Mitochondrial plasticity in response to changing abiotic factors in Antarctic fish and cephalopods

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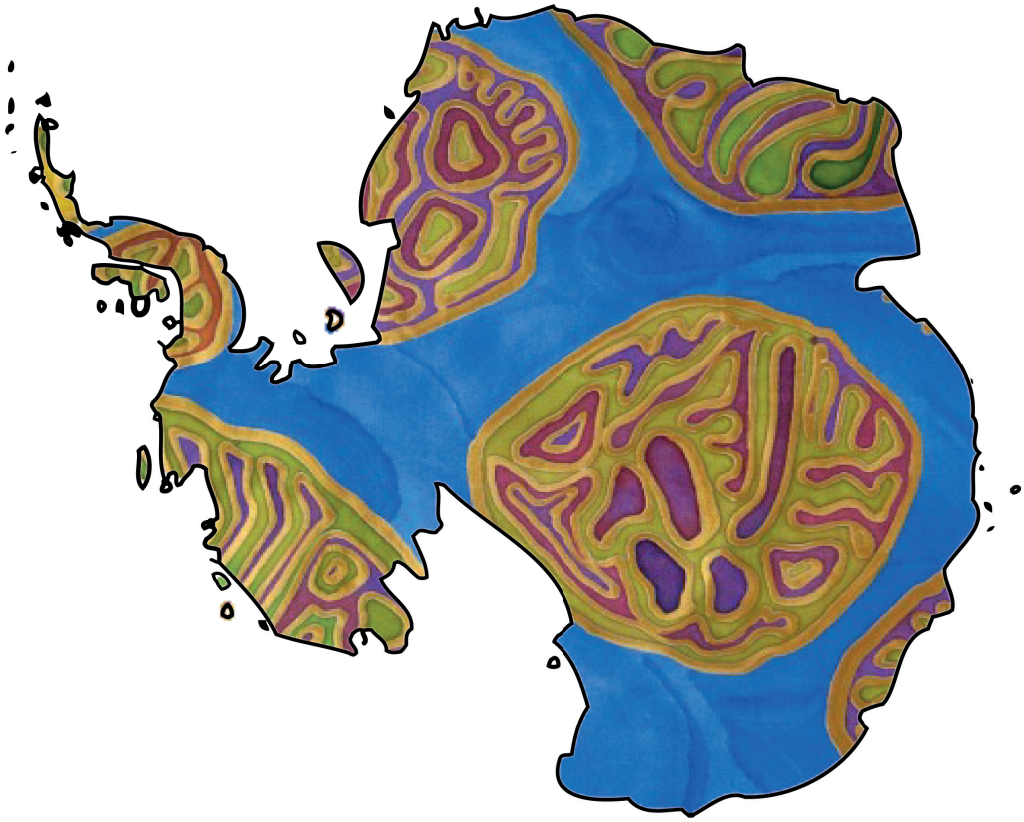

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Mitochondriale Anpassungsfähigkeiten Antarktischer Fische und Cephalopoden unter sich ändernden Umweltbedingungen

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Summary

The Southern Ocean is thermally isolated by the Antarctic Polar Front, which allowed the formation of the very stable, cold Antarctic ecosystem over several millions of years, where temperatures are consistently low from -1.86°C to 1.0°C with little seasonal variation. The local fauna of the Southern Ocean, which evolved in these cold and stable Antarctic waters, possesses unique physiological adaptations that causes them to be particularly sensitive towards even small changes in abiotic environmental conditions.

The effect of anthropogenic CO_2 emissions into the atmosphere, one-third of which are taken up by the oceans (ocean acidification), and an almost linear rise in average global ocean surface-temperatures, is also obvious in Antarctic waters. Cold stenothermal organisms generally possess extremely low metabolic rates and appear to have narrow thermal tolerance ranges. Both ocean warming and acidification have recently been found to reduce the aerobic scope of marine ectotherms, and the combination of these two stressors may further reduce the already very narrow thermal window of optimum performance in Antarctic species.

Up to now, hardly anything is known about the biochemical mechanisms, which determine the acclimation capacities of Antarctic organisms to rising temperature and CO_2 concentrations. At the cellular level, it is the aerobic capacity of individual mitochondria, which shapes the capacity of aerobic energy metabolism of cells or tissues, and thus also plays a crucial role in the acclimation capacity of the whole animal.

The aim of this thesis was to shed light upon the capacities of the aerobic energy metabolism of marine fish and cephalopods from different latitudinal clines to respond to ocean acidification and warming. It particularly focused on mitochondrial metabolism and capacities and considered the functional integration of higher hierarchical structures of organisation such as cellular and systemic levels.

To answer the question on the acclimation capacity of Antarctic fish towards ocean warming and acidification, the Antarctic notothenioid *Notothenia rossii* served as a model organism in this study, and was acclimated for five weeks to warmer temperatures (7°C) and elevated seawater PCO_2 (2000 µatm) (both cold - 1°C, and warm). At the whole animal level, the results showed that *N. rossii* could only partially compensate its routine metabolic rates after long-term warm acclimation. In contrast, routine metabolic rates of cold hypercapnia acclimated *N. rossii* measured at habitat temperature were not different from control animals. Furthermore, *N. rossii* could compensate acid-base disturbances in their blood and cells by a

significant increase in plasma and intracellular HCO_3^- in both the cold and warm hypercapnic groups, which possibly resulted in a shift in 'set points' of acid-base regulation towards slightly alkaline pH values. The maintenance of this new acid-base equilibrium may come with elevated metabolic costs for ion-regulation, which concomitantly challenges the ATP-producing mitochondria.

Yet, isolated liver mitochondria of both cold and warm hypercapnia acclimated fish showed reduced respiration rates and thus reduced ATP-synthesis capacities. Still, the activities of isolated enzymes of the electron transfer system (cytochrome *c* oxidase, COX) and the TCA-cycle (citrate synthase, CS) in the in liver of cold- and warm-hypercapnic fish, namely decreased COX activities at unchanged CS activities, indicate shifts in metabolic pathways. As liver plays an important role as storage organ and in biosynthetic pathways, shunting of TCA-cycle intermediates, e.g. away from the electron transfer system (ETS) towards gluconeogenesis, may serve as a partial compensation for the reduced liver mitochondria capacities in order to support other oxidative tissues. Furthermore, the reduced mitochondrial respiration in liver of hypercapnia acclimated *N. rossii* appeared to be partially compensated by a higher contribution of Complex I within the ETS. Both the shifts in biosynthetic pathways and substrate preferences of the ETS led to the development of two hypotheses, which explain how liver cells and mitochondria of *N. rossii* could be affected by and may compensate for chronically elevated PCO_2:

Permanently elevated bicarbonate concentrations within tissues and mitochondria can acutely inhibit the TCA-cycle e.g. at the level of CS and succinate dehydrogenase (Complex II of the ETS). An increased metabolisation of NADH-linked substrates, such as glutamate and malate, could enhance the flux through Complex I and thereby the energetic efficiency of mitochondrial oxidative capacity. Furthermore, high levels of bicarbonate can activate intracellular and intramitochondrial messengers, which in turn activate metabolic key enzymes by direct phosphorylation by protein kinase A or transcriptional and post-translational modification, which may lead to continuously elevated levels of CS and COX. Nevertheless, it remains open whether these mechanisms are sufficient to support other tissues with a high energy demand, such as heart or red muscle, under chronic hypercapnia in the long run.

In order to estimate general mitochondrial flexibilities towards changing environmental conditions of *N. rossii,* mitochondrial capacities of further sub- and high-Antarctic notothenioids, and of the New Zealand black cod, *Notothenia angustata*, were compared. This analysis revealed relatively low Complex I capacities in *N. rossii* compared to

the other notothenioids, which appears to be a peculiarity of this species and may be one factor that contributes to *N. rossii*'s low acclimation capacities towards seawater warming and acidification. As one would expect, mitochondria of high-Antarctic fish showed limited aerobic capacities in the warmth. Interestingly, the Austral *N. angustata* displayed only modest capacities to increase its aerobic metabolism under rising temperature, which were not much higher than those of the Antarctic notothenioids, and are likely related to their Antarctic heritage.

The last part of the study was dedicated to the comparison of mitochondrial capacities between Antarctic fish and cephalopods. The mitochondrial responses of Antarctic cephalopods to an acute temperature rise suggest that they possess similar mitochondrial flexibilities and capacities towards the warmth as Antarctic fish. Nevertheless, generally more effective capacities for acid-base regulation and larger energy reserves (lipids) in fish compared to cephalopods will putatively make them 'win' the competition for resources over longer time-scales, when seawater temperatures and PCO_2 in the Southern Ocean continue to rise.

In conclusion, the variety of responses in mitochondrial respiration, enzyme activities and shifts in substrate preferences in various tissues of Antarctic fish *N. rossii* during chronic hypercapnia exposure appears to come along with an elevated metabolic demand of highly oxidative tissues and a concomitant reduction of liver energy stores in the long run. By the extremely lowered enzyme activities in heart of warm and hypercapnia acclimated *N. rossii*, this study firstly demonstrates that the synergistic effect of rising seawater temperatures and CO_2-concentrations may cause reductions aerobic heart performance in Antarctic fish. This could further reduce the already very narrow thermal window of *Notothenia rossii* and could hamper whole animal aerobic scope and performance in response to warming and elevated PCO_2. Thereby, survival of various Antarctic species and populations might be put at risk due to continuing climate change in Antarctica in the long run.

Zusammenfassung

Der Antarktische Ozean stellt ein einzigartiges, durch die Polarfront isoliertes Ökosystem dar, welches sich über Jahrmillionen hinweg durch extrem kalte Temperaturen nahe dem Meerwasser-Gefrierpunkt (-1.9°C) auszeichnet. Im Laufe der Evolution hat die dort endemische Fauna spezielle Anpassungen an diese stabilen Umweltbedingungen entwickelt, und ist dementsprechend sehr empfindlich gegenüber bereits kleinsten Veränderungen in ihrer abiotischen Umwelt.

Der anthropogen verursachte, rapide Anstieg atmosphärischer CO_2-Konzentrationen und die daraus resultierende kontinuierliche Erwärmung und Versauerung der Weltmeere wirkt sich auch auf den Antarktischen Ozean aus. Antarktische Arten besitzen aufgrund ihrer geringen Stoffwechselrate und –flexibilität höchstwahrscheinlich ein nur geringes Akklimatisationspotential gegenüber steigenden Meerwasser-Temperaturen und CO_2-Konzentrationen, und vor allem die Kombination der beiden Faktoren hat höchstwahrscheinlich das Potential, das bereits sehr schmale Temperaturfenster Antarktischer Arten zusätzlich einzuengen.

Bisher ist kaum etwas über die biochemischen Mechanismen der Akklimatisationsfähigkeit Antarktischer Tiere gegenüber steigenden Temperaturen und CO_2-Konzentrationen bekannt. Auf zellulärer Ebene ist es vornehmlich der Energiestoffwechsel der Mitochondrien, welcher die Fähigkeiten der Zelle und damit auch des gesamten Organismus, sich an einen ändernden Energiebedarf anzupassen, bestimmt.

Ziel dieser Arbeit war, die Auswirkungen erhöhter Meerestemperaturen und CO_2-Konzentrationen auf den Energiestoffwechsel von Fischen und Cephalopoden verschiedenster geographischer Herkunft zu untersuchen. Neben den Reaktionen und Anpassungsfähigkeiten einzelner hierarchischer Organisationsebenen von der Zelle bis hin zum Ganztier, lag das Hauptaugenmerk dieser Studie auf den Auswirkungen steigender Temperaturen und CO_2-Konzentrationen auf den mitochondrialen Stoffwechsel.

Um die Frage der Akklimatisationskapazitäten Antarktischer Fische zu beantworten, wurde *Notothenia rossii* als Beispielorganismus für Antarktische Fische herangezogen und für mehrere Wochen an erhöhte Temperaturen (7°C) und CO_2-Konzentrationen (2000 µatm) akklimatisiert. Die Studie ergab, dass *N. rossii* seine Stoffwechselrate nur geringfügig an die erhöhte Temperatur anpassen (kompensieren) konnte, während die chronisch erhöhte CO_2-Konzentration keinen ersichtlichen Einfluss auf den Ruhestoffwechsel des Tieres hatte.

Sowohl der extrazelluläre pH im Blut als auch der intrazelluläre pH konnte durch aktive Hydrogencarbonat-Aufnahme im physiologischen Bereich gehalten werden, welcher einen neuen Ausgangs- und Gleichgewichtszustand (‚set point') im Säure-Base Haushalt darstellt. Die Aufrechterhaltung solch einer dauerhaft erhöhte Hydrogencarbonat-Konzentration in den Körperflüssigkeiten zieht einen erhöhten Energiebedarf zur Ionenregulation (Natrium-Kalium-ATPase) nach sich und damit eine erhöhte Energienachfrage an die Mitochondrien.

In den CO_2-akklimierten Fischen ergab die Messung isolierter Lebermitochondrien dramatisch erniedrigte Respirationsraten, was auf Störungen im aeroben Mitochondrien-Stoffwechsel und der Energiebereitstellung (ATP) hindeutet. Der Leber kommt eine wichtige Rolle in der Energiespeicherung und Biosynthese im Stoffwechsel zu. Daher deuten die Messungen mitochondrialer Enzyme (Citratsynthase, Cytochrom *c* oxidase) in CO_2 akklimatisierten *N. rossii* darauf hin, dass durch eine Verschiebung in den bevorzugten Stoffwechselwegen, z.B. hin zu erhöhter Gluconeogenese oder Fettverstoffwechselung, eine Limitierung des aeroben Leberstoffwechsels möglicherweise ausgeglichen und die Versorgung anderer Gewebe teilweise gesichert werden kann. Zudem wurde eine erhöhte Beteiligung des Komplex I am mitochondrialen Elektronentransfer-System (ETS) trotz generell niedriger mitochondrialer Sauerstoffverbrauchsraten in der Leber festgestellt. Zusammen führten diese Befunde zur Entwicklung zweier Hypothesen, wie die gemessenen Defizite in der Lebermitochondrien-Atmung in CO_2-akklimierten *N. rossii* möglicherweise zustande kommen und eventuell ausgeglichen werden könnten:

Eine dauerhafte erhöhte Hydrogencarbonat-Konzentration in Zellen und Mitochondrien könnte die Enzyme Citratsynthase und Succinat-Dehydrogenase des Citratzyklus kompetitiv hemmen. Infolgedessen könnte es durch eine erhöhte Oxidation NADH-liefernder Substrate zu einer erhöhten Komplex I-Aktivität des ETS kommen. Zudem werden möglicherweise intrazelluläre bzw. -mitochondriale Botenstoffe durch hohe Hydrogencarbonat-konzentrationen aktiviert, welche wiederum Komplex I und Komplex IV des ETS aktivieren oder eine beständig erhöhte Transkription oder Translation metabolischer Proteine, so z.B. Citratsynthase und Cytochrom c Oxidase, anregen. Es bleibt jedoch fraglich, ob diese Mechanismen ausreichen, um andere Gewebe mit einem höheren Energiebedarf als Leber, wie z.B. das Herz oder der rote Muskel, langfristig mit Energie zu versorgen.

Um zu vergleichen, wie flexibel die Mitochondrien von *N. rossii* gegenüber Umweltveränderungen sind, wurden die Mitochondrien-Kapazitäten weiterer Antarktischer Fische als auch die von *Notothenia angustata*, einer eng verwandten Art aus kalt-gemäßigten Breiten, gemessen. Diese Analyse ergab dass *N. rossii* vergleichsweise geringe Komplex I

Kapazitäten aufweist, welche möglicherweise für die relativ geringen Anpassungsfähigkeiten gegenüber wärmeren Meerestemperaturen und höheren CO_2-Konzentrationen dieser Art mit verantwortlich sind. Erwartungsgemäß konnten die Mitochondrien Hoch-Antarktischer Fischarten ihre Mitochondrien-Kapazitäten unter akut ansteigenden Temperaturen nicht in dem Maße steigern, wie es für die Aufrechterhaltung eines voll funktionsfähigen mitochondrialen Stoffwechsels nötig wäre. Interessanterweise besaß auch *N. angustata*, welcher in Gewässern um Neuseeland heimisch ist, nur eine geringe Steigerung der Mitochondrien-Kapazitäten während eines akuten Temperaturanstieges. Dies deutet darauf hin, dass diese Art bereits die volle Bandbreite der möglichen mitochondrialen Anpassungsfähigkeiten ausnutzt und zu keiner weiteren Steigerung mehr fähig ist, vermutlich ein Vermächtnis ihrer ursprünglich Antarktischen Herkunft.

Im letzten Teil der Studie wurden die mitochondrialen Kapazitäten Antarktischer Fische mit denen Antarktischer Kraken verglichen. Die Mitochondrien der Antarktischen Kraken zeigten ausreichende Kapazitäten, um ihren Stoffwechsel einem akutem Temperaturanstieg anzupassen, was darauf hindeutet, dass Antarktische Kraken bei einem Temperaturanstieg theoretisch mit Antarktischen Fischen wie *N. rossii* konkurrieren könnten. Allerdings verfügen Fische generell über eine leistungsfähigere Regulation ihres Säure-Base Haushaltes und höhere Energiereserven (Fett) im Vergleich zu Kraken; beides Charakteristika, welche Fischen im Wettbewerb um ähnliche Ressourcen einen Vorteil gegenüber Kraken verschaffen könnten.

Obgleich für den Antarktische Fisch, *N. rossii,* eine beständig erhöhte CO_2-Konzentrationen im Wasser zunächst keine offensichtliche Veränderung in der Stoffwechselrate nach sich zieht, deuten die Vielzahl an Veränderungen in Mitochondrien- und Enzymaktivitäten innerhalb einzelner Geweben des Fisches darauf hin, dass chronisch hyperkapnische Bedingungen zu einem erhöhten Energiebedarf vor allem in Geweben mit einer hoher Stoffwechselaktivität, und zu einer Verringerung der Energiereserven im Speicherorgan Leber führen kann. Zudem konnten durch die vorliegenden Daten ein Hinweis auf eine möglicherweise beeinträchtigte Herzkapazität in warm- und CO_2-akklimierten *N. rossii* erbracht werden. Hierdurch wird in dieser Studie erstmals deutlich, dass das Temperaturfenster des Antarktischen Fisches *Notothenia rossii* durch eine Kombination aus fortschreitender Ozeanversauerung- und -erwärmung möglicherweise weiter verschmälert und die Leistungsfähigkeit des gesamten Organismus eingeschränkt werden kann, was längerfristig auch zu Gefährdung zahlreicher Antarktischer Fischpopulationen führen könnte.

List of abbreviations

ACC	Antarctic circumpolar current
ADP	Adenosine diphosphate
AFGP	Antifreeze glycoprotein
ATP	Adenosine triphosphate
β NB	β non-bicarbonate buffer
BIOPS	Mitochondrial biopsy buffer
BSA	Bovine serum albumin
°C	Degree Celcius
CI	Complex I or NADH dehydrogenase
CII	Complex II or succinate dehydrogenase
CIII	Complex III or cytochrome *c* - oxidoreductase
CIV	Complex IV or cytochrome *c* oxidase
cAMP	Cyclic adenosine monophosphate
CCO2	Total CO_2
CF	Condition factor
CS	Citrate synthase
COX	Cytochrome c oxidase
DIC	Dissolved inorganic carbon
DTNB	5,5'-dithio-bis-(2-nitro-benzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
e.g.	For example
ETF	Electron transferring flavoprotein
ETS	Electron transfer system
F_1F_OATPase	ATP synthase
FCCP	Carbonylcyanide-p-(trifluoromethyl) phenylhydrazone
FW	Fresh weight
h	Hours
H+	Proton
HCO_3-	Bicarbonate
HSI	Hepatosomatic index
i.e.	That is
J_{O2}	Oxygen flux (per fresh weight)
kPA	Kilopascal
µatm	Micro atmospheres
MAM	Mitochondrial assay medium
MCA	Metabolic cold adaptation
MIM	Mitochondrial isolation medium
min	Minutes
MiRO5	Mitochondrial respiration medium
MO2	Oxygen consumption
MS 222	Tricaine methano-sulphonate
MUFA	Mono-unsaturated fatty acids
Mya	Million years ago
NKA	Na^+/K^+ ATPase; sodium potassium ATPase
NBC	Na^+/HCO^{3-} (bicarbonate)-cotransporter

OCLTT	Oxygen and capacity dependent thermal tolerance
Osm	Osmolar
OXPHOS	Oxidative phosphorylation capacity, maximum oxygen flux
PCO_2	Carbon dioxide partial pressure
pH	‚Potential hydrogen', measure for hydrogen ion activity
pH_e	Extracellular pH
pH_i	Intracellular pH
PKA	Protein kinase A
PUFA	Poly-unsaturated fatty acids
Psu	Practical salinity units
Q_{10}	Temperature coefficient
RCR	Respiratory control ratio
RMR	Routine metabolic rate
ROS	Reactive oxygen species
ROX	Residual oxygen consumption
Rpm	Resolutions per minute
S	Salinity
sAC	Soluble adenylyl cyclase
SEM	Standard error of the mean
T	Temperature
T_{crit}	Critical temperature
TCA-cycle	Tricarboxylic-acid cycle
TMPD	N,N,N',N'-tetramethyl-p-phenylendiamine dihydrochloride
TPMP	Triphenyl methyl phosphate
UCP	Uncoupling protein
UI	Unsaturation index

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1 Introduction

The oceans occupy approximately 71% of the Earth's surface. As the principal component of Earth's hydrosphere, the world's oceans are integral to sustaining all known life, they form a part of the carbon cycle and influence climate and weather patterns. A greater total amount of organic matter is produced annually in the ocean than on land and the economic utilization of the marine production is rapidly developing (Lalli and Parsons, 1997). The health of the earth's oceans and the long-term sustainability of ecosystems that support human populations is an emerging issue due to the rising impact of global climate change. This thesis mainly focuses on the unique marine environment of the Southern Ocean.

1.1 The Southern Ocean

The geological origins of Antarctica lie in the complete separation of Antarctica from South America and the opening of the Drake passage around 37 - 34 million years ago (mya). In the course of this separation, a large abyssal basin of more than 4000 m water depths was formed, and Antarctica has been physically isolated from other southern continents ever since. A sharp drop in temperatures led to the formation of an extensive, clockwise moving oceanographic barrier, the Antarctic Circumpolar Current (ACC). The unrestricted flow of the ACC around 25 to 22 mya allowed final thermal isolation and glaciation of the Antarctic continent. With its abrupt change in ocean temperature, salinity and distribution of nutrients, the ACC isolates cold water-masses from the Southern Ocean from lower latitude warmer waters. This is reflected in the Antarctic Polar Front (or Antarctic Convergence) (Falco and Zambianchi, 2011), which is located within the ACC and forms one of the most important features of the Southern Ocean. The Front is circumpolar but occurs at different latitudes around the continent, and acts as a thermal and geographical barrier against migration of fish in either direction (Lutjeharms, 1990). The separation from the Pacific, Indian and Atlantic Oceans allowed the development of a very stable, cold-stenotherm Antarctic ecosystem where temperatures are consistently low from -1.86°C to 1.0°C with little seasonal variation (Olbers et al., 1992; Cheng et al., 2003; Arntz, 2005).

1.2 Physiological adaptations of life in the Southern Ocean

1.2.1 Organismic level

The local fauna of the Southern Ocean could evolve in the cold and stable Antarctic waters, and thus possesses unique adaptations to the life at permanent close-to zero temperatures (Clarke, 1998; Peck, 2005).

In ectothermal animals, body temperature is determined by environmental temperature. Consequently, any changes in seawater temperature directly affect their metabolic rates and internal biological processes. Evolutionary adaptations of organisms to certain thermal regimes (temperatures and variations thereof) are reflected in different thermal optima and width of thermal tolerance ranges over a latitudinal gradient (Peck, 2005; Hofmann and Todgham, 2010). As a result, a given species is characterized by its metabolic capacities, especially those of the ventilatory and circulatory system. The concept of 'oxygen and capacity dependent thermal tolerance' (OCLTT) suggests that a mismatch between oxygen supply and demand at both low and high temperatures sets the limits for whole organism aerobic scope and reduces organismal performance at the borders of their thermal envelope (Pörtner, 2002c, 2010). However, oxygen limitation in the cold may not be seen in sub-polar and polar species. High oxygen solubilities in water and haemolymph, paralleled by falling metabolic rates, may cause oxygen supply in excess of demand in the cold. Despite, functional capacity limitations may remain in the cold (Pörtner, 2012; Wittmann et al., 2012) (c.f. figure 1.1).

