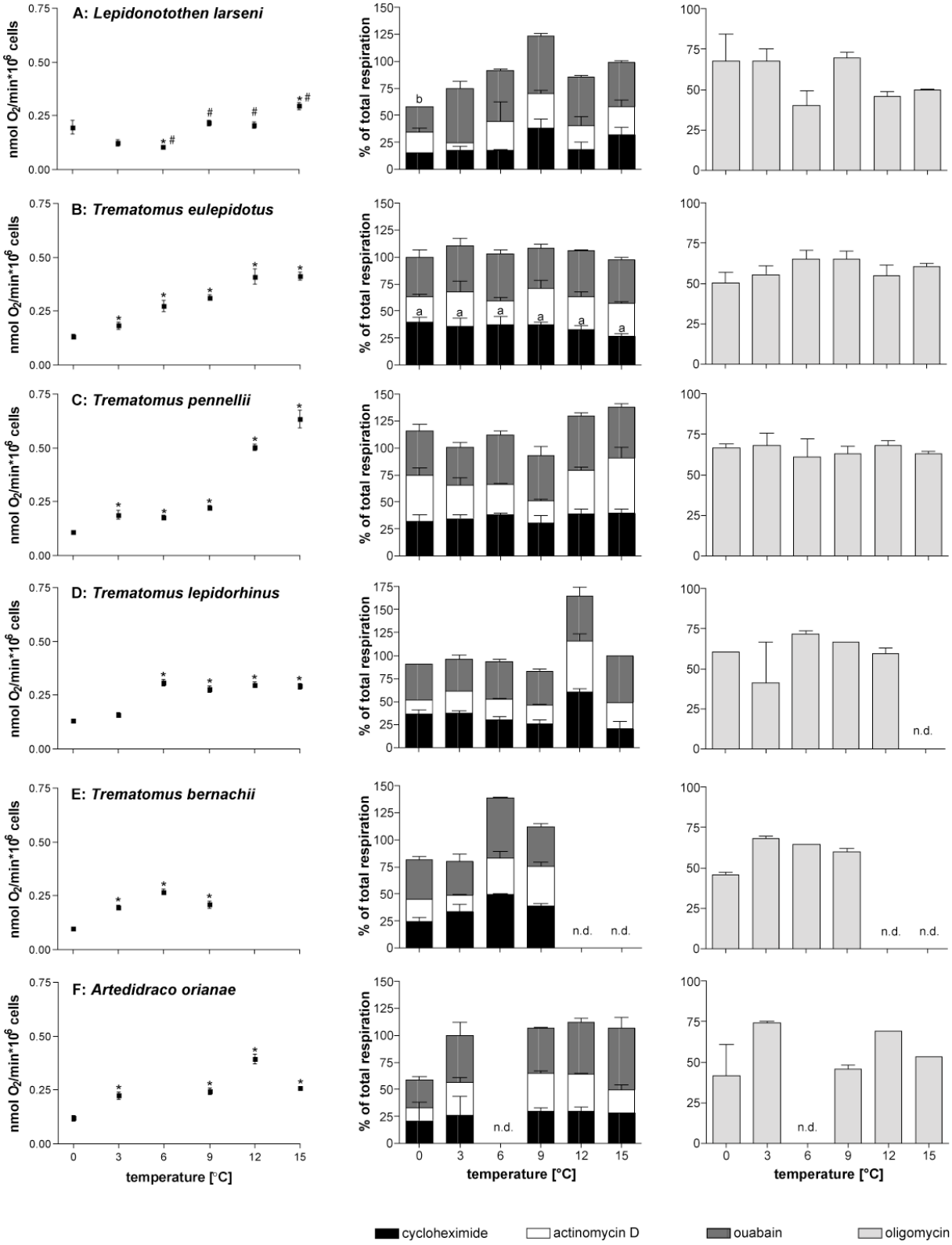
A: Lepidonotothen larseni
B: Trematomus eulepidotus
C: Trematomus pennellii
D: Trematomus lepidorhinus
E: Trematomus bernachii
F: Artedidraco orianae
nmol O2/min*10^6 cells
% of total respiration
temperature [°C]
n.d.
cycloheximide
actinomycin D
ouabain
oligomycin

figure 2

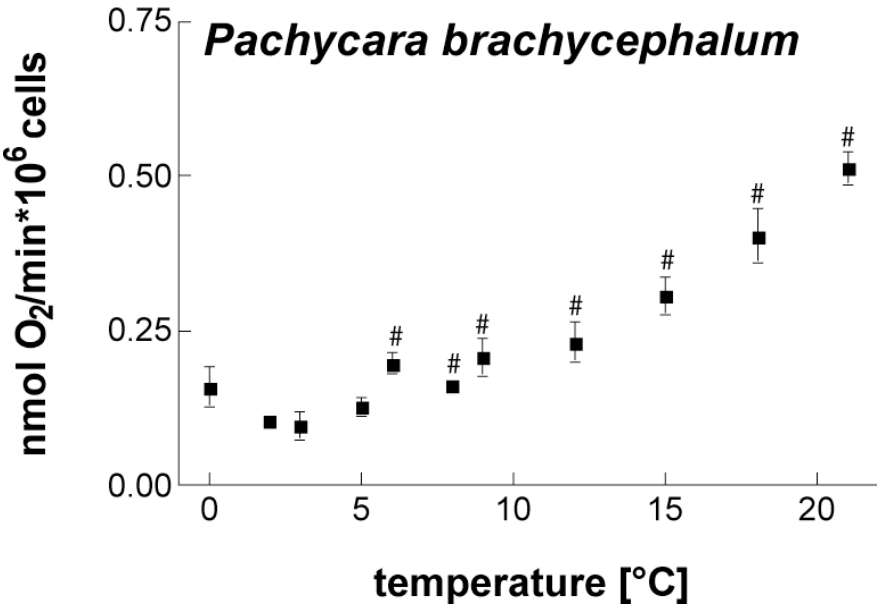
Pachycara brachycephalum
nmol O2/min*10^6 cells
0.75
0.50
0.25
0.00
0
5
10
15
20
temperature [°C]
#

figure 3

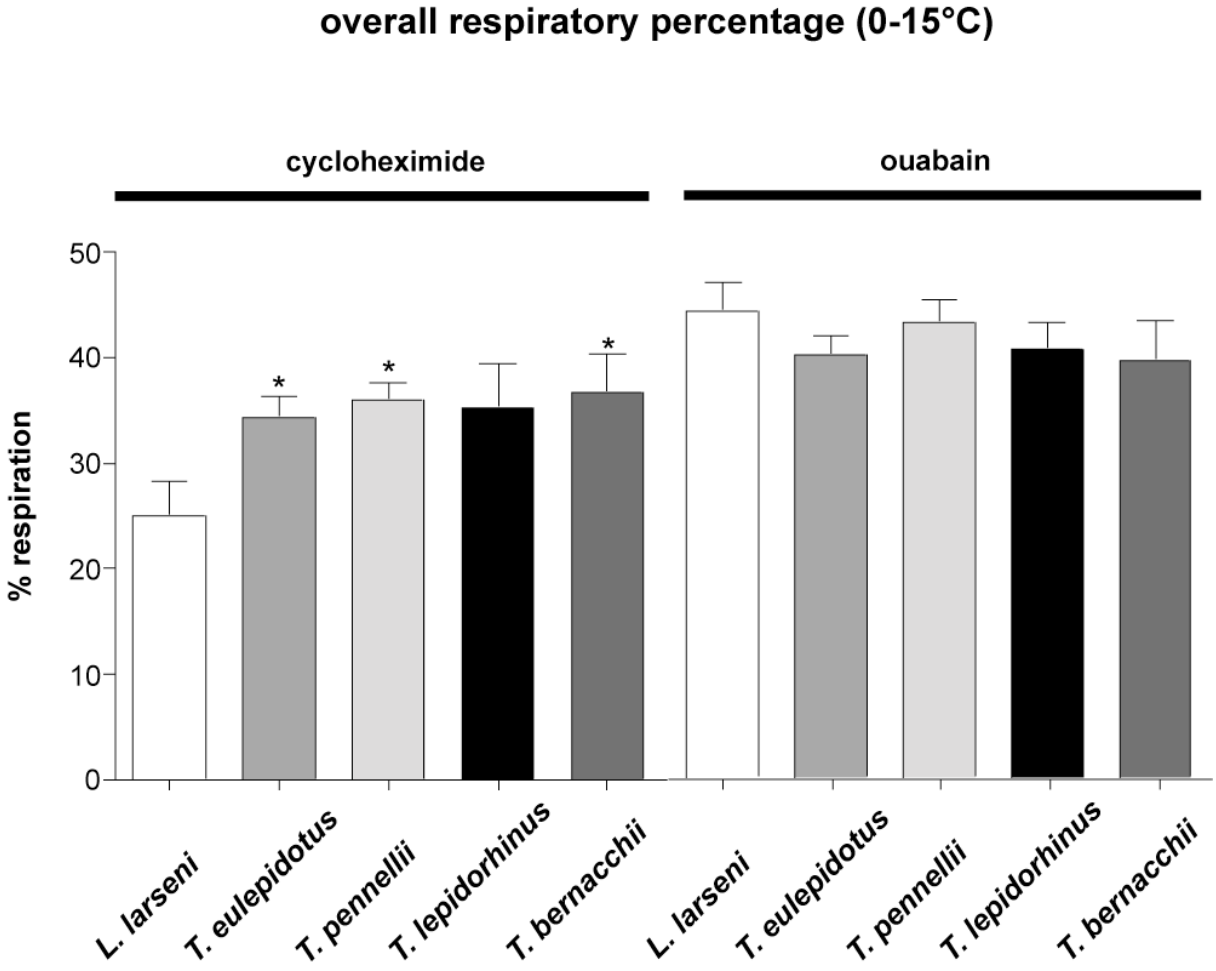
overall respiratory percentage (0-15°C)
cycloheximide
ouabain
% respiration
50
40
30
20
10
0
*
*
*
L. larseni
T. eulepidotus
T. pennellii
T. lepidorhinus
T. bernacchii
L. larseni
T. eulepidotus
T. pennellii
T. lepidorhinus
T. bernacchii

table 1

	cycloheximide												actinomycin D											
	Lepidonotothen larseni		*Trematomus eulepidotus*		*Trematomus pennellii*		*Trematomus lepidorhinus*		*Trematomus bernacchii*		*Artedidraco orianae*		*Lepidonotothen larseni*		*Trematomus eulepidotus*		*Trematomus pennellii*		*Trematomus lepidorhinus*		*Trematomus bernacchii*		*Artedidraco orianae*	
temperature	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	15,1	0,2	39,3	5,1	31,9	6,4	36,6	4,0	24,4	3,9	20,7	17,2	18,9	3,8	23,8	2,7	42,5	7,1	14,8	--	20,2	0,4	12,2	--
3	17,2	4,2	35,9	7,7	34,6	3,2	37,6	2,7	33,8	7,0	26,3	17,3	7,5	0,1	31,9	10,0	30,8	6,9	23,5	0,0	15,3	0,6	30,2	4,6
6	17,3	0,9	37,1	7,7	37,9	2,0	30,4	3,2	49,4	1,2	--	--	27,0	18,4	22,2	2,9	28,3	0,9	21,9	1,2	33,7	6,1	--	--
9	37,9	8,5	37,2	2,3	30,2	6,9	25,5	4,4	39,0	2,2	30,1	3,1	32,4	2,6	33,6	7,7	20,5	1,7	21,1	0,7	36,3	4,2	34,4	2,6
12	18,6	6,4	32,9	3,6	38,9	4,3	60,8	3,0	--	--	29,8	4,0	21,5	8,7	30,2	5,0	40,1	3,1	55,1	7,6	--	--	34,3	1,0
15	32,0	6,6	26,8	1,9	39,7	3,8	20,7	7,9	--	--	28,2	0,0	26,1	6,3	30,7	1,6	51,0	9,9	28,0	0,1	--	--	21,2	4,8
Mean 0-15°C	25,0	3,2	34,4	1,9	35,9	1,8	35,3	4,1	36,6	3,8	26,9	4,3	23,7	3,3	28,4	2,0	35,1	2,9	29,1	4,6	26,4	3,6	28,1	2,9
	ouabain												oligomycin											
temperature	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	24,1	0,2	36,9	6,3	41,2	6,1	39,6	0,0	36,9	3,1	25,9	3,3	67,6	16,7	50,2	6,5	66,4	2,5	60,4	--	45,6	1,8	41,5	19,4
3	49,7	7,2	42,9	6,9	35,1	4,8	34,5	4,6	30,9	7,0	43,4	12,4	67,3	7,7	55,4	5,6	68,1	7,5	41,2	25,2	68,3	1,3	74,4	0,9
6	47,3	1,1	43,7	3,4	46,2	3,1	40,7	2,6	55,3	0,7	--	--	40,0	9,2	65,0	5,7	61,2	10,9	71,8	1,9	64,7	0,1	--	--
9	52,9	2,7	37,1	4,1	42,5	8,5	36,6	2,0	36,8	2,8	42,0	0,9	69,5	3,5	65,2	4,7	63,2	4,5	66,5	0,0	59,8	2,3	45,8	2,3
12	45,2	1,7	42,5	1,0	50,4	3,0	48,8	9,2	--	--	47,5	3,9	45,7	3,0	54,8	6,8	67,8	3,5	59,5	3,5	--	--	69,0	0,3
15	40,7	1,8	40,2	2,3	47,1	3,4	50,9	0,0	--	--	57,3	10,1	49,7	0,8	60,4	2,0	62,9	1,7	--	--	--	--	53,1	0,2
Mean 0-15°C	44,7	2,8	40,5	1,9	43,7	2,1	41,2	2,5	40,0	3,8	43,2	4,2	59,6	3,9	57,6	2,5	65,3	1,8	59,8	5,7	59,6	3,3	56,8	5,2

figure legends

figure 1

Cellular respiration (left) and energy budgets (right) in the temperature range between 0 and 15°C. In the energy budgets, inhibitor sensitive respiration is depicted as follows: black: cycloheximide sensitive respiration (protein synthesis); white: actinomycin D sensitive respiration (RNA/DNA synthesis); dark grey: ouabain sensitive respiration (Na^+/K^+-ATPase); and light grey: oligomycine sensitive respiration (F_OF_1-ATPase). Asteriks indicate cellular respiration rates significantly different from values at 0°C ($P<0.05$), # indicates cellular respiration rates significantly different from values at 3°C ($P<0.05$). a: significant decrease in oligomycin sensitive respiration from 0 to 15°C (*T. eulepidotus*); b: ouabain sensitive respiration significantly lower than at 6 – 15°C (*L. larseni*); n.d.: not determined; n=2-8, all values given as means ± standard error of the mean (SEM), where applicable.

figure 2:

Cellular respiration of hepatocytes of the Antarctic eelpout *Pachycara brachycephalum* in the thermal range between 0 and 21°C; n=3-6; all values given as means ± standard error of the mean (SEM). # indicates cellular respiration rates significantly different from values at 3°C ($P<0.05$).

figure 3:

Mean cycloheximide and ouabain sensitive cellular respiration as measured between 0 and 15°C in the nototheniid species. Asteriks indicate cellular respiration values in the high-Antarctic nototheniids significantly different from the sub-Antarctic *L. larseni* ($P<0.05$), all values given as means ± standard error of the mean (SEM).

table 1:

Inhibition of cellular respiration rates by the four inhibitors used, presented as percent fractions of total respiration rates as in the energy budgets in figure 1. --: not determined; n=2-8, all values given as means ± standard error of the mean (SEM), where applicable.

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Are mitochondrial uncoupling proteins involved in thermal acclimation of polar and temperate fish?

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Abstract

Uncoupling proteins (UCP), especially UCP2, may play a role in the temperature dependent setting of energy turnover in animals and their mitochondria. Therefore, the genes and expression of UCP2 were investigated in the Antarctic eelpout *Pachycara brachycephalum* and a temperate confamilial species, the common eelpout *Zoarces viviparus*. UCP2 full-length cDNA was amplified from liver and muscle using RT-PCR and rapid amplification of cDNA ends (RACE). The UCP2 gene consists of 1906bp in *P. brachycephalum* and of 1876bp in *Z. viviparus*. Both genes contain open reading frames of 939bp, encoding 313 amino acids, with 98 and 99% identity, respectively. Protein sequences of zoarcid UCP2 are closely related to fish and mammalian UCP2. For analysis of temperature dependent expression common eelpouts were cold-acclimated from 10°C to 2°C and Antarctic eelpouts were warm-acclimated from 0°C to 5°C. Identical cDNA probes for both species were developed to investigate UCP2 mRNA expression, and protein expression levels were detected by Western Blot in the enriched membrane fraction. During cold-acclimation in *Z. viviparus*, mRNA levels increased by a factor up to 2.0, protein levels increased up to 1.5, in line with mitochondrial proliferation during cold-acclimation. In Antarctic eelpout, however, UCP2 levels rose upon warm acclimation, by a factor up to 2.0 (mRNA) and 1.6 (protein), respectively. The data indicate an important role for UCP2 expression in thermal adaptation of fish. A function of UCP2 in controlling the mitochondrial membrane potential to balance ROS formation and ATP production during thermal stress is discussed.

Introduction

Since the discovery of the first uncoupling protein (UCP1) in mammalian brown adipose tissue (BAT) (36), the various roles of UCPs have been widely discussed, with particular respect to their implications for energy metabolism. Uncoupling proteins (UCPs) belong to the family of mitochondrial membrane transporter proteins (59) and provide a channel for protons, which flow back in after having been pumped out of the mitochondrial matrix by the enzymes of the electron transport chain. By so dissipating the electrochemical proton gradient, which drives mitochondrial ATP synthesis over the F_oF_1-ATPase, UCPs mediate the so-called proton leak over the inner mitochondrial membrane (22, 38) and influence aerobic ATP formation of the cell.

UCP1 is restricted to mammalian BAT and has a clear role in thermoregulation in hibernators and small mammals, generating heat by the dissipation of membrane potential. Over the last few years, a variety of homologues of UCP1 have been identified in placental and non-placental mammalian tissues (5, 11, 21, 35), suggesting a more central role for the UCP family in metabolism. The specific functions of the different UCP isoforms, however, are not clear.

Up to now, research has concentrated mainly on mammalian UCPs, focussing on obesity and ageing, but UCP homologues can be found in birds (42, 57, 58), plants (16, 24, 30), protists (20) and fungi (19), and thus have been shown to appear in all four eukaryotic kingdoms.

Stuart and coworkers (56) identified homologues of the mammalian uncoupling protein 2 in cDNA libraries of zebrafish (*Danio rerio*) and carp (*Cyprinus carpio*). Recently, a partial sequence of UCP2 isolated from red sea bream (*Pagrus major*) liver tissue was published by Liang *et al.* (27), thereby substantiating that UCP2 is common among fish.

It is unlikely that UCPs of most water breathing ectotherms have a role in thermoregulation: Because of the high thermal capacity of water, any metabolic heat is instantly lost over the gills. Only in tuna red muscle, some heat is conserved inside the body, eg. by *retia mirabilia* systems (55). In their habitats, fish can experience wide fluctuations of ambient water temperature throughout the year, and as the rate of their metabolic reactions follows temperature passively, they have to adjust metabolic energy supply according to energy demand (cf. 15, 40). Assuming a central position in energy metabolism, UCP2 in ectotherms might thus be involved in metabolic processes related to thermal adaptation rather than thermoregulation.

In mammals and birds, UCP1, UCP2 and UCP3 show temperature sensitive expression and their levels increase upon cold exposure (42, 45, 50, 58). Although ectothermal UCP2 might

have a function different from that of mammals and birds, it is conceivable that its expression level is also dependent on temperature.

Very little is known about the temperature sensitivity of UCP expression and *in vivo* physiological regulation of the gene in ectotherms (protists: 18). In part this is due to the historical focus on mammalian UCPs. With this study we investigate a putative role for UCP in temperature adaptation of two closely related members of the ubiquitous fish family Zoarcidae from different thermal habitats, the eurythermal common eelpout (*Zoarces viviparus*) from the Baltic Sea and the stenothermal Antarctic eelpout (*Pachycara brachycephalum*) from Antarctic waters. White muscle and liver were chosen as tissues for the identification of UCP2 homologues and the studies of their temperature dependent expression. These tissues have been widely examined with special regard to thermal adaptation in ectotherms in a number of recent studies (13, 14, 25, 26, 28), which therefore provide a good basis for the evaluation of results presented in this study. In the sluggish benthic zoarcids, white muscle tissue is hypometabolic and does not show large alterations of its characteristics with temperature, while liver is a metabolically very active organ and over a temperature range can undergo large changes in size and function, for example when serving as a lipid depot. With UCP expression examined in tissues of high and low metabolic activity of a temperate eurythermal and a cold adapted fish, after cold or warm acclimation, this study is based on a broad range of physiological preconditions and is the first to provide detailed insight into temperature dependent expression of fish UCP2.