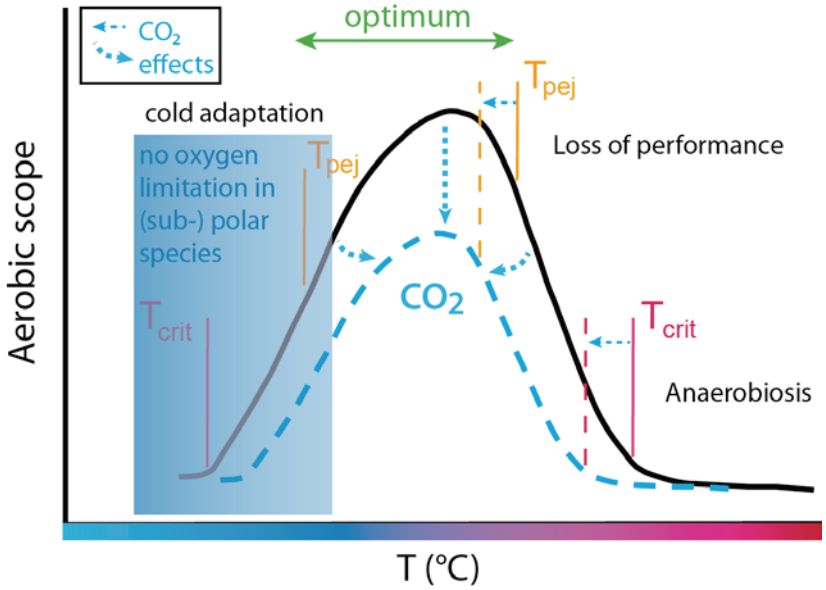

Figure 1.1 Concept of Oxygen limited thermal tolerance (OCLTT) after (Pörtner, 2010). Increasing temperatures result in an increase in the resting oxygen demand. When the ability of the circulatory and ventilator systems to supply oxygen reach their maximum limit, aerobic scope (the difference between maximum and resting metabolic rate) declines as temperature increases. Decreasing temperatures cause a decline in the ability of mitochondria to produce ATP, compromising the function of the ventilatory muscles and circulatory pumps that are needed to supply oxygen to the working tissues at low temperatures. Beyond *pejus* temperatures (*pejus*=worse), a mismatch develops between oxygen supply and temperature-dependent oxygen demand leading to hypoxemia in body fluids. With continued cooling or warming, aerobic scope finally vanishes towards low or high critical threshold temperatures (T_{crit}), where transition to anaerobic mitochondrial metabolism and progressive insufficiency of cellular energy levels occurs. Note that in sub-polar and polar species, oxygen supply to tissues may not be constrained, due to falling oxygen demand and rising oxygen concentrations (Wittmann et al., 2012). Ambient elevated CO_2 levels may cause a narrowing of thermal windows (Pörtner, 2005; Metzger et al., 2007; Walther et al., 2009); Polar species with a low thermal limit may reach *pejus* or T_{crit} at even lower temperatures due to the combined stressors elevated temperature and PCO_2.

Most teleost fish inhabiting the Antarctic ecosystem are considered to be extremely stenotherm specialists, as their physiological performance is restricted to a very narrow thermal range (Somero and DeVries, 1967; Wilson et al., 2002; Johnston, 2003). This specialisation to the most thermostable environment on earth has resulted in very low upper critical temperatures (Pörtner et al., 2000; Peck, 2002). For example, the bottom-dwelling *Trematomus* species die of acute heat death at temperatures between 4 and 6°C, and show physiological performance restrictions already well below this lethal limit (Somero and DeVries, 1967; Robinson and Davison, 2008).

A prominent adaptation of cold stenothermal fish, which enables their survival in ice-laden, subzero marine environments lies in their blood properties. For example, they achieved freezing resistance by the unique evolution of antifreeze glycoproteins (AFGPs, DeVries, 1971; Matschiner et al., 2011). AFGPs adhere to ice crystals that form in the body and arrest further ice growth within their blood, cytosol and other extracellular fluids, thereby keeping

the crystals small enough to prevent cellular damage. In combination with extremely high serum osmolarities up to 600 mOsm*kg^{-1} (Hudson et al., 2008), they effectively lower the freezing point of the blood to -2.1°C (DeVries, 1971).

Another potential problem of the cold is the increased viscosity of Antarctic fish blood. This is mitigated by reduced haematocrit and haemoglobin concentrations compared to temperate species with similar lifestyles as notothenioid fishes (Kunzmann, 1991; Gonzalez-Cabrera et al., 1995; Egginton, 1997). The reduction in viscosity is taken to extremes in members of the family Channichthyidae, the icefish, who completely lack the oxygen carrying pigment haemoglobin. In some species, the intracellular oxygen-binding protein myoglobin is also absent (Sidell and O'Brien, 2006; O'Brien and Mueller, 2010). However, due to the increased physical oxygen solubility in seawater or body fluids in the cold, and also because of cardiovascular modifications such as larger heart ventricles, increased blood volume and cardiac output, aerobic performance is not limited by the low oxygen-carrying capacity in Antarctic fish (Tetens et al., 1984; O'Brien and Sidell, 2000).

Additionally, Antarctic fish possess large intracellular lipid stores, which aid buoyancy control (most Antarctic fish do not have a swim bladder), but also aid in gas diffusion through cells (Eastman and DeVries, 1985). Increased intracellular lipid concentration is thought to reduce problems associated with diffusive exchange of oxygen by assisting in intracellular oxygen-transport and by acting as an intracellular oxygen-storage site (Egginton et al., 2002). Furthermore, these lipids are typically used as energy stores (Kamler et al., 2001), as Antarctic fish preferentially rely on the catabolism of lipid fuels for aerobic metabolism. This also relates to high mitochondrial densities (30%-60% of cell volume, figure 1.3; Johnston, 1987; Clarke and Johnston, 1996) and goes hand in hand with low anaerobic capacities (Crockett and Sidell, 1990).

1.2.2 Mitochondrial function and cold adaptation

Mitochondria are the primary site of cellular oxygen consumption and aerobic energy production. Because oxygen is required for the aerobic production of ATP, mitochondrial function is closely connected to the ventilatory and circulatory capacities of the animal. Accordingly, limitations in mitochondrial energy metabolism contribute to a loss of whole animal aerobic scope (Pörtner, 2001, 2002b). Thus, it is the aerobic capacity of individual mitochondria which shapes the capacity of aerobic energy metabolism of cells or tissues. Conversely, mitochondrial performance is limited by the capacity of the cardiovascular

system to supply oxygen to the respiring mitochondria, i.e. thermal limitations result from a mismatch between oxygen supply and demand at both low and high temperature extremes (Pörtner, 2006). Though, limited oxygen supply to tissues may not be limited in sub-polar and polar species due to high oxygen concentrations and falling oxygen demand in the cold (c.f. figure 1.1).

Mitochondrial respiration depends on a continuous flow of substrates across the inner mitochondrial membrane into the matrix space. Within the mitochondria, various substrates feed electrons into the electron transfer system (ETS), which is comprised of at least six complexes. Complex I (CI, NADH dehydrogenase, also called NADH-ubiquinone oxidoreductase) can oxidise NADH-linked substrates from all NADH (nicotinamide adenine dinucleotide) generating reactions, both from the tricarboxylic-acid cycle (TCA-cycle) or further cytosolic (e.g. glycolysis) and mitochondrial pathways (e.g. glutamate dehydrogenase, fatty acid β-oxidation). The $FADH_2$-linked Complex II (CII, succinate dehydrogenase) is directly connected to the TCA-cycle and delivers additional electrons into the quinone pool, originating from succinate oxidation. The oxidation of reduction equivalents leads to a separation of protons and electrons. The transfer of every electron from Complex I through coenzyme Q to Complex III (Cytochrome bc_1 complex) and Complex IV (Cytochrome *c* oxidase) is energetically coupled to the pumping of protons by Complex I, III and IV into the intermembrane space (Gnaiger, 2012). The ETS is thereby coupled to oxidative phosphorylation (OXPHOS) owing to the creation of a proton gradient across the inner mitochondrial membrane, which is used by the F_OF_1-ATP synthase (also termed Complex V) to synthetize ATP.

Especially Complex I plays a central role in the creation of membrane potential, as in terms of substrate turnover and proton pumping, Complex I exceeds the respective Complex II rates by far. Regarding ATP production per mol substrate (or full cycle of the TCA). Oxidation of one pyruvate yields four NADH, as per oxidation of one NADH two electrons are transported through the ETS, paralleled by four protons pumped by Complex I and Complex III each, and two protons through Complex IV. This equals 40 protons being pumped per molecule of pyruvate, which then yields 10 ATP. In contrast, the oxidation of one succinate by Complex II is six times less efficient: per transport of two electrons along the ETS, a total of six protons is being pumped through Complex III to Complex IV, which in the end only yields 1.5 ATP (Efremov et al., 2010; Mark et al., 2012).

All mitochondria are characterised by a basal level of uncoupling of oxidative phosphorylation, a process called proton leak, which contributes significantly (up to 20-30%)

to cellular metabolic rate (Brand et al., 1993; Rolfe and Brand, 1996). Proton leak reactions compete with the ATP synthase for the same driving force, the mitochondrial electrochemical proton gradient, which is built up by the ETS and constitutes the primary energy source for cellular ATP synthesis (c.f. figure 1.2). These basal leak rates are characterised by an either passive diffusion of protons back from the mitochondrial intermembrane-space into the matrix, or mediated by so-called uncoupling proteins (UCPs), mitochondrial membrane transporter proteins, which provide a channel for protons to flow back into the matrix. Both processes circumvent the ATP synthase by uncoupling the energy available in the electrochemical proton gradient from ATP synthesis, which is lost as thermic energy. In contrast to the passive proton conductance pathways in the inner membrane, UCPs may allow a controlled dissipation of the electrochemical proton gradient, and their regulatory modulation of leak rates has been recently found related to thermal acclimation of Antarctic fish (Mark et al., 2006).

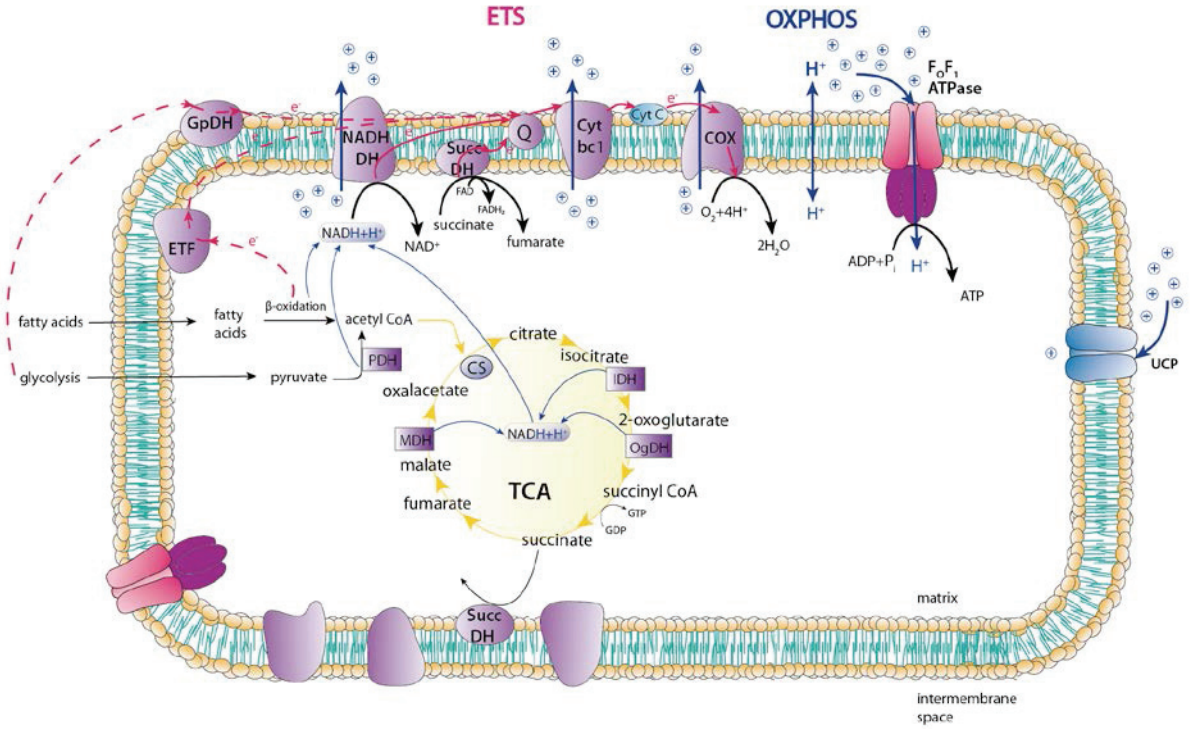

Figure 1.2 Overview of the electron transfer system (ETS) and related pathways feeding into the TCA-cycle.
Oxidation of NADH-linked substrates such as glutamate, malate and pyruvate (and proline in cephalopods) feed electrons into Complex I (NADH DH). Under conditions of a fully functioning TCA, electrons are transported via the Q-junction (Q) in a convergent flow from Complex I, Complex II (Succ DH; following succinate oxidation, $FADH_2$-linked), the electron-transferring flavoprotein (ETF, electrons from fatty acid β-oxidation) and glycerophosphate dehydrogenase (GpDH, electrons from glycolysis), to Complex III (Cyt bc1) and IV (COX), and then transferred to molecular oxygen (O_2) together with four H^+, producing two molecules of water. Membrane potential is built up across the inner mitochondrial membrane, which is dissipated by the F_OF_1 –ATPase to produce ATP

(oxidative phosphorylation, OXPHOS). Protons flowing back through the membrane or mediated by uncoupling proteins (UCP) bypass the ATPase, thereby reducing the electrochemical gradient without ATP synthesis. To simplify the overview, cristae structures were omitted. See text for further explanations. Abbreviations of enzymes: PDH: pyruvate dehydrogenase, IDH: isocitrate dehydrogenase, OgDH: 2-oxoglutarate dehydrogenase, MDH: malate dehydrogenase, Cyt C: Cytochrome C.

Mitochondria in cold-adapted ectotherms possess several specialisations that appear to be related to the restrictive effect of permanent close-to or sub-zero temperatures. Similar to the high viscosity of body fluids described above, the fluidity of membrane lipids may be compromised at cold temperatures. In notothenioid fish, the membrane fluidity is frequently maintained due to an increased content of unsaturated fatty acids to keep membranes fluid at low temperatures. This process is known as homeoviscous adaptation, which can alter mitochondrial proton permeability or the capacity of membrane-bound enzymes in response to temperature changes (Moran and Melani, 2001).

Enzyme kinetics and cytosolic gas diffusion are also temperature dependent processes, which decelerate in the cold (Sidell, 1991). To overcome the negative effects of temperature on enzymes and diffusion, Antarctic fish display ‘mitochondrial proliferation’, i.e. an increase in mitochondrial abundance and ultra-structural density (figure 1.3; Dunn et al., 1989; Guderley and Johnston, 1996). It has been suggested that this proliferation of mitochondria with cold-adaptation, or by cold-acclimation of temperate species, represents a mechanism to decrease the mean free spacing between mitochondria (Johnston et al., 1998). Furthermore, Antarctic species adjust quantities and capacities of intracellular enzymes (Crockett and Sidell, 1990), and by these mechanisms they may reduce diffusion distances between cytosolic metabolites and increase enzyme efficiencies especially of those involved in aerobic metabolism (Pörtner et al., 2000). In many temperate fish species, cold compensation involves enhanced mitochondrial densities and aerobic capacities of individual mitochondria, as it has been demonstrated by enhanced total enzyme activities in cold-acclimated cod (*Gadus morhua*), stickleback (*Gasterosteus aculeatus*) or trout (*Oncorhynchus mykiss*) (Egginton et al., 2000; Guderley et al., 2001; Lannig et al., 2003). In parallel with high mitochondrial densities and enzyme quantities, high Arrhenius activation energies, which are typical in stenotherms, decrease the activities of individual enzymes in the cold. In consequence, enzyme activities of Antarctic fish (per gram tissue fresh weight) are at similar level compared to temperate fish, without increasing the flux through the reactions catalysed by the enzymes (Hardewig et al., 1999b; Pörtner et al., 1999; Pörtner et al., 2000).

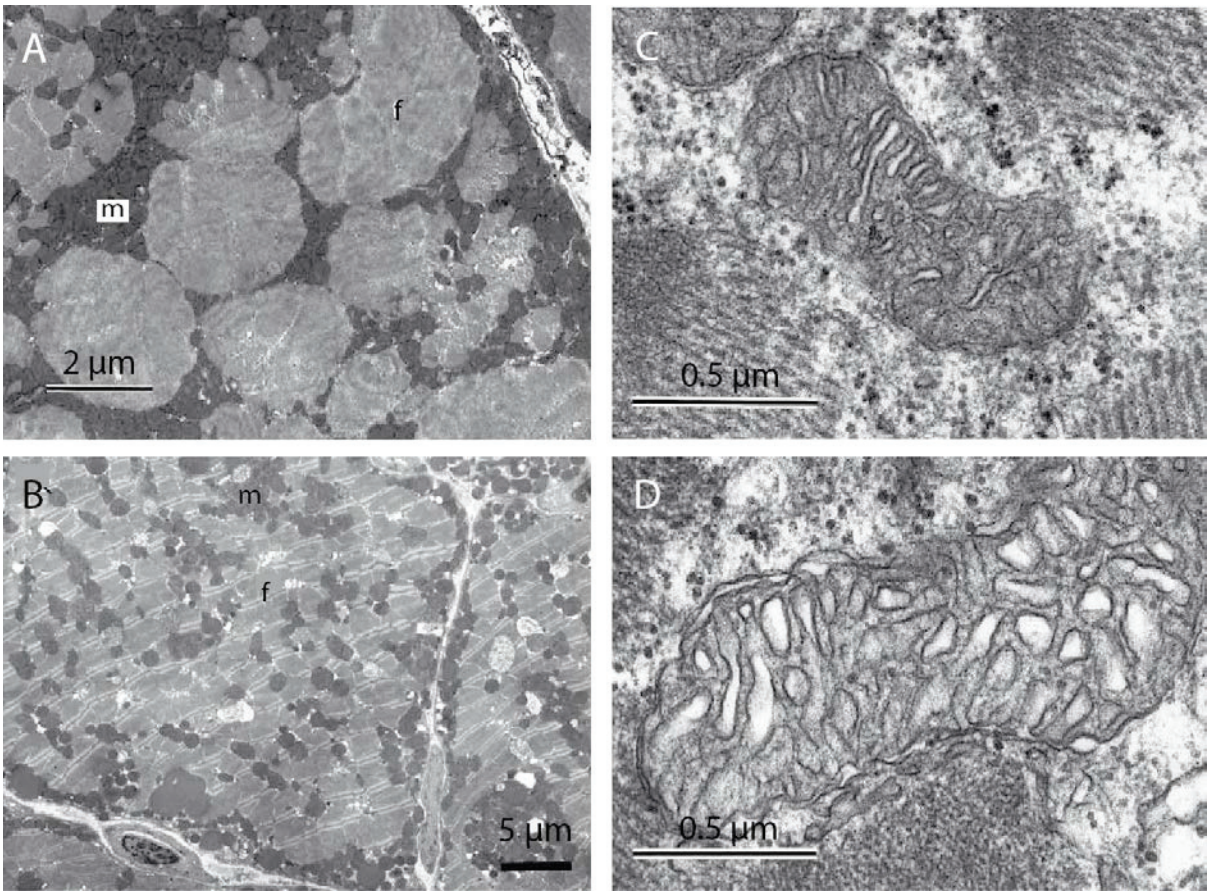

Figure 1.3 Electron micrographs of ultrathin sections of red fibres of Antarctic and temperate fish. Different mitochondrial packing in the red-blooded, Antarctic *Gobionotothen gibberifrons* (A) (O'Brien et al., 2003) and in the temperate rainbow trout *Oncorhynchus mykiss* (B) (St-Pierre et al., 1998). Panel C and D demonstrate that red-blooded notothenioids (*Gobionotothen gibberifrons*) possess a higher surface area of inner mitochondrial membranes per unit volume mitochondria compared to the white-blooded icefish (*Chionodraco rastrospinosus*); conversely, icefish have larger and more mitochondria per gram tissue, so that oxidative capacities within the tissue are similar in red- and white-blooded Antarctic fish (O'Brien Sidell, 2000). m: mitochondria, f: myofibrils.

In this context, some studies suggested the concept of metabolic cold adaptation (MCA) for Antarctic fish with the idea that the metabolic rates of Antarctic fish are elevated when compared with data extrapolated from temperate species (Krogh, 1914; Scholander et al., 1953; Steffensen, 2002). The basis of MCA is suggested to lie in an upward adjustment of ion pump densities to maintain channel/ pump flux ratios at equilibrium in Antarctic fish, and this presumably results in higher metabolic maintenance costs (Hochachka, 1988). This has been proposed to be the reason for increased mitochondrial capacities in the cold, probably mediated rather by proliferation of mitochondria than by up-regulation of mitochondrial oxidative capacities per milligram protein in polar species (figure 1.3; Johnston et al., 1998). However, this may result in a trade-off during warming because they also increase resting oxygen demand at warmer temperatures. Thus, warm-compensation as seen in various

temperate fish would involve reverse mitochondrial proliferation (e.g. Lannig et al., 2003; Lannig et al., 2005; Lucassen, 2006) or high mitochondrial activation energies (see above).

In light of the very stable conditions of the Southern Ocean that lasted several million years, and the peculiar adaptations of Antarctic species to their environment, the onset of rapid global climate change may exert a severe impact on the Antarctic realm, which will be presented in the following sections.

1.3 Ocean warming

From the beginning of the industrial revolution, the accumulation of greenhouse gases has resulted in a continuous warming trend in Earth's climate and an almost linear rise in average global ocean surface-temperatures by about 0.7°C (IPCC, 2007). Particularly the waters around the Antarctic Peninsula have warmed by approximately 1°C since 1955 (Meredith et al., 2005; Turner et al., 2006; Smith et al., 2012), and this trend is predicted to continue by further warming of another 2°C within this century (Murphy and Mitchell, 1995). In some models, warming is predicted to cause serious effects to Southern Ocean circulation patterns (Bi et al., 2001). Increasing seawater temperatures and changing thermohaline circulation patterns significantly contribute to melting and thinning of ice shelves, and also large ice-shelf collapses such as of the Larsen ice shelf in the northwestern Weddel Sea (Turner et al., 2006). Furthermore, it already caused retreat and acceleration of several glaciers (Oppenheimer, 1998; Cook et al.; Clarke et al., 2007), as well as a mass loss of ice-sheets spanning the entire Antarctic continent (Pritchard et al., 2012).

1.4 Ocean acidification

Since the beginning of the industrial revolution in the middle of the 18th century, anthropogenic CO_2 emissions have increased atmospheric CO_2 concentrations to current values of more than 385 µatm/ 0.038 kPa since pre-industrial values of 280 µatm/ 0.028 kPa (Körtzinger, 2010). About one-third of the anthropogenic CO_2 accumulating in the atmosphere is taken up by the oceans (Feely et al., 2004; Sabine et al., 2004).

When CO_2 gets physically solubilized in seawater, it is firstly hydrated to carbonic acid (H_2CO_3), which then dissociates to bicarbonate (HCO_3^-) and one proton (H^+), then sheds another proton to become carbonate (CO_3^{2-}), according to the following equilibrium:

$$H_2O + CO_2 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

At a seawater pH between 7.8 and 8.4, the equilibria between these three species of total dissolved inorganic carbon is comprised of about 90% of bicarbonate ion, 10% of carbonate ion and less than 1% is present as unionised carbon dioxide (Dickson, 2010). Rising atmospheric CO_2 concentrations absorbed by seawater shift the equilibrium towards higher PCO_2 and lower carbonate ion concentration, and concomitantly more protons, which progressively leads to acidification of ocean surface waters.

Models based on the 'business-as-usual' scenario by the Intergovernmental Panel on Climate Change (IPCC, 2007), which assumes rising CO_2 emissions at current rates, predict a drop of the average oceans pH by 0.3 to 0.5 units by the year 2100 and up to 0.8 units by the year 2300 at atmospheric PCO_2 values of 0.1 kPa or 0.2 kPa, respectively (Feely et al., 2004; Caldeira, 2005). Although information on atmospheric CO_2 changes around the Antarctic continent are scarce, local measurements on the Antarctic Peninsula (Carlini Station, Potter Cove) revealed the same trend of continuously rising CO_2 concentrations by 0.003 kPa CO_2 within a time period of 12 years (Ciattaglia et al., 2008).

1.5 Physiological impacts of warming and hypercapnia

1.5.1 Organismic level

Owing to the above-mentioned physiological adaptations for a life in permanently cold Antarctic waters, stenotherm species may prove to be particularly threatened by rising seawater temperature and PCO_2. As cold stenothermal organisms generally possess extremely low metabolic rates and appear to have narrow thermal tolerance ranges (Clarke, 1991; Peck, 2005; Pörtner, 2006), even small increases in temperature could alter individual aerobic performance. For example, high enzymes quantities and high activation energies in mitochondria of Antarctic species cause a large increase in metabolic flux only at a small rise in temperature, and thereby contribute to the low upper critical temperatures in these species (Pörtner et al., 2000). Not only for cold-adapted species, a reduced capacity for aerobic performance at high temperatures has consequences for activity levels, growth rates, reproduction and ultimately limiting an organism's thermal niche and geographical expansion, which in the end may endanger the whole population's sustainability (Pörtner and Farrell, 2008).

The OCLTT hypothesis states that metabolic performances are linked with an ectotherm's ability to undergo physiological adjustments to temperature in order to maintain aerobic scope, and therefore define the whole-organism capacities to cope with thermal challenges, i.e. their acclimation capacities (c.f. figure 1.1). Although Antarctic marine organisms have extraordinarily narrow ranges over which temperature acclimation can occur, the thermal window, and thereby temperature limits, can be shifted by increasing heat tolerance in several species (Seebacher et al., 2005; Lowe and Davison, 2006; Franklin et al., 2007; Pörtner and Lannig, 2009). These acclimations are frequently based on incomplete metabolic compensations involving e.g. an increased net use of storage compounds such as carbohydrates and lipids, and metabolic rearrangements towards enhanced protein catabolism and reduced lipid biosynthesis (Brodte et al., 2006; Windisch et al., 2011). However, there is considerable variation in acclimation capacity among Antarctic fish species: some Antarctic notothenioids, such as *Pagothenia borchgrevinki*, possess a wide thermal window (Robinson and Davison, 2008), whereas the potential for acclimation is apparently low for many others, such as high Antarctic *Trematomus* species (Robinson and Davison, 2008; Enzor et al., 2013).

A recent study in different Antarctic invertebrate species (e.g. limpets, bivalves, ascidians or urchins) revealed highest tolerable acclimation temperatures of 1°C to 6°C over longer time periods, while other species like the brittle star *Ophionotus victoria* only tolerated acclimation temperatures 2°C above their habitat temperature (Peck et al., 2009). Also tropical species are predicted to have limited thermal acclimation capacities owing to their evolution in thermally stable environments (Munday et al., 2012). For example, some coral reef fishes of the Great Barrier Reef have greatly reduced aerobic scope at temperatures only a few degrees above summer mean temperatures, which highlights limited capacities particularly of stenothermal fish species to respond to a rapidly changing climate (Nilsson et al., 2010).

All these examples emphasize how various fish and invertebrate species may be differently sensitive to increases in water temperature. While some cold-adapted, more eurytherm temperate species may be able to shift or extend their geographical distribution range along a latitudinal cline towards the poles as a reaction towards a warming ocean, this is not possible for all species, particularly those already living at their limits in the Southern Ocean (Somero, 2010).

In light of the ongoing ocean acidification of warming oceans, the synergistic effects of both ocean warming and acidification have recently been found to reduce aerobic scope of marine ectotherms by further increasing their aerobic demand or suppressed efficiency of

oxygen supply (c.f. Pörtner, 2010; Pörtner, 2012). As a result, the capacity of an animal to increase its rate of aerobic energy turnover is likely to be reduced possibly even at temperatures within the optimal range of thermal tolerance (Pörtner and Farrell, 2008). Thereby, the combination of these two stressors may further reduce the already very narrow thermal window of optimum performance in Antarctic species (c.f. figure 1.1), with consequences for activity levels, growth rates and probably population survival (Pörtner, 2010; Munday et al., 2012).

Due to the enhanced CO_2 solubility in cold waters and body fluids, ocean acidification along with warming may become particularly threatening to polar ectotherms. Many stenotherm notothenioids live already close to their upper thermal tolerance limits. In line with this, it has been demonstrated that the already high thermal sensitivity of Antarctic *Trematomus* species and *P. borchgrevinki* can be enhanced by the synergistic impact of warming and elevated CO_2 concentrations (Enzor et al., 2013). Similarly, it has been confirmed for tropical fish, which already live at the edge of their thermal range, that only moderately increased CO_2 concentrations of 0.1 kPa can reduce whole animal aerobic scope (Munday et al., 2009).

Yet, the effects of elevated CO_2 levels on adult marine fish have received little attention compared to those of rising temperature. Some previous studies revealed that most adult fish are not particularly vulnerable to ocean acidification, as they usually regulate intracellular pH (pH_i) and, to various degrees, extracellular pH (pH_e) by the accumulation of bicarbonate ions in body fluids, mediated through ion exchange via the gills in order to compensate for rising seawater PCO_2 (Larsen et al., 1997; Pörtner et al., 1998; Brauner et al., 2004; Pörtner, 2005; Melzner et al., 2009). Hence acid-base and ion equilibria reach new steady state values and while tissue pH_i may fully recover, blood pH_e does not necessarily do the same (e.g. *Sparus aurata*, Michaelidis et al., 2007), which may cause specific, long-term shifts in metabolic equilibria (Deigweiher et al., 2010).

In the long run, the maintenance of permanently elevated bicarbonate levels for a new steady-state condition may represent a continuously higher energy demand of the animal for the maintenance of ion gradients via the cellular membranes. This could result in a higher fraction of metabolic energy needed for acid-base regulation. In line with this, fish show elevated activities of Na^+/K^+-ATPase (NKA) or Na^+/HCO_3^- cotransporter under hypercapnia (Deigweiher et al., 2008), and NKA is considered a key enzyme involved in compensation of acid-base disturbances (Choe and Evans, 2003).

Marine invertebrates are hypothesized to be among the organisms most sensitive to ocean acidification, for example due to constraints in their metabolic rates, growth or calcification efficiencies (Jensen et al., 2000; Langenbuch and Pörtner, 2002; Michaelidis et al., 2005). Especially less mobile animal groups such as pteropods (Orr et al., 2005), echinoderms (Kurihara et al., 2004), bivalves (Kurihara et al., 2007) and particularly corals (Hoegh-Guldberg et al., 2007) are suggested to suffer more under hypercapnia than actively swimming animals with higher metabolic rates, such as crustaceans or cephalopods (Spicer et al., 2007; Gutowska et al., 2009; Melzner et al., 2009). Primary responses of the more sensitive marine organisms could be acid-base imbalances, which hamper calcification and the formation of calcareous shells, lead to metabolic depression (a condition expected to retard growth and reproduction), reduced activity, in severe cases to a loss of consciousness due to disruption of oxygen-transport mechanisms, and, if persistent, death (Reipschläger and Pörtner, 1996; Seibel and Walsh, 2001). For temperate crustaceans, experimental evidence is already available which indicates a narrowing of the thermal tolerance window of the edible crab *Cancer pagurus* and the spider crab *Hyas araneus*, by environmental hypercapnia (Metzger et al., 2007; Walther et al., 2009).

1.5.2 Mitochondrial level

With changing metabolic demand of an organism, e.g. under chronic hypercapnia or rising temperatures, the energy demand of tissues follows according to the metabolic role of the tissue. Previous studies suggest that whole animal thermal limits are mainly governed by capacity limitations of the circulatory system rather than a general failure of cellular energy metabolism, and that organelles cover a wider thermal tolerance window than those of the whole organism (Mark et al., 2002; Mark et al., 2005).

Rising standard metabolic rates and mitochondrial respiration during warming go hand in hand with higher leakiness of biological membranes (Hazel, 1995) and thus need an appropriate adjustment of aerobic capacities. Especially the persistent occurrence of mitochondrial proton leak plays a physiologically important role in thermal tolerance, as it can account for up to 20-25% of the whole animal basal metabolic rate (Brand, 2000; Chamberlin, 2004). At high temperatures, excessive oxygen demand through enhanced proton leakage rates is followed by a rise of baseline oxygen demand, as observed in mitochondria of Antarctic bivalves and fish (Hardewig et al., 1999a; Pörtner et al., 1999). Such a drastically elevated mitochondrial oxygen demand, which is paralleled by progressively decreasing ATP

synthesis capacities, may at a certain point exceed the capacity of oxygen supply by the circulatory system and thus lead to a restriction or loss of aerobic mitochondrial metabolism (Pörtner, 2001, 2002b; Brand and Esteves, 2005).

As already pointed out, cold-adapted membranes frequently posses a high content of unsaturated fatty acids to maintain membrane fluidity in the cold (e.g. fish - Hazel, 1995; cephalopods - Turner et al., 2005). On the other hand, the content of unsaturated fatty acids in the membranes of cold-adapted animals need to be reduced upon warming (Brand et al., 1994; Porter et al., 1996; Brookes et al., 1998) – otherwise, membranes would become too fluid during warming, which may affect various membrane-associated proteins and processes, such as ETS complexes or the electrochemical proton gradient across the inner mitochondrial membrane (higher proton leakage, Brand et al., 1994; Porter et al., 1996; Lee, 2004).

Rising ambient temperatures can further lead to an increase in the production of reactive oxygen species (ROS) in cold adapted marine animals (Heise et al., 2003; Keller et al., 2004). It is postulated that higher ROS formation in the ETS can be prevented by controlled mild uncoupling by mitigating the proton motive force (Guderley, 2004). Consequently, a control of proton leak would allow adjustments of the mitochondrial metabolism in response to temperature changes on the molecular level.

Functional responses to changes in tissue-specific aerobic energy demand include concomitant adjustments of it's metabolic demand, such as shifts in substrate turnover (e.g. seasonal shifts in glycogen and lipid usage in *Arenicola marina*, Sommer and Pörtner, 1999), or changes in mitochondrial abundance and/ or mitochondrial aerobic metabolism. Mitochondrial adjustments can include changes in the activities of their enzymes such as citrate synthase (CS), which catalyses the first step of the TCA-cycle, and cytochrome *c* oxidase (COX), a mitochondrial trans-membrane protein and component of the electron transport system (ETS). The activities of these enzymes are thus commonly used as a parameter reflecting the metabolic responses to warming and hypercapnia (Guderley, 1998; Windisch et al., 2011), and the changes in mitochondrial amount or structure: COX activities relate to mitochondrial membrane structure (Wodtke, 1981; O'Brien, 2011), and CS activity to the mitochondrial matrix volume (e.g. Hardewig et al., 1999b; Guderley and St-Pierre, 2002; Guderley, 2004). To monitor the processes involved in temperature-related mitochondrial proliferation and their acclimation capacities, measurements of CS and COX activities have been used in several, eurythermal temperate and stenothermal Antarctic fish species (e.g. sea bass (Egginton and Sidell, 1989), cod (Lucassen, 2006) or trout (Battersby and Moyes, 1998)), and also in cephalopods (Driedzic et al., 1990; Oellermann et al. 2012). However, the

temperature-related patterns of enzymatic responses are not necessarily the same in all species and particularly not in different tissue types (e.g. Dalziel et al., 2005; Hulbert et al., 2006). Enzymatic responses to higher PCO_2 are poorly studied in fish (tuna, Greco et al., 1982; sea bass, Michaelidis et al., 2007), and not at all in cephalopods.

Up to now, only few studies have demonstrated a compensation of mitochondrial oxygen demand in response to warm-acclimation, and they were conducted mostly on non-Antarctic fish (e.g. Dahlhoff and Somero, 1993; Sloman et al., 2008; but see Lannig et al., 2005). In contrast, stenotherm Antarctic fish analysed so far appear not to possess any mitochondrial compensation abilities in response to chronic warmth-exposure (Weinstein and Somero, 1998). Further studies on mitochondrial warm-acclimation capacities are rare, and data on the effects of chronic hypercapnia on mitochondrial capacities in both marine vertebrates and invertebrates are completely lacking so far.

1.6 Cephalopods *vs.* Fish: convergences and limitations

While Antarctic fish physiology has been generally studied in more detail, the Antarctic cephalopods have received only little attention. However, cephalopods play a significant role in the ecology of the Southern Ocean (linkage between macro-zooplankton and higher predators such as small whales, seals and albatross), and share similar ecological niches with both pelagic and benthic notothenioids (Collins and Rodhouse, 2006).

Evolution of marine fish and cephalopods is consistently described as convergent. It likely had its origin in the selection for locomotory capabilities during the Cretaceaous period, as both phyla developed similar, e.g. in terms of lifestyle and predation behaviour, since then (Packard, 1972; O'Dor and Webber, 1986).

In the marine environment, cephalopods are the sole large invertebrates that have reached sufficient complexity in terms of sensory and locomotive ability that allowed them to conquer the pelagic and benthic zone and to occupy the same ecological niche as marine fish (Sidell et al., 1987; Pörtner, 2002a).

Despite their high efficiency in vision, mobility and energy metabolism performance, their molluscan heritage goes along with several constraints. The jet propulsion, for example, is an energetically less efficient mode of locomotion than the undulatory swimming style of their competitors, and the oxygen carrying capacity of their blood (1-2 $mmol*l^{-1}$) is clearly below that of fish (4-5 $mmol*l^{-1}$) (Pörtner et al., 1996). The reason lies in the means of oxygen transport to tissues in cephalopods by the extracellular respiratory pigment

haemocyanin. Despite displaying the highest haemocyanin concentrations in the animal kingdom, the level of oxygen bound by haemocyanin is only about half of that bound by the cellular haemoglobin of vertebrates (Brix et al., 1989; Pörtner, 2002a). In order to optimize the oxygen transport efficiency, blood-oxygen binding and release in cephalopods is highly pH sensitive. This high pH sensitivity of oxygen transport is the result of a large Bohr-effect (Bohr-factors > -1), which describes a reduced oxygen-affinity with lowering of pH (Brix et al., 1981). Therefore, it is very important for these animals to tightly regulate their blood pH under varying environmental conditions.

To compensate for – compared to fish – inadequate oxygen carrying capacities, cephalopods meet their oxygen demand by a highly efficient circulatory system pumping large volumes of blood. Thus, cephalopods possess respiratory and circulatory systems with great capacities at the highest rates of oxygen consumption observed for aquatic ectotherms. These effective systems allow oxygen transport and aerobic metabolism at comparable or even higher rates than those of similarly active fish (O'Dor and Webber, 1986; Wells, 1992).

Compared to non-polar fish, temperate cephalopods have less anaerobic capacities which are linked to their very small glycogen (and lipid) stores. These serve as substrates for (time-limited) anaerobic energy production (with octopine as the end product) and are mainly reserved for burst activity (Storey and Storey, 1979).

The resting metabolism in cephalopods is highly dependent on aerobic metabolism normally fuelled with dietary carbohydrate and amino acids in temperate species. The coupling of glycogen catabolism to amino acid (especially proline) oxidation allows an additional entry point of carbon into the TCA-cycle via 2-oxoglutarate. This can increase ATP production by 50% and strongly raises energy efficiency of aerobic metabolism (Lee, 1994). Cephalopods only possess extremely low lipid reserves (commonly less than 5% of body weight), which are degraded quickly during fasting (O'Dor and Webber, 1986). In contrast, calculations of the energy budget of the Antarctic octopus *Pareledone charcoti* indicate that they may utilize a slightly higher amount of their lipid or carbohydrate stores compared to temperate octopods (Daly and Peck, 2000).

While temperate fish fuel their aerobic energy metabolism with carbohydrates and fat (both entering the TCA-cycle via acetyl-CoA), Antarctic fish are highly reliant on aerobic and lipid catabolising pathways (Crockett and Sidell, 1990), which has been partly related to high mitochondrial densities in the cold. A high cellular mitochondrial density can minimise the activation of carbon flux through the glycolytic pathway (Crockett and Sidell, 1990). Thus,

preferences of oxidation of non-carbohydrate substrates appear a common feature for Antarctic fish and octopods.

1.7 Aim and outline of the thesis

Although active organisms like fish and cuttlefish are believed to possess adequate capacities to cope with hypercapnia-induced acid-base disturbances, shifts in energy demand and intracellular pathways for acid-base regulation during chronic hypercapnia exposure may exacerbate the effects of rising seawater temperature on cellular and whole animal metabolism (Munday et al., 2012; Pörtner, 2012). The response of fish to warming or hypercapnia has until now mainly been investigated for temperate species, and no study analysed the interaction of warming and hypercapnia on Antarctic fish, such as *Notothenia rossii*, a representative for Antarctic notohenioids.

In light of the above-outlined physiological adaptations of Antarctic fish to their minimally fluctuating environment, it is highly questionable if *N. rossii* displays similar acclimation or acid-base regulation capacities as temperate fish. To predict the future fate of this unique group of fish, it is therefore important to identify the capacities of their circulatory and ventilatory system to supply tissues with sufficient oxygen during chronically elevated temperature and PCO_2. Conversely, the capacity of mitochondria to produce energy aerobically is one of the mechanisms supporting and restricting performance including that of ventilation and circulation. The characterization of how exactly mitochondrial complexes of the ETS, enzymes or the mitochondrial structure of *N. rossii* respond to long-term elevated CO_2 levels will therefore increase the knowledge about the capacity of *N. rossii* to respond or acclimate to rising temperature and PCO_2. Measuring extra- and intracellular acid-base parameters after long-term cold and warm hypercapnia acclimation would finally help to elucidate if Antarctic fish possess the capacity to maintain the pH of their body fluids in an physiological optimum range, amongst others to ensure mitochondrial functioning.

However, the ability for warm acclimation has been reported to vary between Antarctic fish species (Podrabsky and Somero, 2006). The determination of mitochondrial characteristic in different Antarctic fish, but also in such species living in thermally more fluctuating habitats, would result in a better understanding on the capacities of Antarctic fish to cope with environmental challenges.

Mitochondrial respiration in Antarctic octopods has never been analysed, and other studies on cephalopod mitochondrial capacities are scarce (but see Mommsen and Hochachka,

1981; Oellermann et al., 2012). Moreover, knowledge on the effect of hypercapnia on cephalopod mitochondria is completely lacking. The determination of acute thermal effects and susceptibility towards warming and hypercapnia in two competing animal phyla, fish and cephalopods, may reveal unifying principles of metabolic responses to ocean warming and acidification in vertebrates and invertebrates. In case of the peculiar Antarctic ecosystem, such physiological knowledge will also help to elucidate if, and in favour of which species, the fragile ecological balance between benthic octopods and notothenioids might be put at risk by future climate change.

Overall, this thesis aims to shed light upon the capacities of the aerobic energy metabolism of marine fish and cephalopods from different latitudinal clines to respond to ocean acidification and warming. It will particularly focus on mitochondrial metabolism and capacities and consider the functional integration to higher hierarchical structures such as cellular and systemic levels by approaching the following three questions:

a) *How does increased seawater PCO_2 affect the thermal acclimation capacity of Antarctic fish?*

Thermal tolerance and acclimation capacities are very low in most Antarctic teleost fish and sensitivities to combined stressors, i.e. higher PCO_2 and temperature, are likely to be increased in cold-adapted animals (Pörtner, 2010). Little information exists on the acid-base regulating machinery in highly stenothermal Antarctic fish (Deigweiher et al., 2010), and nothing is known about mitochondrial responses to chronic environmental hypercapnia in teleost fish at all. To address the question above, **publication I** investigated the response of single complexes of the mitochondrial electron transfer system, and of the mitochondrial membrane structure in detail in warm- and hypercapnia acclimated Antarctic fish, *N. rossii*. Routine metabolic rate (RMR), extra- and intracellular acid-base parameters and mitochondrial capacities are presented in **publication II**. Measurements of the aerobic enzymes, CS and COX, were performed in various tissues of acclimated *N. rossii* in **publication III**, to compare tissues-specific mitochondrial characteristics and responses towards warming and hypercapnia. It is hypothesized that Antarctic fish possess limited warm-acclimation capacities and that CO_2 would exacerbate the effects of increased temperature at the mitochondrial, cellular and whole organism level.

b) Do high-Antarctic and sub-Antarctic/ Austral notothenioids display mitochondrial capacities of different thermal sensitivity, and in which way does this influence sensitivity to ocean warming and acidification?

This part of the study is dedicated to the question of whether mitochondrial capacities can serve as an indicator for the sensitivity of various notothenioid fish to warming or acidification. It is assumed that notothenioids living in thermally more fluctuating habitats may be more tolerant to changing environmental conditions than stenotherm notothenioids living in the cold and stable Antarctic Ocean. Therefore, sub-Antarctic and cold-eurytherm Austral species are expected to possess higher mitochondrial capacities with lower thermal sensitivities than their stenotherm relatives.

Besides the ability of an organism's ventilatory and circulatory system to supply oxygen to respiring mitochondria at both low and high temperatures, mitochondrial capacities to produce ATP are believed to form the basis of the sensitivity of aerobic scope towards changing energetic demands, e.g. due to warming or elevated PCO_2.

Thus, mitochondrial oxidative phosphorylation capacities and their ability to respond to acute thermal challenges are compared between Austral (*Notothenia angustata*), Sub-Antarctic (*Lepidonotothen squamifrons*), Antarctic (*Notothenia rossii* & *Notothenia coriiceps*) and High-Antarctic (*Trematomus nicolai* & *Chionodraco hamatus*) notothenioids as a possible indicator for metabolic flexibility towards future climate change (additional data).

Another focus lies on the elaboration of chronic hypercapnia tolerances of Antarctic *versus* Austral notothenioids. p**ublication IV** presents results from heart fibre respiration experiments of long-term hypercapnia acclimated *N. angustata*, which are compared to the hypercapnia tolerance of *N. rossii*.

c) Do mitochondrial acclimation and regulatory capacities to warming and hypercapnia provide competitive advantages to fish or cephalopods?

The overall goal of this part of the study is to compare metabolic capacities between (Antarctic) teleost fish and cephalopods. Ocean acidification may lead to an elevated energy demand for acid-base regulation by shifts in acid-base status and intracellular pathways (Pörtner, 2010). The sensitivity of cephalopods to ocean warming and acidification may vary, depending on several physiological characteristics, such as pH sensitivities (and

concentration) of their respiratory proteins. It is further related to the cephalopod's thermal window, metabolic rate and mitochondrial capacities to sustain them during environmental challenges (Seibel and Walsh, 2001).

In this respect, it is hypothesized that octopods, which have the lowest metabolic rates and capacities among coleoid cephalopods, may be more sensitive to warming and hypercapnia than the more active cuttlefish. In **publication V**, aerobic metabolic capacities are therefore compared between two benthic octopods of different latitudinal origin (and thus putatively different thermal tolerance), the Antarctic *Pareledone charcoti* and the sub-tropical *Eledone moschata,* and then related to the well-studied bentho-pelagic cuttlefish *Sepia officinalis.*

Limitations in cephalopod physiology by different metabolic preferences, lower energy stores (O'Dor and Webber, 1986), and a less efficient acid-base regulation machinery, are expected to render cephalopods more susceptible to warming or acute and chronic hypercapnia exposure in comparison to fish.

The importance of different metabolic and acclimation capacities of Antarctic notothenioid fish and cephalopods will be discussed in light of the fragile balance between these ecotypically similar and competing groups of animals of different evolutionary origin.

2 Material and Methods

2.1 Experimental animals

Notothenioid fish inhabit polar, sub-polar and in part cold temperate waters and therefore are good comparative model organisms for studies of thermal plasticity among closely related Antarctic fish species. This holds also true for the octopods (class Cephalopoda), which are found from tropical to polar latitudes. In Antarctic waters, these highly developed animals share the same spatial and ecological niche as benthic notothenioids and thus directly compete for the same resources in the ecosystem.

The experiments conducted in this study aimed to characterise thermal tolerances and susceptibility towards global climate change in these competing animals by measuring acute and chronic responses to rising temperatures and PCO_2 on mitochondrial, cellular and systemic levels.

The suborder Notothenioidei comprises eight families, from which five are exclusively found in the Southern Ocean. The other three families (Bovichtidae, Pseudaphritidae and Eleginopidae) comprise nine non-Antarctic species that occur almost exclusively outside Antarctic waters (Matschiner et al., 2011). Two further cold-eurytherm species, *Notothenia angustata* and *N. microlepidota*, do not belong to these three basal temperate notothenioid families, but entered temperate water habitats via a different evolutionary path. These species had an Antarctic origin, but diverged from their Antarctic ancestors 11 million years ago, which is coincident with the northward advance of the Antarctic Convergence over New Zealand. After the southern retreat of the Antarctic Convergence, these species remained in their New Zealand habitats (Cheng et al., 2003).

In this thesis, the following five fish species, which are all endemic to the Antarctic region were used: *Notothenia rossii*, *Notothenia coriiceps, Lepidonotothen squamifrons* and *Trematomus nicolai* (Nototheniidae, notothens) and *Chionodraco hamatus* (Channichthyidae, icefishes). A sixth species was the non-Antarctic, Austral black cod, *Notothenia angustata*.

N. rossii and its sympatric sister species *N. coriiceps* are widely distributed around the Antarctic continent between 45° and ~ 62°S, and also occur in the seasonal sea ice zone (Everson, 1969; DeWitt et al., 1990; Gon and Heemstra, 1990). They preferably live from 5 to 50 m depths on rock bottoms and mainly prey on gammaridean amphipods. At the Antarctic Peninsula, water temperatures range from -1.9°C in winter to maximum 2°C in summer

(Schloss et al., 2008). Despite several functional and ecological resemblances, different life cycles and habitat preferences may reflect different physiological adaptations. *N. coriiceps* is more demersal and sedentary, while *N. rossii* is semipelagic and also feeds on water column prey, such as krill (North, 1996).