Materials and methods

Animals

Eurythermal common eelpouts *Z. viviparus* from the Baltic Sea (mass: 100.9g ± 35.3 g) were caught during summer 2001 in the Kieler Förde. Fish were kept at 13 ‰ salinity, and were acclimated to 2.0 ± 0.5 °C (cold-acclimated) or 10.5 ± 0.5 °C (habitat temperature) for at least 2 months. Benthic Antarctic eelpouts, *P. brachycephalum,* were caught at a depth of 500 m close to King George Island (Antarctic Peninsula) during the cruise ANT XIX of the German research vessel "Polarstern" in April/May, 2001. Fish (mass: 58.9g ± 7.3g) were transferred to Bremerhaven and kept in well-aerated water of 0.0 ± 0.5 °C (habitat temperature) and 5.0 ± 0.5 °C (warm-acclimated) at 32-34 ‰ salinity for at least 2 months. All fish were kept under a 12:12-h light-dark cycle and were fed shrimps *ad libitum* once a week. Feeding was terminated 7 days prior to experimentation.

RNA-Isolation

Animals were anaesthetized with MS-222 (3-Amino-benzoic-methanosulfonate, 0.5 g/l) before being killed. Samples of different tissues were quickly removed, placed in sterile 1.5 ml tubes and were frozen immediately in liquid nitrogen. Until used for RNA or protein isolation, the samples were stored at -80°C.

For quantitative isolation of total RNA from frozen tissue the peqgold TriFast kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) was used. For the preparation of cDNA, total RNA was isolated with the RNeasy kit, and mRNA was isolated using the Oligotex mRNA kit (both kits from Qiagen, Hilden, Germany). The TriFast protocol gave slightly higher yields and could easily be scaled up for larger amounts of tissue, whereas the faster RNeasy protocol was preferred for cDNA construction. The RNA was quantified spectrophotometrically in triplicate samples. A_{260}/A_{280} ratios were always >1.9. Formaldehyde agarose gel electrophoresis according to Sambrook (49) was used to verify the integrity of the RNA.

Characterisation of the UCP2 genes

Fragments of the UCP2 gene were isolated by means of reverse transcription followed by PCR (RT-PCR). Primers were designed using the MacVector 7.0 program package (Oxford Molecular Ltd., Oxford, UK), using highly conserved regions of published sequences of the carp and zebra fish UCP2 gene (56) as a reference. Reverse transcription was performed with Superscript RT (Invitrogen, Karlsruhe, Germany) and the reverse primer 2 (for all primer

details, see table 1) using mRNA as templates. The components were incubated for 1 h at 37 °C in 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 10 mM DTT and 2.5 mM $MgCl_2$. For the amplification of the resulting single strand cDNA, forward primer 1 was used in combination with the reverse primer 2 in a PCR reaction resulting in a 440-nucleotide fragment (cf. figure 1). The procedure was repeated with a second set of primers (primers 3/4, cf. table 1) to yield a second fragment of 550 nucleotides. The design of primers followed the suggestions of the MacVector primer design software on the basis of the UCP2 sequence for *D. rerio*, which were then compared with conserved regions of other vertebrate UCP2 sequences.

The cDNA was amplified with *Taq*-Polymerase (Invitrogen, Karlsruhe, Germany) with 1.5 mM MgCl2, 1 min denaturation at 94°C, 1 min annealing at 59°C and 1 min elongation at 72°C. After amplification within 30 cycles a final prolonged elongation step of 8 min at 72°C was introduced to prepare the PCR fragments for cloning. All fragments were purified by gel electrophoresis and eluted with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany).

For cloning of the fragments, the TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany) was used according to the manual. After separation of some clones plasmids were isolated from overnight cultures using the Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany). To verify the presence and size of inserts, the isolated plasmids were analysed by restriction digestion with *Eco*RI. For each fragment, the DNA sequences of positive clones were determined for both strands by MWG Biotech (Ebersberg, Germany) using an automatic sequencer. Sequences were analysed by alignment in MacVector and a BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The full-length cDNA was determined by means of the RACE technique (rapid amplification of cDNA ends), using the RLM-RACE kit (Ambion, Austin, Texas, USA) according to the manual. The isolated cDNA fragments were used to design 3' RACE forward primers and 5' RACE reverse primers with sequences, identical for both eelpout species, and giving access to RACE fragments with a sufficient overlap to the first set of cDNA clones (cf. figure 1). Sequences of the RACE primers are listed in table 1 (no. 5-9).

Cloning and sequencing of the fragments was done following the same protocols as outlined above. Sequences were assembled in MacVector to yield the full-length cDNA sequence of UCP2 for *P. brachycephalum* and *Z. viviparus*. The cDNA sequences have been submitted to Genbank and can be obtained under following accession numbers: Genbank AY625190 (ZvUCP2); Genbank AY625191 (PbUCP2). To locate putative transmembrane helices, analyses of hydrophilicity after van Heijne and Kyte-Doolittle were carried out using the MacVector program package, which was also used for analysis of phylogenetic relationships within a number of UCP homologues.

Construction of probes

For the construction of species-specific probes for *Z. viviparus* and *P. brachycephalum* cDNA, clones for the UCP2 gene and β-actin were isolated using RT-PCR. Reverse transcription was performed following the protocol outlined above with the reverse primer 11, again using mRNA as templates. The cDNA was amplified as outlined above, using forward primer 10 in combination with the reverse primer 11 in a PCR reaction resulting in a 137-nucleotide fragment. Primers were designed with the MacVector program package, within a given region of 150 bp that was identical in both species.

A 215bp cDNA fragment of the β-actin gene from both organisms was isolated from an existing fragment of 377bp (position 161-372, cf. ref. 28) with essentially the same protocol using primer 12 as forward primer and primer 13 as reverse primer (cf. table 1). All fragments were purified by gel electrophoresis and eluted with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and then cloned using the TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany).

Quantification of UCP2 mRNA

Ribonuclease protection assays (RPA) were performed with the RPA-III kit from Ambion (Austin, USA). Total RNA (10 μg) was simultaneously hybridized at 42 °C to antisense probes for UCP2 (UCP2) and β-actin (ACT-B), in case of liver RNA, or UCP2 and 18S-rRNA (18-S), for muscle RNA, respectively. Probes were synthesized by in vitro transcription with T7 or T3 RNA Polymerase (Invitrogen, Karlsruhe, Germany) with the plasmids, containing the respective cDNA fragments (described above). For 18S-rRNA, a commercial plasmid containing a highly conserved 80bp fragment (pTRI RNA 18S, Ambion, Austin, Texas, USA) was used. All probes were labelled with α-^{32}P uridine 5´-triphoshate (Amersham Biosciences, Freiburg, Germany). To equalize protected fragment intensities, a specific radioactivity of 570 Ci/mmol was used for UCP2, 45 Ci/mmol for ACT-B and 0.1 Ci/mmol for 18S, respectively. The probes were always prepared freshly and purified by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (8 M urea, 5 % acryl amide gel with 1xTBE running buffer; (49)) using a vertical slab apparatus (Protean II xi, Bio-Rad, München, Germany). The DNA templates were removed prior to electrophoresis by DNase I treatment (Invitrogen, Karlsruhe, Germany).

The RPA was optimized according to manufacturers' instructions with an RNaseA/T1 dilution of 1:50. After RNase treatment the RNA:RNA hybrids were co-precipitated with

yeast RNA. The RNA was dissolved in 6 µl loading dye and separated by denaturing PAGE (8 M urea, 5 % acryl amide gel with 1xTBE running buffer). The size of the protected fragments corresponded to the size of the cloned PCR fragments. All probes were tested in separate lines to ensure that no background bands interfered with another probe. Primary assays have been performed to ensure the specificity of the signal by means of unrelated RNA; concentration serials were used to determine the amount of probe needed for a linearly correlated signal. After drying the gel radioactivity was detected and quantified with a phosphorous storage image system (FLA-5000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany).

Protein isolation, gel electrophoresis and Western blot analysis

Membrane enrichments were prepared from frozen tissue (about 100 mg) by disruption with a hand homogenizer in 15 vol. ice-cold buffer (50 mM imidazole, pH 7.4, 250 mM sucrose, 1 mM EDTA, 200 µg/ml PMSF (phenyl methylsulfonyl fluoride), protease inhibitor cocktail (P-2714, Sigma-Aldrich, Deisenhofen, Germany), 0.1% Na^+-desoxycholate). Cellular debris was removed by low-speed centrifugation (1020 g for 10 min at 0°C). The membranes were pelleted from the supernatant (crude extract) by final high-speed centrifugation (40 min, 200,000 g at 4°C). The membrane pellets were resuspended in a minimum volume of homogenisation buffer (~ 1/5 of the starting volume). The supernatant (cytosolic proteins) was kept for comparison. Total protein was measured using the method of Bradford (1) and a BSA standard.

Protein samples (50 µg for liver, 22.2 µg for muscle) were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (23), using a vertical mini-slab apparatus (Bio-Rad, München, Germany) and a 12 % gel. As heating of the samples has led to high molecular mass aggregates in previous experiments, the samples were mixed with Laemmli´s buffer and applied directly to the gel without boiling. For the determination of molecular size, a prestained marker was used (Bio-Rad, München, Germany). After electrophoresis, the proteins were transferred to nitrocellulose membranes (0.2 µm, Sartorius, Göttingen, Germany) using a trans-blot cell (Bio-Rad, München, Germany) according to the manufacturer´s protocol. Blots were stained with Ponceau S to control for equal loading and successful transfer (49). After de-staining blots were blocked in Blotto (5 % non-fat dry milk in Tris-buffered Saline with 0.1% (v/v) Tween, TBST, pH 7.4) for 1 h at room temperature. A monoclonal rabbit anti-human UCP2 antibody was used (UCP23-S; Alpha Diagnostic International, San Antonio, TX, USA) for immunodetection. The blots were incubated under

agitation with primary antiserum diluted in Blotto (1:2500) at 4°C over night. Following a series of washes with TBST, blots were incubated with mouse anti-rabbit antibody conjugated to horseradish peroxidase (1:2500, diluted in Blotto; Amersham Biosciences, Freiburg, Germany) for 1 h at 37°C. Antibody binding was visualized by the ECL-system (Amersham Biosciences, Freiburg, Germany). Chemiluminescence was detected and quantified with a cooled CCD-camera system (LAS-1000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany). Normal rabbit serum (Pierce, Rockford, IL, USA) was substituted for primary antibodies to assess non-specific immunoreactivity. Membrane preparations were used to determine the optimal concentration ratio for antigen over primary and secondary antibody. For quantification, a protein concentration was used in a range, where the signal changed linearly with antibody binding.

Statistical analysis

Statistical analyses of differences among treatments by t-tests were carried out using Prism 4.0a (GraphPad Software, Inc.). Differences were considered significant if $P < 0.05$. All data are presented as values ± standard deviation (SD), unless stated otherwise.

Results

UCP2 sequence and protein specifications

RT-PCR using the primer pairs 1/2 and 3/4 yielded the expected 440bp and 550bp fragments, respectively. Completion of the sequences by RACE using the specific 3' forward primers 5, 6, 7 and the specific 5' backward primers 8, and 9 (see table 1 and figure 1) resulted in a number of overlapping fragments, which were assembled to receive the complete sequence of the transcripts. In *P. brachycephalum*, the UCP2 transcript consists of 1906bp; the gene of *Z. viviparus* is somewhat shorter and consists of 1876bp. Both genes contain an open reading frame of 939bp, encoding 313 amino acids. The complete transcript sequences are 95% identical, the coding regions 98% (925/939bp), and the deduced protein sequences are 99% identical with only two exchanged amino acids in 313 (*P.b./Z.v.*: Phe259Leu and Thr311Ile) (cf. figure 2). The three mitochondrial transporter protein signature motifs found in all members of the mitochondrial transporter protein family (59) are present in *P. brachycephalum* and *Z. viviparus* UCP2 (cf. figure 2) and identical to the motifs found in rat UCP2 and the three known fish UCP2s (27, 56). The zoarcid UCP2 consists of three repeated motifs of about 100bp, each containing two membrane helices, again typical for this protein family. Six putative membrane helices (predicted by MacVector and www.predictprotein.org) are indicated in figure 2. Interestingly, three of the predicted helix structures (Network Protein

Sequence Analysis, (3)) are situated directly in front of the mitochondrial transporter protein signature motifs (Swiss Institute of Bioinformatics, www.isb-sib.ch/; www.predictprotein.org) and overlap into the motifs by two amino acids each (cf. figure 2). The total protein sequence of zoarcid UCP2 is 77% identical to rat UCP2 (85% similarity) and 75-79% to those of zebrafish (*D. rerio*), carp (*C. carpio*) and red sea bream (*P. major*) (85-87% similarity). The phylogenetical reconstruction in figure 3 depicts the close relationship to carp and zebrafish UCP2 proteins and to the mammalian UCP2s. The encoding cDNA regions bear 73-75% similarity to carp and zebrafish cDNA and 71% similarity to rat UCP2 cDNA, while the complete transcripts bear 47-50% similarity to carp and zebrafish cDNA and 34% to rat cDNA.

Temperature dependent UCP expression

Z. viviparus were acclimated to 2 °C and 10.5 °C over a period of at least two months. The confamilial cold-adapted *P. brachycephalum* were acclimated to 0°C and 5°C, respectively. Total RNA was isolated from muscle and liver. Total RNA concentrations in liver and muscle were comparable to earlier results (14, 28) and did not change significantly during acclimation. Also, liver sizes did not change during acclimation. Specific expression of the UCP2 genes was determined using ribonuclease protection assays (RPA) and the probes with identical sequences in both species. Figure 4 depicts a typical autoradiography of an RPA of UCP2 mRNA expression in the liver of cold and warm acclimated common eelpout, *Z. viviparus*. UCP2 mRNA expression was detected with the 137bp UCP2 probe relative to the expression of β-actin (215bp probe), and expression levels were normalised to the particular habitat temperatures of the fish. During cold acclimation, relative expression levels in *Z. viviparus* liver tissue rose two-fold from 1.0±0.34 at 10°C to 2.07±0.56 at 2°C (figure 5A), while they were increased in muscle tissue by a factor 1.5 (1.0±0.05 to 1.55±0.19) (figure 5B). In contrast, in the Antarctic eelpout *P. brachycephalum*, a 2-fold increment was detectable after warm acclimation in muscle (1.0±0.16 to 1.98±0.15), and a less pronounced increment in liver (1.0±0.09 at 0°C to 1.33±0.20 at 5°C) (figures 6A and 6B). All increases were statistically significant ($p<0.05$).

To determine, whether these increments in transcript levels have led to functional shifts, the respective protein levels were quantified with monoclonal antibodies raised against the human UCP2 protein. Figure 6 represents a typical Western Blot of liver protein extracts of *Z. viviparus* and *P. brachycephalum* acclimated to extreme and habitat temperatures. The antibody was able to specifically cross-react with a single prominent protein band of 37 kDa, which is in

good agreement to the predicted size of the deduced amino acid sequence (33,4 kDa), and could be enriched in the membrane fraction.

UCP2 protein expression levels in *Z. viviparus* liver tissue rose during cold acclimation by a factor of 1.45 (1.0±0.07 at 10°C to 1.45±0.01 at 2°C, figure 5C), and increased in muscle tissue by a factor of 1.3 (1.0±0.14 to 1.28±0.07) (figure 5D). In the Antarctic eelpout *P. brachycephalum*, we found protein levels to increase during warm acclimation to 5°C by a factor 1.6 (1.0±0.13 at 0°C to 1.58±0.001 at 5°C) in liver (figure 5C). Therefore, protein expression levels were in line with mRNA expression levels, differences in expression were all significant, although not quite as prominent as on mRNA level. Protein levels in *P. brachycephalum* muscle were only barely detectable, possibly due to lower affinity of the antibody to the UCP of Antarctic eelpout and/or too low UCP2 concentrations in the white muscle tissue. These data had therefore to be excluded.

It should be noted that data presented here were normalised to visualise the differences between organs and species, thus the graphs in figure 5 do not represent actual concentrations but normalised ratios. In *P. brachycephalum*, constitutive UCP2 mRNA levels were up to 6.5 times lower than in *Z. viviparus*, in muscle even more so than in liver (data not shown). This is also reflected at the protein level (cf. figure 6), although interspecies comparisons using antibodies have to be analysed with care.

Discussion

Molecular adaptations to low temperature

In this study, we were able to identify an uncoupling protein homologous gene in muscle and liver tissue of the two zoarcid species *P. brachycephalum* and *Z. viviparus*. Gene and associated protein were found to belong to the mitochondrial transporter protein family, showing highest similarities to fish and mammalian homologues of UCP2, and therefore were designated zoarcid UCP2.

Independent of the used algorithm, the zoarcid proteins are clustered together with the cyprinid and the mammalian UCP2 in the phylogenetic tree (figure 3). These UCP2 form a branch together with the mammalian UCP3. Interestingly, the UCP2 fragment (224 amino acids) from *Pagrus major* seems to be less related to the other fish UCP2 than the mammalian UCP3, even if the tree reconstruction was limited to the *P. pagrus* fragment. Therefore, it remains questionable, whether this picture is due to the missing N- and C-termini of the *P. pagrus* protein. Alternatively, further isoform(s) have to be postulated for fish.

Its high degree of identity (99%) within the zoarcids and considerable similarity to fish and mammalian UCP2 (>85%) suggest that UCP2 has been much conserved over evolutionary timescales and therefore holds a position of significant importance in cellular energy metabolism. These findings are in line with similarly high degrees of conservation in other functionally important genes like citrate synthase, cytochrome-c oxidase (28) and Na^+/K^+-ATPase and Na^+/H^+ exchanger (29) in teleosts and other vertebrates. The two amino acid exchanges observed (*P.b./Z.v.*: Phe259Leu and Thr311Ile) may play a significant role in cold adaptation of the protein. According to the secondary structure model, the first is located in a variable loop sequence on the matrix side between the two helices of repeat 3, the latter close to the C-terminal end on the cytosolic side of the protein (figure 2). For cold-adapted proteins a reduction of hydrophobicity and improved solvent interactions with a more hydrophilic surface have been postulated (7, 32), which support increased structural flexibility at lower temperature. Whereas Thr311 is in line with these assumptions, the Phe259 in the postulated loop of the cold-adapted protein is thought to reduce the flexibility of this loop and increase hydrophobicity, and may therefore have been introduced for different reasons. Yet, identical amino acid sequences do not necessarily bring about identical properties in the entire protein: the work by Fields and Somero on A_4-lactate dehydrogenase of gobys and notothenioids has shown that alternative conformational structures of identical amino acid sequences (conformers) can lead to different thermal sensitivities of the native proteins and thus be a means of temperature adaptation (8-10). To date, it remains unclear whether similar

conclusions can be drawn for membrane bound proteins, and further functional and structural studies are clearly necessary for a clearer picture.

Temperature dependent UCP2 expression and function

UCP2 expression in fish is clearly temperature dependent; in this study we found a general up-regulation with temperature adaptation beyond habitat temperature in the stenothermal Antarctic and the eurythermal common eelpout. Up-regulation includes both mRNA and protein expression levels.

After cold acclimation, UCP levels have been reported to show enhanced expression in endothermic animals like mammals and birds (33, 58), and UCP2 mRNA increases after cold acclimation in chicken (42). Higher levels of UCP can simply be the result of an overall increase in mitochondrial capacity frequently found during cold acclimation (12, 54), either by mitochondrial proliferation (be it in number, volume or cristae surface area), or by changing the biochemical properties of particular enzymes (4). Mitochondrial proliferation involves enhanced expression of aerobic enzymes such as cytochrome-c oxidase and its respective RNA (39). For *Z. viviparus*, there is clear evidence for mitochondrial proliferation in the cold, the key enzyme of the electron transport chain, cytochrome-c oxidase has been found to increase at both message and functional levels in muscle after cold acclimation (14). Activity levels of liver citrate synthase were also enhanced in the cold (28), implying a general augmentation of mitochondrial capacity following cold adaptation. This is corroborated by our findings for *Z. viviparus*, in which UCP2 message and protein levels were up-regulated significantly upon cold acclimation.

In the warm, one would expect a corresponding reduction of mitochondrial capacity, thus enhancing temperature tolerance by reducing mitochondrial maintenance costs (39). This is the case in *Z. viviparus*, and in isolated mitochondria of winter flounder *Pleuronectes americanus* acclimated from 0°C to 9°C, Rosenberger and Ballantyne (48) observed reduced proton leak in the warm. Yet winter flounder is a seasonally cold adapted fish, comparable to *Z. viviparus*. Both increase their mitochondrial capacities and thus proton leakage or UPC2 protein levels, respectively, in the cold, while the Antarctic eelpout *P. brachycephalum* adapted to the cold on evolutionary timescales and hence has to be considered permanently cold adapted. In contrast to extremely stenothermal high Antarctic fish, some of which cannot survive temperatures exceeding 6°C (53), the thermal tolerance range of the Antarctic eelpout, however, is somewhat wider. Upon warming, its cardiovascular capacity can be increased within a thermal window of 0 to 7°C, where first limitations of aerobic capacity set in (31). These findings suggest that *P. brachycephalum* is not an extreme stenotherm and may be able to in part adapt to

higher temperatures than nowadays found in Antarctic waters by accordingly adjusting its metabolism.