The sub-Antarctic, semipelagic *L. squamifrons* is a typical member of the ice-free zone on the sub-Antarctic island shelves. In terms of its ecology, it is similar to *N. rossii*. *L. squamifrons* is distributed between 45° and 55°S, where water temperatures range from about 0.7°C in winter (e.g. Ward, 1989) to about 3.5°C in summer (Pakhomov et al., 2006).

The high-Antarctic genus *Trematomus* include benthic or epibenthic species and are the dominant fish species of the continental shelf regions of Antarctica, most of them with a circum-continental distribution. The small, benthic *T. nicolai* (about 36 cm total length) is known from high-Antarctic localities around Antarctica at depths to 420 m, for example in East Antarctica and the Ross Sea. If feeds primarily on amphipods, other fishes and molluscan larvae.

The white-blooded icefish *C. hamatus* lacks both haemoglobin and myoglobin. Characteristically, it occurs in circum-Antarctic on the continental shelf in a depth range from 4 to 600 m. Little is known about the biology of this species; adult fish are known to mostly feed on krill and fish (Gon and Heemstra, 1990; Mintenbeck et al., 2012).

The New Zealand black cod (or Maori Chief), *N. angustata,* can be found in the cool temperate shallow waters around the southern coast of New Zealands South Island at depths down to 100 m. Their environmental temperature ranges from 2°C in winter to 18°C during summer, and it feeds on a variety of invertebrates, small fish and seaweed (Guynn et al., 2002; Cheng et al., 2003) (see figure 2.1 for species distribution).

Cephalopods are the largest, most active invertebrates and there is considerable evidence for their convergent evolution with fish (Packard, 1972). Particular consideration was paid on the comparison of acute temperature and hypercapnia responses in polar and temperate octopods to identify the susceptibility of octopods to climate change. The cuttlefish *Sepia officinalis,* which shares large parts of its habitat with the sub-tropical *Eledone moschata,* was used to gain insight into the hypercapnia vulnerability of cephalopods.

The experimental animals used in this thesis belonged to both the superorders decapodiformes and the octopodiformes. The decapod common cuttlefish *Sepia officinalis* is widespread on continental shelves from the temperate North Atlantic to the sub-tropical Mediterranean and in Atlantic coastal waters off Senegal, and one of the most abundant

cephalopods (von Boletzky, 1983; Jereb and Roper, 2005). This neritic and demersal, eurythermal species is exposed to broad temperature ranges, which can range from 10 up to 30°C, depending on the habitat (Artegiani et al., 1997).

The octopods investigated were first the benthic, Sub-tropical musky octopus *Eledone moschata*, which is abundant throughout the Mediterranean Sea and off the coast of Portugal (Costello et al., 2001). The second octopus species was the benthic, Antarctic octopus, *Pareledone charcoti*, which can be found on continental shelves around the Antarctic Peninsula and in the Ross Sea in depths from 0 to 800 meters, at habitat temperatures from -2 to 2°C (Allcock, 2005) (figure 2.1).

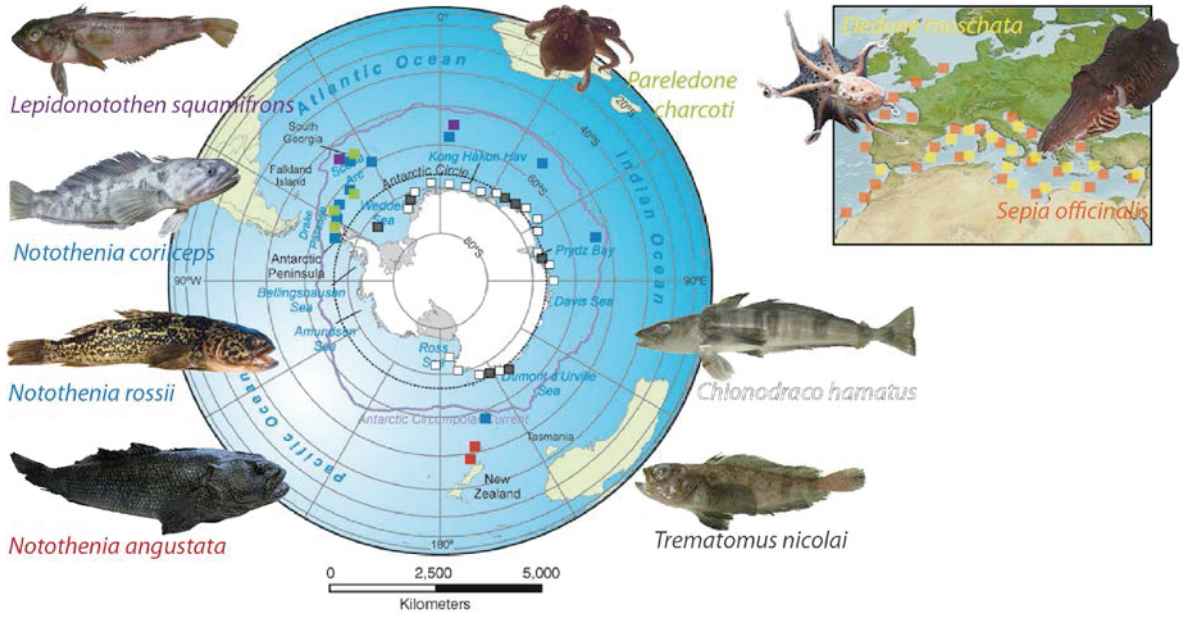

Figure 2.1 Species distribution pattern.
Lepidonotothen squamifrons (violet), *Notothenia rossii* (blue), *Notothenia coriiceps* (blue), *Notothenia angustata* (red), *C hamatus* (white), *Trematomus nicolai* (grey) and *Pareledone charcoti* (green). *Sepia officinalis* (orange) and *Eledone moschata* (yellow). Data derived from von Boltezky, 1983; Mangold 1983; Gon Heemstra 1990; Allcock 2005, and FishBase (http://www.fishbase.org). Pictures have been taken by F. Mark at Carlini Station in 2009 and by M. Damerau during ANTXXVII/3 in 2011.

N. rossii were caught in December 2009 in Potter Cove, King George Island, Antarctic Peninsula (KGI; 62°14'S; 058°41'W). Fish were collected using baited traps (length 124 cm, width 64 cm, height 56 cm, mesh size 25 mm) and trammel nets (length 15 m, inner mesh 25 mm) at depths of 5 to 15 m and a seawater temperature of 0.8±0.9°C and a salinity of 33.5±0.2 psu. Animals were reared and acclimated in the aquaria facilities at Dallmann Laboratory, Carlini Station (KGI) with direct seawater supply from the cove, under natural light conditions (figure 2.2). Animals were fed to satiation twice per week on chopped fish

muscle and snails. All experiments that required live specimens or fresh blood/ tissue samples were performed in the laboratories of the Dallmann Laboratory.

Another batch of *N. rossii* (*n*=4. mass 397 – 481 g, standard length 33.0 - 36.0 cm) and speciem of *N. coriiceps* (*n*=4. mass 370 – 472 g, standard length 28.5 - 34.0 cm) were caught in January 2011 in Potter Cove, King George Island, using the same capture methods as described above for *N. rossii*, at an environmental temperature ranging from 1.4-2°C and a salinity of 33.2-34psu. They were picked up by the research vessel *RV Polarstern* in February 2011 and kept on board, with direct seawater supply, in well-aerated 500 liter tanks at 0°C, salinity 34.5-35.5psu, and dim light (12h light/ dark). They were transported to and maintained at 0°C in the Aquaria facilities of the Alfred Wegener Institute, Bremerhaven. These animals were exclusively used for measurement of permeabilised heart fibre respiration in January 2012 in the laboratories of the Alfred Wegener Institute (see below), and the data are not published so far.

Specimens of *L. squamifrons, T. nicolai* (*n*=4, mass 90 – 332 g, standard length 17.7 – 25.3 cm) and *C. hamatus* (*n*=5, mass 192 – 279 g, standard length 27.5 – 30.5 cm), and octopods of the species *Pareledone charcoti* were caught in Agassiz trawls and bottom trawls between February and March 2011 during the cruise ANT XXVII/3 of the German research vessel *RV Polarstern* (figure 2.2). *P. charcoti* (*n*=4-9, mass 43 – 92 g) were caught close to the South Orkney Islands (61°0'S; 44°2'W) and King George Island (62°18'S; 58°41'W) at depths of 350 m at a local water temperature of 0.1°C and a salinity of 34.4 psu. *L. squamifrons* were caught at 300 m water depth off South Georgia (53°24.54'S; 42°40.55'W, local water temperature 2.1°C), the two other species in the eastern Weddell Sea at 250 m depth and a local water temperature of -1.5°C (close to Kap Norvegia, 70°52.55'S; 10°35.99'W). The icefish were used for the experiments within the first 24 hours after capture. All other animals were maintained onboard the research vessel in an air-conditioned container equipped with aquaria and aerated recirculated natural seawater at 0.1°C±0.5°C for maximum 1 week prior to the experiments, all of which were conducted in the laboratories onboard.

N. angustata were caught close to Otago harbour, New Zealand (45°52.26'S; 170°30.12'E) using baited traps at depths of 8 m in November 2011 at a local temperature of 13°C (figure 2.2). They were maintained for three weeks until used for the experimentation at the Portobello Marine Station, Portobello, New Zealand, with direct supply of seawater at local water-temperature of 14.5°C (*n* = 11, mass 733 – 3360 g, standard length 39.0 – 51 cm).

Wild–laid *S. officinalis* egg clusters were collected by fisherman in the Venice Lagoon, Chioggia, Italy, in May 2009 at a local temperature of 16°C. They were transported to and raised in a closed, re-circulating aquarium system at the Alfred Wegener Institute in Bremerhaven, Germany, on a diet of mysids (*Neomysis integer*) and brown shrimp (*C. crangon*) at constant temperature (16 ± 0.1°C), salinity (30-32 psu) and pH (>8.0) (control group - 16°C, 0.04 kPa, *n*=6, 88-146g). Water quality parameters were determined three times weekly and maintained at levels suitable for cephalopod culture.

Adult specimens of *E. moschata* from the Adriatic Sea were caught in Mai 2010 at a water temperature of 16°C by local fisherman in Chioggia, Italy (figure 2.2), and directly transported to the Alfred Wegener Institute, Bremerhaven.

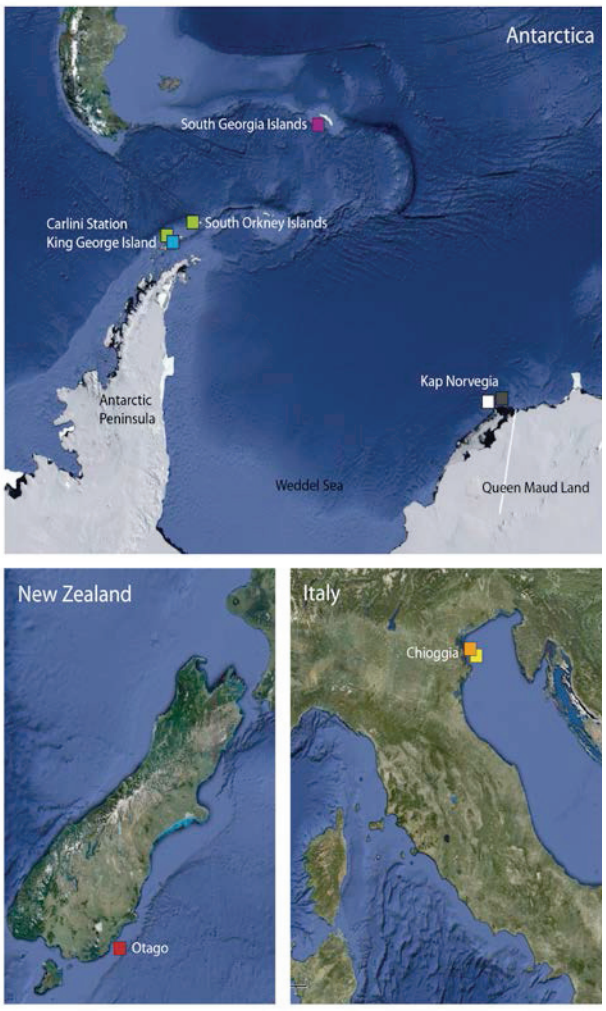

Figure 2.2 Species sampling areas. *Lepidonotothen squamifrons* (violet) was caught off the coast of South Georgia, *Notothenia rossii* (blue) and *Notothenia coriiceps* (blue) in Potter Cove, King George Island, *Notothenia angustata* (red) close to Otago Harbour (Zew Zealand), *C hamatus* (white) and *Trematomus nicolai* (grey) in the Eastern Weddel Sea and *Pareledone charcoti* (green) close to King George Island and the South Orkney Islands. *Sepia officinalis* (orange) and *Eledone moschata* (yellow) were both collected in the Venice lagoon, Italy. Maps are derived from Google Maps (http://maps.google.de/maps).

2.2 Acclimation setup

2.2.1 Fish

N. rossii. The acclimation of *N. rossii* was conducted at Dallmann Laboratory (Carlini Station, KGI), in December 2009 until January 2010. The acclimation setup consisted of well-aerated (>95% O_2 saturation) 150 liter aquaria tanks, fed by an additional 150 liter header tank equilibrated to the appropriate PCO_2 and temperature. This header tank was used for a daily water exchange of 150 liter to avoid alteration of the conditions in the acclimation tanks. For the warm normocapnia/ hypercapnia acclimations, temperature was kept constant using a 250 W heating element (Jaeger, EHEIM GmbH, Germany), controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). For the cold/ warm hypercapnia acclimations, elevated seawater- PCO_2 was regulated by an iks aquastar system (iks Computer Systeme GmbH, Germany). This system maintained a constant pH (accuracy ± 0.05 pH units) by controlling a solenoid valve (Aqua Medic GmbH, Germany) in order to equilibrate the acclimation and header-tanks with pure CO_2.
For the acclimation trials (29-36 days), the experimental animals were randomly selected and exposed to the following four conditions:

1) 1°C, 0.04 kPa CO_2 (control group, n=9, mass 155 - 804 g; standard length 25.0 - 39.4 cm)
2) 1°C, 0.2 kPa CO_2 (cold hypercapnic group, n=10, mass 144 - 510 g; standard length 23.8 - 32.8 cm)
3) 7°C, 0.04 kPa CO_2 (warm normocapnic group, n=5, mass 151 - 412 g; standard length 23.6 - 33.9 cm)
4) 7°C, 0.2 kPa CO_2 (warm hypercapnic group, n=10, mass 137-504 g; total standard 21.4 - 31.3 cm).

L. squamifrons. The fish were acclimated in well-aerated 150 liter aquaria tanks at a salinity 34.4±0.15 psu in aquaria containers on board of *RV Polarstern* at a light regime with a 12 h/ 12 h light/dark-cycle. Two thirds of the water in the experimental tanks was exchanged daily with water from a pre-warmed (2 or 9°C) reservoir tank to maintain high water quality within the system. Animals were kept for 39-46 days at:

1) 2°C±0.45, 0.04 kPa CO_2 (control; *n*=7, mass 182.0 – 292.0 g, standard length 22.0 – 25.4 cm)
2) 9°C±0.26, 0.04 kPa CO_2 (warm acclimation; *n*=9, mass 107.4 – 255.2 g, standard length 19.8 – 24.9 cm).

Temperature in the aquaria tanks was maintained with a 250 W heating element (Jaeger, EHEIM GmbH, Germany) controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). Fish were fed to satiation every other day with isopods.

N. angustata were acclimated to 0.2 kPa CO_2 for 15 days in two well-aerated, 200 liter aquarium tanks, each of which contained three individuals (mass 158 to 1470 g, standard length 20.0 – 40.5 cm), with seawater (14°C) directly pumped from Portobello Bay. During the acclimation period, the animals were not fed and kept under dim light conditions during the days. The acclimation tanks were continuously equilibrated with pure CO_2 using the same pH-feedback system as described for *N. rossii* above.

2.2.2 Cephalopods

The Adriatic cuttlefish were acclimated to 16°C, 1.12 kPa CO_2 (hypercapnia acclimation, *n*=7, mass 33 – 46 g), Adriatic octopods were kept under control conditions at 16°C, 0.04 kPa CO_2 (control; *n*=4, mass 54 - 76 g) or warm-acclimated to 21°C, 0.04 kPa CO_2 (*n*=4, mass 25 - 31 g), for a time-period of five months in three recirculating seawater systems maintaining the respective acclimation conditions, at the Alfred Wegener Institute, Bremerhaven. Each animal was placed in individual closed incubation boxes of 8.5 liter volume, fed by a reservoir and header tank containing well-aerated, pre-equilibrated seawater (16°C, 1.12 kPa CO_2, 16°C, 0.04 kPa CO_2 or 21°C, 0.04 kPa CO_2, respectively). Seawater from the reservoir and header tank was constantly adjusted by injection of a gas mixture of compressed air, CO_2 and N_2 using a mass flow controlled, automatic gas mixing-facility (HTK Hamburg GmbH, Germany), and temperature adjusted by several 250 W heating elements (Jaeger, EHEIM GmbH, Germany) controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). Cuttlefish were fed every other day with live shrimp (*Palaemon sp.* or *C. crangon*).

During long-term hypercapnia acclimation of fish and cephalopods, the seawater pH of all acclimation systems was measured every other day with a WTW 340i pH meter (WTW,

Germany. Electrode: WTW SenTix HWS) and calibrated daily with NIST certified buffer (WTW, Germany). Total CO_2 (C_{CO2}/ DIC) in the seawater was determined with a carbon dioxide analyser (Corning 965, CIBA, Corning Diagnostics, UK) in the field or by gas chromatography at the Alfred Wegener Institute, Bremerhaven. Seawater carbonate chemistry parameters were calculated from the measured pH_{NIST} and DIC with the software CO2SYS (Pierrot et al., 2006).

Table 2.1 summarizes all experimental animals, acclimations and conducted experiments of the present work.

Table 2.1 Experiments and animals used in the present thesis

species	origin	treatment	experiment/ parameters measured	publication
L. squamifrons	South Georgia	control; 9°C 0.04 kPa	RMR; OXPHOS & ETS capacities; CS & COX activity; mitochondrial fatty acids	I discussion
N. rossii	KGI	control; 1°C 0.2 kPa; 7°C 0.04 kPa; 7°C 0.2 kPa	RMR; extra/- intracellular pH, DIC; OXPHOS capacities; CS & COX activity; mitochondrial fatty acids	I, II, II discussion
N. coriiceps	KGI	control	OXPHOS & ETS capacities	discussion
C. hamatus	Weddel Sea	control	OXPHOS & ETS capacities	discussion
T. nicolai	Weddel Sea	control	OXPHOS & ETS capacities	discussion
N. angustata	Otago	control; 15°C 0.2 kPa	RMR; OXPHOS & ETS capacities	IV
S. officinalis	Adria	control; 16°C 0.11 kPa	RMR; haemolymph pH, DIC; mantle pH_i, DIC_i; OXPHOS capacities	V
E. moschata	Adria	control; 21°C 0.04 kPa	RMR; haemolymph pH, DIC; branchial heart pH_i, DIC_i; OXPHOS capacities	V
P. charcoti	KGI	control	RMR; OXPHOS capacities	V

2.3 Whole animal oxygen consumption

In all experiments with fish and cephalopods, routine metabolic rate (RMR) was measured via intermittent-flow respirometry. Feeding was terminated seven days prior to experimentation to ensure that only RMR was measured. Each animal was placed in a cylindrical respirometer in an experimental aquarium equilibrated to the respective control/ acclimation conditions.

Before starting the experiments, animals were allowed to recover from handling stress for 24 hours. A constant, circulating water flow in the respirometer was guaranteed with an aquarium pump. Resting oxygen consumption of each individual was measured for 24 hours in total. During the measurement period in the intermittent-flow system, several 15 or 30 minutes oxygen consumption runs were completed. In each cycle, the animal depleted the oxygen within the chamber by maximum 10 - 15%, depending on the size of the animal, then oxygen concentration was replenished to 100% by flush pumps. Oxygen concentration within the chamber was detected once per minute using a temperature-compensated FiBox2 (PreSens – Precision Sensing GmbH, Germany) oxygen meter and flouroptic sensors (optodes), which were connected to the tubing of the recirculation pump. The optodes were calibrated before each measurement in well-aerated seawater at the respective acclimation temperature, zero calibration was conducted with nitrogen-bubbled seawater. Blank measurements for determination of bacterial respiration within the respirometer were carried out for each control/ acclimation group, oxygen consumption was corrected accordingly.

Oxygen consumption of the animal was calculated by using the linear declining rate of oxygen content within the respiration chamber (ΔP_{O_2}), with the following formula:

$$M_{O2} = \frac{\Delta P_{O2} \times \beta_{O2} \times V}{W}$$

M_{O2} = oxygen consumption rate [µmol * kg^{-1} * min^{-1}]

ΔP_{O_2} = change in oxygen partial pressure over the measurement [µmol * min^{-1} * Torr]

β_{O2} = oxygen capacity of seawater at a given temperature [µmol * L^{-1} * $Torr^{-1}$] according to Boutilier (1984)

V = volume of water in the respirometer – mass of animal [L]

W = mass of animal [kg]

2.4 Sampling and acid-base parameters

Fish were anaesthesized with 0.5 g/l tricaine methano- sulphonate (MS 222). Blood was taken with a syringe from the caudal vein, the liver and heart (ventricle) were taken for mitochondrial isolation or heart fibre permeabilisation, respectively. Parts of liver and muscle samples were quickly freeze-clamped and instantaneously frozen in liquid nitrogen for pH_i

analysis, as described by Pörtner (Pörtner, 1990). Further samples of heart, liver, red and white muscle were shock-frozen in liquid nitrogen and stored at -80°C for enzyme assays. The animal was then killed by a spinal cut behind the head plates.

Cephalopods were anaesthesized in 2.5% v/v ethanol. Afterwards, the mantle cavity was opened on the ventral side, samples of haemolymph were taken from the anterior vena cava (vena cephalica) and the animals were sacrificed by decapitation. The branchial hearts and a piece of funnel musculature were immediately excised, freeze-clamped and stored in liquid nitrogen. The systemic heart was transferred in modified respiration buffer and used for the heart fibre respiration experiments (section 2.5).

During the measurement period of blood/ haemolymph pH, the sample and sensor were placed on ice or in a thermostatted water bath at the respective acclimation temperatures. The pH electrode (InLab Routine Pt1000®, Mettler Toledo GmbH, Germany) was calibrated with IUPAC pH standards (pH 6.856 & 7.413 at 25°C; WTW, Germany) to measure pH in closed 0.5 ml Eppendorf tubes to avoid contract with environmental air. Total CO_2 (DIC) was either measured with a carbon dioxide analyser (Corning 965, CIBA, Corning Diagnostics, England) in the field, or in case of the Adriatic cephalopods, by gas chromatography (6890N Network GC System, Agilent Technologies, Germany) at the Alfred Wegener Institute, Bremerhaven, Germany.

Measurement of the intracellular pH was carried out according to the homogenization technique described by Pörtner (Pörtner, 1990) using the tissue samples stored at -196°C. Homogenate medium parameters, i.e. potassium (KF) and nitrilotriacetic acid (NTA) concentrations, were adjusted for fish or cephalopods according to Pörtner (1990) (c.f. publication II). pH of the tissue homogenate was measured with a pH optode (PreSens Needle-Type-Housing-pH-Mircosensor, PreSens GmbH, Germany) in a water bath adjusted to acclimation temperature. Before measurement, the optode was calibrated using a pH-meter with a glass electrode (InLab Routine Pt1000®, Mettler Toledo GmbH, Germany) and IUPAC pH standards (pH 6.865 & 7.413 at 25°C; WTW, Germany). DIC was measured in the supernatant of the homogenate by gas chromatography (6890N Network GC System, Agilent Technologies, Germany).

2.5 Heart fibre and mitochondrial isolation

2.5.1 Heart fibre preparation

In the fish *L. squamifrons, T. nicolai, C. hamatus, N. angustata* and all cephalopods, the heart fibres were prepared following a modified protocol by Kuznetsov et al. (2008). After dissection of the heart, a piece of heart tissue mechanically dissected in ice-cold biopsy buffer (modified respiration buffer/ BIOPS, Table 2.2) using scissors and forceps and stored on ice in medium.

Table 2.2 Composition of heart fibre biopsy buffer for notothenioids and cuttlefish (BIOPS; modified after Lassnig et al., 1998-2008; Kuznetsov, et al., 2008). pH was adjusted with KOH to 7.01 (fish) or 7.4 (cephalopods) at 24.8°C.

Chemical	**Final concentration [mmol l^{-1}]**	
	Fish	**Cephalopods**
CaK_2EGTA	2.77	2.77
K_2EGTA	7.23	7.23
Na_2ATP	0.32	5.77
$MgCl_2$	0.13	6.56
Taurine	0.25	20
Na_2Phosphocreatine	0.38	-
Imidazole	0.14	20
Dithiothreitol (DTT)	0.001	0.5
MES	1.0	50
Sucrose	8.9	588
Glycine	-	252
Final osmolarity [mOsm l^{-1}]	435	1000

For each respiration experiment, a subsample of the heart fibre bundles was permeabilised for 30 min with 10 µg ml^{-1} saponin by gentle mixing on ice for 30 min. After this incubation, fibres were washed three times for 10 min by gentle mixing on ice in 2 ml of modified assay medium (Mitochondrial Respiration Medium, MiRO5; Table 2.3).

Table 2.3 Composition of heart fibre assay medium for notothenioids and cephalopods (MiRO5; modified after (Mommsen and Hochachka, 1981; Kuznetsov, et al., 2008). pH was adjusted with KOH to 7.02 (fish) or 7.4 (cephalopods) at 24°C.

Chemical	Final concentration [mmol l^{-1}]	
	Fish	Cephalopods
HEPES	20	50
KH_2PO_4	10	25
KCl	-	50
NaCl	-	50
$MgCl_2$	3	10
Taurine	20	20
Lactobionate	60	50
Sucrose	215	350
Glycine	-	150
EGTA	0.5	
BSA fatty acid free	1%	1%
Final osmolarity [mOsm l^{-1}]	435	1000

Then, the subsample was blotted, weighed, and immediately used for respirometric analysis. Respiration of each subsample was measured at three different, incrasing assay temperatures in 2 ml air-saturated assay medium containing 300U/ml catalase for reoxygenation with hydrogen-peroxide in glas-chambers of an Oroboros Oxygraph-2k^{TM} respirometer (Oroboros Instruments, Austria) at the respective acute assay temperature for respirometric analsis. The heart fibre respiration was converted to nmol O_2 min^{-1} $mg_{fresh\ weight}^{-1}$.

In case of the cephalopods and *N. angustata,* heart fibre respiration assays were measured in the standard, normocapnic respiration buffer (MiRO5) of 0.04 kPa CO_2, and in hypercapnic respiration buffer with 1.6 (cephalopods) and 2 (fish) kPa CO_2. The hypercapnic MiRO5 was equilibrated in a closed vial on a magnetic stirrer with the appropriate gas mixture of N_2, O_2 and CO_2 supplied by a gas-mixing pump (DIGAMIX®, Wösthoff Messtechnik GmbH, Germany) in case of the experiments at the Alfred Wegener Institute. In

New Zealand, MiRO5 was CO_2 equilibrated by using a Multi-Channel Flow Ratio/Pressure Controller (MKS Instruments GmbH, Germany), which received pressurized air and pure CO_2.

2.5.2 Mitochondrial isolation

Immediately after excision, liver of *N. rossii* or *L. squamifrons* was cleaned and total liver weight was taken before a subsample of liver tissue was taken, weighed and washed with 5ml/g ice-cold isolation buffer (MIM, Table 2.4). The liver tissue was then finely minced with scissors, suspended in 10 volumes ice-cold isolation buffer, and then put into a 30 ml Potter-Elvehjem glass homogenizer (VWR International, Germany) and slowly homogenised with three strokes at 80 revolutions/minute. The homogenate was centrifuged (600 g, 10 min., 0°C), the supernatant collected and the pellet vigorously resuspended in isolation buffer and centrifuged for a second time. Supernatants were then joined and centrifuged for 10 min at 11.000 g (0°C). The supernatant was discarded, any remaining droplets of fat removed with a cotton swab and the pellet resuspended in isolation buffer and centrifuged again. In the last step, supernatant was discarded again, and the pellet was resuspended in ice-cold mitochondria assay buffer (MAM, Table 2.4) at a dilution of 1 ml/g initial liver weight. This mitochondria preparation was kept on ice; 50µl of the mitochondrial extract were stored at -80°C for further experiments.

Table 2.4 Composition of mitochondrial isolation medium (MIM) and mitochondrial assay medium (MAM), modified after (Hardewig et al., 1999a). pH was adjusted with KOH to 7.1 at 20°C.

Chemical	Final concentration [mmol l^{-1}]	
	MIM	MAM
HEPES	50	50
EGTA	5	-
EDTA	5	-
KCl	85	85
KH_2PO_4	-	5
Sucrose	80	80
BSA fatty acid free	1%	1%

In the case of *N. rossii,* mitochondrial respiration was detected in two thermostatted perspex respiration chambers of 3 ml volume (World Precision Instruments, Inc., USA). They were equipped with an adjustable stopper and ports for TPMP (triphenylmethylphosphonium) and reference electrodes, as well as a titration port for metabolites and inhibitors and one for a TX micro-optode (PreSens – Precision Sensing GmbH, Germany). In addition to the fluoroptic measurement of mitochondrial respiration, membrane potential was measured with a TPMP electrode (after Brand, 1995) and a Dri-Ref reference electrode (World Precision Instruments, Inc., USA). The electrodes were connected to a PHM220 voltmeter (Radiometer analytical, France). The voltage output was recorded simultaneously with the oxygen traces by means of a PowerLab recording unit and the Chart v5.5.6 software (ADInstruments GmbH, Germany) (see example trace, figure 8.1, in Appendix).

The respiration of each mitochondrial sample of *L. squamifrons* was measured in 2 ml glass-chambers of an Oroboros Oxygraph-2k^{TM} respirometer (Oroboros Instruments, Austria). For both species, the mitochondrial respiration was converted to nmol O_2*min^{-1}*$mg_{protein}^{-1}$.

2.6 Substrate - inhibitor protocol

To assess mitochondrial respiratory states and maximum capacities, a combined substrate-inhibitor protocol was applied on permeabilised heart fibres or isolated liver mitochondria. A specific series of substrates and inhibitors allowed the experimental determination of the contribution of single ETS complexes to oxidative phosphorylation, maximum ETS capacities and mitochondrial efficiencies, e.g. by measuring leak respiration and calculating respiratory control ratios (RCR).

In the experiments using permeabilised heart fibres, the titration protocol started with addition of Complex I substrates and ADP to induce state III. By addition of Complex II substrates and ADP, maximum OXPHOS capacity was achieved. To inhibit mitochondrial ATP synthesis, oligomycin was used to estimate leak respiration (state IV^{+}). The capacity of the electron transfer system (ETS) was evaluated by titration with the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which relieves the proton gradient across the inner mitochondrial membrane, thereby uncoupling ETS electron flow from ATP generation/oxidative phosphorylation. Afterwards, Complex I was inhibited with rotenone and Complex II with malonate, to test the flux through the respective complexes. Inhibition of Complex III by Antimycin revealed the oxygen uptake due to residual oxidative side reactions

(non-mitochondrial respiration, ROX). In the last step of the protocol, Ascorbate and TMPD served as artificial substrates for Complex IV (figure 2.3; see Table 8.1 and 8.2 in the Appendix for substrate and inhibitor concentrations applied).

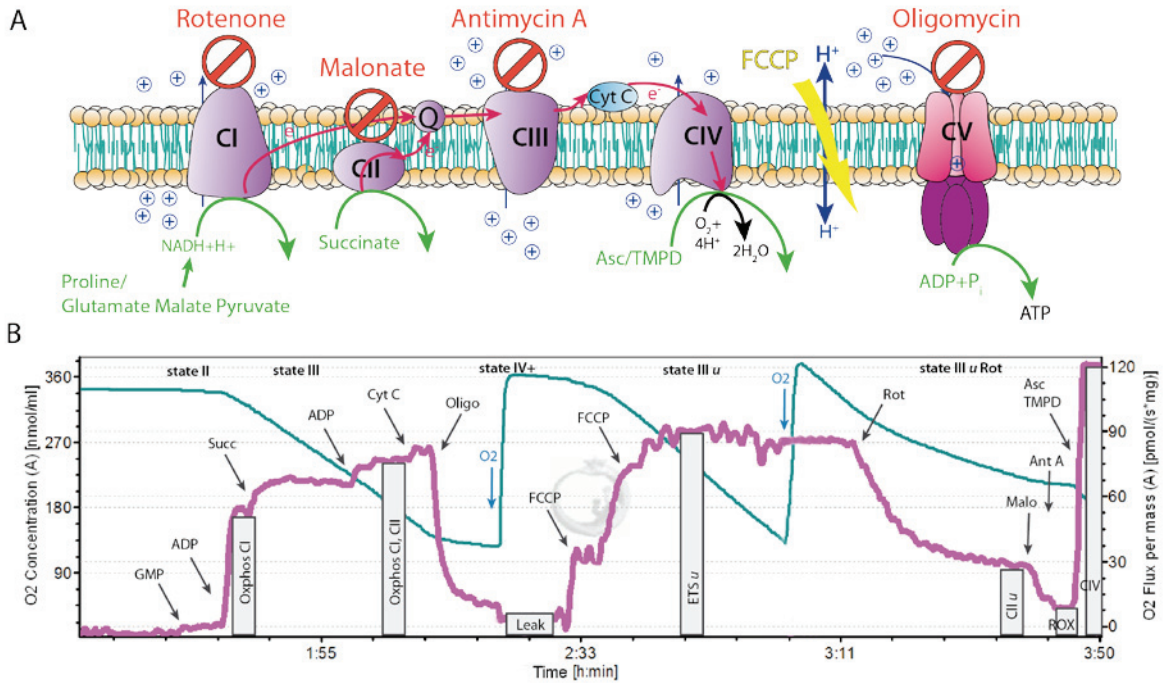

Figure 2.3 Example of a substrate inhibitor titration oxygraphic protocol for step-by-step analysis of various segments of the respiratory chain.
A: Schematic electron transport system constisting of Complexes I (CI, NADH dehydrogenase), II (CII, succinate dehydrogenase), III (CIII, cytochrome *c* oxidoreductase), IV (CIV, cytochrome *c* oxidase) and V (CV, ATP synthase). The specific substrates (green) used were: Proline+pyruvate (cephalopods) or GMP - glutamate+malate+pyruvate (fish); Succ – succinate; Asc TMPD – Ascorbate+TMPD; Cyt C – cytochrome *c*. Maximal respiration capacity (state III) was achieved by stimulating with ADP at saturating concentration. The inhibitors (red) were Oligo – oligomycine, Rot – rotenone, Malo – malonate, Ant A – antimycin A. FCCP (yellow) dissipates the proton gratient across the inner mitochondrial membrane. B: Representative oxygraph trace of permeabilised fish heart fibres. Slope of declining oxygen concentration (green line; nmol ml^{-1}) and rate of oxygen consumption (J_{O2}, purple line; pmol s^{-1} mg^{-1}) over time (min) obtained from the oxygraph. *Arrows* indicate the point of addition of each substrate/ inhibitor, or of reoxygenation. For details on substrate/ inhibitor concentrations and termination of the different respiratory states see Table 8.1 and 8.2 in the Appendix).

With the isolated liver mitochondria of *N. rossii*, the sequence of analyses was slightly modified: first, Complex I state III respiration was induced with substrates and ADP, then state IV respiration was recorded after complete ADP depletion (non-saturating ADP concentrations). After that, Complex I was inhibited with rotenone (state IV^{+}) and succinate + ADP added to yield Complex II state III. State IV^{+} was then initiated with oligomycin and proton leak titrated with malonate. The protocol ended with mitochondrial uncoupling by FCCP (see Table 8.3 in the Appendix for details).

2.7 Enzyme assays

For extraction, frozen samples were ground into powder by mortar and pestle under liquid nitrogen. The fine tissue powder was weight into a microcentrifuge tube and then homogenized in a glass homogenizer in 9 volumes ice-cold extraction buffer. Afterwards, the pre-homogenate was transferred into a 2 ml microcentrifuge tube and homogenized three times using an Ultra Turrax (Silent Crusher M (Heidolph Instruments, Germany), followed by 10 min centrifugation at 1,000 *g* at 4°C. The supernatants were readily used for the protein assays. The enzyme activities of each sample extraction were measured in two concentrations at 0, 6, 9 and 12°C in a UV/VIS spectrophotometer (Beckman, Fullerton, CA, USA) equipped with a thermostatted cell holder.

Citrate Synthase (CS) activity was measured according to Sidell et al. (1987) as the increase of absorbance at λ =412 nm. The method is based on a colour reaction by the transfer of sulphur groups (SH) from coenzyme A (CoA) to 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), which can be detected by the UV/VIS spectrophotometer.

Cytochrome *c* oxidase (COX) activity was determined according to a protocol modified from Moyes et al. (1997). The activity was measured by the decrease in extinction at λ = 550 nm through oxidation of cytochrome *c*. Heart and red muscle samples were diluted 1:10 with extraction buffer before the assays.

Protein concentration of each tissue extract was determined after Bradford (Bradford, 1976) by measuring the absorbance at λ=595 nm at 20°C in a spectrophotometer (Pharmacia LKB Biochrom 4060, Pharmacia, UK) to refer enzyme activities to amounts of cellular protein (μmol min^{-1} mg $protein^{-1}$). Furthermore, enzyme activities were calculated per gram tissue fresh weight (μmol min^{-1} mg FW^{-1}) using the initial weight after the first homogenisation step.

2.8 Lipid extraction

In the liver mitochondrial preparation described in section **2.5**, mitochondrial membrane lipids lipids were extracted following a protocol modified after Folch et al. (1957). The samples were analysed using gas-chromatography with a flame ionization detector (Agilent 6890N GC, Agilent Technologies, USA).

The unsaturation index (UI) of the mitochondrial membranes was calculated following Grim et al. (2010) according to the formula:

$$UI = \sum_{n=24}^{n=0} n * \% \text{ of fatty acids with } n \text{ double bonds.}$$

2.9 Statistics

Appropriate statistical analysis was performed using GraphPad Prism Version 5.0b (GraphPad Software, Inc., US) by employing tests as followed: Kolmogorov-Smirnov test to assess homogeneity of variance or normality distribution. One-way independent analysis of variance (ANOVA) and an additional post-hoc Tukey test, or two-tailed t-tests for comparison between assay temperatures, acclimation conditions or between species. $p \leq 0.05$ was considered the significance threshold

Outliers were detected and excluded using the Nalimov test with a significance level of $p<0.01$. See respective publications for detailed descriptions. If not stated otherwise, data are presented as mean values± standard error of the mean (SEM).

3 Publications

List of publications and declaration of the candidate's contribution towards them.

Publication I

Anneli Strobel, Martin Graeve, Hans O. Pörtner, Felix C. Mark (2013)

Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*

Plos One (in revision)

The design of the experiments was developed by the candidate and the last author. Parts of the experiments were conducted in cooperation with the last author, the other part was carried out by the candidate alone on board RV Polarstern. The candidate analysed the data and wrote the manuscript, which was revised together with the last two authors.

Publication II

Anneli Strobel, Swaantje Bennecke, Elettra Leo, Katja Mintenbeck, Hans O. Pörtner, Felix C. Mark (2012)

Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2

Frontiers in Zoology 2012, 9:28

The outline for the study was designed by the candidate, the next to the last and the last author. All experiments were carried out either by the candidate or in close collaboration with the second, third and last author. The first draft of the manuscript was written by the candidate and revised together with the last thee authors.

Publication III

Anneli Strobel, Elettra Leo, Hans O. Pörtner, Felix C. Mark (2013)

Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*

Comparative Biochemistry and Physiology B – Biochemistry & Molecular Biology (submitted)

The ideas for the experiments were developed by the candidate and the last author. The experiments were conducted in close cooperation with the second author. The candidate

analysed the data and wrote the manuscript, which was revised in cooperation with the second to the last and the last author.

Publication IV

Anneli Strobel, Felix C. Mark, Daniel W. Baker, Michael Oellermann, Fathima I. Iftikar, Anthony J.R. Hickey, Hans O. Pörtner

Compensation capacities for ocean acidification in the Austral nototheniid *N. angustata*

(*to be submitted*)

Together with the second and third author, the candidate planned the concept of this study. The candidate carried out the experiments together with the second author and in cooperation with the third and fourth author. The second author and the candidate contributed equally to realisation and writing of this manuscript.

Publication V

Anneli Strobel, Hans O. Pörtner, Felix C. Mark (2013)

Metabolic capacities in relation to temperature and hypercapnia in cephalopods from various climate zones

American Journal of Physiology – Regulatory, Integrative and Comparative Physiology

(in review)

The experiments were designed together with the co-authors, all experiments and data analysis was carried out by the candidate. The manuscript was written by the candidate, and revised together with the co-authors.

The candidate is co-author on two further publications that are included in the Appendix.

Publication AI

Felix C. Mark, Magnus Lucassen, **Anneli Strobel**, Esteban Barrera-Oro, Nils Koschnick, Lorenzo Zane, Tomaso Patarnello, Hans O. Pörtner, Chiara Papetti

Mitochondrial Function in Antarctic Nototheniids with ND6 Translocation

PLoS ONE 7(2): e31860

The concept of this study was developed by the two first authors and the last author. The experiments were carried out in close cooperation by the first, second, fifth and last author

and the candidate. The draft of the manuscript was written by the first author and revised together with all authors.

Publication AII

Katja Mintenbeck, Esteban R. Barrera-Oro, Thomas Brey, Ute Jacob, Rainer Knust, Felix C. Mark, Eugenia Moreira, **Anneli Strobel**, Wolf E. Arntz

Impact of Climate Change on Fishes in Complex Antarctic Ecosystems

Advances In Ecological Research, Vol. 46, 2012, pp. 351-426

The first author developed the concept of this review and wrote the outline of the manuscript. All other authors including the candidate contributed equally to the design and realisation of this review.

PUBLICATION I

Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic notothenиid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*

Anneli Strobel, Martin Graeve, Hans O. Pörtner, Felix C. Mark

2013

Plos One

(in revision)

Plos One

Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*

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Running head:

Mitochondrial complex function in Antarctic fish

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Abstract

Antarctic notothenioid fish are characterized by their evolutionary adaptation to the cold Southern Ocean, which is putatively associated with low acclimation capacities, making them highly sensitive to environmental change. This study compares the capacities of mitochondrial acclimation to ocean warming and acidification between the Antarctic nototheniid *Notothenia rossii* and the sub-Antarctic *Lepidonotothen squamifrons*, which share a similar ecology, but different habitat temperatures. After acclimation of *L. squamifrons* to 9°C and *N. rossii* to 7°C (normocapnic/ hypercapnic - 0.2 kPa CO_2) for 4-6 weeks, we compared the capacities of their mitochondrial respiratory complexes I (CI) and II (CII), their P/O ratios, proton leakage rates and mitochondrial membrane fatty acid compositions.

Our results reveal generally elevated CI capacities after warm acclimation, with higher total rates in *L. squamifrons* than in *N. rossii*. CII respiration rate was reduced in warm acclimated *L. squamifrons* and in both cold and warm hypercapnia acclimated *N. rossii*. Membrane unsaturation was not altered by warm or hypercapnia acclimation in both species, but membrane fatty acids of warm acclimated *L. squamifrons* were less saturated than in warm normocapnia/ hypercapnia acclimated *N. rossii.* Proton leak capacities were elevated in both cold and warm hypercapnic *N. rossii*.

We conclude that warm acclimation and chronic hypercapnia reduce mitochondrial capacities, which might be partially compensated for by higher CI activities supported by enhanced utilization of anaplerotic substrates (via oxidative decarboxylation reactions) feeding the citrate cycle. *L. squamifrons* possesses higher relative CI capacities than *N. rossii*, which may facilitate the usage of energy efficient NADH-related substrates under conditions of elevated energy demand, possibly induced by ocean warming and acidification. The observed adjustments of electron transport system complexes with a higher flux through CI under warming and acidification suggest a metabolic acclimation potential of the sub-Antartctic *L. squamifrons,* but only limited acclimation capacities for *N. rossii.*

Key words

Electron transport system, tricarboxylic acid cycle (TCA), mitochondrial complexes, proton leak, membrane lipids, fatty acids, hypercapnia, temperature

Introduction

Rising temperatures and P_{CO_2} values around the Antarctic Peninsula [1-3] warrant investigation of the physiological flexibility of Antarctic species to respond to these environmental changes [4]. Adaptations of Antarctic teleost fish to their cold environment include for example higher mitochondrial densities and changes in mitochondrial christae surface [5,6], as well as higher levels of unsaturated fatty acids in the biological membranes (termed 'homeoviscous adaptation') [7-9], when compared to temperate zone fish.

Mitochondria are suggested to play a central role in defining the thermal responses of aerobic energy metabolism of ectothermic animals [10,11]. Only few studies have investigated the effects of warming on the contribution of the different respiratory complexes to mitochondrial state III respiration [12,13]. They reported limitations in complex I (CI, NADH dehydrogenase) respiration at higher temperatures in more stenothermal species of crustaceans and temperate fish. Conversely, variability in CI contribution can be an indicator for eurythermy in ectothermal fish [13]. This indicates an important role for CI capacities in setting thermal tolerances of both invertebrates and vertebrates and makes it an important parameter for the comparison of acclimation capacities between fish species.

A recent study on the physiological function of the electron transport system (ETS) complexes I and II (CII, succinate dehydrogenase) in the Antarctic fish *N. rossii* and *N. coriiceps* presents a functioning CI despite translocation of its coding gene (*ND*6, [14,15]), with a higher thermal sensitivity for *N. rossii* [16]. Furthermore, they reported a marginally increasing CI contribution to state III respiration with rising temperatures in *N. rossii,* and an increasing CII contribution in *N. coriiceps,* suggesting differences in mitochondrial responses towards warming between the two species.

Protons leaking through the inner mitochondrial membrane without concomitant ATP production account for a significant amount of the metabolic rate in isolated cells (20-25%) [17-20]. Typically, proton leak is adjusted in parallel to changes in metabolic rate, in that it is increased with rising standard metabolic rate and mitochondrial state III respiration, e.g. during acute thermal challenges [21,22]. These adjustments are driven by modifications in the ETS activity [23], and a higher proton leakage would therefore result in reduced mitochondrial capacities and P/O ratios (amount of ATP produced per total oxygen consumed) at higher temperatures [18,24]. Thus, at a higher temperature more oxygen is required by the ETS to maintain ATP supply, which has been found in ectothermal invertebrates [25,26], and vertebrates, such as temperate [13] and Antarctic fish [22,27].

Additionally, temperature changes (both cold and warm) can modify saturation or fatty acid composition of the membrane phospholipids [7,17,21,28]. This may affect various membrane-associated proteins and processes, such as ETS complexes or altered proton permeability [7,29], up to a complete loss of mitochondrial function [30]. For example, a recent study of long-term warm-acclimated trout (*Oncorhynchus mykiss*) reported a restructuring of membrane phospholipid classes, but a limited effect on membrane desaturation [31]. Therefore, acclimation-induced modulations in the fatty acid composition of mitochondrial membranes may become a critical aspect under altered environmental conditions.

Some studies investigated the temperature or hypercapnia acclimation capacities in Antarctic fish at the whole animal level, however, little is known about the biochemical mechanisms involved [32-34]. Most studies at the mitochondrial level in fish address mitochondrial proliferation, changes in cristae volume or enzyme capacities (e.g. [35] for review; [36]). A few relate to mitochondrial respiration in Antarctic fish during acutely increasing temperature (e.g. [22,27,37], and only for the extreme stenotherm Antarctic *Trematomus bernacchii*, an unaffected mitochondrial metabolism is reported after two weeks warm exposure [38]. To our knowledge, very few studies have included the effect of long-term hypercapnia acclimation at whole animal level [39,40] and only one the mitochondrial level [40].

The nototheniid Antarctic fish species *N. rossii* and *L. squamifrons* (Notothenioidei, Perciformes) are frequently found in coastal Antarctic communities [41-43]. Both species are similar in terms of their ecology [44], but strongly differ in their geographical distribution, and therefore environmental temperature exposure. The sub-Antarctic *L. squamifrons* faces summer temperatures up to 3.5°C, while the more southerly *N. rossii* experiences maximum habitat temperatures of 2°C during summer. This makes them excellent models to compare physiological acclimation capacities towards increased temperatures and PCO_2.

We hypothesize that due to its distribution in warmer waters, the sub-Antarctic fish *L. squamifrons* possesses higher thermal acclimation capacities than the Antarctic fish *N. rossii.* Thus, the first aim of the study was to compare the effect of long-term warm-acclimation (4-6 weeks; *L. squamifrons*: 9°C, *N. rossii*: 7°C) on liver mitochondrial capacities between these two species. The second aim of this study was to compare effects of warm (7°C) and/or hypercapnia acclimation (5 weeks; 0.2 kPa CO_2) on liver mitochondria of *N. rossii*. In our analysis, we focused on the contribution of the mitochondrial respiratory complexes I and II, P/O ratio and proton leakage in *N. rossii* and *L. squamifrons*. In particular, we measured

mitochondrial respiration related to mitochondrial fatty acid composition, the two complexes, and leak respiration (state IV^+, after inhibition with oligomycin) at three acute assay temperatures of 0, 6 and 12°C.

Methods

Animal collection, acclimation and sampling

Animal collection & acclimation. Using baited traps and trammel nets, specimens of *N. rossii* were caught in December 2009 in Potter Cove, King George Island (62°14'S; 058°41'W) during the Antarctic summer season (seawater temperature 0.8°±0.9°C, salinity 33.5±0.2 psu).