However, when acclimating Antarctic eelpout *P. brachycephalum* to 5°C, Lannig *et al.* (26) found only small decreases in hepatosomatic index, mitochondrial protein content and ATP synthesis or proton leakage capacities in the liver. In the present study, UCP2 expression during warm acclimation in the Antarctic eelpout did also not follow the general pattern of mitochondrial down regulation in the warm; mRNA and protein levels were increased instead. Among investigated mitochondrial properties, only UCP2 levels appear to be significantly up-regulated. One might ask whether this pattern is adaptive or indicates a pathological change in response to heat stress. Up-regulated message and protein levels on the one hand might suggest the potential for higher mitochondrial proton leak rates in warm acclimated *P. brachycephalum*. However, proton leakage rates of isolated mitochondria remained more or less unchanged upon warm acclimation (26). The reason for the discrepancy between increased UCP levels and seemingly constant proton leak rates after warm acclimation might be located in homeoviscous adaptation (60), according to which a cold adapted membrane should be more fluid than a warm adapted membrane, when measured at the warm acclimation temperature and above (61). The more fluid a membrane becomes, the less restricted and hence more active are membrane bound proteins like cytochrome c oxidase (62). The same might apply to UCP2, and consequently cold adapted (0°C) mitochondria should show higher proton leak rates per mg protein than warm acclimated (5°C) mitochondria, when measured at the warm acclimation temperature. In this line of thought, the observation that proton leak rates of cold adapted mitochondria are comparable to those of warm acclimated, when measured at 5°C, suggests higher UCP protein levels in the warm, which have been found in this study (cf. figure 5).

On the other hand, only marginally down regulated mitochondrial capacities in the warm (see above) lead to the question, whether mitochondria lack the adaptive plasticity to fully compensate for warming to 5°C in this species and proton leak is used to control a partly unbalanced increase in energy turnover during warming. In captivity, *Pachycara brachycephalum* can survive for years at these temperatures and still display positive growth (pers. observation), but may only do so at the expense of elevated metabolic costs and reduced growth and reproduction rates.

Functional role of UCP 2

UCP2 appears to play a prominent role in thermal adaptation in fish, but the general function of fish UCP2 remains still speculative - why would an ectothermal animal tolerate such an apparently wasteful process as proton leak if not used for thermogenesis?

UCP1 is an accepted mediator of proton leak and in this function decouples the respiratory chain, dissipating membrane potential (44). For the other homologues, this function is less well established. In endothermal animals further mitochondrial anion carriers appear to be involved in proton leak, such as the adenine nucleotide translocase (ANT)(63), the glutamate/aspartate antiporter and the dicarboxylate carrier (51) as well as the transhydrogenase (17, 41). They may all play a role in proton leakage and can contribute at least to its basal rates.

Potential for higher proton leak rates in warm acclimated Antarctic eelpouts point to increased membrane potentials, which may build up in the warm. On top of the other abovementioned processes, UCP2 may act as a 'safety valve' for membrane potential, either with a regulative (by controlling ATP synthesis) or protective function, preventing production of reactive oxygen species (ROS). Skulachev (52) was one of the first to suggest a role for mammalian UCP2 in the prevention of ROS formation by mild uncoupling, a theory that was adopted by a number of authors (2, 37, 43). The cold adapted Antarctic eelpout might not be able to adapt entirely during warm acclimation and, as a consequence, its mitochondrial capacities might remain too high in the warm, exceeding ATP demand or supply of oxygen as final acceptor of electrons in the respiratory chain. That would lead to a high membrane potential and high reducing capacities in the respiratory chain, conditions which facilitate ROS formation. By cutting the electrochemical gradient short, UCP2 could ameliorate the situation – at the cost of increased mitochondrial energy consumption. This protective process at the mitochondrial level may therefore become detrimental during further warming by demanding a large fraction of the organism's oxygen budget.

In a parallel study of the two eelpout species with comparable acclimation temperatures to the present study (0 and 5°C for *P. brachycephalum* and 12 and 6°C for *Z. viviparus*), Heise *et al.* (26) found patterns of oxidative stress parameters to correspond to our observed levels of UCP2 expression: ROS production might have increased in *P. brachycephalum* in the warm and in *Z. viviparus* in the cold, indicating that elevated levels of UCP-2 might well be a reaction towards oxidative stress (6), but neither *P. brachycephalum* nor *Z. viviparus* appear to have been able to fully compensate for elicited thermal stress and ROS formation by increasing levels of UCP2.

In a more regulative fashion, UCP2 levels might also be increased in the warm to enhance the plasticity of mitochondrial energy metabolism. It is conceivable that *P. brachycephalum*,

belonging to the globally distributed Zoarcidae, has kept some features of its eurythermal confamilials, which - within certain limits - it can revert to upon warming. It may switch to a more eurythermal metabolic mode, keep high mitochondrial capacities and regulate them by controlled uncoupling for the sake of spontaneous ATP supply. Although being energetically more expensive, it may be quicker and easier to either transcriptionally (34) or translationally (37) regulate a single protein like UCP2 instead of the suite of proteins of the electron transport chain, especially under stress conditions, when flexibility of energy supply is needed. A further indication of a regulative function has been published by Brand and coworkers (46, 47): In mammalian resting skeletal muscle they found proton leak rates to be higher than in working muscle. It is possible that by regulating the degree of mitochondrial coupling, UCP controls both ATP synthesis and the prevention of ROS formation; the more flexible mitochondrial metabolism has to be, the higher the rate of control. By increasing UCP levels, switching from cold-stenothermality to cold-eurythermality thus may be accomplished in animals lacking the necessary adaptive mitochondrial adaptability, provided they stay within the limits set by oxygen supply to the mitochondria. Flexibility of mitochondrial energy metabolism might therefore be closely linked to UCP expression, which is in line with a theory brought forward by Hardewig *et al.* (13), who assumed that 'proton leakiness may be lower in mitochondria from Antarctic fish than in temperate fish mitochondria'. Although we found native UCP2 levels to be somewhat lower in *Pachycara brachycephalum* than in *Zoarces viviparus* (cf. figure 6), there is still no evidence to unambiguously prove this hypothesis and further investigation is needed.

Conclusion

To our knowledge, this is the first study to demonstrate temperature dependent UCP2 expression in fish at transcript and protein levels, possibly even the first such study in ectothermic vertebrates.

Upon cold and warm acclimation, we found two different phenomena. Following cold acclimation, there was a general up-regulation of UCP2 expression levels in the common eelpout *Z. viviparus*, in line with evidence for cold-induced mitochondrial proliferation provided by earlier studies (13, 26, 28). During warm acclimation of the cold-adapted Antarctic eelpout *P. brachycephalum*, UCP2 expression underwent as yet undocumented changes; in muscle and liver tissue we found a putatively regulative increase in UCP2 levels, both at message and protein levels, while other enzymes involved in mitochondrial energy metabolism such as cytochrome-c oxidase and citrate synthase have been reported to remain constant (or even slightly decrease in activity) upon warming (26).

Our findings are in line with the hypothesis that UCP2 holds an important position within mitochondrial energy metabolism of ectotherms, and especially during thermal stress may function as a regulative protein, controlling the mitochondrial membrane potential to balance ROS formation and ATP production. There is, however, no evidence for a change in baseline mitochondrial proton leakage upon enhanced UCP2 expression. Further work should therefore focus on a functional characterisation of UCP homologues within mitochondria. It remains to be investigated whether an evolutionary conservation of function can be found within this protein family, which is indicated by its widespread occurrence in the eukaryotic kingdom.

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Figure legends

Table 1: A list of all primers used in this study

figure 1: Schematic overview of the *Z. viviparus* UCP2 cDNA. The open reading frame is shaded in dark grey, the 5' and 3' untranslated regions in lighter grey. The arrows indicate the positions of the primers used (see material section).

figure 2: Alignment of UCP2 peptide sequences of the two zoarcids *P. brachycephalum* and *Z. viviparus*, carp (*C.carpio*) and zebrafish (*D. rerio*) and rat (*R. norvegicus*). Amino acid exchanges are highlighted in grey, exchanges between *P. brachycephalum* and *Z. viviparus* are additionally marked with an asterisk. Solid boxes indicate signature motifs of the mitochondrial transporter protein family, dashed boxes depict putative transmembrane helices.

figure 3: Phylogenetic reconstruction of the fish uncoupling proteins in relation to the mammalian isoforms. The tree was calculated using the Neighbour Joining method (best tree; tie breaking = Systematic; Distance: Poisson-correction; Gaps distributed proportionally) with the invertebrate *Chaenorhabditis elegans* protein (NM_073013), related to the mammalian UCP4 isoforms, as outgroup. The proteins from *P. brachycephalum* and *Z. viviparus* group with all other known fish proteins, and are closest related to the mammalian UCP2. The position of the red sea bream (*Pagrus major*) UCP2 (AF487341) is questionable, since its sequence is not complete. Accession numbers of the respective genes: *Homo sapiens* UCP1: U28480; UCP2: U82819; UCP3: U84763; UCP4: AF110532; UCP5: AY358099. *Rattus norvegicus* UCP1: M11814; UCP2: AB006613; UCP3: AB006614; UCP4: AJ300162. *Sus scrofa* UCP2: AF036757; UCP3: AF095744. *Danio rerio* UCP2: AJ243250. *Cyprio carpio* UCP2: AJ243486.

figure 4: Ribonuclease protection assay of liver RNA samples of *Z. viviparus*, acclimated to 2°C and 10°C. Each lane was run with 10µg RNA, lanes 1-3 represent triplicates of pooled RNA (n=5). The size of the protected beta actin fragment was 215bp, the length of the UCP2 fragment was 137bp.

figure 5: UCP2 mRNA and protein expression levels in liver and muscle of the two zoarcids *P. brachycephalum* (Pb) and *Z. viviparus* (Zv), acclimated to 0 and 5°C and 10 and 2°C, respectively. A: mRNA expression in liver. B: mRNA expression in muscle. C: protein expression in liver. D: protein expression in muscle. *: significantly different from Zv. 10°C; #: significantly different from Pb. 0°C, (P=0.05). Error bars represent standard deviation (SD).

figure 6: Western Blot detection of UCP2 in enriched mitochondrial fractions from liver of the two zoarcids *P. brachycephalum* and *Z. viviparus*, acclimated to 0 and 5°C and 10 and 2°C, respectively. Each lane contained 50µg of protein pooled from five individuals, lanes were run in duplicates. The UCP2 antibody bound to a protein band of approximately 37kDa.

Figures

Table 1

Primer	*Name*	*Sequence (5' to 3')*	*Origin*	*Reference*
1	UCP2-F1	CCACTGGACACYGCAAAAGTTAG	410-432 of *D. rerio* UCP2	Stuart *et al.*, 1999
2	UCP2-B2	CAAACCACGAAACCCCTCTTCC	841-820 of *D. rerio* UCP2	Stuart *et al.*, 1999
3	UCP2-F8	GATTCKGTCAAGCAGTTYTACACC	617-640 of *D. rerio* UCP2	Stuart *et al.*, 1999
4	UCP2-B12	CATAACCACATTCCAGGAGCCC	1177-1156 of *D. rerio* UCP2	Stuart *et al.*, 1999
5	UCP2-RACE-F2	CGATTTCATCAAGGATTCCCTC	1067-1088 of *Z. viviparus* UCP2	--
6	UCP2-RACE-F3	AAGTCCACTCCCCTGACAGACAAC	1092-1115 of *Z. viviparus* UCP2	--
7	UCP2-RACE-F4	CTGCCCTGCCACTTTGTATC	1117-1135 of *Z. viviparus* UCP2	--
8	UCP2-RACE-B2	TGTAGGCTGAGCAAAAGCAACC	884-863 of *Z. viviparus* UCP2	--
9	UCP2-RACE-B6	CCACGGATGCCTTCTTCTTTAGC	997-975 of *Z. viviparus* UCP2	--
10	UCP-Pb/Zv-F2	GCCATGGCGGTTGCTTTTGCTC	856-877 of *Z. viviparus* UCP2	--
11	UCP-Pb/Zv-B1	ATGCCTTCTTCTTTAGCAATGGTCTTG	992-966 of *Z. viviparus* UCP2	--
12	βACT-F4	CTGTCCCTGTATGCCTCTGGTC	161-182 of *Z. viviparus* ACT-B	Lucassen *et al.*, 2004
13	βACT-B11	GTCACGCACGATTTCCCTCTC	372-351 of *Z. viviparus* ACT-B	Lucassen *et al.*, 2004

figure 1

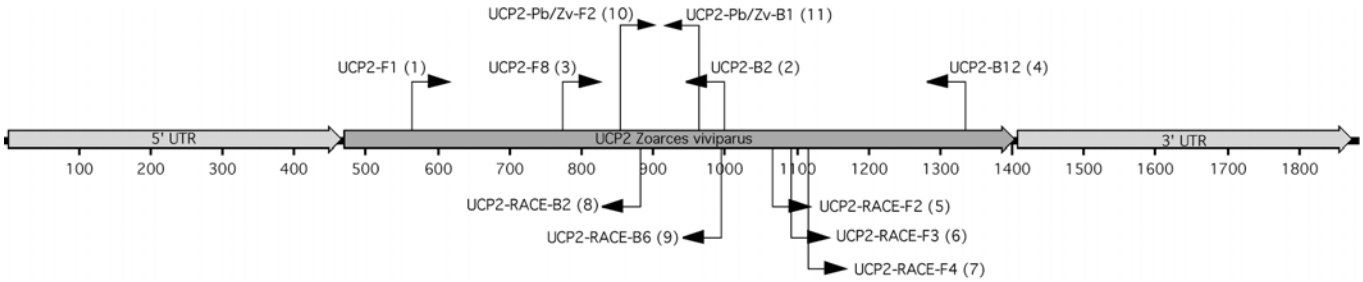

UCP2-Pb/Zv-F2 (10)
UCP2-Pb/Zv-B1 (11)
UCP2-F1 (1)
UCP2-F8 (3)
UCP2-B2 (2)
UCP2-B12 (4)
5' UTR
UCP2 Zoarces viviparus
3' UTR
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
UCP2-RACE-B2 (8)
UCP2-RACE-F2 (5)
UCP2-RACE-B6 (9)
UCP2-RACE-F3 (6)
UCP2-RACE-F4 (7)

figure 2

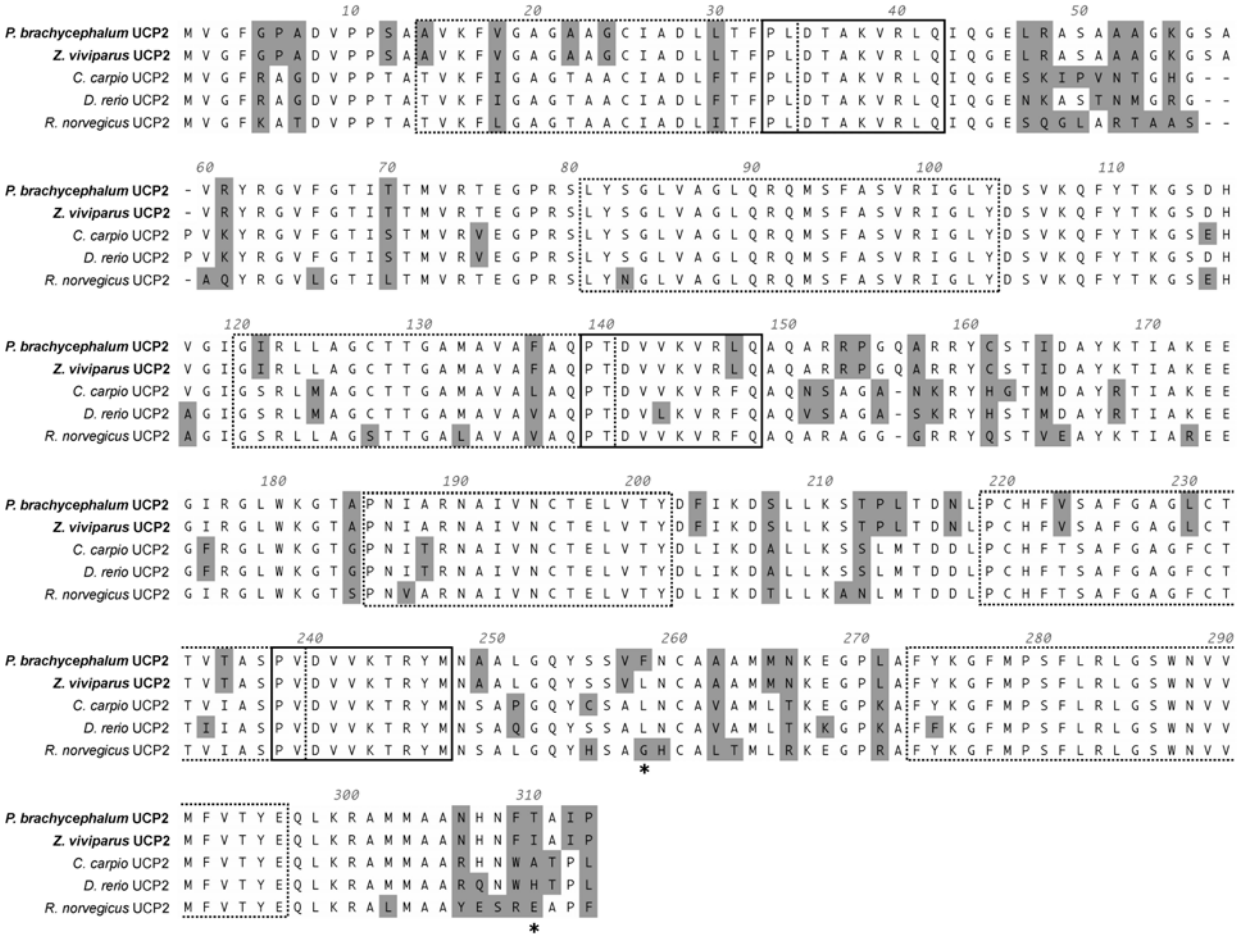

P. brachycephalum UCP2
Z. viviparus UCP2
C. carpio UCP2
D. rerio UCP2
R. norvegicus UCP2

figure 3

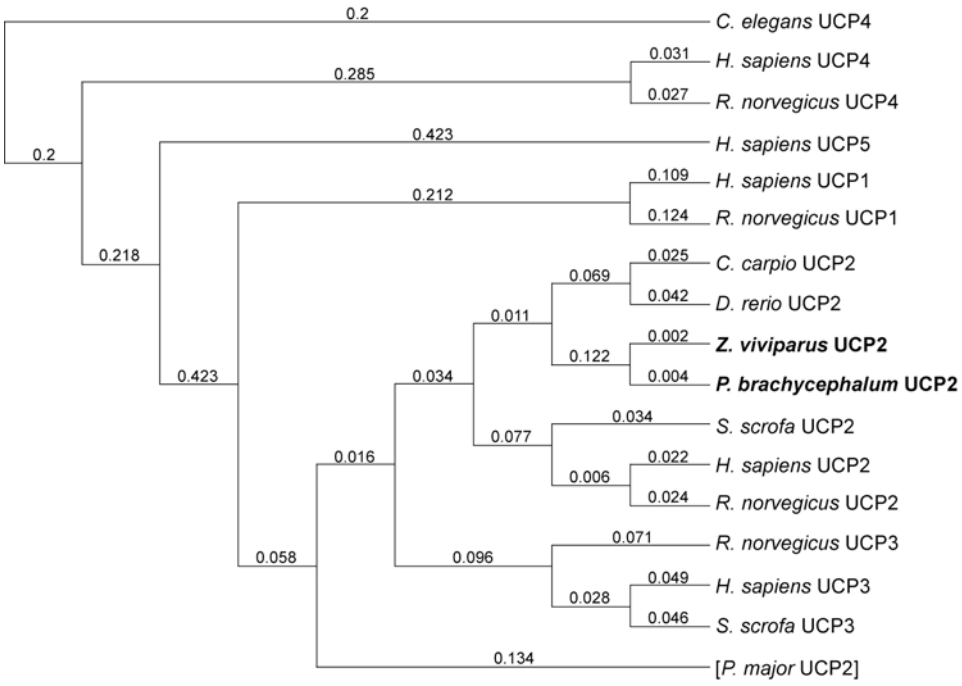

0.2
C. elegans UCP4
0.031
H. sapiens UCP4
0.285
0.027
R. norvegicus UCP4
0.423
H. sapiens UCP5
0.2
0.109
H. sapiens UCP1
0.212
0.124
R. norvegicus UCP1
0.218
0.025
C. carpio UCP2
0.069
0.042
D. rerio UCP2
0.011
0.002
Z. viviparus UCP2
0.122
0.423
0.034
0.004
P. brachycephalum UCP2
0.034
S. scrofa UCP2
0.077
0.016
0.022
H. sapiens UCP2
0.006
0.024
R. norvegicus UCP2
0.071
R. norvegicus UCP3
0.058
0.096
0.049
H. sapiens UCP3
0.028
0.046
S. scrofa UCP3
0.134
[P. major UCP2]