For the acclimation trials (29-36 days), animals were randomly selected and exposed to:

1) 1°C, 0.04 kPa CO_2 (control group, *n*=9, mass 155-804 g; total length 25-39.4 cm)
2) 1°C, 0.2 kPa CO_2 (cold hypercapnic group, *n*=10, mass 144-510 g; total length 23.8-32.8 cm)
3) 7°C, 0.04 kPa CO_2 (warm normocapnic group, *n*=5, mass 151-412 g; total length 23.6-33.9 cm)
4) 7°C, 0.2 kPa CO_2 (warm hypercapnic group, *n*=10, mass 137-504 g; total length 21.4-31.3 cm).

Fish were acclimated in well-aerated 150 liter tanks, in turn fed by a 150 liter header tank. Acclimation temperature was controlled using a 250 W heating element (Jaeger, EHEIM GmbH, Germany), and a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). For the CO_2 acclimations, PCO_2 was regulated by an iks aquastar system (iks ComputerSysteme GmbH, Germany). pH of all acclimation systems was measured daily with a WTW 340i pH meter (WTW, Germany. Electrode: WTW SenTix HWS) and calibrated daily with NIST certified buffer (WTW, Germany). Total CO_2 (C_{CO2}) in the seawater was determined with a carbon dioxide analyser (Corning 965, CIBA, Corning Diagnostics, UK). Seawater carbonate chemistry was calculated with the measured pH_{NIST} and C_{CO2} using the CO2sys software [45]. For details on seawater physicochemistry, see [40]. Animals were fed to satiation every other day with chopped fish.

Sub-Antarctic *L. squamifrons* were caught in February 2011 during RV Polarstern cruise ANT XXVII/3 by means of bottom trawls at 300 m water depth off South Georgia (53°24.54'S; 42°40.55'W). Animals were kept in well-aerated 150 liter tanks (salinity

34.4±0.15 psu) in aquaria containers on board of RV Polarstern. Animals were kept for 39-46 days at:

1) 2°C±0.45, 0.04 kPa CO_2 (control; *n*=7, mass 182.0 – 292.0 g, standard length 22.0 – 25.4 cm)
2) 9°C±0.26, 0.04 kPa CO_2 (warm acclimation; *n*=9, mass 107.4 – 255.2 g, standard length 19.8 – 24.9 cm).

Temperature was maintained with a 250 W heating element (Jaeger, EHEIM GmbH, Germany) controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). Fish were fed to satiation every other day with isopods.

Sampling & Ethics statement. Animals were anaesthetised with 0.5 g/l tricaine methane sulphonate (MS 222), and the liver and the heart excised. After that, animals were killed by a spinal cut behind the head plates. All sampling of fish was conducted according to the ethics and guidelines of German law. The experiments have been approved according to § 8 animal welfare act (18.05.2006; 8081. I p. 1207) by the veterinary inspection office 'Senatorin für Arbeit, Frauen, Gesundheit, Jugend und Soziales, Abt. Veterinärwesen, Lebensmittelsicherheit und Pflanzenschutz', Bahnhofsplatz 29, 28195 Bremen, Germany, under the permit number Az.: 522-27-11/02-00 (93) on January 15th, 2008 (permit valid until Jan 14th 2013).

Mitochondria isolation, respiration assays

Isolation of liver mitochondria. In both fish species, the liver was cleaned of blood and total liver weight was taken before a subsample of liver tissue was taken, weighed and washed in 5 ml/g tissue ice-cold wash buffer (80mM sucrose, 85mM KCl, 5mM EGTA, 5mM EDTA, 50mM HEPES, pH 7.1 at 20°C). Then, the liver tissue was cut into small pieces, suspended in 10 volumes ice-cold isolation buffer, and then put into a 30 ml Potter-Elvehjem glass homogenizer (VWR International, Germany) and slowly homogenised with three strokes at 80 revolutions/ minute. The homogenate was centrifuged (600 g, 10 min, 0°C), the supernatant collected and the pellet resuspended in isolation buffer and centrifuged again. Joined supernatants were centrifuged for 10 min at 11,000 g (0°C). After discarding the supernatant, the pellet was resuspended in isolation buffer and centrifuged again. In the last step, supernatant was discarded again, and the pellet was resuspended in ice-cold mitochondrial assay buffer (80 mM sucrose, 85 mM KCl, 5 mM KH_2PO_4, 50 mM HEPES,

1% w/v BSA (fatty acid free), pH 7,1 at 20°C) at a dilution of 1 ml/g initial liver weight. This mitochondrial preparation was kept on ice and away from light. The mitochondrial protein concentration was determined according to Bradford [46] using a bovine serum albumin (BSA) standard, also accounting for the protein content of the mitochondrial assay buffer.

Respiration assay - N. rossii. Measurements were carried out in assay buffer with a final volume of 1200 µl with mitochondrial concentrations adjusted to about 3 mg mitochondrial protein per ml, at 0, 6, and 12±0.1°C, respectively. Chamber temperature was maintained with a thermostat (LAUDA, Germany). Initial respiration was recorded and malate and pyruvate added to a final concentration of 1.3 mM and 1.6 mM, respectively, as substrates for complex I (CI, state II). Then ADP (final conc. 0.16 mM) was added to measure state III (max. slope) and state IV (ADP depleted) respiration. After that, CI was inhibited with 0.01 mM rotenone (state IV^{+}) and 1.6 mM succinate added (state II respiration, complex II (CII)) followed by 0.16 mM ADP (state III and IV after ADP exhaustion, complex II). State IV^{+} was initiated with 1.3µg/ml oligomycin and proton leak titrated stepwise with 0.1 mM malonate. Finally, mitochondria were uncoupled with 0.05 mM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).

Respiration assay - L. squamifrons. Respiration of each liver mitochondrial sample was measured at 0, 6 and 12°C in 2 ml assay medium + 300 U/ml catalase (for reoxygenation with hydroxen-peroxide), in glass-chambers of an Oroboros Oxygraph-2k^{TM} respirometer (Oroboros Instruments, Austria). The mitochondrial respiration was converted to nmol O_2*mg^{-1}*min^{-1}. Resting respiration (state II) was measured with CI substrates, 2 mM glutamate, 1 mM malate and 1 mM pyruvate. State III respiration of CI was induced by 0.4 mM ADP, state III respiration of CI and II by adding 2 mM succinate and 0.1 mM ADP. Leak respiration (state IV^{+}) was evaluated by adding 0.002 µg/ml oligomycin; stepwise titration with the uncoupler FCCP (2 mM stock) revealed maximum capacity of the electron transport system. After inhibition of CI with 5 µM rotenone (state III*u* of CII), non-mitochondrial respiration (residual oxygen consumption, ROX) was detected by adding 2.5 µM antimycin A, and all values were ROX corrected later on in the data analysis.

Lipid extraction

Mitochondrial membrane lipids of control/acclimated *N. rossii* and *L. squamifrons* were extracted after Folch [47]. The extract of liver mitochondria was diluted with 3 ml methylene chloride/methanol (2:1). Following ultra-sonication (20°C, 10% of power (Sonorex Digital 10P, Bandelin electronic GmbH&Co, Germany), 10 min), the extraction mixture was further diluted with 2 ml of 0.8% KCl and sonicated again as described above. Then, the mixture was centrifuged (5 min at 1000 rpm) and the separated, aqueous phase carefully removed. After complete evaporization with nitrogen, the raw extract was resuspended with 250 µl hexane and 1 ml of derivatisation reagent (methanol, 3 % H_2SO_4) and incubated at 80°C for 4 h. After the incubation, the solution was dissolved in 4 ml of water and the lipids separated with 3 x 3 ml hexane. Then, the hexane was completely evaporated under nitrogen and the lipid extracts resuspended in 50 µl hexane prior to analysis. The samples were analysed using gas-chromatography with a flame ionization detector (Agilent 6890N GC, Agilent Technologies, USA).

The unsaturation index (UI) of the mitochondrial membranes was calculated following Grim [48] according to the formula:

$$\mathrm{UI} = \sum_{n=24}^{n=0} n * \% \text{ of fatty acids with } n \text{ double bonds.} \quad (1)$$

Data and statistical analysis

All data were tested for outliers at the 95% significance level using Nalimov's test [49] as well as for normality (Kolmogorov-Smirnov) and homogeneity of variance. Statistical differences in mitochondrial state III/ leak respiration and P/O ratio between assay temperatures (0, 6 and 12°C), and mitochondrial membrane lipid composition (different lipid classes and membrane unsaturation) was evaluated by unpaired t-test/ analysis of variance (ANOVA) followed by a Tukey test. Values of the acclimated animals were compared to the control group. All data are presented as means ± standard error of the mean (SEM). Differences were considered significant if $p \leq 0.05$.

Results

Complex I/ II contribution to mitochondrial state III respiration

Here we contrast the effects of acute changes in mitochondrial assay temperature with those of long term changes in temperature and CO_2 levels during whole animal acclimation.

N. rossii. State III respiration in all groups comprised 21-41% CI and 59-79% CII. In the control group, mitochondrial state III respiration increased with rising assay temperature and CI and CII respiration were significantly elevated at 12°C in comparison to the respective CI and CII respiration in the 0°C assay (habitat temperature of the fish; Figure 1). In the warm normocapnic *N. rossii*, state III respiration showed a slower acute rise with increasing assay temperature, but was not significantly lower compared to the control group. Only CII respiration was significantly elevated at 6°C in comparison to 0°C.

In the cold hypercapnic group, CI and CII respiration increased significantly with rising assay temperature. However, total state III respiration at 6 and 12°C was significantly lower than in control animals at these assay temperatures (Figure 1). Similar to the cold hypercapnic group, state III respiration in the warm hypercapnic group rose slightly with assay temperature, and total state III respiration was lower than in the control animals (Figure 1). In contrast to the control group, all acclimated *N. rossii* showed a higher CI contribution to total state III respiration, reflected by a significantly elevated mean CI/CII ratio (Figure 5) in the warm normocapnic and cold hypercapnic groups.

L. squamifrons. In control and warm-acclimated *L. squamifrons*, CI and CII state III respiration rose significantly with rising assay temperatures (0-12°C, Figure 2). In the warm-acclimated group, both CI and CII respiration rates were significantly lower than in the control group at 12°C assay temperature.

In both groups, CI contributed increasingly to total state III respiration with rising assay temperature (control CI: 0°C-40%, 6°C-54%, 12°C-64%; warm normocapnic CI: 0°C-45, 6°C-52%, 12°C-75%). This effect was significantly stronger in the warm acclimated fish, and also reflected in a significant rise in the CI/CII ratio (Figure 5) due to warm-acclimation. At warmer assay temperatures, the CI/CII ratios of *L. squamifrons* were significantly higher than in *N. rossii*.

P/O ratio and RCR$^+$

N. rossii. In all control/ acclimation groups, P/O ratios were higher for CI than for CII (Figure 3), and stable over the whole acute thermal range. The mean P/O ratios for each group (over all 3 assay temperatures, 0, 6 & 12°C) were a) CI: control 2.49±0.12, warm normocapnic 3.00±0.42, cold hypercapnic 3.34±0.16, warm hypercapnic 2.42±0.04; b) CII: control 1.86±0.08, warm normocapnic 2.33±0.21, cold hypercapnic 1.69±0.05, warm hypercapnic 1.82±0.11. In the cold hypercapnic group, the P/O ratio for CI related respiration (3.34) was significantly higher than for CII related respiration (1.69) in comparison to the control *N. rossii*.

The respiratory control ratio (mean RCR$^+$ over all three assay temperatures, calculated as state III/ state IV$^+$ (oligomycin)) was significantly reduced in the cold hypercapnic (4.82±0.4) and the warm hypercapnic (4.30±0.6) compared to the control group (6.05±0.2), caused by lower state III respiration rates and slightly elevated proton leak rates (see below).

L. squamifrons. The RCR$^+$ of the warm-acclimated *L. squamifrons* (6.42±1.1) was similar to the control animals (8.25±1.2). The RCR$^+$ of *L. squamifrons* was higher than in *N. rossii,* but not significantly different between the two species.

Proton leak in N. rossii and L. squamifrons

In all control and acclimation groups of *N. rossii* and *L. squamifrons*, the capacities for proton leak (state IV$^+$) rose with increasing assay temperature and in parallel to increasing state III respiration. In the control and warm-acclimated *L. squamifrons*, net leak respiration was significantly elevated at 12°C above those in the 0°C assays (Figure 4).

In the cold/ warm hypercapnic *N. rossii*, the % fraction of state IV$^+$ respiration in relation to state III respiration tended to be higher (significant only in the warm hypercapnic group, 27.1±2.1%) compared to the *N. rossii* control group (20.4±2.1%), while it was lower in the warm normocapnic group (17.5±1.7%) (Table 1). In control *L. squamifrons*, mean state IV$^+$ fraction of 12.77±1.1% represented a significantly lower fraction of state III respiration than in control *N. rossii*. The mean percent fraction of state IV$^+$ in the warm-acclimated *L. squamifrons* (12.83±2.2%) was significantly higher than in their control group.

Lipid composition of mitochondrial membranes

Mitochondrial membrane fatty acid composition influences mitochondrial membrane permeability with consequences for ETS function and proton leakage. The mitochondrial membrane of *N. rossii* control animals had significantly more saturated and less n-3 fatty acids than *L. squamifrons* control animals (Table 2). In the cold and warm hypercapnic *N. rossii,* the mitochondrial membranes consisted of more poly-unsaturated fatty acids (PUFAs) than in the control. The unsaturation index (UI) was not altered by either warm and/ or hypercapnia acclimation, neither in *N. rossii*, nor in *L. squamifrons*. However, UI of the warm normocapnia acclimated *L. squamifrons* was significantly higher than in the warm normocapnia/ hypercapnia acclimated *N. rossii.*

Discussion

In this study, we used the contributions of CI (NADH dehydrogenase) and CII (Succinate dehydrogenase) to mitochondrial state III respiration as indicators of temperature acclimation capacities in the Antarctic fish *N. rossii* and the more sub-Antarctic fish *L. squamifrons*. Additionally, we focused on the effect of long-term cold/warm hypercapnia acclimation on mitochondrial function in *N. rossii.*

Warm normocapnic acclimated N. rossii vs. L. squamifrons

CI plays an important role in aerobic metabolism, as it creates a major amount of the protonmotive force used for ATP production in vertebrates [50]. In the *N. rossii* control group, CI comprised about 25% of state III respiration, which equals to a CI/CII ratio of 0.3 (Figure 5), and is coherent with CI/CII ratios found in *N. rossii* and *N. coriiceps* at their habitat temperature [16].

According to the theoretical stoichiometry for the P/O ratio, which is 2.5 ATP (CI) *vs.* 1.5 ATP (CII) per pair of electrons translocated/ mol O consumed [51], the P/O ratio in the control *N. rossii* were 2.5 (CI) and 1.8 (CII). These values, which were stable over the whole thermal range investigated (Figure 3), support a high thermal stability for CI and CII in *N. rossii* at habitat temperature, similar to findings in the Antarctic fish *L. nudifrons, N. coriiceps* and *N. rossii* [16,22]. The constant maximum proton leak capacities as a percentage of total state III respiration (18-22%; see Table 1 and Figure 4) [18,51] at all assay temperatures

further indicate full functional integrity of coupled mitochondria across a range of temperatures.

Although total state III respiration of the warm normocapnia acclimated *N. rossii* was similar to the control group, CI contribution to state III respiration was increased at all assay temperatures, leading to a higher CI/CII ratio than in the control fish (0.5 in warm-acclimated fish *vs*. 0.3 in control fish, Figure 5).

In terms of ATP production per mol of substrate, CI is more efficient than CII. Per NADH, 2 electrons are transported via CI and CIII to CIV, paralleled by 4 protons pumped through CI and CIII each, and 2 protons through CIV = 10 protons pumped per NADH. Oxidation of 1 pyruvate yields 4 NADH, which equals 40 protons being pumped by CI, III and IV. Oxidation of 1 succinate equals transport of 2 electrons through CIII to CIV and a total of 6 protons being pumped through complex III and IV [16,24,50].

At least under *in vitro* conditions, increased CI capacities after warm acclimation become obvious. *In vivo*, CI and CII turnover are connected by the TCA-cycle, and it would take further sources of NADH from non-TCA-cycle related origin to fully use the enhanced CI capacities. For example, an increased metabolization of glutamate and aspartate could result in an anaplerotic enhancement of the TCA-cycle intermediates and also in a higher amount of NADH. During thermal challenges, proton leakage is frequently increased in parallel to standard metabolic rate, and thus could result in reduced mitochondrial capacities [22,52].

Therefore, an enhanced flux through CI (more H^+ pumped per electron) in warm-acclimated *N. rossii* may act as a compensation in order to increase membrane potential and to maintain phosphorylation capacities.

This was also reflected by the slightly elevated P/O ratios for CI at warmer assay temperatures (mean P/O over all three assay temperatures: 3.0 in the warm normocapnic group, higher than 2.5 of the control group) and thus an enhanced energetic efficiency of mitochondrial oxidative capacity in warm-acclimated *N. rossii*. In fact, triplefin fish (*Bellapiscis medius*) also increase the flux through CI with warming to meet the elevated (uncompensated) whole animal energy demand at warmer temperatures [13].

Also in Antarctic fish, warmer ambient temperatures also lead to acute increments in metabolic rates (and thus ATP demand) [53,54]. This increase in energy demand can be partly or fully reversed during acclimation, depending on the fish species (e.g. [55,56]). Long-term acclimation to 7°C only led to an incomplete compensation of whole animal routine metabolic rate in *N. rossii* (Precht Type III) [40]. Maintenance of an elevated routine metabolic rate at

warmer temperatures may thus be supported by higher a mitochondrial phosphorylation efficiency, as described above.

As already mentioned above, mitochondrial responses to elevated energy demand can involve a slight reduction in the level of proton leak [28] in relation to state III respiration in warm-acclimated fish compared to controls (warm-acclimated: 17.5±1.7% *vs.* control: 20.4±2.1%; Table 1). Despite the reduced proton leak fraction in warm normocapnia acclimated *N. rossii*, the mitochondria of these fish did not show any sign of homeoviscous adaptation (Table 2), fatty acid composition remained unchanged. Similarly, warm or cold exposed sea bass *Dicentrarchus labrax* showed unaffected mitochondrial membrane composition while mitochondrial respiration was affected by temperature [57]. Lower proton leak rates could therefore be mediated by modifications in either the activities or abundances or both of proteins involved in membrane uncoupling, such as uncoupling proteins (UCPs) or adenine nucleotide translocase (ANT) [58], and the associated regulation of the P/O ratio [59,60]. These proteins contribute to total proton leakage to a much larger extent (95%) than any protons leaking directly through the membrane [58]; and UCP expression in fish is clearly temperature dependent [20,61]. Although the molecular mechanisms which lead to a higher CI contribution in total state III respiration after warm acclimation are not clear at present, our data reveal that compensation of metabolic rate after warm acclimation of *N. rossii* may be accomplished by an improved P/O ratio due to increased CI activity (per milligram mitochondrial protein), generating a higher membrane potential than in the control fish.

In the sub-Antarctic *L. squamifrons* control group, the increasing state III respiration with rising assay temperature was mainly driven by an increase in net CI respiration, while CII respiration was not elevated concomitantly at all temperatures. This was reflected in the increasing CI/CII ratio (Figure 5) with rising acute temperature, which was even more pronounced in the warm-acclimated group. Thus, warm-acclimated *L. squamifrons* shows a high capacity to increase flux through CI, potentially to compensate for an elevated energy demand and proton leakage at higher temperatures.

In the warm-acclimated *L. squamifrons,* CI and CII respiration rates at 12°C assay temperatures were significantly reduced compared to the control group at 12°C. Similar to other studies, the lower oxidative capacity in warm-exposed fish can relate to lower mitochondrial content (mitochondrial proliferation), changes in the activity of membrane-bound proteins, such as lower cytochrome *c* oxidase activity in warm-exposed carp (*Cyprinus carpio*) [62] and eelpout (*Zoarces viviparus*) [22], paralleled by a significantly elevated

proton leak capacity in relation to state III respiration (control *L. squamifrons*: 12.77±1.1%, warm-acclimated: 18.83±2.2%; Table 1). The higher proton leak fraction in the warm-acclimated fish could partially be related to the thermal stimulation of UCPs (see above; [63]) or to the levels of PUFAs and n-3 FAs elevated after warm acclimation, which are factors that can increase the amount of protons leaking through the inner mitochondrial membrane [17] (Table 1). As a consequence, state IV^+ respiration rate of the warm-acclimated *L. squamifrons* was similar to that of the control group but showed a more pronounced increase during acute warming (Figure 4). This was also mirrored in the coupling efficiency, which was higher in the control group (RCR^+ 8.2±1.2) than after warm acclimation to 9°C (RCR^+ 6.4±1.2). The higher CI contribution in warm–acclimated *L. squamifrons*, suggests a compensation for the increased state IV^+ respiration in the sub-Antarctic species, similar to that of the warm normocapnic acclimated *N. rossii* (see above and Figure 2).

In both *L. squamifrons* and *N. rossii*, UI of the mitochondrial membranes was not altered by warm acclimation. In many temperate zone fish, the percentage of unsaturated fatty acids increases in response to cold temperatures [64], e.g. in goldfish (*Carassius auratus*) [65] and shorthorned sculpin (*Myoxocephalus scorpius*) [66]. However, this pattern cannot be generalized for all phylogenetic groups. Similar to *L. squamifrons* and *N. rossii,* unsaturation of heart and liver membrane lipid composition in sea bass (*Dicentrarchus labrax*) is not affected by temperature [57]. The mismatch between unsaturation and acclimation temperature observed in our study might relate to a limited ability for homeoviscous adaptation in both fish species and could in turn hamper the function of membrane bound proteins (e.g. [62]) in a warming Southern ocean.

Considerable differences exist between the two nototheniid fish species in the contributions of respiratory complexes to total mitochondrial respiration. Control *L. squamifrons* showed a larger dynamic response (Q_{10} 3.1, range 0-12°C) in mitochondrial respiration during acute temperature rise than control *N. rossii* (Q_{10} 1.7, range 0-12°C) and a greater stability of the CI contribution after warm acclimation. A study on several temperate triplefin fish found that high mitochondrial capacities are related to a high tolerance of the whole animal to temperature change and hypoxia. CI contributions were reduced with acutely rising temperatures in more stenotherm as compared to more eurytherm triplefin fish species [13]. In line with these findings, *L. squamifrons* may possess a generally higher scope for adjustment/ acclimation of their mitochondrial capacities towards changing environmental conditions than *N. rossii*.

In *L. squamifrons*, total state III respiration comprised a significantly higher fraction of CI than in *N. rossii,* indicated by a much higher mean CI/CII ratio (over the whole assay thermal range, per treatment; Figure 5) in the sub-Antarctic (control: 1.4±0.9) than in the Antarctic fish (control: 0.3±0.1). Such a distinctly larger fraction of CI suggests higher mitochondrial capacities in the sub-Antarctic species. Furthermore, the warmer maximum seawater temperatures experienced by *L. squamifrons* may support a higher usage of NADH-linked CI substrates possibly to compensate for a higher energy demand at warmer more variable temperatures. Similarly, warm acclimation led to a higher mean CI/CII ratio in *N. rossii* (0.5±0.2, this study; *L. squamifrons*: 1.9±1.2) and in heat tolerant (eurythermal) triplefin species, as compared to the more stenothermal among the triplefin species, c.f. [13].

Effect of hypercapnia acclimation on N. rossii

In the cold hypercapnia acclimated animals, CII respiration rate was significantly reduced at warmer assay temperatures in comparison to the control group (Figure 1), and also the warm hypercapnia acclimated *N. rossii* showed the same trend. In both groups, this resulted in elevated CI/CII ratios, especially in the mitochondria from the cold hypercapnic fish (mean CI/CII ratio: control 0.3±0.1; cold hypercapnic group 0.5±0.1; warm hypercapnic group 0.4±0.0; Figure 5). Nevertheless, total mitochondrial capacities (state III respiration) were significantly reduced, although CI activities seemed to be elevated after long-term cold/warm hypercapnia acclimation. This relative shift in favour of CI, but lower rates of state III may reflect a role for CO_2 in depressing aerobic scope in response to environmental stress, in similar ways as seen in marine invertebrates [67].

When exposed to acutely elevated ambient PCO_2, teleost fish compensate for this rise via an active extra- and intracellular accumulation of bicarbonate [40,68,69]. A new steady-state in acid-base balance includes permanently elevated bicarbonate and is established within the blood and intracellular milieu [40]. Acid-base regulation may bring about a continuous elevation in energy demand to maintain ion gradients across cellular membranes paralleled by an increase in the abundance of ion exchangers, e.g. Na^+/K^+-ATPase or Na^+/HCO_3^- cotransporter, during acclimation to hypercapnic conditions [70].