Figure 4

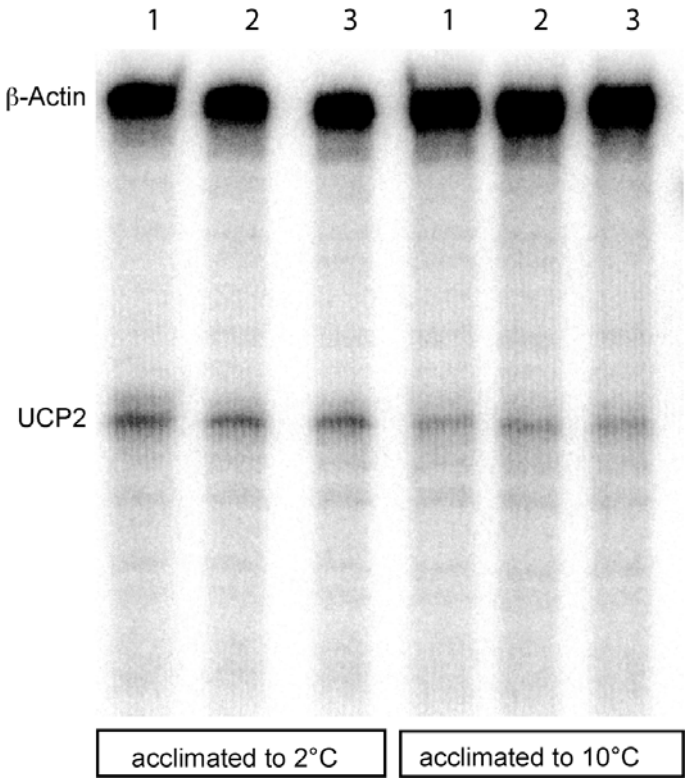


figure 5

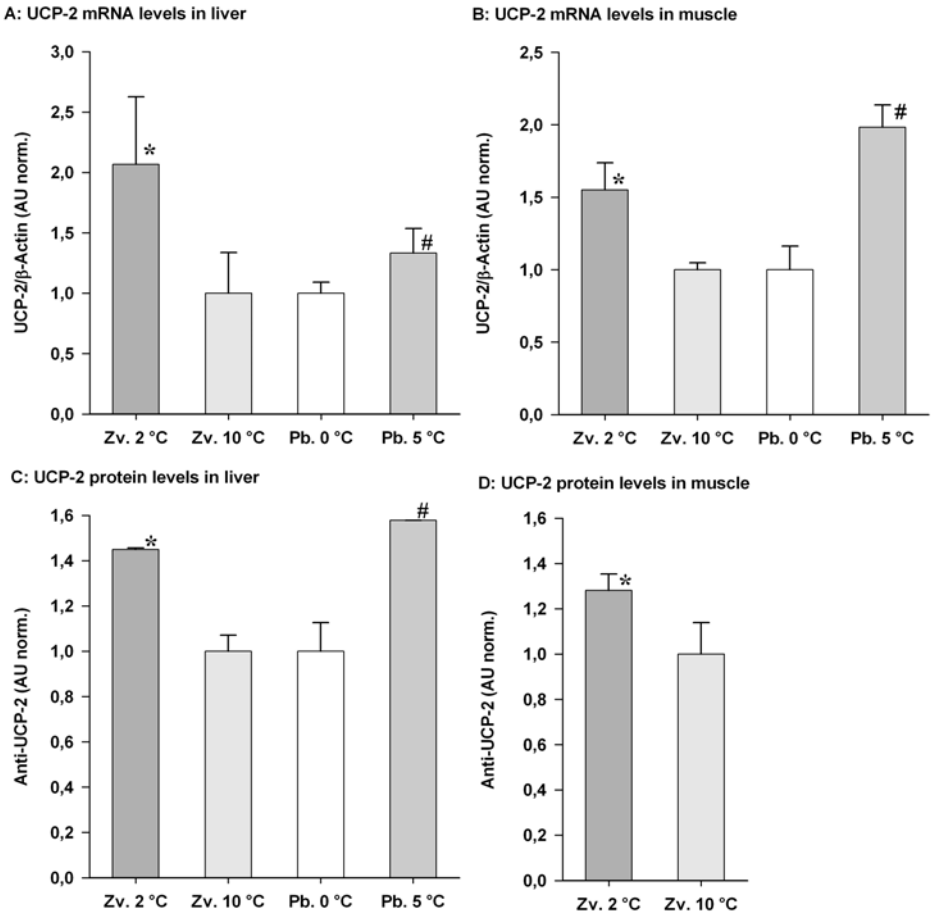
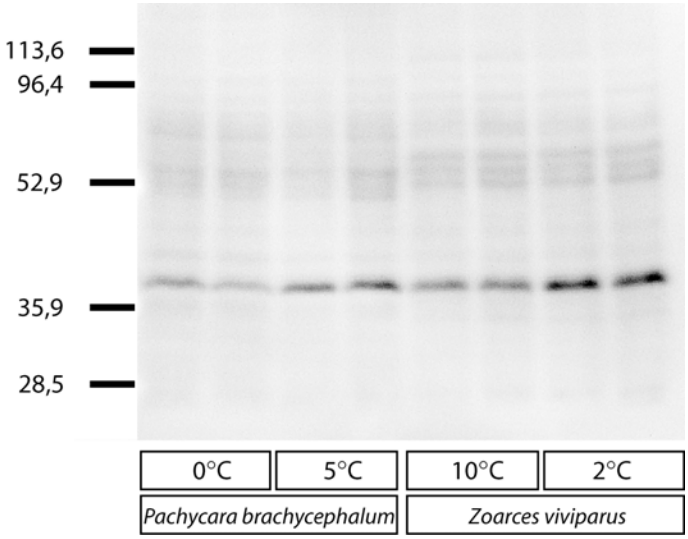

A: UCP-2 mRNA levels in liver
UCP-2/β-Actin (AU norm.)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
#
Zv. 2 °C
Zv. 10 °C
Pb. 0 °C
Pb. 5 °C
B: UCP-2 mRNA levels in muscle
UCP-2/β-Actin (AU norm.)
2,5
2,0
1,5
1,0
0,5
0,0
*
#
Zv. 2 °C
Zv. 10 °C
Pb. 0 °C
Pb. 5 °C
C: UCP-2 protein levels in liver
Anti-UCP-2 (AU norm.)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
#
Zv. 2 °C
Zv. 10 °C
Pb. 0 °C
Pb. 5 °C
D: UCP-2 protein levels in muscle
Anti-UCP-2 (AU norm.)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
Zv. 2 °C
Zv. 10 °C

figure 6


113,6
96,4
52,9
35,9
28,5
0°C
5°C
10°C
2°C
Pachycara brachycephalum
Zoarces viviparus

PUBLICATION IV

Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques

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Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques

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Abstract

In various phyla of marine invertebrates limited capacities of both ventilatory and circulatory performance were found to set the borders of the thermal tolerance window with limitations in aerobic scope and onset of hypoxia as a first line of sensitivity to both cold and warm temperature extremes. The hypothesis of oxygen limited thermal tolerance has recently been investigated in fish using a combination of non-invasive nuclear magnetic resonance (NMR) methodology with invasive techniques. In contrast to observations in marine invertebrates arterial oxygen tensions in fish were independent of temperature, while venous oxygen tensions displayed a thermal optimum. As the fish heart relies on venous oxygen supply, limited cardio-circulatory capacity is concluded to set the first level of thermal intolerance in fish. Nonetheless, maximized ventilatory capacity is seen to support circulation in maintaining the width of thermal tolerance windows. The interdependent setting of low and high tolerance limits is interpreted to result from trade-offs between optimized tissue functional capacity and baseline oxygen demand and energy turnover co-determined by the adjustment of mitochondrial densities and functional properties to a species-specific temperature range. At temperature extremes, systemic hypoxia will elicit metabolic depression, thereby widening the thermal window transiently sustained especially in those species preadapted to hypoxic environments.
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Keywords: Fish; Teleost; Hypoxia; Thermal tolerance; Metabolism; Cold adaptation; Temperature; Thermal tolerance; Hypoxia

1. Introduction: a role for hypoxia in thermal limitation?

The physiological mechanisms setting thermal tolerance and defining thermal sensitivity have recently come into focus due to rising interest in the effects of climate change on organisms and ecosystems. In this context, the question has regained interest whether limitations in oxygen availability or supply are involved in thermal limitation. Early evidence collected in marine invertebrates (annelids and sipunculids) demonstrated a transition to anaerobic metabolism (including mitochondrial anaerobiosis) at both cold and warm temperature extremes (Zielinski and Pörtner, 1996; Sommer et al., 1997), later on confirmed in crustaceans (Frederich and Pörtner, 2000) and molluscs, i.e. bivalves, gastropods and cephalopods (Pörtner and Zielinski, 1998; Pörtner et al., 1999; Peck et al., 2002; Sokolova and Pörtner, 2003). Studies in a sipunculid (*Sipunculus nudus*,

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Zielinski and Pörtner, 1996) and the spider crab (*Maja squinado*, Frederich and Pörtner, 2000) investigated the pattern of coelomic fluid/haemolymph oxygen tensions in relation to warming and/or cooling and demonstrated development of hypoxia which preceded the onset of anaerobic metabolism towards both cold and warm temperature extremes. A temperature dependent optimization of oxygen supply capacity was visible in the form of maximized arterial P_{O_2} within a limited temperature range, equivalent to the window of mean temperatures in the natural environment (Frederich and Pörtner, 2000). Limitation of both ventilatory and circulatory capacities towards thermal extremes was found to explain these patterns of body fluid oxygenation (Zielinski and Pörtner, 1996; Frederich and Pörtner, 2000).

Such a restriction of optimized oxygen supply to a limited thermal window demonstrated that temperature extremes are suitable to induce hypoxia in the organism despite ample oxygen supply from the environment. Work on temperate invertebrates and their populations in a latitudinal cline demonstrated a shift of oxygen dependent thermal thresholds depending on the ambient climate regime and winter or summer seasons (Sommer et al., 1997; Sommer and Pörtner, 1999, 2002). Inclusion of Antarctic marine invertebrates in this picture revealed very narrow windows of thermal tolerance in these organisms, in a temperature range just above freezing. An early transition to "heat" induced anaerobiosis between 2 and 6 °C seen in bivalves reflected the permanently low temperatures of Antarctic seas (Pörtner et al., 1999; Peck et al., 2002).

The work of Zielinski and Pörtner (1996), Sommer et al. (1997), Pörtner et al. (1999) and Frederich and Pörtner (2000) in marine invertebrates led to the concept of oxygen-limited thermal tolerance, which suggests that towards cold or warm extremes progressively inadequate oxygen supply and thus, decreasing body fluid oxygen levels finally lead to temperature induced anaerobiosis. Terminology was applied by adopting the one used in Shelford's law of tolerance (Shelford, 1931). In the spider crab *Maja squinado*, early limits of thermal tolerance during both heating and cooling were indicated by a set of low and high *peius* temperatures (T_p), which denote the beginning of insufficient oxygen supply to an organism, or in other words, the onset of mildly hypoxic conditions associated with a progressive loss in aerobic scope. This transition occurs in fully oxygenated environments. The point of transition where increasing internal hypoxia at more extreme temperatures finally leads into anaerobiosis, was termed *critical* temperature, T_c (Frederich and Pörtner, 2000).

Evidently, adaptations to ambient temperature and oxygen levels were found closely related in the marine invertebrate species studied. The question arose early on whether the concept of an oxygen limitation of thermal tolerance is applicable to (aquatic) vertebrates, especially marine teleost fish. Initial evidence demonstrated that the indicator of mitochondrial anaerobiosis, succinate, accumulated in liver of North Sea eelpout, *Zoarces viviparus*, during heat stress (Van Dijk et al., 1999). Particularly in fish, however, further study of temperature dependent oxygen limitation (or vice versa, oxygen limited thermal tolerance) proved difficult, firstly due to more limited hypoxia tolerance and stress resistance of most fish compared to invertebrates and secondly due to limited accumulation of anaerobic mitochondrial end products like succinate, especially in bulk tissues like white muscle. To monitor the onset of temperature induced hypoxia, particularly the early stages of transition from normoxic to hypoxic conditions, and in order to overcome these constraints, non-invasive whole animal experiments or, alternatively, a combination of non-invasive and invasive techniques proved useful. Obtaining the respective evidence in fish was supported by recent developments in the non-invasive techniques of nuclear magnetic resonance imaging and spectroscopy (MRI and MRS) and their applicability to unrestrained, non-anaesthetized aquatic animals. The present study is intended to review these accomplishments and the available information on thermal limitation in (marine) fishes. From a wider perspective, it also examines to what extent hypoxia induced hypometabolism may support survival at thermal extremes. The respective findings are in line with the results obtained in invertebrates and suggest that thermal limitations in oxygen supply occur in fish, however, with an emphasis on a limiting role for circulation rather than ventilation. These comparative analyses have thus supported and are in line with a unifying conceptual framework of the physiological principles setting thermal tolerance windows and of the key mechanisms of thermal adaptation and limitation (Pörtner, 2001, 2002a,b).

2. Methodological developments

Over the last 2 decades magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) techniques have extensively been used to study hypoxia with its immediate and drastic effects on cellular energy metabolism and acid-base regulation. In vivo experiments were carried out in invertebrates like lugworms (Kamp et al., 1995), mussels (Tjeerdema et al., 1991; Shofer et al., 1998, Shofer and Tjeerdema, 1998) or prawns (Raffin et al., 1988; Thébault and Raffin, 1991) and fish. However, the latter were anaesthetized and restrained during early studies. Various review articles about NMR applications in comparative physiology already exist in the literature (e.g. Ellington and Wiseman, 1989; Van den Thillart and Van Waarde, 1996). Our emphasis in this review is on the contribution of MRS and MRI combined with invasive techniques to studies of temperature hypoxia interactions, in the context of testing the concept of oxygen limited thermal tolerance in fish.

The early NMR investigations of the effects of environmental hypoxia on fish muscle energy metabolism started by Van den Thillart et al. (1989a). They developed a flow through probe for in vivo ^{31}P-NMR spectroscopy in a 9.4 T vertical NMR spectrometer (Van den Thillart et al., 1989b). The set-up allowed long term online recordings of energy metabolism in muscle of carp, tilapia and goldfish during anoxia and hypoxia by means of ^{31}P-NMR spectroscopy. Data recordings at 10 min intervals were characterized by high signal to noise ratios. Control values indicated by a high phosphocreatine to inorganic phosphate ratio (PCr/Pi) were reached after 2 h and did not change significantly over an experimental period of 8 h. Hypoxia induced a rapid decline in the PCr/Pi ratio accompanied by a drop in intracellular pH (Van den Thillart et al., 1989a). Return to normoxic control conditions occurred within 3 h in carp as well as in goldfish. Besides the determination of high energy phosphate concentrations, of inorganic phosphate and of intracellular pH, free ADP concentrations were calculated from the equilibrium of creatine kinase (Van Waarde et al., 1990) and provided insight into the functional coupling of phosphocreatine utilisation and glycolysis in these three species in vivo. Nevertheless, the animals had to be anaesthetized prior to experimentation and were fixed in a vertical position inside the probe, limiting experiments to more robust fish species like carp or eel.

Blackband and Stoskopf (1990) reported the first combined MR imaging and spectroscopy studies in marine, albeit anaesthetized, fish. They focused mainly on the feasibility of NMR experiments with marine animals and did not describe any dynamic observations or metabolic patterns. Eight years later, Borger et al. (1998) reported in vivo ^{31}P-NMR experiments with common carp using a similar approach as the one described by van den Thillart. However, this time the fish was placed in a horizontal MR scanner at a magnetic field strength of 7 T. These experiments investigated the combined effects of temperature (acclimation as well as rapid change) and hypoxia on fish energy metabolism over several hours with a temporal resolution of minutes. These in vivo ^{31}P-NMR observations confirmed a negative correlation between temperature and intracellular pH in fish muscle in accordance with the alphastat pH regulation hypothesis developed by Reeves (1972). However, these experiments again involved the shortcomings of pre-experimental anaesthesia and fixation of the animal. Such experiments preclude long term analyses (for days or even weeks) especially of delicate organisms like polar animals or of animals displaying some of their normal physiological activities under resting conditions.

The shortcomings involved in studies of anaesthetized or immobilized animals were finally overcome when MRI and MRS experiments were successfully carried out in non-anaesthetized, unrestrained marine teleosts like benthic zoarcids (eelpout) or demersal gadids like Atlantic cod (Bock et al., 2001; Mark et al., 2002; Sartoris et al., 2003a,b). The experimental set-up (Fig. 1) allowed long term MRS and MRI experiments for more than 8 days. Polar organisms like Antarctic eelpout (*Pachycara brachycephalum*) were studied under controlled and stable conditions allowing extensive physiological monitoring with high localized and temporal resolutions. Animals were not even anaesthetized prior to experimentation, thereby excluding possible long-term effects of narcotics (Iwama et al., 1989). The fish usually recovered from handling stress within 1 h. Excellent resting conditions were reflected by extremely high and constant PCr/Pi ratios and stable intracellular pH values. The fish was free to move inside the chamber, but were imperturbable even during

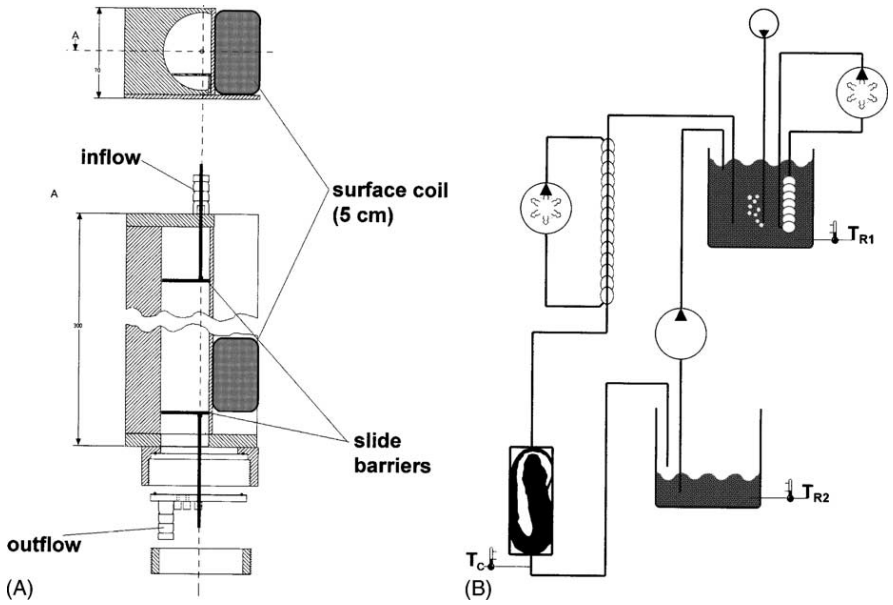


Fig. 1. (A) Experimental set-up for in vivo NMR and MR studies of un-anaesthetized and un-restrained fish (adapted from Bock et al., 2001). The animal is positioned inside the chamber with slide barriers parallel to a NMR surface coil, ensuring the mobility of the fish. (B) Water was continuously supplied from a temperature controlled water reservoir (minimum 50 l, T_{R1}) by hydrostatic pressure. Temperature stability in the chamber and reservoirs was ±0.5 °C as confirmed by continuous T-measurements (T_C, T_{R1}, T_{R2}).

periods of MR scanner sounds; therefore MR images were obtained with high anatomical resolution allowing for localized MR spectroscopy of different organs (Bock et al., 2001, 2002a). For example, Fig. 2 depicts perfectly localized in vivo ^{1}H-NMR spectra obtained in embryos of the North Sea eelpout *Z. viviparus*.

This methodology proved applicable to demersal, more mobile fish. Fig. 3 presents a stack plot of in vivo ^{31}P-NMR spectra from North Sea cod *Gadus morhua* during hypoxia and recovery. The time interval was 5 min between each spectrum. Under control conditions, only NMR signals from the high-energy phosphates PCr and ATP could be detected, inorganic phosphate signals did not even reach noise levels, indicating minimal activity levels and undisturbed resting conditions of the fish. Onset of hypoxia resulted in an immediate increase of inorganic phosphate levels at the expense of phosphocreatine. Values returned to control levels within 15 min of post-hypoxic recovery (Fig. 3). In the meanwhile, this technology has been developed even further to allow online study of tissue energetics of unrestrained swimming cod in swim tunnels fed through the NMR system (Pörtner et al., 2002; Bock et al., 2002b).

3. Evidence for temperature induced hypoxia in fish

In fish, heart rate and both ventilation frequency and amplitude have frequently been reported to increase in association with a temperature-induced rise in oxygen consumption in order to compensate for elevated oxygen demand by progressively enhanced oxygen supply (Barron et al., 1987; Graham and Farrell, 1989; Mark et al., 2002). According to the concept of oxygen limited thermal tolerance (see Section 1) onset of thermal limitation should be elicited by limited capacity of oxygen supply mechanisms to match oxygen demand beyond low or high peius temperatures (T_p, see above), thereby eliciting a drop in aerobic scope. In NMR experiments monitoring of blood flow changes by a flow weighted MR imaging sequence was combined with localized ^{1}H-NMR spectroscopy in the North Sea eelpout *Z. viviparus* at different tem-

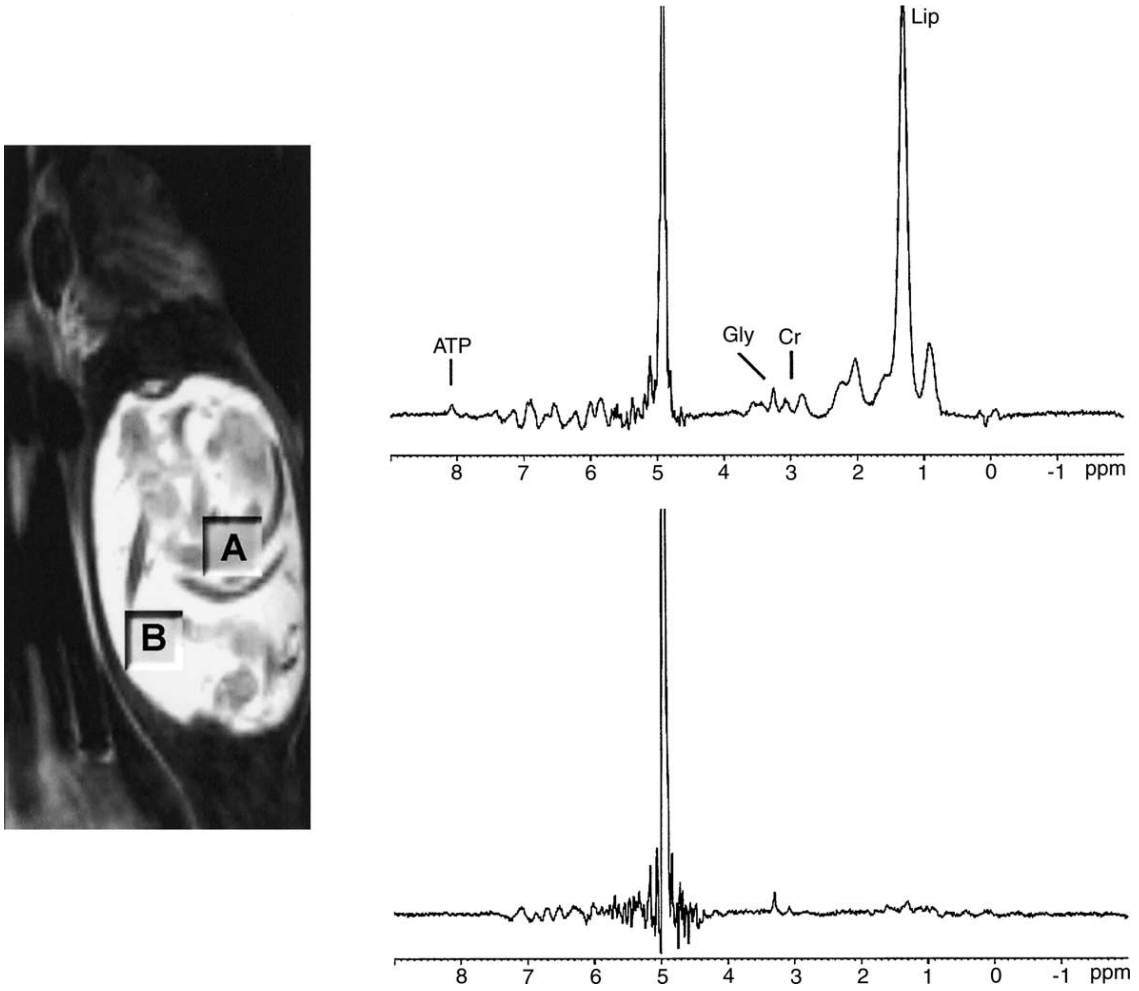


Fig. 2. Anatomical MR image of a pregnant specimen of the North Sea eelpout *Zoarces viviparus*. Localized in vivo ^{1}H-NMR spectra obtained in two voxels are presented on the right. Metabolites in spectrum (A) originated from embryonic fish, whereas almost no signal (except for residual water) arose in spectrum (B) where no embryo was present (after Bock et al., 2002a).

peratures. Progressive warming resulted in increased arterial and venous blood flow until it remained unchanged despite a continued rise in oxygen demand (Van Dijk et al., 1999; Zakhartsev et al., 2003). Above a critical temperature invasive work reported succinate accumulation in liver (Van Dijk et al., 1999). In the MR studies a sudden drop in blood flow was observed and lactate accumulation in the white muscle of the fish was detected in localized ^{1}H-NMR spectra (Fig. 4). Lactate as an anaerobic end product is a more indirect marker for cellular hypoxia than succinate. It nonetheless indicates that critical metabolic conditions were reached at this temperature; consequently, the animal died.

These findings already indicated closely coordinated adaptation to ambient temperature and oxygen levels as derived for the marine invertebrates. In consequence, Zakhartsev et al. (2003) studied the temperature dependence of the critical oxygen tension (P_c) in eelpout, *Z. viviparus*. The P_c was determined as the oxygen tension below which the rate of oxygen consumption fell below the regulated value when the animal was exposed to progressive hypoxia. Zakhartsev et al. reported that the P_c rose linearly depending

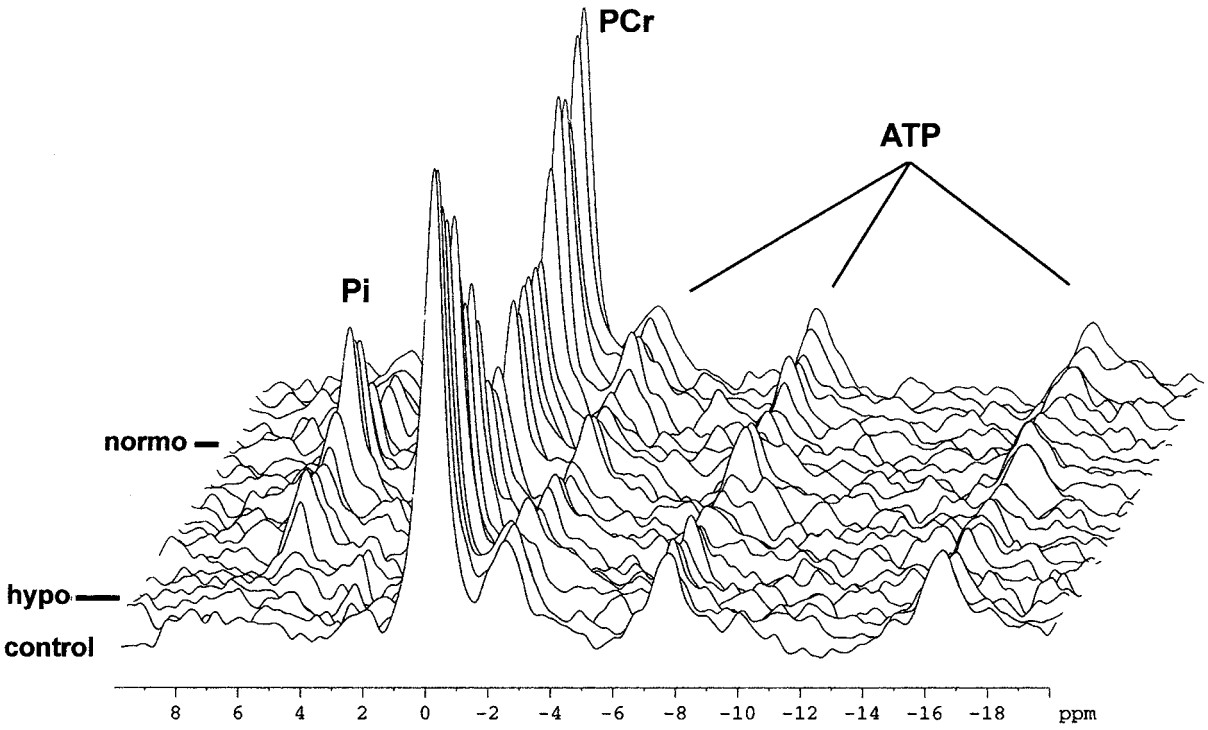
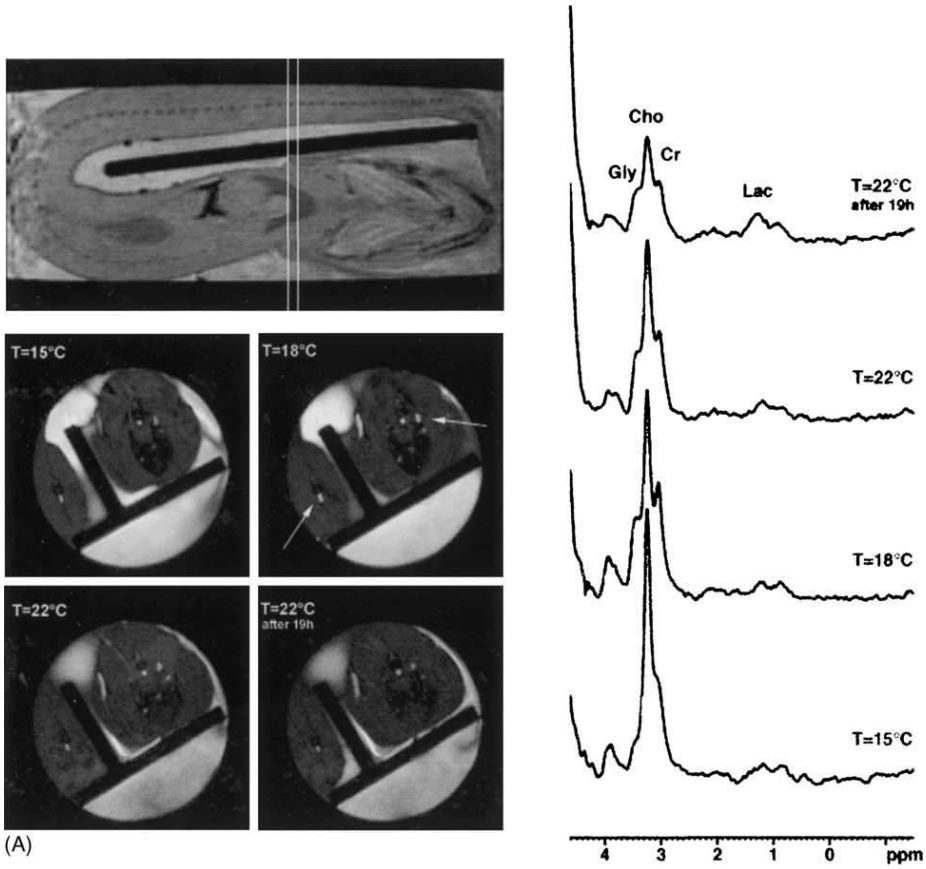


Fig. 3. Stack plot of in vivo ^{31}P-NMR spectra collected in Atlantic cod *G. morhua* during normoxia (front), hypoxia and post-hypoxic normoxia (after Bock et al., 2002a). Time resolution between spectra was 5 min. Note the drastic decrease of phosphocreatine and increase of inorganic phosphate under hypoxia. Switching to normoxia caused values to return to control levels within 15 min.

on water temperature and would reach normoxic levels at those temperatures where Van Dijk et al. (1999) had found the critical temperature and onset of mitochondrial anaerobiosis.

Mark et al. (2002) tested whether temperature induced hypoxia was alleviated by ambient hyperoxia in the Antarctic eelpout *P. brachycephalum*. They monitored the effects of temperature on oxygen demand, ventilatory effort and blood flow at normoxic and hyperoxic oxygen levels (Fig. 5). Under normoxia arterial blood flow rose distinctly between 0 and 7 °C. It reached a plateau above 7 °C, possibly due to a limitation of heart and/or vascular capacity. In contrast, experiments carried out under hyperoxia (P_{O_2} = 45 kPa, ~2-fold normal O_2 tension) did not cause a significant rise in blood flow in the *Aorta dorsalis*, in line with an alleviation of temperature induced oxygen shortage by hyperoxia. At elevated temperatures this effect was also clear from significantly lower oxygen consumption rates under hyperoxia than seen under normoxia. In fact, hyperoxia alleviated the "typical" exponential increase in oxygen consumption with temperature,

Fig. 4. (A) Axial views of flow weighted MR images of eelpout (*Zoarces viviparus*) from the North Sea at different temperatures. Blood flow in vessels, visible as bright spots, increased with temperature (see arrows). At a water temperature of 22 °C blood flow dropped abruptly after 19 h accompanied by an increase of lactate (Lac) in localized ^{1}H-NMR spectra from white muscle, indicating that the critical temperature was reached. (B) Development of arterial blood flow between 10 and 22 °C showed an early increment but no further rise despite increased oxygen demand. Similar to observations in Antarctic eelpout (Fig. 5) and in cod (Lannig et al., 2004) transition to saturated blood flow velocity is interpreted to reflect the peius temperature which indicates onset of a loss in aerobic scope. In *Z. viviparus*, T_p was found at 14 °C while the critical temperature was reached at 22 °C, in line with earlier observations of succinate accumulation (Van Dijk et al., 1999; modified after Bock et al., 2002a and unpublished).


T=15°C
T=18°C
T=22°C
T=22°C
after 19h
Cho
Cr
Gly
Lac
T=22°C
after 19h
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T=18°C
T=15°C
4
3
2
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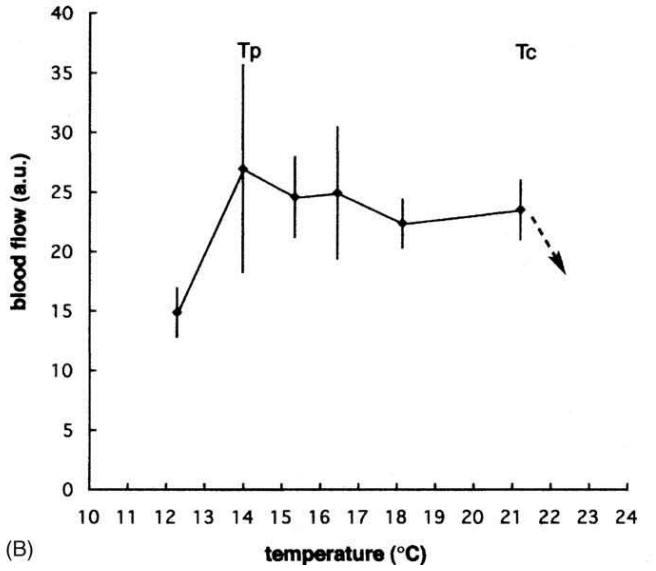
0
ppm

(A)


Tp
Tc
blood flow (a.u.)
40
35
30
25
20
15
10
5
0
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
temperature (°C)

(B)

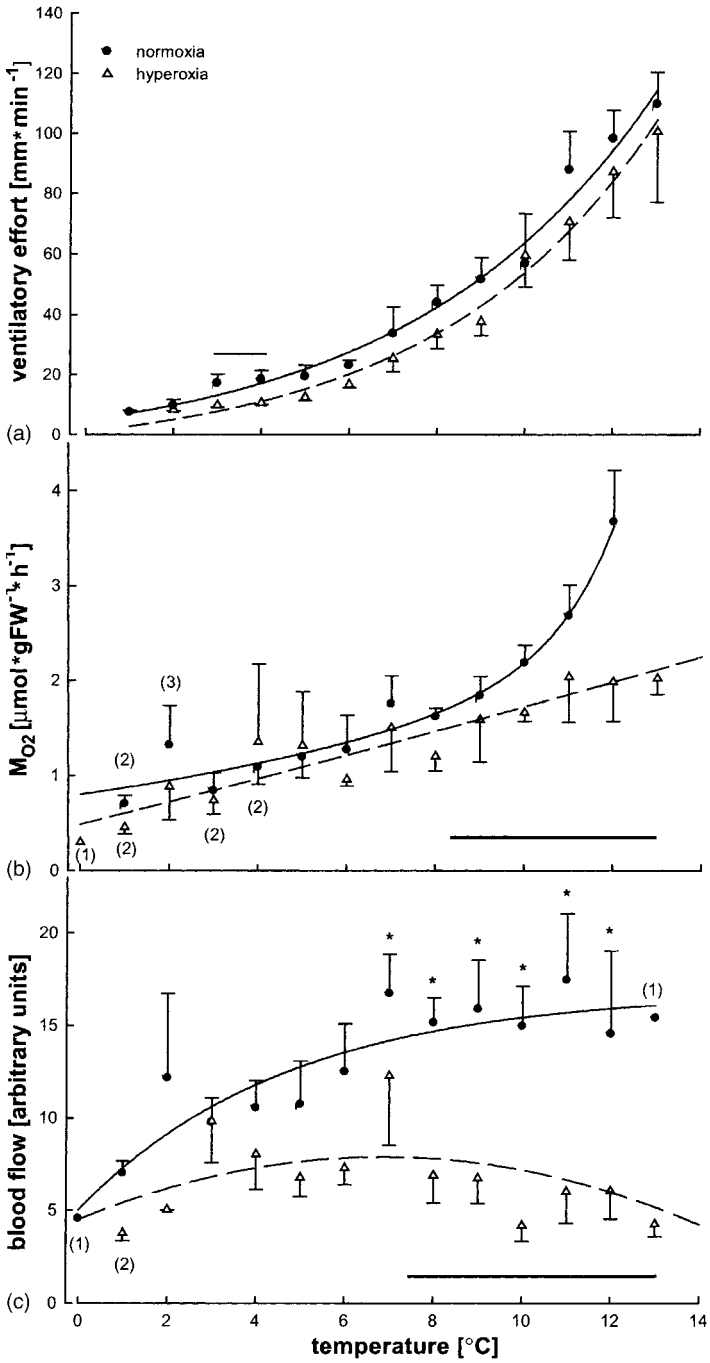


Fig. 5. Ventilatory effort (a), oxygen consumption (b) and blood flow (c) of Antarctic eelpout *P. brachycephalum* at different temperatures and external oxygen levels. In contrast to ventilation and oxygen consumption blood flow levelled off at higher temperatures. Interestingly, hyperoxia alleviated the effect of warming on systemic parameters (after Mark et al., 2002). The drop in oxygen consumption observed under hyperoxia at high temperatures indicates reduced cost of circulation due to ample oxygen supply. In contrast to *Z. viviparus*, T_p under normoxia was reached at about 7 °C in *P. brachycephalum*.

very likely due to energy savings in the cardiocirculatory system due to enhanced oxygen availability. Ventilatory effort under both treatments did not reveal a significant effect of hyperoxia as it followed the same exponential increment, regardless of ambient oxygen levels.

These findings indicated that the Antarctic eelpouts became hypoxic because of a temperature induced systemic limitation of oxygen supply at rising oxygen demand, which was alleviated by hyperoxia. At similar ventilation rates higher oxygen consumption and limited blood flow under normoxia on the one hand and reduced oxygen consumption and blood flow at elevated temperatures under hyperoxic conditions on the other hand suggested that the cardiovascular rather than the ventilatory system responds to changes in systemic oxygen availability and may thus play a key role in thermal tolerance in these Antarctic fish (Mark et al., 2002). Nonetheless, although hyperoxia likely widened the temperature range of available aerobic scope in the Antarctic eelpout, it did not cause large shifts of the limits of passive heat tolerance. A recent treatment of thermal limits in various systematic groups from prokaryotes via unicellular eukaryotes to metazoa suggested a systemic to molecular hierarchy of thermal limitation with the narrowest windows found at the highest levels of organisational complexity (Pörtner, 2002a). At the same time, the concept of symmorphosis indicates that the functional capacities of individual components contributing to the performance capacity of the higher unit, i.e. the organism, are usually not expressed in excess. With respect to the functional capacities setting thermal tolerance this would mean that, once the limits at the highest level are alleviated (in this case by hyperoxia), those at a lower (i.e. cellular or molecular) level of complexity may now predominate in limiting whole organism thermal tolerance at a slightly but not hugely widened window of thermal tolerance.

Studies carried out in Atlantic cod, *G. morhua* from the North Sea and in rainbow trout, *Oncorhynchus mykiss*, support a crucial role of the circulatory system in thermal limitation and temperature dependent aerobic scope and suggest that this may be a general pattern in fish. Early data by Heath and Hughes (1973) would also match this interpretation. They found that heart rate in rainbow trout decreased at temperatures above 24 °C, whereas ventilation remained virtually unchanged until death of the animals occurred. In cod implanted with micro-optodes in gill blood vessels, Sartoris et al. (2003b) demonstrated that arterial oxygen tensions (P_{a,O_2}) remained unaffected by progressive warming. However, venous oxygen tension (P_{v,O_2}) dropped progressively during warming, in line with limited cardiac rather than ventilatory performance. It was concluded that in resting cod at elevated temperatures, circulatory performance cannot fully compensate for excessive oxygen extraction from the blood (Pörtner et al., 2001; Sartoris et al., 2003b). Functionally, this pattern can seriously hamper myocardial oxygen supply, as most teleost fish lack or only possess a weak coronary circulation and hence almost exclusively rely on the venous oxygen reserve to provide the heart with oxygen (Farrell, 1993).

Farrell and Clutterham (2003) measured venous oxygen tension in the *ductus Cuvier* of rainbow trout, *O. mykiss*, during exercise at different acclimation temperatures. They discussed that a specific threshold P_{v,O_2} is required in fish in order to maintain sufficient oxygen supply to the myocardium and support cardiac output. Accordingly, a reduction in aerobic scope is likely to result when a temperature dependent decrease in venous oxygen tension (P_{v,O_2}) sets in. In line with these findings and with a limited temperature window of optimum oxygen supply, Farrell (2002) found maximum cardiac output of exercising salmonids within the optimal temperature range. Similarly, cod make use of their full aerobic capacity and use both the glycolytic and oxidative capacities of their musculature to support endurance swimming under 'normal' thermal conditions (Martinez et al., 2003).