The significantly lower rates of CII respiration, coupled to lower CI respiration as well, indicate limitations in mitochondrial metabolism, including the TCA-cycle, as a response to chronic hypercapnia in *N. rossii*. Furthermore, a reduction in complex IV activity of the ETS was reported for *N. rossii* long-term acclimated to a PCO_2 of 0.2 kPa [40],

highlighting the reductions in mitochondrial capacities of these hypercapnia acclimated fish, possibly related to changes in gene expression. An increased energy demand for maintenance of the acid-base balance, combined with a decrease in mitochondrial capacities would support the hypothesis that ambient hypercapnia initiates a decrease in aerobic scope [67].

High bicarbonate levels competitively inhibit citrate synthase function (Figure 6, [71]). During chronically elevated bicarbonate and PCO_2, TCA activity therefore may be reduced. Instead, net oxidative decarboxylation of dicarboxylic acids, such as aspartate and glutamate (after transamination of asparagine/ glutamine) may be enhanced as an anaplerotic mechanism to fuel the TCA-cycle, thereby at least partially displacing the competitive inhibitor bicarbonate (Figure 6, [72,73]). However, these anaplerotic mechanisms may not be sufficient to fully compensate for TCA-inhibition, as reflected by the reduced CII respiration in hypercapnia acclimated *N. rossii.* A similar stimulating effect of acute high bicarbonate concentrations on glutamate, pyruvate or palmitoyl carnitine oxidation is observed in mammalian liver mitochondria [74,75]. These reactions could on the one hand help to reduce the proton load in mitochondria (by proton consumption during oxidative decarboxylation), maintaining bicarbonate concentrations in the mitochondrial matrix. On the other hand, in oxidative decarboxylation reactions NAD^+ is reduced to $NADH+H^+$, which fuels CI. This excess in non-TCA-linked NADH can support the ETS to build up the proton gradient across the inner mitochondrial membrane (Figure 6). As CII does not actively pump protons across the inner mitochondrial membrane, while CI directly supports the proton gradient, the higher CI capacities could be the result of a shift towards a higher amount of NADH, while the TCA-cycle could not maintain full capacities. By this mechanism, mitochondrial capacities of hypercapnia-acclimated *N. rossii* may compensate for a higher ATP demand e.g. to maintain a new acid-base equilibrium, under chronic hypercapnia and at decreased mitochondrial state III capacities, at least to some extent.

As a corollary, exposure to long-term elevated PCO_2 levels involves rearrangements in mitochondrial functions. This may not affect proton leakage, which remained similar to control conditions in cold or warm hypercapnic mitochondria (following an almost linear increase with temperature, Figure 4). However, in light of depressed state III respiration its relative contribution (cold hypercapnic: 25.9±3.2%, warm hypercapnic: 27.1±2.1%; Table 1) to state III respiration may result significantly higher in the hypercapnic animals than in the controls (20.4±2.1%). Nothing is known about the expression of uncoupling proteins, which mediate proton leak to a great extent (see above), under chronic hypercapnia. However, their expression is clearly temperature dependent in Antarctic fish (e.g. up-regulation of UCP2

after warm acclimation of *P. brachycephalum* [76]). According to these capacities for up- or down-regulation as a response to environmental stress, they might be also involved in mediating proton leakage in warm hypercapnia acclimated *N. rossii*. Next to the lower state III respiration in the hypercapnia acclimated fish, this elevation in proton leak capacity can also contribute to the reduced mitochondrial coupling ratio (RCR^+ control: 6.1±0.2, cold hypercapnic group: 4.8±0.4, warm hypercapnic group 4.3±0.6), as commonly seen in animals with highly flexible energy demand [22,52].

Proton leakage is frequently correlated with membrane phospholipid composition, i.e. the UI and involvement of PUFAs [17,28]. Membrane saturation in the mitochondrial extracts of *N. rossii* was not significantly altered by hypercapnia, but a clear trend towards more PUFAs and n-6 FA's was visible in the cold/ warm hypercapnia acclimated animals. The activity of UCPs might also respond to such changes. They may also mediate the effects of long-term elevated P_{CO_2} on other membrane bound proteins, such as cytochrome *c* oxidase [40]. This suggests a remodelling of mitochondrial membrane structure-function relationships following acclimation to long-term hypercapnia, involving proton leakage and reducing mitochondrial coupling capacities. However, these findings are not reflected in whole animal respiration, which remained unaffected in long-term hypercapnia-acclimated *vs.* control *N. rossii* [40]. Overall, our data support limitations in aerobic energy metabolism in the tissues of *N. rossii* chronically exposed to higher P_{CO_2}. Next to the changes in mitochondrial metabolic pathways (see above), they might be partially compensated by a higher mitochondrial volume density, cristae surface or proliferation [6] in order to increase the reduced tissue mitochondrial capacities, an aspect that remains to be explored.

Conclusion

In this study, the variable contribution of CI and CII to mitochondrial state III respiration was found to reflect differential acclimation of the Antarctic fish *N. rossii* and the sub-Antarctic fish *L. squamifrons* to warming and hypercapnia. Long-term warm-acclimation of both *N. rossii* and *L. squamifrons* led to an elevated CI/CII ratio in comparison to their control. The resulting higher contribution of CI to total state III respiration, accompanied by reduced proton leakage capacities (percentual fraction of state III), could contribute to increase membrane potential and enhance mitochondrial oxidative capacity in order to cover for an elevated energy demand in the warmth.

Warm acclimation did not significantly affect the mitochondrial membrane unsaturation index in both species compared to their controls, suggesting a limited ability to react to temperature changes. Nevertheless, warm-acclimated *L. squamifrons* possess more polyunsaturated fatty acids in their mitochondrial membranes than warm normocapnia/ hypercapnia acclimated *N. rossii*, and thus possibly a higher flexibility in their thermal response.

A much higher CI/CII ratio, particularly in warm-acclimated *L. squamifrons,* probably relates to elevated CI capacities in the sub-Antarctic species compared to *N. rossii*. A higher flux through CI, which directly contributes to the proton gradient over the inner mitochondrial membrane, supported by an enhanced utilization of anaplerotic substrates (via oxidative decarboxylation reactions) may result in mitochondria with a higher flexibility to respond to thermal challenges in warm- and hypercapnia acclimated *N. rossii* and, to a larger extent, in *L. squamifrons*. In hypercapnia acclimated fish, high bicarbonate levels may inhibit the TCA-cycle, thus a trend towards non-TCA-linked NADH, used by CI, may partially compensate for the reduced aerobic scope indicated by lower state III capacities to a certain extent. The questions whether these changes are adaptive or not and whether change in mitchondrial densities occur at the same time, remain to be investigated.

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References

1. Turner J, Colwell SR, Marshall GJ, Lachlan-Cope TA, Carleton AM, et al. (2005) Antarctic climate change during the last 50 years. Int J Climatol 25: 279-294.
2. Turner J, Lachlan-Cope TA, Colwell S, Marshall GJ, Connolley WM (2006) Significant warming of the Antarctic winter troposphere. Science 311: 1914-1917.
3. Meredith, PM, King, CJ (2005) Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. Geophys Res Lett 32: L196004.
4. Fuller A, Dawson T, Helmuth B, Hetem RS, Mitchell D, et al. (2010) Physiological Mechanisms in Coping with Climate Change. Physiol Biochem Zool 83: 713-720.
5. Egginton S, Sidell BD (1989) Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. Am J Physiol Regul Integr Comp Physiol 256: 1-9.
6. Johnston IA, Calvo J, Guderley H, Fernandez D, Palmer L (1998) Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. J Exp Biol 201: 1-12.
7. Hazel JR (1995) Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? Annu Rev Physiol 57: 19-42.
8. Dey I, Buda C, Wiik T, Halver JE, Farkas T (1993) Molecular and structural composition of phospholipid membranes in livers of marine and freshwater fish in relation to temperature. Proc Natl Acad Sci 90: 7498-7502.
9. Verde C, Parisi E, di Prisco G (2006) The evolution of thermal adaptation in polar fish. Gene 385: 137-145.
10. Duchen MR (1999) Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. J Physiol 516: 1-17.
11. Poertner HO (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp Biochem Physiol A 132: 739-761.

12. Iftikar FI, MacDonald J, Hickey AJR (2010) Thermal limits of portunid crab heart mitochondria: Could more thermo-stable mitochondria advantage invasive species? J Exp Mar Bio Ecol 395: 232-239.

13. Hilton Z, Clements KD, Hickey AJ (2010) Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. J Comp Physiol B 180: 979-990.

14. Papetti C, Lio P, Ruber L, Patarnello T, Zardoya R (2007) Antarctic fish mitochondrial genomes lack ND6 gene. J Mol Evol 65: 519-528.

15. Zhuang X, Cheng CH (2010) ND6 gene "lost" and found: evolution of mitochondrial gene rearrangement in Antarctic notothenioids. Mol Biol Evol 27: 1391-1403.

16. Mark FC, Lucassen M, Strobel A, Barrera-Oro E, Koschnick N, et al. (2012) Mitochondrial Function in Antarctic Nototheniids with ND6 Translocation. PLoS Porter RK, Hulbert A, Brand MD (1996) Allometry of mitochondrial proton leak: influence of membrane surface area and fatty acid composition. Am J Physiol Regul Integr Comp Physiol 271: R1550-R1560.

18. Brand M (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Exp Gerontol 35: 811-820.

19. Chamberlin ME (2004) Top-down control analysis of the effect of temperature on ectotherm oxidative phosphorylation. Am J Physiol Regul Integr Comp Physiol 287: R794-R800.

20. Jastroch M, Buckingham JA, Helwig M, Klingenspor M, Brand MD (2007) Functional characterisation of UCP1 in the common carp: uncoupling activity in liver mitochondria and cold-induced expression in the brain J Comp Physiol B 177: 743-752.

21. Brookes PS, Buckingham JA, Tenreiro AM, Hulbert AJ, Brand MD (1998) The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition. Comp Biochem Physiol B 19: 325-334.

22. Hardewig I, Peck LS, Poertner HO (1999) Thermal sensitivity of mitochondrial function in the Antarctic Notothenioid *Lepidonotothen nudifrons*. J Comp Physiol B 169: 597-604.

23. Trzcionka M, Withers KW, Klingenspor M, Jastroch M (2008) The effects of fasting and cold exposure on metabolic rate and mitochondrial proton leak in liver and skeletal muscle of an amphibian, the cane toad Bufo marinus. J Exp Biol 211: 1911-1918.

24. Brand MD, Esteves TC (2005) Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. Cell Metab 2: 85-93.

25. Keller M, Sommer A, Poertner HO, Abele D (2004) Seasonality of energetic functioning and production of reactive oxygen species by lugworm (*Arenicola marina*) mitochondria exposed to acute temperature changes. J Exp Biol 207: 2529.

26. Heise K, Puntarulo S, Poertner HO, Abele D (2003) Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. Comp Biochem Physiol C 134: 79-90.

27. Mueller IA, Grim JM, Beers JM, Crockett EL, O'Brien KM (2011) Inter-relationship between mitochondrial function and susceptibility to oxidative stress in red-and white-blooded Antarctic notothenioid fishes. J Exp Biol 214: 3732-3741.

28. Brand M, Chien L, Ainscow E, Rolfe D, Porter R (1994) The causes and functions of mitochondrial proton leak. Biochim Biophys Acta 1187: 132.

29. Hazel JR, Williams E (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog Lipid Res 29: 167.

30. Lee AG (2004) How lipids affect the activities of integral membrane proteins. Biochim Biophys Acta 1666: 62-87.

31. Kraffe E, Marty Y, Guderley H (2007) Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. J Exp Biol 210: 149.

32. Deigweiher K, Koschnick N, Poertner HO, Lucassen M (2008) Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am J Physiol Regul Integr Comp Physiol 295: R1660-R1670.

33. Eckerle LG, Lucassen M, Hirse T, Poertner HO (2008) Cold induced changes of adenosine levels in common eelpout (Zoarces viviparus): a role in modulating cytochrome c oxidase expression. J Exp Biol 211: 1262-1269.

34. Windisch HS, Kathoever R, Poertner HO, Frickenhaus S, Lucassen M (2011) Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways. Am J Physiol Regul Integr Comp Physiol 301: R1453-R1466.

35. Guderley H, St-Pierre J (2002) Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. J Exp Biol 205: 2237.

36. Lucassen M, Koschnick, N., Eckerle, L. G., Poertner, HO (2006) Mitochondiral mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. J Exp Biol 209: 1410-1420.

37. Urschel M, O'Brien K (2009) Mitochondrial function in Antarctic notothenioid fishes that differ in the expression of oxygen-binding proteins. Polar Biol 32: 1323-1330.

38. Weinstein RB, Somero GN (1998) Effects of temperature on mitochondrial function in the Antarctic fish Trematomus bernacchii. J Comp Physiol B 168: 190-196.

39. Enzor LA, Zippay ML, Place SP (2012) High latitude fish in a high CO2 world: Synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids. Comp Biochem Physiol A 164: 154-161.

40. Strobel A, Bennecke S, Leo E, Mintenbeck K, Poertner HO, et al. (2012) Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO2. Front Zool 9: 28.

41. Sosinski J, S. K (1987) Biomass estimate of commercial fish on the shelf of South Georgia by the swept area method. Bull Sea Fish Inst 5-6: 8-14.

42. Duhamel G (1985) Distribution and abundance of fish on the Kerguelen Island shelf. Fifth Congress of European Icthyologists. Stockholm.

43. Casaux R, Mazzotta A, Barrera-Oro E (1990) Seasonal aspects of the biology and diet of nearshore nototheniid fish at Potter Cove, South Shetland Islands, Antarctica. Polar Biol 11: 63-72.

44. Gon O, Heemstra P (1990) Fishes of the Southern Ocean. Grahamstown, South Africa: J.L.B. Smith Institute for Ichthyology. 462 pp.

45. Pierrot D, Lewis E, Wallace DWR (2006) MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a ed. Oak Ridge, Tennessee Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy.

46. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

47. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226: 497-509.

48. Grim J, Miles D, Crockett E (2010) Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic

antioxidants or susceptibility to lipid peroxidation in fish muscle. J Exp Biol 213: 445-452.

49. Noack S (1980) Statistische Auswertung von Menue- und Versuchsdaten mit Taschenrechner und Tischcomputer: De Gruyter; 1980.
50. Efremov RG, Baradaran R, Sazanov LA (2010) The architecture of respiratory complex I. Nature 465: 441-445.
51. Brand MD, Harper ME, Taylor HC (1993) Control of the effective P/O ratio of oxidative phosphorylation in liver mitochondria and hepatocytes. Biochem J 291: 739.
52. Poertner HO, Hardewig I, Peck LS (1999) Mitochondrial Function and Critical Temperature in the Antarctic Bivalve, *Laternula elliptica*. Comp Biochem Physiol A 124: 179-189.
53. Johnston I (2003) Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. Comp Biochem Physiol B 136: 701-713.
54. Mark FC, Hirse T, Poertner HO (2005) Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol 28: 805-814.
55. Franklin CE, Davison W, Seebacher F (2007) Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinki*. J Exp Biol 210: 3068-3074.
56. Robinson E, Davison W (2008) The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption. Polar Biol 31: 317-326.
57. Trigari G, Pirini M, Ventrella V, Pagliarani A, Trombetti F, et al. (1992) Lipid composition and mitochondrial respiration in warm-and cold-adapted sea bass. Lipids 27: 371-377.
58. Azzu V, Brand MD (2010) The on-off switches of the mitochondrial uncoupling proteins. Trends Biochem Sci 35: 298-307.
59. Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, et al. (2001) Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 105: 745-755.
60. Jastroch M, Wuertz S, Kloas W, Klingenspor M (2005) Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis. Physiol Genomics 22: 150-156.

61. Mark FC, Lucassen M, Poertner HO (2006) Thermal sensitivity of uncoupling protein expression in polar and temperate fish. Comp Biochem Physiol D 1: 365-374.

62. Wodtke E (1981) Temperature adaptation of biological membranes: Compensation of the molar activity of cytochrome c oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). Biochim Biophys Acta 640: 710-720.

63. Echtay KS, Esteves TC, Pakay JL, Jekabsons MB, Lambert AJ, et al. (2003) A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. Embo J 22: 4103-4110.

64. Kamler E, Krasicka B, Rakusa-Suszczewski S (2001) Comparison of lipid content and fatty acid composition in muscle and liver of two notothenioid fishes from Admiralty Bay (Antarctica): an eco-physiological perspective. Polar Biol 24: 735-743.

65. Hazel JR (1972) The effect of temperature acclimation upon succinic dehydrogenase activity from the epaxial muscle of the common goldfish (*Carassius auratus* L)-I. Properties of the enzyme and the effect of lipid extraction. Comp Biochem Physiol B 43: 837-861.

66. Guderley H, Johnston I (1996) Plasticity of fish muscle mitochondria with thermal acclimation. J Exp Biol 199: 1311.

67. Poertner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881-893.

68. Brauner CJ, Wang T, Wang Y, Richards JG, Gonzalez RJ, et al. (2004) Limited extracellular but complete intracellular acid-base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*. J Exp Biol 207: 3381-3390.

69. Michaelidis B, Spring A, Poertner HO (2007) Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparu saurata*. Mar Biol 150: 1417-1429.

70. Deigweiher K, Hirse T, Bock C, Lucassen M, Poertner HO (2010) Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. J Comp Physiol B 180: 347-359.

71. Simpson DP (1967) Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. J Clin Investig 46: 225.

72. Poertner HO (1987) Contributions of anaerobic metabolism to pH regulation in animal tissues: theory. J Exp Biol 131: 69-87.

73. Langenbuch M, Poertner HO (2002) Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid-base variables. J Exp Biol 205: 1153-1160.

74. Robinson BH, Oei J, Cheema-Dhadli S, Halperin M (1977) Regulation of citrate transport and pyruvate dehydrogenase in rat kidney cortex mitochondria by bicarbonate. J Biol Chem 252: 5661-5665.

75. Wanders RJA, Mejer AJ, Groen AK, Tager JM (1983) Bicarbonate and the pathway of glutamate oxidation in isolated rat-liver mitochondria. Eur J Biochem 133: 245-254.

76. Mark FC, Lucassen M, Poertner HO (2006) Thermal sensitivity of uncoupling protein expression in polar and temperate fish. Comp Biochem Physiol D 1: 365-374.

Figures

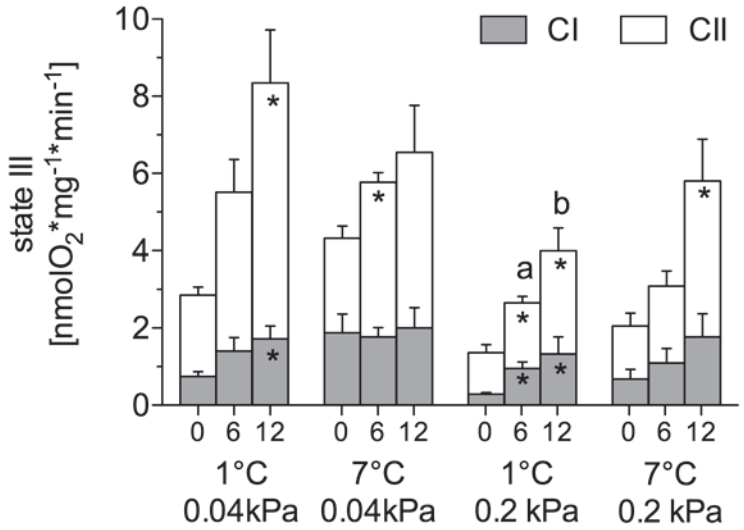

Figure 1: State III respiration rate of liver mitochondria at various assay temperatures of 0, 6, 12°C. Mitochondria isolated from *N. rossii* acclimated to 1°C, 0.04 kPa (control), *n*=9; 7°C, 0.04 kPa (warm normocapnic), *n*=5; 1°C, 0.2 kPa (cold hypercapnic), *n*=10; and 7°C 0.2 kPa (warm hypercapnic), *n*=10. The total state III rate comprises the involement of complex I (CI, grey part of stacked bars) and II (CII, white part of stacked bars). Values are given as means ± SEM. * indicates significantly increased CI or CII state III respiration over the rate at 0°C within a control/ acclimation group ($p<0.05$, t-test); letters (a, b) indicate significant changes in CII state III respiration from the control group at the respective assay temperature ($p<0.05$, t-test).

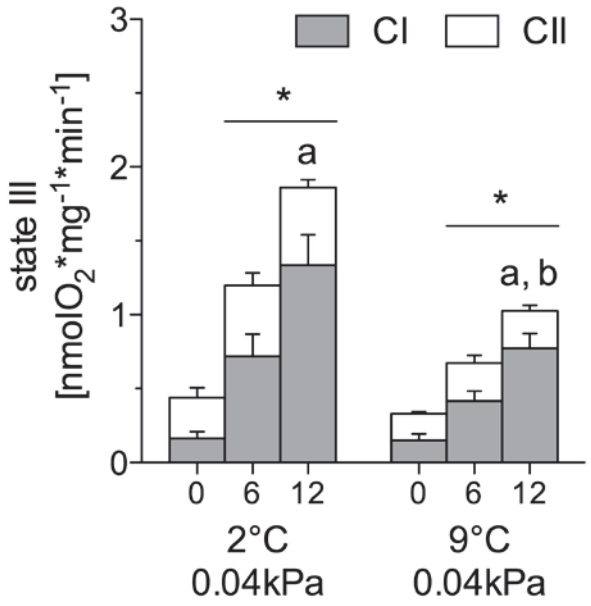

Figure 2: State III respiration rate (isolated liver mitochondria) assayed at 0, 6, 12°C in *L. squamifrons*. State III respiration comprises complex I (CI, grey part of stacked bars) and II (CII, white part of stacked bars) in control (2°C, 0.04 kPa CO_2), *n*=7, and warm acclimated (9°C, 0.04 kPa CO_2), *n*=5, *L. squamifrons*. Values are given as means ± SEM. * depicts a significantly elevated CI and CII state III respiration rate in comparison to the respective rate at 0°C in the control/ acclimation group. [a] denotes a significantly lower CII than CI contribution at the respective assay temperature within a treatment ($p<0.05$, t-test). [b] incidates a significantly lower CI and CII rate in comparison to the control group ($p<0.05$, t-test) at the respective assay temperature.

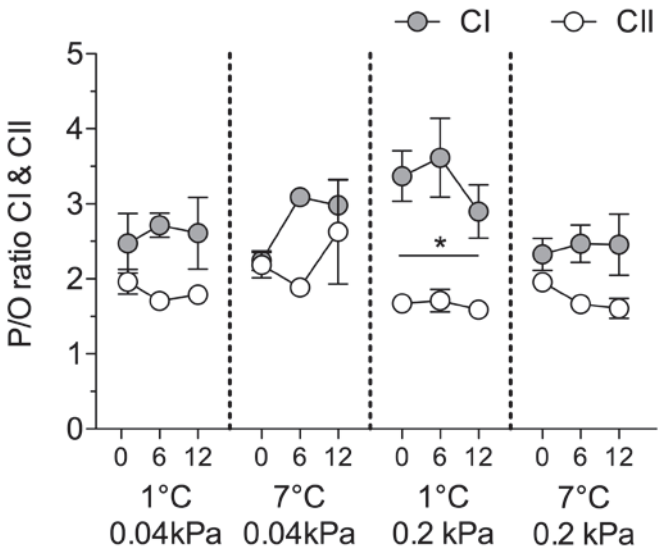

Figure 3: P/O ratio of acclimated *N. rossii*. Ratio of ADP produced per oxygen consumed (P/O ratio) by complex I & II (CI & CII) in *N. rossii* acclimated to 1°C, 0.04 kPa (control), n=9; 7°C, 0.04 kPa (warm normocapnic), n=5; 1°C, 0.2 kPa (cold hypercapnic), n=10; and 7°C 0.2 kPa (warm hypercapnic), n=10. Values are given as means ± SEM. * indicate significantly different P/O ratios at the respective assay temperature within an control/acclimation group (p<0.05, t-test).