At constant levels of arterial oxygen tension (P_{a,O_2}) temperature dependent patterns of venous oxygen tension (P_{v,O_2}) should therefore delineate the window of thermal tolerance in fish in similar ways as previous recordings of arterial oxygen tensions in a crustacean (Frederich and Pörtner, 2000). In a combination of invasive oxygen analyses and measurements of blood flow by MRI, Lannig et al. (2004) found a decrease in venous P_{O_2} towards both sides of the thermal optimum. Resting heart rate in cod (*G. morhua*) rose exponentially upon warming from 10 to 16 °C. However, arterial and venous blood flow rose only slightly and did not compensate for the drop in venous P_{O_2} in the warm. Hence, loss of optimized oxygen sup-

ply to the heart and thus, decreased aerobic scope of the whole organism seems to be the first limiting factor in these fish. The maintenance of arterial P_{O_2} seen during warming in cod is in line with an excess capacity of the ventilatory system for oxygen uptake, if compared to oxygen distribution via circulation. However, arterial P_{O_2} fell drastically above 16 °C (Sartoris et al., 2003b), presumably indicating the temperature at which a minimum threshold P_{v,O_2} is reached in cod with the result of cardiac failure and organismic collapse.

As a corollary, the capacity of the teleost circulatory system likely becomes insufficient in the warm to match the rising oxygen demand. The question then arises how oxygen limitation develops in the cold. In the cold, oxygen provision appears facilitated due to high oxygen solubility in ambient water and body fluids. Moreover, oxygen diffusion should be facilitated in relation to oxygen demand as diffusion decreases less with temperature than baseline metabolic costs which are reflected in the level of standard metabolic rate (Q_{10} of ~1.1 versus ~2–3). The data obtained under hyperoxia in Antarctic eelpout (Mark et al., 2002) suggest that enhanced oxygen availability as in cold waters should allow for a reduction in the energy cost of circulation and ventilation and thereby support the reduction in metabolic rates typically seen in Antarctic stenotherms. Energy savings are also supported by the increasing importance of cutaneous uptake of O_2 in the cold, culminating in a 30% contribution of cutaneous oxygen uptake to standard metabolic rate (SMR) in the Antarctic icequab *Rigophila dearbornii* (Wells, 1986). Low metabolic rates at enhanced oxygen solubility in body fluids enable icefish (*Chaennichthyidae*) to survive without red blood cells that greatly contribute to blood viscosity (Davison et al., 1997)—an alternative way to cut cardiovascular costs at low temperatures.

Nonetheless, oxygen supply capacity becomes limiting in temperate water breathers exposed to cold temperatures, observed in cod (Lannig et al., 2004) in similar ways as seen in a temperate crustacean (Frederich and Pörtner, 2000) or in annelids and sipunculids (Zielinski and Pörtner, 1996; Sommer et al., 1997). The drop in venous P_{O_2} (in fish) or arterial P_{O_2} (in the crustacean) and the transition to anaerobic metabolism in the cold indicate a limited functional capacity of oxygen supply mechanisms likely elicited by cold induced slowing. Functional capacity of oxygen supply mechanisms and the muscular tissues involved falls below the one to cover metabolic requirements at low ambient temperatures, thereby setting the first limit to cold tolerance. The upregulation of mitochondrial densities in the cold discussed below indicates that loss in mitochondrial functional capacity contributes to the limited functional capacity of cells and organs and is therefore compensated for in the cold. Again, the symmorphosis concept would predict that cold induced limitations in oxygen supply are likely to be closely followed by limitations in the capacity of other systemic, cellular and molecular functions such a general functional collapse of the organism occurs beyond but close to the limits set by insufficient oxygen supply. These relationships warrant further investigation.

4. Trade-offs in thermal adaptation setting functional limits

The question arises which mechanisms cause animals and their oxygen supply systems to specialize on a limited range of thermal tolerance which matches the thermal range which a species usually experiences in its natural habitat. In particular, invertebrates and fish adapted to the stable temperatures of Antarctic waters rely on narrow thermal windows. As oxygen limitations set in at both sides of the temperature window it appears likely that mechanisms are involved which define oxygen demand in relation to the capacity of oxygen delivery by circulation and ventilation such that tissue functional capacities (esp. of the heart) are set to a level sufficient to match maximum oxygen demand between the average highs and lows of environmental temperatures. The responsible mechanisms should also characterize the trade-offs involved in thermal adaptation, i.e. they should explain why a downward shift of the oxygen limited cold tolerance threshold coincides with an increase in heat sensitivity and vice versa.

The mitochondrial metabolic background of setting both tissue and organismic functional capacity and oxygen demand at various temperatures has contributed to an understanding of the links between low and high thermal limits and thus, the trade-offs in thermal adaptation (Pörtner et al., 1998, 2000; Pörtner, 2002a). In ectothermic species, especially

fish a plethora of studies have identified mechanisms of seasonal and latitudinal cold versus warm adaptation which are suitable to modulate the capacity of aerobic metabolism. As outlined above, a rise in aerobic capacity permits maintenance of cell functions in the cold (for review Guderley, 1998; Pörtner et al., 1998, 2001). Associated processes are rising enzyme capacities (Crockett and Sidell, 1990; Guderley, 1990; Lannig et al., 2003), increased mitochondrial or capillary densities (Sisson and Sidell, 1987; Guderley and Blier, 1988; Ressel, 2001), changes in mitochondrial structure (Gaebel and Roots, 1989; St.-Pierre et al., 1998) and/or alterations in membrane composition (Miranda and Hazel, 1996; Logue et al., 2000). Mitochondrial densities are found esp. large in pelagic notothenioid fishes of the Antarctic, where densities in red muscle result beyond 50% (Dunn et al., 1989). Recent evidence indicates that the thermal adaptation of marine invertebrates follows similar principles (Sommer and Pörtner, 2002) which therefore appear unifying in thermal adaptation.

In the context of the concept of oxygen limited thermal tolerance, these mechanisms contribute to a unidirectional shift of both low and high peius and critical temperatures. Such a shift is associated with a change in mitochondrial density, which drops as temperature rises and increases as ambient temperature falls. The main advantage of an increase in mitochondrial density and activity in the cold is an increase in aerobic functional capacity. The associated increase in the network of intracellular membrane lipids constitutes a significant facilitation of intracellular oxygen diffusion in the cold (Sidell, 1998; cf. Pörtner, 2002b). In the cold, the improvement of aerobic energy production also supports the capacities of ventilation and circulation (Pörtner, 2001). As a consequence of elevated mitochondrial densities, however, baseline oxygen demand by mitochondria is enhanced, set by the level of mitochondrial proton leakage. This will cause earlier problems during warming, where associated with a rise in other baseline costs the overall increment in oxygen demand can no longer be met by the capacity of oxygen supply mechanisms. A trade-off results between the compensation of functional capacity in the cold and the resulting increase in baseline oxygen demand which contributes to lower the limits of heat tolerance. In this context, recent evidence indicates that these mechanisms of cold adaptation are likely modulated in Antarctic stenotherms in order to minimize the cost of cold adaptation below the one seen in cold adapted eurytherms (Pörtner et al., 2000). Especially in temperate to high latitudes of the Northern hemisphere several species are found eurythermal and, thus, experience high costs of cold adaptation (e.g. Sommer and Pörtner, 2002) associated with trade-offs in energy budget and their likely ecological consequences (Pörtner et al., 2000, 2001, 2004). Such differences will also have their bearing with respect to the sensitivity of animal species to climate and associated temperature change.

The mechanistic and regulatory bases of the processes setting thermal tolerance and defining thermal adaptation as well as their integration into whole animal functioning are still incompletely understood. Thermal adaptation is linked to temperature dependent gene expression, for example of key aerobic enzymes, as seen during seasonal as well as latitudinal cold adaptation (e.g. Hardewig et al., 1999; Lucassen et al., 2003). Rearrangements of aerobic metabolism also occur with a shift to lipid accumulation and energy storage (cf. Pörtner, 2002b, for review). The fine tuning of these processes on a temperature scale or their functional consequences at the whole animal level remain to be quantified and the regulatory signals to be identified.

The mitochondrial trade-offs addressed above will relate to changes in functional capacity and oxygen demand of more or less all cells of the organism and these patterns transfer to the next hierarchical level, the functional capacity of tissues like the cardiovascular system and finally of the organism. Further trade-offs apply at the organismic level, like for the cardiovascular system of fish which supplies oxygen to tissues on the one hand but on the other hand relies on supply from residual oxygen in venous blood. Therefore, it is cost-effectively designed to consume rather small amounts of oxygen itself (Farrell and Clutterham, 2003). This constraint limits the development of functional capacity and may be the key reason why in fish the circulatory system appears more crucial in thermal limitation than the ventilatory system. However, compared to invertebrates and higher vertebrates, where cardiac supply is via arterial blood or haemolymph, the excess ventilatory capacity observed in fish may in fact be related to the unusual pattern of venous oxygen supply to the fish heart. This

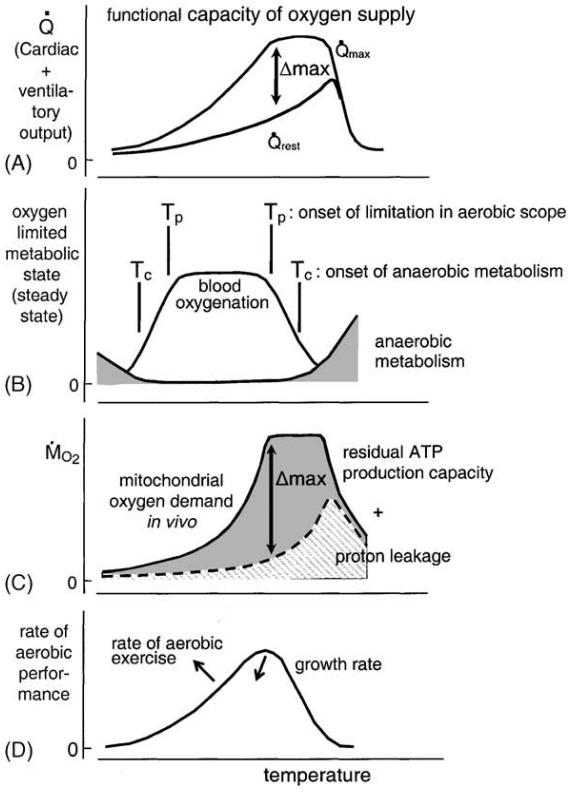


Fig. 6. Schematic model of oxygen limited thermal tolerance and performance capacity in fish and other metazoa, set by the capacity of oxygen supply mechanisms. (A) Functional reserves in oxygen supply result as combined ventilatory and cardiac output (Q) and are maximum at the upper pejus temperature T_p, before aerobic scope becomes thermally limited (B). Maximum scope (Δ_{max}) between resting and maximum output in oxygen supply is likely correlated with the one in mitochondrial ATP generation such that the functional capacity of the (ventilatory and circulatory) muscles is co-defined by the capacity of mitochondria to produce ATP which is limited by oxygen supply in vivo (C). Part of this limitation is elicited by the temperature dependent rise in oxygen demand by the cost of mitochondrial proton leakage which is no longer available to ATP formation. Low ATP formation capacity in the cold and high proton leakage in the warm contribute to insufficient oxygen supply, loss of aerobic scope and finally, transition to anaerobic metabolism (B). Maximum scope in ATP generation at the upper T_p not only supports maximum capacity of organismic oxygen supply by circulatory and ventilatory muscles, but also an asymmetric performance curve of the whole organism (D, after Angilletta et al., 2002) with optimal performance (e.g. growth, exercise) again expected at the upper peius temperature T_p. Here, functions are supported by both high temperatures and optimum oxygen supply in relation to baseline oxygen demand. As a trade-off in eurythermal cold adaptation (e.g. upper limits constant, lower limits shifting to colder temperatures), standard metabolism and, in consequence, aerobic exercise capacity may increase in the cold (cf. Pörtner, 2002b), while temperature specific growth performance is reduced likely due to enhanced mitochondrial proton leakage (Pörtner et al., 2001). These contrasting changes in exercise capacity vs. those in growth rate are indicated by arrows in (D) (for further explanations, see text).

excess ventilatory capacity likely supports wider tolerance windows. Maximized arterial oxygen supply regardless of temperature would help to prevent an earlier drop in venous P_{O_2} below critical values and thus support the cardiovascular system in counteracting thermal limitation. From this point of view, a coordinated thermal limitation by integrated ventilatory and circulatory capacities as observed in a crustacean (Frederich and Pörtner, 2000) exists in teleosts as well; however, limitation is first experienced by the circulatory system. The evolutionary constraint of venous oxygen supply to the heart in fish leads to specific patterns of temperature dependent oxygenation in arterial versus venous blood. In conclusion and in similar ways as in marine invertebrates, the integrated cardiovascular and ventilatory capacities of oxygen supply to tissues appear as the first thermally sensitive functional level that defines a fish's thermal limits of biogeography.

The principle trade-offs leading to thermal optimization of cellular and tissue function will not only be valid for the circulatory system but for many tissues and finally the intact organisms although details and trade-offs at the tissue and organism levels need to be investigated. These principle relationships between temperature dependent capacities and limits of organismal performance have been summarized in Fig. 6 (based on studies across phyla, mostly in aquatic ectotherms such as sipunculids, annelids, crustaceans, molluscs and fishes, cited above). Trade-offs as outlined above support optimized performance only within a limited temperature window. Minimum and maximum peius temperatures (T_p) delineate the first level of thermal limitation and indicate onset of a loss in aerobic scope, as the capacities of oxygen supply (integrated capacities of circulation and ventilation) become progressively unable to meet oxygen demand. Once aerobic scope is reduced towards thermal extremes, critical temperature thresholds (T_c) delineate the transition to an anaerobic mode of metabolism or

passive tolerance. Extended exposure of animals to temperatures above high or below low critical temperatures finally leads to death of the animal unless thermal acclimatization, i.e. a shift of T_c values occurs (Zielinski and Pörtner, 1996; Sommer et al., 1997). Limitation of survival is associated with a drop of the cellular Gibb's free energy change of ATP hydrolysis to a low, possibly critical value (Zielinski and Pörtner, 1996). In accordance with a hierarchy of thermal tolerance limits, a failure in oxygen delivery system at the whole-organism level occurs prior to a failure in mitochondrial and then molecular functions, thereby setting the ecologically relevant thermal tolerance thresholds of the intact organism (Pörtner, 2002a).

Critical temperatures as discussed here border the temperature range that permits performance on top of baseline energy expenditure and are likely reached before the onset of spasms (Zakhartsev et al., 2003) which are traditionally used to define critical thermal maxima (Lutterschmidt and Hutchison, 1997a,b). Within the thermal tolerance window aerobic performance increases with temperature to a maximum and then decreases at higher temperatures yielding a species-specific asymmetric bell shaped curve, which shifts depending on thermal adaptation (Angilletta et al., 2002). Fig. 6 predicts that optimum performance occurs close to upper peius values linked to the maximum scope for ATP formation by mitochondria. The relationship between temperature dependent growth rates and aerobic scope may follow this pattern. Aerobic scope and growth rate were found related in a population of cod (Claireaux et al., 2000). Growth curves similarly shaped as in Fig. 6D were found in invertebrates (Mitchell and Lampert, 2000; Giebelhausen and Lampert, 2001) and in fish (Jobling, 1997). Moreover, protein synthesis rates will set the pace for organismic growth. Recent findings indicate that low blood oxygen tensions limit protein synthesis rates as seen in feeding crabs (Mente et al., 2003), thereby supporting the concept layed out in Fig. 6.

5. Temperature induced hypoxic hypometabolism?

The question arises whether temperature induced hypoxia and finally anaerobiosis at the edges of the thermal tolerance window have consequences other than reducing performance capacity of the organism. In principle, all processes will become involved that characterize survival strategies in hypoxia tolerant animals mainly through metabolic depression and associated passive tolerance of adverse environmental conditions as seen with respect to survival of turtles, frogs or fish during winter cold (Jackson, this volume). However, while transition to anaerobic metabolism has been clearly demonstrated in the turtles or goldfish other hibernating animals may succeed to use metabolic depression strategies while being fully aerobic. This is likely true for many hibernating amphibians and includes mammalian hibernators, where at least the brain remains fully aerobic, despite extremely cold body temperatures (Bock et al., 2002c). Passive hibernation is thus interpreted to be a strategy which allows the animal to survive at minimal cost and thereby escape from the costly mechanisms of eurythermal cold adaptation outlined above (Pörtner, 2004). The factors and mechanisms eliciting metabolic depression in excess of the one elicited by cold temperature itself are currently unknown.

Heat induced hypoxia will also elicit such responses which are likely beneficial to counteract the temperature induced acceleration of baseline metabolic costs. As the interdependence of thermal tolerance and aerobic scope have only recently been discussed as a unifying principle among animals (Pörtner, 2001) these relationships have not been systematically investigated. However, invertebrate examples from the intertidal zone where they may be exposed to midday sunshine and heat, would most adequately illustrate that extreme heat goes hand in hand with anaerobic metabolism and passive survival (Sokolova and Pörtner, 2003) and thus very likely involves a metabolic depression scenario which contributes to energy savings and thereby extends the period during which heat beyond the critical temperature can be tolerated.

6. Ecological perspectives

Temperature and global climate patterns have frequently been proposed as the most important factors governing marine zoogeography (Angel, 1991). Compared with terrestrial fauna, marine organisms cover larger ranges of geographical distribution and

exhibit distinct latitudinal zonation more frequently, especially in the near-shore environment where physical barriers prevent the migration of littoral species (Pielou, 1979; Rapoport, 1994). Along the same lines of thought identifying mechanisms of temperature adaptation (Johnston and Bennett, 1996) and their contribution to adjusting and limiting both cold and heat tolerance are considered important in the light of global warming (e.g. Wood and MacDonald, 1997; Pörtner et al., 2001) and the associated shifts in geographical distribution and/or physiological performance of ectothermic animals (Pörtner et al., 2001).

The ecological relevance of the physiological principles discussed in this chapter is emphasized by the observation that in the spider crab, *M. squinado*, upper and lower peius temperatures are more or less equivalent to the mean highs and lows of ambient temperature in the natural environment of this species (Frederich and Pörtner, 2000). This indicates that peius temperatures are prime candidates to relate to temperature dependent limits of geographical distribution. Due to the recent nature of the concept of oxygen limited thermal tolerance such patterns need to be investigated in more metazoan taxa and species including vertebrates and fish. The cellular and organismic principles of thermal limitation and adaptation outlined here may be influenced by overlying phylogenetic constraints of specific groups which may then contribute to modulate oxygen dependent thermal limits. For example, enhanced sensitivity to the anaesthetizing effect of magnesium reflects such a phylogenetic constraint in the special case of marine reptant decapod crustaceans (anurans and brachyurans). High magnesium levels in the haemolymph of this group likely limit its capability to adapt to extremely cold temperatures below 0 °C by the mechanisms outlined above and, thereby, excludes them from the respective temperature regimes in polar areas (Frederich et al., 2001). In contrast, marine ectothermic teleosts exist at all temperatures of the ocean. Although venous oxygen supply to the heart is an evolutionary constraint in teleost fish, it has not been found to limit cold adaptation capacity or biogeography of the whole group. Nonetheless, this constraint may limit the thermal range of individual species according to the trade-offs discussed above.

Some evidence indicates that adjustment to cold rather than warm temperatures is a more severe challenge for organismic physiology, in other words, small temperature changes on the cold side of the temperature spectrum may elicit larger effects than similar temperature changes in the warm. For example, warming by just 1 or 2 °C above ambient average temperatures will be fatal for extreme Antarctic stenotherms (Pörtner et al., 1999). The cost of adaptation to the same degree of cooling appears much larger at cold than at higher temperatures unless the width of thermal windows is minimized (Pörtner et al., 2000). This may be one reason for the impression that true stenotherms in the marine realm may only be found in polar esp. Antarctic cold (Pörtner, 2002a). Moreover, the decrease in biodiversity of extant marine macrofauna towards high, esp. Northern latitudes (Roy et al., 1998) may be due to the limited capacity of species to adapt to low but unstable temperatures. The picture is less clear for the southern hemisphere, where temperature oscillations are less expressed. However, a clear temperature dependent decrease in crustacean biodiversity has been shown by Astorga et al. (2003). The anaesthetizing effect of Mg^{2+} in decapod crustaceans is progressively increased during cooling and allows reptant decapods to live at 0 °C but no longer at −1 to −1.9 °C. In earth history decreasing winter temperatures by about 4 °C were associated with mass extinctions of marine invertebrates at the Eocene/Oligocene boundary despite constant summer temperatures (Ivany et al., 2000). During climate change scenarios alleviation of winter cold may, therefore, play a key role in changes of ecosystem structure and functioning, esp. in Northern temperate zones. As an example, in the North Sea, warmer water species immigrate including both invertebrates and fish (Hummel et al., 2001; Von Westernhagen and Schnack, 2001) while cold-water species like cod move further North (Fischer, 2002). Associated changes in biodiversity will have to be investigated.

Although the contribution of physiological constraints and capacities to these patterns remains to be clearly elaborated insight is only just emerging that the physiological basis for such ecological patterns may be associated with the width of the thermal tolerance window and its location on the temperature scale (Pörtner, 2002a,b). Temperature dependent shifts in geographical distribution may be related to the mismatch phenomena elaborated above and may be due to the limited phenotypic plasticity of the species involved, i.e. the limited capacity to shift the window of

oxygen limited thermal tolerance. A rising width of the thermal tolerance window, especially in the cold, was suggested to be associated with enhanced energy turnover due to the cost of cold adaptation. These relationships may elicit climate dependent trends in lifestyles and, last not least, biodiversity in ecosystems and will have to be considered in future analyses of such patterns.

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4 Discussion

This thesis addresses the mechanisms of thermal tolerance in temperate, sub-polar and polar fish. As laid out in the introductory chapter, the thesis consists of three main studies (publication I to III), which examine thermal tolerance at the systemic, cellular and molecular level, respectively. In this chapter, the most important results of publications I-III will be revisited in brief and completed with some additional data, followed by an integrative discussion of the patterns of thermal and energetic limitations observed at whole animal, systemic and cellular levels and thermally induced adaptive processes at the molecular level. They will be discussed in an attempt to address the question of where particular mechanisms limiting thermal tolerance are located and how they are interlinked between the various levels of organisational complexity.

4.1 Systemic thermal tolerance

In the experiments on the hypothesis of an oxygen limited thermal tolerance (publication I), an alleviating influence of additional oxygen (ie. hyperoxia) on limits of thermal tolerance was investigated at the systemic level in *Pachycara brachycephalum*. Animals remained in a flow-through system under normoxia and hyperoxia, respectively, throughout the experimental period, when temperature was increased by 1°C once every 12 hours.

Only some of the examined parameters showed a hyperoxia sensitive reaction during warming, whilst others did not. Of the cardio-respiratory system (publication I, figure 2), ventilatory effort (the product of ventilation frequency and amplitude) remained insensitive towards oxygen concentration and showed similar exponential increments under both normoxia and hyperoxia. Whole animal oxygen consumption rose with temperature under both normoxia and hyperoxia, but displayed a much steeper, more exponential incline under normoxia. Normoxic bloodflow increased steeply until about 7°C and then levelled off, while hyperoxic bloodflow had lower starting levels and rose only slowly (and not significantly) up to 7°C and from there on slightly decreased.

Ventilation frequency and amplitude did only react to temperature changes and not to ambient or blood oxygen levels, the latter of which can only be assumed to have been higher due to increased physically dissolved oxygen under hyperoxia (in case that blood P_{O2} is not kept constant). Arterial oxygen contents have been reported to be nearly always close to saturation in cod (Sartoris et al., 2003b; Lannig et al., 2004) and trout (Heath and Hughes, 1973), also during warming. At high temperatures above 24°C, Heath and Hughes (1973) observed ventilation in trout to remain virtually unchanged while heart rate already decreased. In the light of the recent oxygen limitation hypothesis, this excess ventilatory capacity suggests

that ventilation is regulated independent of the cardio-vascular system and, moreover, is less sensitive to thermal extremes than the heart.

Although the picture of location, function and control of oxygen receptors in fish is still incomplete and warrants further research, a number of studies have confirmed the existence of branchial chemoreceptors in the gill arches of fishes. They monitor both internal and external O_2 concentrations and accordingly drive ventilation (Burleson and Smatresk, 1990a; b) and heart innervation (Reid et al., 2000; Sundin et al., 2000). Apparently, in *Pachycara brachycephalum* ventilation frequency and amplitude was not adjusted to external oxygen concentration, although ventilation volume has been reported to be greatly reduced during hyperoxic exposure in catfish, cyprinids, salmonids and Antarctic notothenioids (Wood and Jackson, 1980; Fanta et al., 1989; Takeda, 1990; Soncini and Glass, 2000). It is possible that ventilatory effort was not sufficient as a proxy for all processes involved in ventilation. In addition to the opercular pump, fish use the buccal pump to modify ventilation flow and pressure independent of the opercular amplitude (Hughes, 1984) and thus water flow over the gill arches could have been lower under hyperoxia without being accounted for.

Interestingly, in the trout, hyperoxia as well as hypoxia is associated with a marked bradycardia, evoked by probably the same receptor on the first gill arch (Daxboeck and Holeton, 1978), which corroborates the observed lower levels of blood-flow under hyperoxia. In preventing the organism to be flushed with too much oxygen it may constitute a protective function against oxidative stress (whilst under hypoxia, circulatory reduction can be a sign of metabolic reduction). It is interesting to find this behaviour conserved in *Pachycara brachycephalum*, as environmental hyperoxia is seldomly experienced by Antarctic fish.

In the fish circulatory system, the heart is situated far downstream on the venous side and constitutes the last oxygen consumer before venous blood is replenished with oxygen in the gills. In addition, three quarters of the teleost species lack a coronary circulation supplying the myocard with blood (Farrell, 1993) and the heart has to exclusively rely on supply of residual oxygen from venous blood. It is therefore designed to keep its costs low and consume rather small amounts of oxygen (Farrell and Clutterham, 2003), which on the other hand sets clear limitations to its functional capacities and may explain the particular thermal sensitivity of the circulatory system in fish. Under thermal or physical stress (ie. exercise), first circulation is increased to augment oxygen supply until the limits of cardio-vascular capacity are reached. As a second step, further oxygen demand will then lead to an intensified oxygen extraction, resulting in progressively reduced venous P_{O2} ($P_{v,O2}$) and aerobic scope of the animal, as has been demonstrated for cod (Sartoris et al., 2003b; Lannig et al., 2004) and trout (Farrell and Clutterham, 2003). Oxygen availability to the heart becomes thus more and more restrained,

until a critical $P_{v,O2}$, is reached, as suggested by Farrell and Clutterham (2003), and heart function becomes hampered. Cardiac failure and organismic collapse are the consequence (see publication IV for a more detailed discussion). Within the model of thermal tolerance (see introduction) reaching of the limit of cardio-vascular capacity would correspond to T_p, whereas the critical threshold $P_{v,O2}$, causing collapse, would correspond to T_c.

Blood flow is a measure of cardiac output, and in the experiments of publication I (cf. publ. I figure 2) it reached a maximum at 7°C under both hyperoxia and normoxia, although its overall level was much lower under hyperoxia. The distinctly lower perfusion under hyperoxia indicates good oxygen supply to the tissues and it is astounding to find such a substantial alleviation by hyperoxia, as the actual oxygen carrying capacity is only – if at all – increased by a fraction. With a hematocrit of about 13, *Pachycara brachycephalum* (as most Antarctic fish, cf. introduction) finds itself at the lower end of blood haemoglobin (Hb) content in fish, which ranges between 30 and 150g /l (Urich, 1990). 1g Hb binds 1.34ml oxygen, thus 30g Hb would hold about 40ml oxygen. At 0°C, fish blood contains about 10ml physically dissolved oxygen per litre (at 760 Torr / 101,3kPa and an αO_2 of 2.589 $\mu mol \cdot l^{-1} \cdot Torr^{-1}$), which accounts for about 20% of the total oxygen content. Since arterial blood can be considered saturated to 100% even under normoxia (see above), under hyperoxia only the physically dissolved amount of oxygen increases (i.e. blood P_{O2}). In the experiments of publication I, a hypothetical two-fold rise in blood P_{O2} would cause physically dissolved oxygen concentration to double from 10ml to 20ml per litre, an increase in total oxygen of only 20% (which will become even smaller with rising temperature). Reduced blood flow under hyperoxia indicates that the organism tries to compensate and keep its blood P_{O2} close to normoxic levels. These considerations mirror the role of physically dissolved oxygen (reflected in partial pressure) in being the key parameter driving diffusion. Higher water P_{O2} will also cause increased cutaneous respiration, thereby supporting further reductions in circulatory work and associated oxygen consumption. Under resting conditions and normoxia, cutaneous oxygen uptake can already comprise up to 35% of total respiration in the Antarctic eelpout *Rhigophila dearborni* (Wells, 1986) and is likely increased under hyperoxia. Fish possess a so-called 'secondary circulation' of low blood pressure and reduced hematocrit (Farrell, 1993), which extends from the gills to skin, scales and peripheral organs and has a primarily nutritive function but may be used for additional oxygen uptake and transport – especially of physically dissolved oxygen. Synergistically, the reduced oxygen consumption of the ventilatory and circulatory system likely contributes to lower total oxygen demand in a feedback reaction. This may in particular be the case in a benthic fish like *Pachycara brachycephalum*, which exclusively relies on active ventilation.

Muscle tissue oxygenation (publication I, figure 3) decreased above 5 or 6°C under both incubation regimes, slightly more pronounced under hyperoxia. Liver oxygenation rose in both treatments, yet the effect was not as marked as the changes in muscle oxygenation and individual oscillations were large. Although intracellular pH (publication I, figure 4) proved hyperoxia sensitive in displaying higher initial pH_i values due to a presumably hyperoxia-induced respiratory acidosis and elevated bicarbonate levels in the blood (Gilmour and Perry, 1994), pH_i regulation was not affected by hyperoxia. In both treatments, alpha-stat pH_i regulation ceased above 6°C – indicating that oxygen limitation may be the first, but not the only factor regulating thermal tolerance. It is for example conceivable that thermally induced modifications in membrane lipid domains around membrane-located proteins like those of cellular ion regulation may be responsible for shifts in specific activity levels and in the contributions of ion channels involved in pH regulation (homeoviscous adaptation: Wodtke, 1981; Hochachka and Somero, 2002). Moreover, temperature directly affects expression levels of ion pumps: in *Z. viviparus*, Lucassen et al. (submitted) found Na^+/K^+-ATPase expression and activity to increase in liver and gills during cold adaptation. Associated with shifts in activity are often changes in blood plasma ion levels, which have been reported to occur upon thermal acclimation in carp (Metz et al., 2003) and notothenioids (Guynn et al., 2002). Especially in Antarctic notothenioids, they may lead to new steady states in blood plasma ion contents, as Antarctic fish possess serum osmolarities nearly twice as high as temperate teleosts (about 600 mOsm/kg opposed to 330 mOsm/kg; O'Grady and DeVries, 1982) and accordingly show greater temperature dependent osmolality changes. Higher osmolalities are accomplished by elevated Na^+, K^+ and Cl^- levels and are thought to reduce the freezing point of blood (Somero and DeVries, 1967) as well as the energetic costs of maintaining the ionic gradient between blood and seawater in the cold. In the nototheniid *Trematomus bernacchii*, warm-adaptation to 4°C leads to a decrease of serum osmolality to values comparable to eurythermal temperate teleosts, associated with a two fold rise in Na^+/K^+-ATPase activity (Guynn et al., 2002). This may affect pH_i regulation and also appears feasible in *P. brachycephalum*, although serum osmolalities (and changes therein) have never been studied in that species and thus these speculations have to be considered hypothetical.

What elicits death of the animal under hyperoxia? During both treatments, the animals displayed very similar behaviour; spontaneous activity could be monitored online by MR imaging and was equally rare. At low temperatures, ambient oxygen concentration appeared to secure adequate oxygen supply to the animals. Yet arterial blood-flow was not further increased beyond 7°C under both normoxia and hyperoxia, although it would have been possible at least under hyperoxia where levels of blood-flow were always considerably lower

than under normoxia. Thus, the same breakpoint of blood-flow suggests failure of further processes beyond a reduction of aerobic scope under normoxia that was considered a first line of thermal sensitivity. Several scenarios as to the origin and location of a second line of factors limiting thermal tolerance are feasible: as suggested, they might be located on a lower level of organisational complexity and underlie nervous, hormonal or even cellular and molecular control. With the data for cellular respiration and cellular energy budgets presented for *Pachycara brachycephalum* and the Antarctic notothenioids in publication II, the latter two limiting factors can be securely excluded. Once oxygen and metabolic substrate provision is secured, cellular metabolism has been shown to function properly over a thermal range by far surpassing that of the intact individual. Moran and Melani (2001) have demonstrated for peripheral nerves of Arctic fish that their conduction properties begin to change only beyond lethal temperatures of the animals, so thermally elicited nervous failure appears rather improbable. A putative role for hormones is hard to define; moreover it is questionable whether it would be sensible to cause death on a hormonal basis as long as systemic and cellular parameters are still functional.

Within the context of the studies by Lannig et al. (2004), Sartoris et al. (2003b) and Farrell and Clutterham (2003), the results presented here for normoxia clearly indicate an oxygen limited thermal tolerance in fish. During warming, a progressive undersupply of oxygen (and to some extent also for all other metabolic substrates) leads to an increasing reduction of aerobic scope and finally to the onset of anaerobic metabolism. Although arterial oxygen saturation might not be limiting, it is possibly a combination of insufficient circulatory capacities on the one hand and insufficient blood volume and vascularisation on the other hand that might prevent adequate oxygen supply to tissues and organs at high temperatures. Lower costs of blood circulation and improved systemic oxygen supply under hyperoxia have probably increased aerobic scope of the animals and therefore may even have shifted *pejus* and critical temperatures. Yet, this was not observed, possibly due to the closely connected further mechanisms of thermal tolerance discussed above.

Under normoxia and presumably also under hyperoxia, upper *pejus* temperatures for *Pachycara brachycephalum* were supposedly located around 6°C, as this was the temperature where blood-flow levelled off and muscle tissue oxygenation decreasing, indicating first limitations of aerobic scope. Exact critical temperatures are hard to define, since in laboratory studies they are affected by experimental design. Several variables can affect individual critical temperatures, including rate of heating, duration of exposure to high temperature and acclimation history of the specimens (Hochachka and Somero, 2002). This becomes obvious when comparing experimental methodology and critical temperatures determined for *P.*

brachycephalum in publication I with the respective parameters in the studies of Van Dijk et al. (1999), who observed lower critical temperatures due to faster warming.

4.2 Cellular thermal tolerance

Based on the conclusions of publication I, publication II set out to investigate the potential role of cellular processes in thermal limitation. Cellular respiration and energy allocation to the four most important energy-consuming processes in the cell, RNA/DNA, protein and ATP synthesis, as well as ion regulation by the Na^+-K^+-ATPase were examined in isolated liver cells of Antarctic notothenioids fish between 0 and 15°C (publication II, figure 1). The fish species used for the experiments comprised the sub-Antarctic nototheniid *Lepidonotothen larseni*, four high-Antarctic species of the gender *Trematomus*, and the high-Antarctic Artedidraconid *Artedidraco orianae* (cf. figure 3). Results were complemented by cellular respiration data recorded for *Pachycara brachycephalum* in previous experiments (publication II, figure 3).

Sub-Antarctic and high-Antarctic cells displayed different respiration characteristics at low temperatures, reflecting species-specific thermal optima in minimized overall cellular energy demand. Cells of the sub-Antarctic notothenioid *L. larseni* and of *P. brachycephalum* showed minimal oxygen consumption between 3 and 6°C, whilst cells of the high-Antarctic species displayed a cellular energetic minimum at 0°C with progressively rising respiration rates upon warming. In line with this finding, sub-Antarctic notothenioids possess lower cellular protein synthesis and somewhat larger active ion regulation capacities than their high-Antarctic confamilials (publication II, figure 2), which is typical for more eurythermal species (Smith and Haschemeyer, 1980; Pörtner and Sartoris, 1999; Sartoris et al., 2003a; Storch et al., submitted).

Energy budgets remained by and large constant with first signs of changes in energy allocations that may have been caused by cellular energetic restrictions in the sub-Antarctic species *L. larseni* at low temperatures and the high-Antarctic species *T. eulepidotus* at elevated temperatures. Parallel with a rise in cellular oxygen consumption below 3°C, actinomycin D sensitive respiration was decreased at 0°C in *L. larseni*, reflecting decreased RNA and DNA synthesis rates. In *T. eulepidotus*, a progressive decline in cycloheximide sensitive respiration with rising temperatures could be observed and indicated decreasing protein synthesis rates due to energy allocation to processes more important to cellular homeostasis like ion regulation during warming (publication II, figure 1).

Maintenance of unchanged energy allocation over a wide range of temperatures in all species supports the notion that thermal limits are set by oxygen availability and associated

energy limitations of the whole animal level, whilst isolated cells are less thermally sensitive. Wider windows of tolerance at even lower levels of complexity are also reflected in the work of Somero and De Vries (1967), who found some enzymes of *Trematomus bernacchii* to show linearly increasing activity up to 30°C but the intact animals to die beyond 6°C. Hepatocytes of the sub-Antarctic species *L. larseni* and of *P. brachycephalum* showed signs of cold-eurythermy. Their thermal optimum in relation to habitat temperature indicates that the species may live close to their lower limit of thermal tolerance.

The focus of this study was on thermally induced shifts of energy allocation, and not on shifts directly provoked by reductions in cellular energy status. Unlike in the experiments of Buttgereit & Brand (1995) and Wieser & Krumschnabel (2001), where the authors reduced cellular energy charge by stepwise titration with the inhibitor myxothiazol, which blocks the electron transport chain by inhibiting complex III, the in vitro conditions of the medium used for cell storage in publication II were chosen not to be limiting – neither in oxygen, nor in metabolites. Oxygen levels were always above 4 kPa, which has been shown to be the threshold extracellular P_{O2}, below which cellular energy metabolism becomes limited in trout hepatocytes (Pannevis and Houlihan, 1992). Intracellular critical P_{O2} are difficult to measure and consequently rarely published. In canine red muscle cells, minimum intracellular P_{O2} for maximal cytochrome turnover has been estimated to be 0,23 kPa (1.7 torr) and O_2 becomes effectively limiting at 0,04 to 0,07 kPa in these cells (Honig et al., 1992). At the whole animal level it is even more difficult to examine whether cells are oxygen or substrate limited, because one has to also account for the systemic constraints of substrate and oxygen supply. There was probably no decrease in cellular energy charge in the experiments of publication I over a wide thermal range, as is illustrated by the adenylate data presented in figure 4: beyond 6°C, individual variability in ratios of high-energy phosphates increased, but generally ratios remained stable until the very end of the experiments, when ATP and phosphocreatine (PCr) concentrations collapsed virtually minutes before the animals died (cf. publication I). Yet a second way of interpretation would be that constant concentrations of high-energy phosphates have highest priority in cellular metabolism and ATP consumption is accordingly adjusted to grant sufficient ATP provision to selected processes, again leading to a hierarchic priority in those processes. Nonetheless, stable patterns of energy allocation during warming illustrate that the isolated hepatocytes used in the experiments of publication II were not limited in oxygen or metabolites, especially as they were free of the constraints imposed by a complex organism.

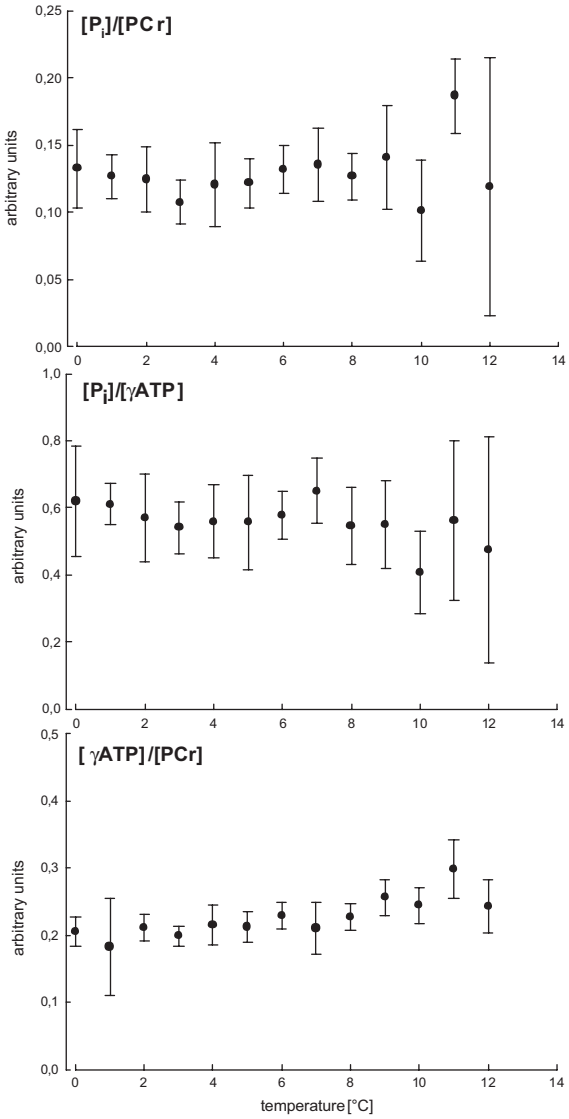

Figure 4: Ratios of concentrations of various high-energy phosphates under normoxia during warming. Levels were comparable under hyperoxia. PCr: phosphocreatine; P_i: inorganic phosphate; γATP: ATP, as measured from the signal of the γ-phosphate moiety. Data are presented as means±SD (unpublished data, taken from MR spectroscopic experiments of publication I, see publ. I for further details).

As mentioned in publication III, the data presented by Lannig et al. (submitted) for membrane bound cytochrome c oxidase activities after warm acclimation in *P. brachycephalum* suggested effects of homeoviscous adaption of mitochondrial membranes for this particular protein. Interestingly, thermally induced changes in membrane fluidity did not appear to play a significant role in the isolated hepatocytes in publication II, in contrast to the conceivable effects discussed above for systemic adaptation in *Pachycara brachycephalum* and in publication III for UCP2 expression. Although effects of homeoviscous adaptation have been reported for Na^+-K^--ATPase activity in erythrocytes of cold-acclimated trout (Raynard and Cossins, 1991), Na^+-K^--ATPase activity in the isolated cells of publication II did not appear to be

influenced by these effects, which may be indicative of an absence or slow reaction times of membrane adaptive processes during short-term warming (minutes to hours) and in isolated cells.

4.3 Thermally induced molecular adaptations

Publication III investigated the adaptive capacities of mitochondrial uncoupling protein 2 during warm and cold acclimation, respectively, in the Antarctic eelpout *Pachycara brachycephalum* and the common eelpout *Zoarces viviparus*. Mitochondrial UCP2 was chosen for the study because of its potential role in enhancing energetic plasticity of the mitochondria during thermal acclimation. Objective of the study was to characterise zoarcid UCP2 cDNA and, secondly, to examine mRNA and protein expression levels during thermal acclimation.

In muscle and liver tissue, well-conserved homologues of mammalian UCP2 could be characterised in *Pachycara brachycephalum* and *Zoarces viviparus* with a high degree of identity between each other and with further fish and mammalian UCP2, which is indicative of an important function of UCP also in ectotherms. The proteins consist of 313 amino acids each (publication III, figure 2), the complete cDNA sequences are depicted in figure 5.

Upon warm acclimation from 0°C to 5°C, mRNA and protein expression increased in *Pachycara brachycephalum*, which stands in contrast to *Zoarces viviparous*, where expression levels rose during cold acclimation from 10°C to 2°C. In both muscle and liver tissue, similar patterns of thermally sensitive expression of UCP2 could be observed (publication III, figure 5).

Higher levels of UCP after cold acclimation in *Z. viviparus* are most probably the result of an overall increase in mitochondrial capacity frequently found during cold acclimation (St-Pierre et al., 1998; D'Amico et al., 2002; Guderley and St-Pierre, 2002; Lannig et al., submitted). For *Z. viviparus*, there is clear evidence for mitochondrial proliferation in the cold, cytochrome c oxidase has been found to increase at both message and functional levels in muscle after cold acclimation (Hardewig et al., 1999b; Pörtner, 2002a), as well as activity levels of liver citrate synthase (Lucassen et al., 2003). The significantly up-regulated UCP2 message and protein levels upon cold acclimation in *Z. viviparus* corroborate these findings.

In the sub-Antarctic species *P. brachycephalum*, the picture was different: UCP2 mRNA and protein expression increased upon warm acclimation, instead of showing the expected decline. This species is apparently not able to sufficiently decrease its mitochondrial capacities in the warm, which has been discussed in publication III in the light of the data presented by Lannig et al. (submitted). Overly high mitochondrial capacities and turnover rates in the warm might lead to high mitochondrial membrane potentials, which increase the risk of reactive

oxygen species (ROS) formation. Increased levels of oxidative stress in warm acclimated *P. brachycephalum* observed by Heise et al. (2004) are in line with this hypothesis. In acting as a security valve, high levels of UCP2 could 'take the edge off' high membrane potentials and thus minimise the risk of ROS formation. Therefore, enhancing expression levels of UCP2 would be a means on the molecular level to support mitochondrial adaptive plasticity during thermal acclimation.

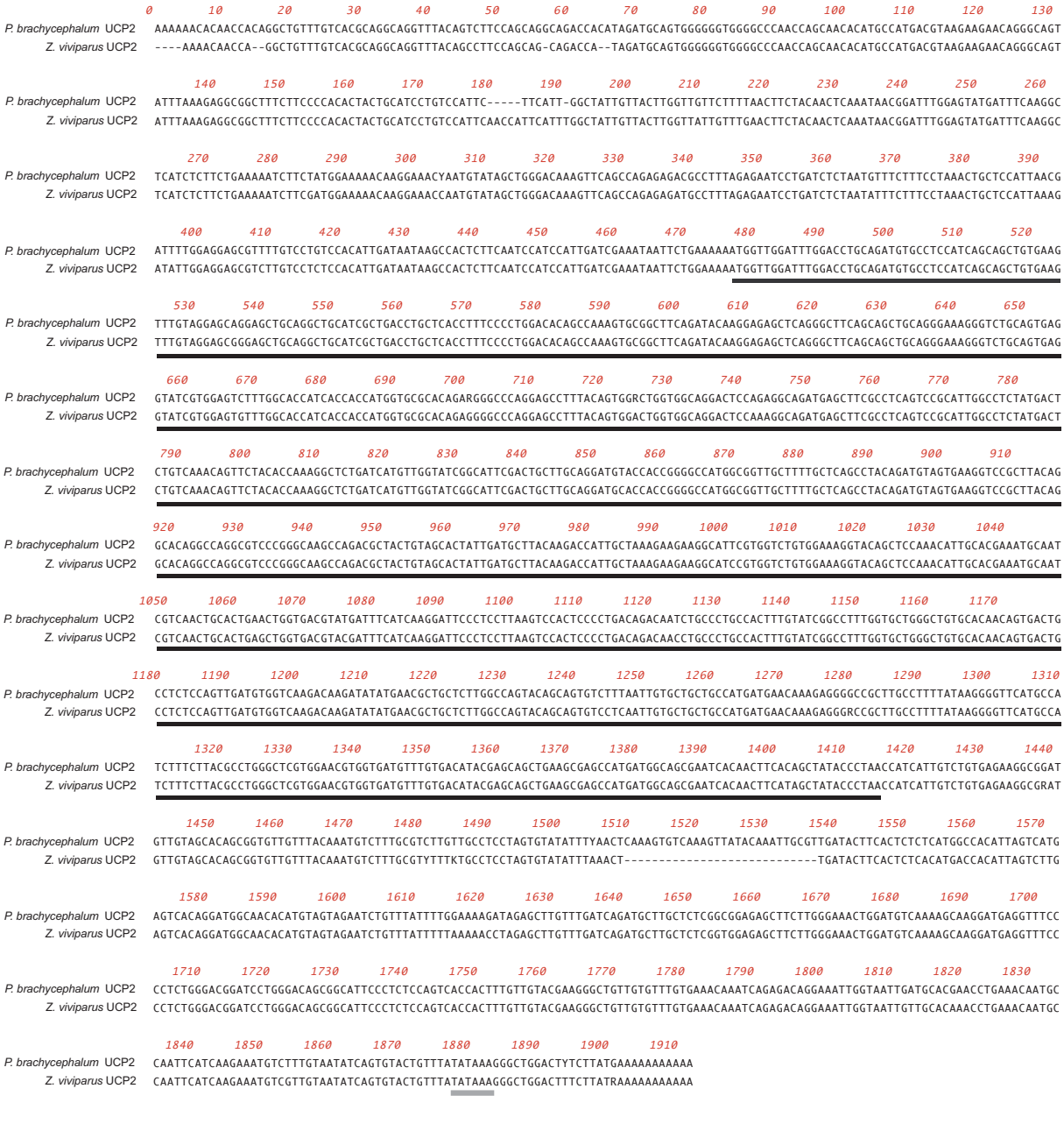

Figure 5: Complete cDNA sequences of the UCP2 genes of *P. brachycephalum* and *Z. viviparus* in 5'-3' orientation. The black line depicts the open reading frame, they grey line the poly A signal sequence (Proudfoot and Brownlee, 1976). The sequences can also be obtained from the Internet by the Genbank accession numbers AY625190 (ZvUCP2) and AY625191 (PbUCP2) (www.ncbi.nlm.nih.gov/Genbank/index.html).

4.4 Conclusions

This thesis investigated a number of aspects of thermal sensitivity and thermal acclimation from the whole animal down to the molecular level, the most important of which have been illustrated above. This section will provide an integrative synopsis of these results followed by an outlook suggesting further possible research in this field.

On the ecological scale it is growth and reproduction, which define survival of a species in a given thermal environment (provided living conditions are optimal) (Brett and Groves, 1979). To secure species survival, animals are therefore confined to the thermal optimum range depicted in figure 1. Growth and reproduction can only occur when surplus energy is available (Pitcher and Hart, 1982; Wieser, 1986). When food availability and uptake rates are high, energy is thus channelled into growth rather than locomotion (Boutilier, 1998). In situations of limited energy availability or limited aerobic scope, growth has less priority in energy allocation and hence growth and reproduction will be stopped first, followed by reductions in activity and movements of the animal (Brett and Groves, 1979).

This is in line with the hypothesis of a hierarchy in ATP consuming processes in the cell suggested by Atkinson (1977). In accordance with their functional importance, ATP consuming processes show different sensitivities towards limitations in cellular energy availability. The findings of Buttgereit & Brand (1995) and Wieser & Krumschnabel (2001) support this notion. In rat thymocytes and fish hepatocytes, respectively, the authors inhibited mitochondrial complexes III and IV and found decreasing sensitivity to cellular energy limitation in the following metabolic processes: protein synthesis was most sensitive, followed by RNA/DNA synthesis. Na^+-cycling and Ca^{2+}-cycling were less sensitive; the least sensitivity was shown by proton leak and further unidentified ATP consumers. Thus, the hierarchies proposed for whole animal thermal tolerance also become visible at the cellular level. Driven by the hierarchies of cellular energy allocation, growth and reproduction are only found in the optimum range at full aerobic scope and less sensitive processes like pH_i regulation extend into the *pejus* range of decreasing aerobic scope. In this light, the animal forms a functional unity, in which energy metabolism is adjusted from molecular to systemic level. Ultimately, the cellular level controls thermal tolerance, but the energetic restrictions leading towards a limited thermal tolerance are not elicited by capacity limitations at the cellular but at a higher, systemic level. The most complex, systemic level appears to be the most sensitive and in terms of thermal tolerance, it is the cardio-vascular system that initiates limitation of aerobic scope in fish. In accordance with the concept of symmorphosis (Taylor and Weibel, 1981), the cardio-vascular system is found to be fine-tuned to function optimally under normal environmental

conditions. At oxygen demands beyond, under-supply of oxygen caused by limited cardio-vascular capacities evokes a hypoxia-induced limitation to energy turnover at the cellular level. The consequences are shifts in cellular energy allocation that become manifest on all levels of organisational complexity up to the final, ecological level. The fact that limitations appear first at the systemic level is also in agreement with the concept of a systemic to molecular hierarchy of thermal tolerance (publication IV; Pörtner, 2002b), in which the most complex organisational level is also the most thermally sensitive, whilst thermal tolerance windows can slightly increase towards lower levels of complexity – as has been observed for cellular thermal tolerance in publication II.

Yet, there are exceptions to the rule, as has been demonstrated by the breakpoints in blood-flow and pH_i regulation in publication I, which were similar under normoxia and hyperoxia and thus appeared independent of systemic oxygen supply. It has therefore to be kept in mind that it may not exclusively be oxygen supply capacity that limits thermal tolerance. Oxygen supply can only be considered the first line of sensitivity, behind which others follow that are closely connected and integrate into tissue functional capacity (Pörtner, 2001). Under long-term acclimation, changes in membrane properties may for example play a role, as has been suggested in publication I and III.

Acclimation of functional capacity compensates for thermal limitations. This appears possible within phylogenetic limits, but it remains to be elucidated how they are defined and where they are set. Many fish species undergo vast biochemical and systemic adaptations during seasonal acclimatisation, yet it remains questionable, whether these adaptive abilities are lost or still possible in Antarctic fish, where seasonal acclimatisation has not been needed for millions of years. Systemic short-term reactions, such as variations of hematocrit, could immediately alleviate limitations in oxygen supply: Decreasing blood viscosity during warming offers the opportunity to improve oxygen provision by a rise in hematocrit, which is generally low in Antarctic fish (partly because of high blood viscosity in the cold, cf. introductory chapter). Erythrocytes are sequestered in the spleen of fish and can be released rather spontaneously in situations of increased oxygen demand, also in Antarctic fish (Egginton and Davison, 1998). Longer-term thermal adaptation leads to capacity increases of the cardio-vascular system due to capillary growth, this has been observed during cold acclimation in carp (Johnston, 1982) and is generally found during altitude acclimation and as a response to exercise in mammals (Taylor and Weibel, 1981). Judging from the role that the cardio-vascular system plays in the current picture of oxygen limited thermal tolerance in fish, a capacity increase would also make sense at the warm end of the thermal tolerance range: increased vascularisation would improve oxygen supply on the venous side and at the same time easen the workload of the

circulatory system, in this way even reducing systemic oxygen demand. Improving the venous oxygen reserve appears crucially important to secure myocardial oxygen supply – independent of the existence of a coronary circulation. Myocardial function is dependent on venous oxygen supply and to assure survival during thermal acclimation, venous oxygen levels must always remain above a certain critical threshold of venous P_{O2} illustrated by Farrell (2002) and examined by Farrell & Clutterham (2003).

Cold adaptation is also reflected on the cellular level by increased mitochondrial and enzymatic capacities and a cold adapted RNA and protein synthesis machinery in Antarctic fish and in sea-urchin embryos (Smith and Haschemeyer, 1980; Marsh et al., 2001; Storch et al., submitted). However, warm acclimation has seldomly been investigated in Antarctic fish and seems to cause problems in the Antarctic species *Pachycara brachycephalum*: The cardio-vascular system appears to form the 'bottleneck' in thermal tolerance by limiting oxygen supply during hyperthermia (cf. publication I) and it is currently not known, whether long-term warm acclimation could lead to increased capacities of the cardio-vascular system. At first sight, mitochondrial adaptation appears constrained in *P. brachycephalum* and can only partly compensate for rising metabolic turnover during warming (publication III, Lannig et al., submitted). But this might also reflect an alternative way of warm adaptation specific for Antarctic fish. As suggested in publication III, proteins like UCP2 might join the adaptive processes at the molecular and protein levels to form an integrative mitochondrial adaptive response, keeping elevated mitochondrial capacities to increase aerobic scope and shift to a more eurythermal mode of life in the warm. By uncoupling of the mitochondrial membrane potential UCP2 may assist mitochondrial adaption and in addition to changes in membrane properties (see publication III) minimise the risk of ROS formation. In this light, it would be very interesting to investigate how far this mode of adaptation can take *P. brachycephalum* or Antarctic fish in general, which shall be discussed in the following.

Recently, Lowe et al. (2004) conducted warm-acclimation experiments with a high-Antarctic notothenioid fish, *Pagothenia borchgrevinki*, which was acclimated from –1 to 4°C for 4-5 weeks. The authors found cytochrome c oxidase activity, lactate dehydrogenase activity and swimming ability to rise and cardiac and aerobic scope increased, too. Thermal performance breadth investigated in swim-tunnel experiments increased from 3 to 9°C. Warm adaptation in notothenioids may hence not only lead to shifts to osmolarities typical for eurythermal fish (see above; Guynn et al., 2002) but also to a more eurythermal mode of life with increased capacities for exercise. This was previously considered impossible for notothenioids (Eastman, 1993), Weinstein & Somero (Weinstein and Somero, 1998) had reported maximal survival time of 4-5 weeks at 4°C for *Trematomus bernachii* and *Trematomus*

newnesi. These results show that the adaptive capabilities to warmth of the so-called 'extremely stenothermal' Antarctic fish have to be re-evaluated.

In a recently conducted 'extremely' long-term warming experiment with *P. brachycephalum*, several specimens were kept at 6°C for several months (instead of weeks) and then progressively warmed to 13°C over a period of two weeks. The fish survived for four weeks (data not published). Compared to the results of publication I, where the animals died after several hours at 12 to 13°C, this is preliminary evidence for increased heat tolerance and maybe even shifts in threshold temperatures elicited by long-term acclimation to intermediate temperatures in this species.

It has to be mentioned though, that members of the Zoarcidae are cosmopolitan and have been able to adapt to cold-water habitats all around the world. In the Antarctic, zoarcids by and large do not enter similarly cold water as the notothenioids, even in the high Antarctic. Species of the sub-order Notothenioidei diversified in Antarctic waters and can be considered extremely cold adapted, although some notothenioid species have been able to adapt to non-Antarctic cold water habitats near New Zealand. Still, their overall warm-adaptation capabilities may be less expressed than in Antarctic zoarcids.

In further experiments carried out by E. Brodte, growth maxima and food conversion optima were found to be at 4°C in *P. brachycephalum* (E. Brodte, personal communication), which is in line with minimum cellular oxygen consumption (cf. publication II, figure 3) and constitutes a further sign that *P. brachycephalum* must be considered less cold adapted than many notothenioids, with optimum temperatures beyond those commonly found in large bodies of high-Antarctic surface waters. Though lower than at 4°C, growth was still present at 6°C, where in the experiments of publication I the characteristics of pH_i regulation changed after acute warming. This might be indicative of an adaptive response; unfortunately, physiological parameters like pH_i regulation were not measured to allow for a direct comparison with the data presented in publication I to locate conceivable acclimation effects and putative shifts of *pejus* temperatures. To shed some light on these processes, slow long-term warming of *P. brachycephalum* and high-Antarctic notothenioids might be promising, examining growth and changes in vascularisation as well as mitochondrial densities and composition and enzymatic capacities over a range of temperatures. These findings could be compared to adaptive mechanisms – and maybe also expression of potentially regulative proteins like UCP2 – occurring during extreme warming in the eurytherm *Z. viviparus*, which frequently undergoes seasonal acclimation.

In conclusion, the data presented in this thesis have shown that thermal tolerance of the various levels of organisation in fish may differ if studied on their own but in a complex

organism are in mutual control of each other, with the highest organisational level showing the highest thermal sensitivity, as depicted in figure 6 (Pörtner, 2002b).

In a narrow window, warm acclimation appears possible also for Antarctic fish (figure 6), yet the data are too few and insubstantial to support generalisations. Nonetheless, they indicate that stenothermal fish species might not be as thermally limited as they appear on first sight. It emerges that at least some Antarctic fish species could keep pace with a slow and moderate warming of Antarctic waters, especially deep water species as *P. brachycephalum* that have invaded high-Antarctic regions but avoid the cold surface waters and stay in warmer deep water layers. Extremely cold-adapted fish like icefish (*Channichthyidae*), which – lacking hemoglobin – are more dependent on cold and stable temperatures that ensure high oxygen solubility (Wells et al., 1990; Di Prisco, 2000) will probably not be among those species. These assumptions are clearly hypothetical and focus on the species level. With interdependent food webs and inter-specific relationships, the ecological level proves even more sensitive and complex and could accordingly be considered the ultimate level defining thermal tolerance (figure 6; Pörtner et al., 2004). Thermally induced changes at the ecosystem level will thus be much more dramatic and will evoke changes from re-arrangement of the inhabiting species to a reorganisation of the complete ecosystem, which has not yet been studied in detail for Antarctic ecosystems, but could have deleterious consequences for individual species (Fraser and Hofmann, 2003; Schmitz et al., 2003; Winder and Schindler, 2004).

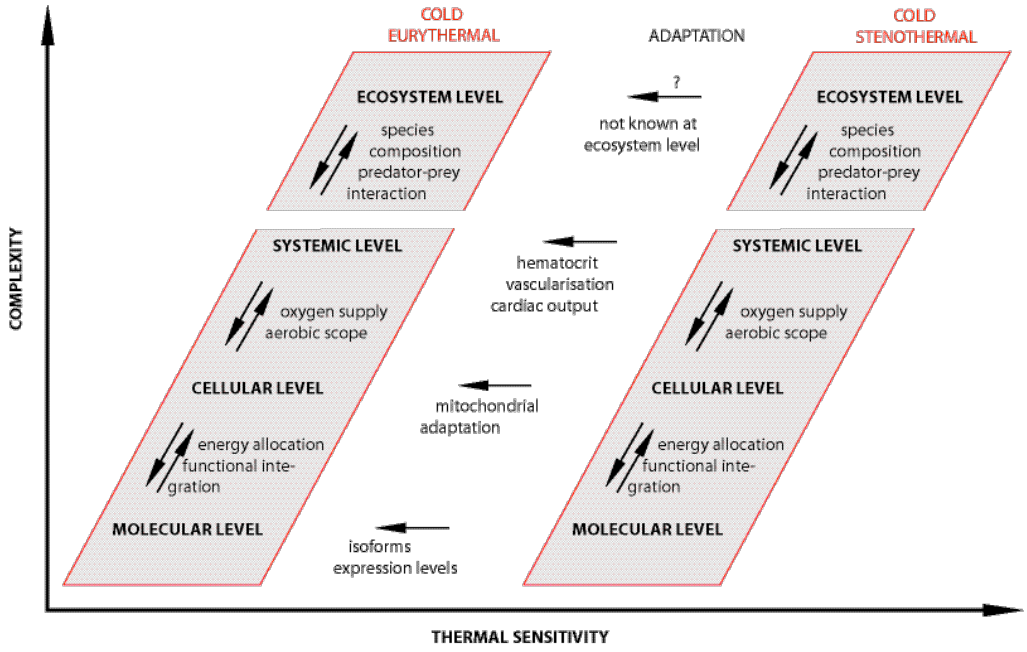

Figure 6: Scheme of the hierarchies and functional integrities of the organisational complexity levels as proposed here for Antarctic fish. Adaptation upon warming may lead to an integrated response of all levels of complexity, causing a shift from cold stenothermy to cold eurythermy. Associated decreased thermal sensitivity could produce higher aerobic scopes and wider thermal tolerance windows.

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Erklärung gem. § 5 (1) Nr. 3 PromO

Ich erkläre hiermit,

1. dass ich mich vor dem jetzigen Promotionsverfahren keinem anderen Promotionsverfahren unterzogen habe

und

2. dass ich außer dem jetzt laufenden Promotionsverfahren auch kein anderes beantragt habe.