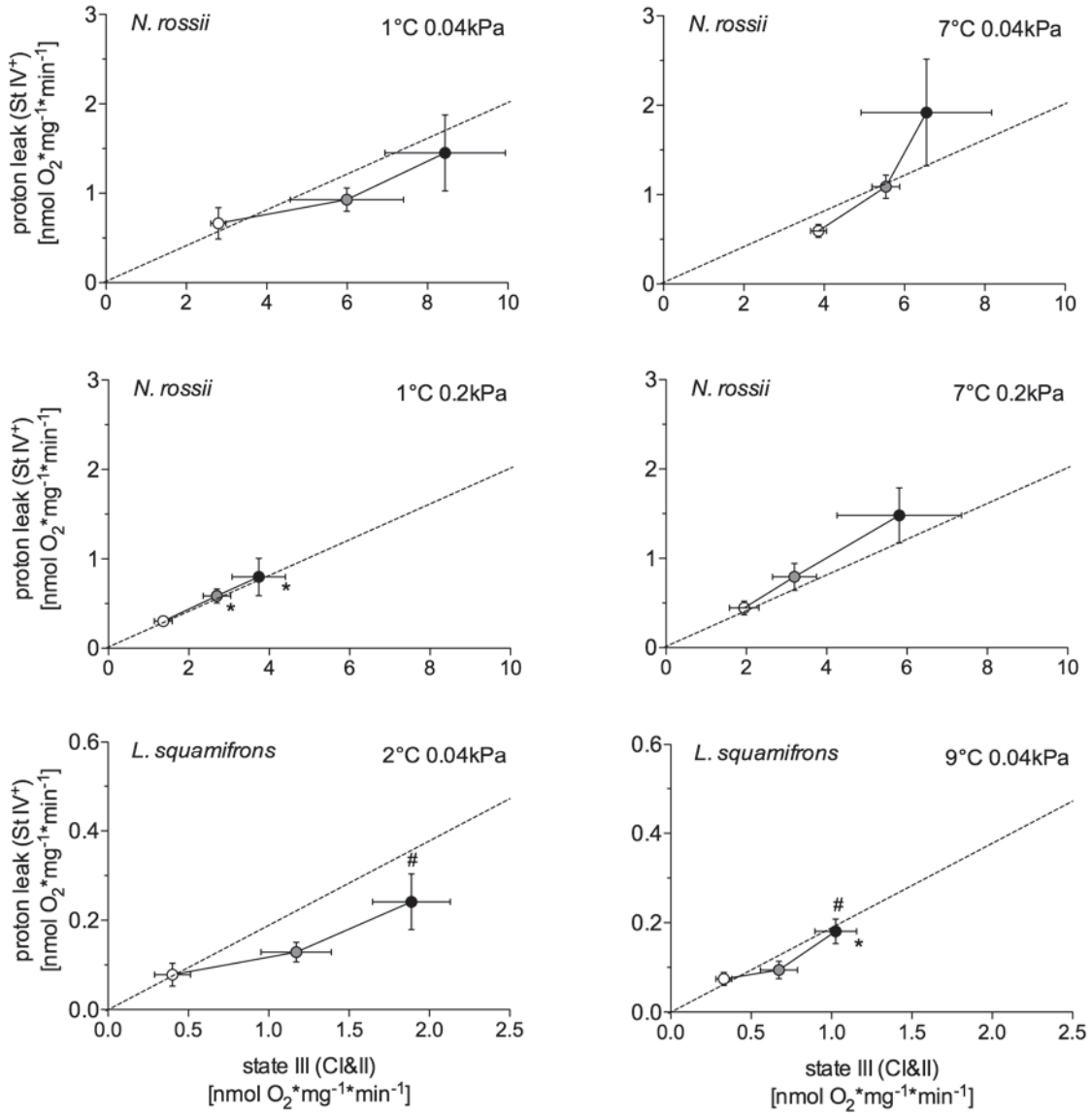

Figure 4: Proton leak (St IV$^+$) plasticity in relation to complex II (CII) in state III respiration. Isolated liver mitochondria from *N. rossii* acclimated to 1°C, 0.04 kPa (control), *n*=9; 7°C, 0.04 kPa (warm normocapnic), *n*=5; 1°C, 0.2 kPa (cold hypercapnic), *n*=10; and 7°C 0.2 kPa (warm hypercapnic), *n*=10, and in mitochondria from control (2°C, 0.04 kPa CO_2, *n*=7) and warm acclimated (9°C, 0.04 kPa CO_2, *n*=5) *L. squamifrons*. White dots are values at 0°C, grey at 6°C and black at 12°C acute assay temperature. Values are given as means ± SEM. * indicates a significant difference of state III respiration at 6°C/12°C assays from the rate at 0°C within a control/acclimation group (p<0.05, t-test). # depicts a significant difference of mitochondrial proton leak from the rate assayed at 0°C within a control/acclimation group (p<0.05, t-test). The dotted line represents 20% leak of the given state III respiration.

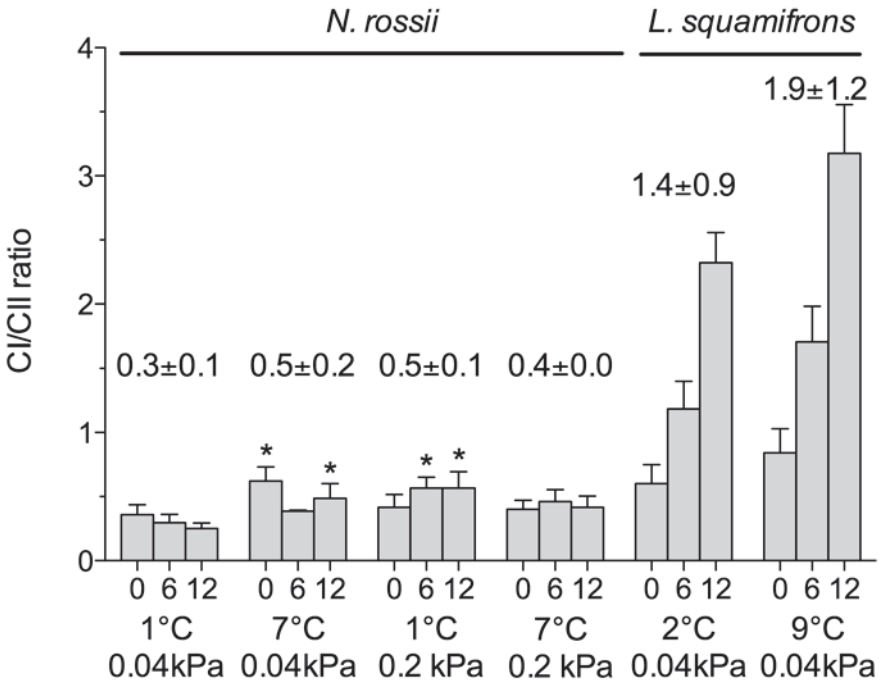

Figure 5: CI/CII ratio in liver mitochondria from warm/ hypercapnia acclimated *N. rossii* and *L. squamifrons*. *N. rossii* acclimated to 1°C, 0.04 kPa (control), *n*=9; 7°C, 0.04 kPa (warm normocapnic), *n*=5; 1°C, 0.2 kPa (cold hypercapnic), *n*=10; and 7°C 0.2 kPa (warm hypercapnic), *n*=10, and in mitochondria from control (2°C, 0.04 kPa CO_2, *n*=7) and warm acclimated (9°C, 0.04 kPa CO_2, *n*=5) *L. squamifrons*. Values are given as means ± SEM.

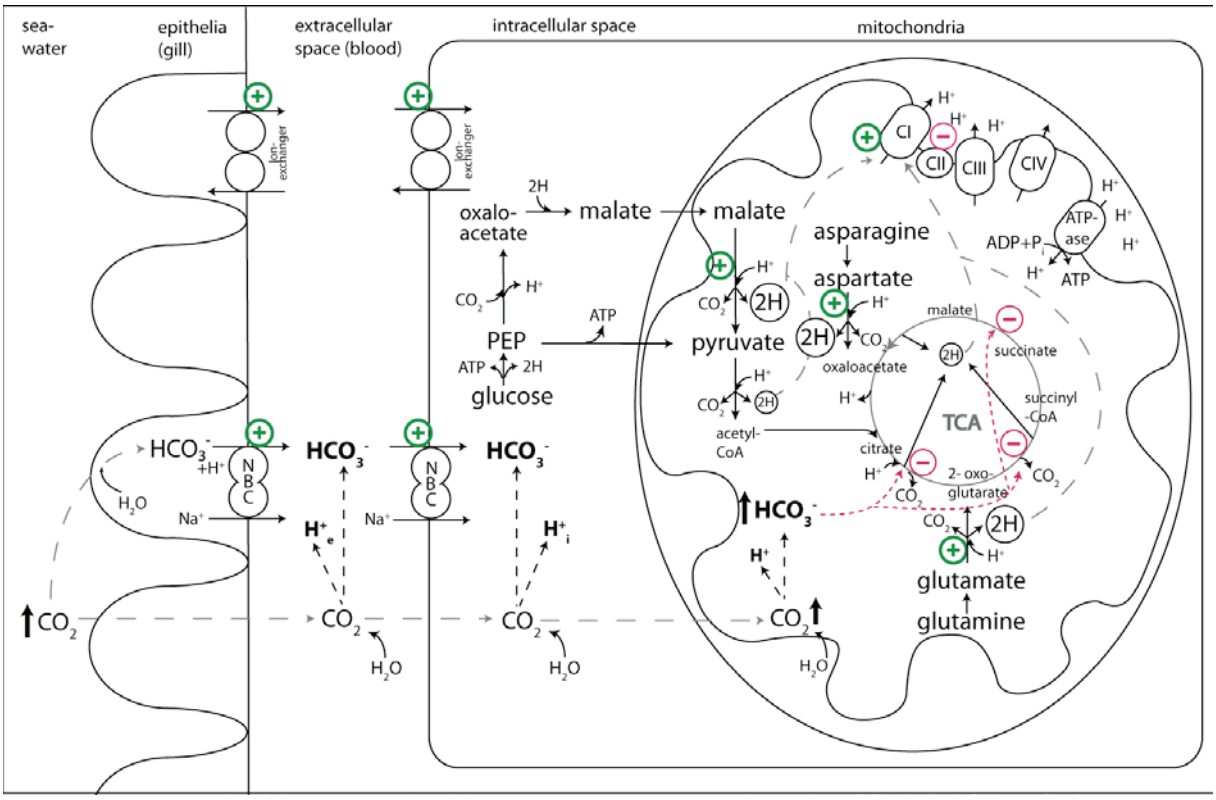

Figure 6: Overview of the proposed effects of long-term elevated ambient PCO_2 at different organisational levels in the Antarctic teleost, *N. rossii*. Long-term hypercapnia acclimation leads to a shift to a new acid-base equilibrium by active accumulation of bicarbonate (HCO_3^-, extra- and intracellular). The new 'set point' for acid-base regulation [40] is maintained via an increase (+) in abundance of the Na^+/HCO_3^- cotransporter (NBC) and further ion transporters (for more details on ion-exchange processes in fish gill tissue under hypercapnia, see [70]). The diffusive entry of CO_2 causes higher levels of H^+ and HCO_3^- inside the mitochondria. During chronically elevated PCO_2 of 0.2 kPa, elevated HCO_3^- competitively inhibits the TCA-cycle (-), as a result complex II (CII) respiration is reduced (-). H^+ are buffered by an increase of oxidative decarboxylation reactions (+) (malate, glutamate/aspartate [72]), leading to an increase in $NADH+H^+$ production and consecutively to enhanced complex I (CI) capacities and membrane potential, partially compensating the reduced TCA-capacities. PEP = phosphoenolpyruvate, "2H" indicates reduction of NAD^+ to $NADH+H^+$.

Tables

Table 1: Maximum proton leak capacities (state IV^+) as a putative fraction of total mitochondrial state III respiration (complex I and II, liver) in *N. rossii* and *L. squamifrons*.

Species	acclimation T (°C)	acclimation CO_2 (kPa)	assay T (°C)	leak (state IV^+) % of state III per assay	leak (state IV^+) % of state III acclimation mean
N. rossii	1	0.04	0	22.6±6.3	
			6	18.1±3.0	20.4±2.1
			12	20.6±3.4	
N. rossii	7	0.04	0	15.1±2.6[b]	
			6	19.8±2.4	17.5±1.7
			12	17.4±3.3	
N. rossii	1	0.2	0	26.1±3.9	
			6	25.3±5.1	25.9±3.2
			12	26.1±7.4	
N. rossii	7	0.2	0	25.7±2.8	
			6	26.6±5.0	27.1±2.1[a]
			12	29.1±1.9	
L. squamifrons	2	0.04	0	15.7±2.1[b]	
			6	14.2±1.9	12.77±1.1[a]
			12	12.8±2.3	
L. squamifrons	9	0.04	0	23.2±4.8	
			6	14.2±1.9[c]	18.83±2.2[c]
			12	18.2±2.8	

Values are given for the three acute mitochondrial assay temperatures 0, 6, 12°C of control/acclimated *N. rossii* (control: 1°C, 0.04 kPa CO_2, n=9; warm normocapnic: 7°C, 0.04 kPa CO_2, n=5; cold hypercapnic: 1°C, 0.2 kPa CO_2, n=10; warm hypercapnic 7°C, 0.2 kPa CO_2, n=10) and *L. squamifrons* (control: 2°C, 0.04 kPa CO_2, n=7; warm normocapnic 9°C, 0.04 kPa CO_2, n=5).

[a] indicates a significant difference in comparison to *N. rossii* controls. [b] indicates a significant difference in comparison to 0°C assay of *N. rossii* controls. [c] indicates a significant difference in comparison to the 0°C assay within the acclimation group. T= temperature.

Table 2: Fatty acid composition of phospholipids in liver mitochondria from control, warm and hypercapnia-acclimated *N. rossii* and *L. squamifrons*

	L. squamifrons		***N. rossii***			
	2°C 0.04 kPa CO_2	**9°C 0.04 kPa CO_2**	**1°C 0.04 kPa CO_2**	**7°C 0.04 kPa CO_2**	**1°C 0.2 kPa CO_2**	**7°C 0.2 kPa CO_2**
SFA	30.4±6.2	28.6±3.6	**42.4±2.0[b,c]**	**37.6±6.2[c]**	**30.9±6.7[a]**	**38.1±3.0[b,c]**
MUFA	23.4±3.9	21.9±1.6	34.4±13.23	23.9±4.2	25.9±5.4	**21.1±4.2[a]**
PUFA	46.2±10.1	54.0±4.1	**36.9±12.7[c]**	**42.4±6.6[c]**	47.8±7.2	**45.2±2.6[c]**
n-3	39.5±10.3	45.9±4.5	**33.0±12.1[b,c]**	**29.7±5.9[c]**	**34.1±8.8[c]**	**32.2±4.1[c]**
n-6	2.6±0.5	2.9±0.6	5.5±1.9	**5.4±2.4[b]**	**6.7±2.3[b,c]**	**8.0±1.8[b,c]**
UI	254.6±54.2	291.5±26.1	221.5±65.3	**224.7±41.6[c]**	231.1±48.2	**239.8±9.6[c]**

Treatments: *N. rossii c*ontrol: 1°C, 0.04 kPa CO_2; warm normocapnic: 7°C, 0.04 kPa CO_2; cold hypercapnic 1°C, 0.2 kPa CO_2; warm hypercapnic: 7°C, 0.2 kPa CO_2. *L. squamifrons* control: 2°C, 0.04 kPa CO_2, warm normocapnic: 9°C, 0.04 kPa CO_2. Units are percentages of total fatty acids within a control/acclimation group of *N. rossii* and *L. squamifrons*. Data are presented as means ± SEM, *N. rossii*: control n=4, warm normocapnic n=4, cold hypercapnic n=7, warm hypercapnic n=8; *L. squamifrons*: control n=7, warm normocapnic n=5.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n-3: fatty acids with 3 double bonds in the carbon chain; n-6: fatty acids with 6 double bonds in the carbon chain

UI: unsaturation index = $\sum_{n=24}^{n=0} n$ * % of fatty acids with n double bonds (adopted from [4848]).

All significances are highlighted bold. [a] indicates a significant difference to the *N. rossii* control group (highlighted in grey). [b] indicates a significant difference to *L. squamifrons* controls.[c] indicates a significant difference to *L. squamifrons* acclimated to 9°C, 0.04 kPa CO_2.

PUBLICATION II

Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2

Anneli Strobel, Swaantje Bennecke, Elettra Leo, Katja Mintenbeck, Hans O. Pörtner, Felix C. Mark

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FRONTIERS IN ZOOLOGY

RESEARCH Open Access

Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2

Anneli Strobel[1*], Swaantje Bennecke[1], Elettra Leo[1,2], Katja Mintenbeck[1], Hans O Pörtner[1] and Felix C Mark[1]

Abstract

Introduction: Ongoing ocean warming and acidification increasingly affect marine ecosystems, in particular around the Antarctic Peninsula. Yet little is known about the capability of Antarctic notothenioid fish to cope with rising temperature in acidifying seawater. While the whole animal level is expected to be more sensitive towards hypercapnia and temperature, the basis of thermal tolerance is set at the cellular level, with a putative key role for mitochondria. This study therefore investigates the physiological responses of the Antarctic *Notothenia rossii* after long-term acclimation to increased temperatures (7°C) and elevated PCO_2 (0.2 kPa CO_2) at different levels of physiological organisation.

Results: For an integrated picture, we analysed the acclimation capacities of *N. rossii* by measuring routine metabolic rate (RMR), mitochondrial capacities (state III respiration) as well as intra- and extracellular acid–base status during acute thermal challenges and after long-term acclimation to changing temperature and hypercapnia. RMR was partially compensated during warm- acclimation (decreased below the rate observed after acute warming), while elevated PCO_2 had no effect on cold or warm acclimated RMR. Mitochondrial state III respiration was unaffected by temperature acclimation but depressed in cold and warm hypercapnia-acclimated fish. In both cold- and warm-exposed *N. rossii*, hypercapnia acclimation resulted in a shift of extracellular pH (pH_e) towards more alkaline values. A similar overcompensation was visible in muscle intracellular pH (pH_i). pH_i in liver displayed a slight acidosis after warm normo- or hypercapnia acclimation, nevertheless, long-term exposure to higher PCO_2 was compensated for by intracellular bicarbonate accumulation.

Conclusion: The partial warm compensation in whole animal metabolic rate indicates beginning limitations in tissue oxygen supply after warm-acclimation of *N. rossii*. Compensatory mechanisms of the reduced mitochondrial capacities under chronic hypercapnia may include a new metabolic equilibrium to meet the elevated energy demand for acid–base regulation. New set points of acid–base regulation under hypercapnia, visible at the systemic and intracellular level, indicate that *N. rossii* can at least in part acclimate to ocean warming and acidification. It remains open whether the reduced capacities of mitochondrial energy metabolism are adaptive or would impair population fitness over longer timescales under chronically elevated temperature and PCO_2.

Keywords: Notothenioid, Oxygen consumption, Routine metabolic rate, Extracellular pH (pH_e), Intracellular pH (pH_i), Mitochondrial respiration, Acclimation, Acid–base

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Introduction

Recent studies have demonstrated warming of the worlds' oceans, and the Antarctic Peninsula also experiences a continuous increase in temperature [1-6]. Additionally, anthropogenic CO_2-emissions accumulate in the atmosphere and the oceans [7] and result in a decrease in seawater pH (ocean acidification) [8-10]. Both ocean warming and acidification exert their specific effects on the marine fauna. Studies identifying the capacity of Antarctic fish to cope with thermal challenges [11-17] contributed to the concept of oxygen and capacity limited thermal tolerance, which explains the limits of thermal tolerance through limitations in tissue functional capacity and the associated oxygen limitation at high and low temperatures [13,18,19].

Changes in seawater temperature particularly affect cold stenothermal organisms, which generally possess extremely slow metabolic rates and poor acclimation capacities, when compared to temperate species [20,21]. While temperate ectothermic vertebrate and invertebrate species are apparently capable of shifting their temperature limits by acclimation [22], this feature and the potential interaction between effects of warming and increasing ocean acidification have been insufficiently explored in Antarctic fauna [11,23-25]. As stenothermal Antarctic fish are assumed to perform well only within their narrow environmental temperature range [15,26], the question arises whether these species can acclimate to increasing temperature and rising ocean PCO_2.

Whole animal oxygen consumption rates reflect the energy demand of the organism as the sum of all physiological costs, including ion and acid–base regulation [20]. Under changing abiotic conditions, the rate of all or some of these processes may change, causing an increased or decreased overall demand for ATP. Therefore, the functional properties of mitochondria as the sites of energy (ATP) production may play a key role in shaping whole organism thermal tolerance and limits of aerobic metabolism [13,27,28]. However, the studies on mitochondrial respiration and capacities of Antarctic invertebrates [29,30] and fish [12,28,31-33] have so far not addressed changes in mitochondrial respiration after long-term acclimation to increased temperature or PCO_2.

Increasing seawater PCO_2 levels are hypothesized to narrow an organism's thermal windows, possibly by limiting the ability to compensate for changes in acid–base status at thermal extremes [34-36]. Changes in acid–base status affect whole organism, cellular and molecular functions. Findings in teleost fish range from high compensatory abilities for rising seawater PCO_2 [37-39], to incomplete compensation with a long-term reduction in pH_e [40].

Less is known about the response of intracellular pH (pH_i) to changing temperature or hypercapnia in both marine vertebrates and invertebrates [41-44]. The intracellular proton buffering capacity of vertebrates is found to vary markedly between animal species, tissue type and aerobic capacity [45-48]. Especially studies on intracellular acid–base status in Antarctic fish are scarce [49].

The Antarctic fish species *N. rossii* is an abundant member of coastal Antarctic communities [50-52] and is widely distributed between 45° and ~ 62°S [53,54]. Water temperatures around the Antarctic Peninsula, e.g. in Potter Cove at King George Island, range from −2°C in winter to 2°C in summer [55]. *N. rossii* is adapted to this narrow thermal range and may display a limited resistance and acclimation capacity to warming compared to more eurythermal fish species.

The aim of this paper is to investigate the acclimation capacities of relevant components in aerobic metabolism of the Antarctic notothenioid *N. rossii* at increased seawater temperature and PCO_2. In an integrative approach, we investigated acclimation to warming (7°C) and hypercapnia (0.2 kPa CO_2) at different organisational levels, the whole animal, extracellular (blood) and intracellular, and the mitochondrial level. We exposed the animals to various abiotic conditions and then focused on changes in the fish's condition and haematological parameters, and on oxygen consumption as a measure of routine metabolic rate (acute and after long-term acclimation). As a next step, we analysed mitochondrial acclimation and adaptation capacities (mitochondrial state III respiration, cytochrome c oxidase activity) as indicators of the plasticity of whole animal metabolic rate. Finally, we determined extra- and intracellular acid–base parameters in *N. rossii* as a measure of acid–base regulation patterns and capacities, and related them to the findings of reduced mitochondrial capacities under hypercapnia.

Material and methods

Animal capture and acclimation

Demersal marbled rockcod, *N. rossii*, were caught in December 2009 in Potter Cove, King George Island, Antarctic Peninsula (62°14'S; 058°41'W) during the Antarctic summer season (seawater temperature 0.8°±0.9° C, salinity 33.5±0.2 psu). Fish were collected using baited traps (length 124 cm, width 64 cm, height 56 cm, mesh size 25 mm) and trammel nets (length 15 m, inner mesh 25 mm).

Animals were reared and acclimated in the aquaria facilities at Dallmann Laboratory, Carlini Station (formerly Jubany Station, King George Island) with direct seawater supply from the cove, under natural light conditions. Following the Intergovernmental Panel on Climate Change's "business-as-usual" scenario, atmospheric CO_2-concentrations may exceed 0.2 kPa by the year 2200 [8,56]. Therefore, we chose 0.2 kPa CO_2 for our hypercapnia acclimation of *N. rossii*. For acclimation, animals

were randomly selected and acclimated to 1°C, 0.04 kPa CO_2 (control group, n=9, mass 155–804 g; total length 25–39.4 cm), 1°C, 0.2 kPa CO_2 (cold hypercapnic group, n=10, mass 144–510 g; total length 23.8-32.8 cm), 7°C, 0.04 kPa CO_2 (warm normocapnic group, n=5, mass 151–412 g; total length 23.6-33.9 cm) and 7°C, 0.2 kPa CO_2 (warm hypercapnic group, n=10, mass 137–504 g; total length 21.4-31.3 cm). Animals were fed to satiation twice per week with chopped fish muscle and snails.

For all acclimations, seawater temperature (from 1° to 7°C) and PCO_2 (from 0.04 kPa CO_2 to 0.2 kPa CO_2) were both increased stepwise (1°C/4 hours; 0.01 kPa CO_2/h) over 24 hours. Total acclimation time was 29–36 days. Experimental animals were acclimated in well-aerated (>95% O_2 saturation), 150 liter tanks, fed by an additional 150 liter header tank. This header tank was used for a daily water exchange of 150 liter to avoid alteration of the conditions in the acclimation tanks. For the warm normocapnia/ hypercapnia acclimations, temperature was kept constant using a 250 W heating element (Jaeger, EHEIM GmbH, Germany), controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). For the cold/ warm hypercapnia acclimations, higher PCO_2 was regulated by an iks aquastar system (iks ComputerSysteme GmbH, Germany). The system maintained constant pH (accuracy ± 0.05 pH units) by controlling a solenoid valve (Aqua Medic GmbH, Germany), which bubbles the acclimation tanks with pure CO_2. Specific seawater conditions are given in Table 1. pH of all acclimation systems was measured daily with a WTW 340i pH meter (WTW, Germany. Electrode: WTW SenTix HWS) and calibrated daily with NBS (WTW, Germany) buffer. Total CO_2 (C_{CO2}) in the seawater was determined with a carbon dioxide analyser (Corning 965, CIBA, Corning Diagnostics, England). Seawater carbonate chemistry was calculated with the measured pH_{NBS} and C_{CO2} using the CO2sys software [57]. All experiments were conducted at Dallmann Laboratory (Carlini Station), King George Island, Antarctic Peninsula.

Routine metabolic rate

Routine metabolic rate (RMR) of *N. rossii* (control/ after acute temperature elevation/ long-term acclimated) was measured via intermittent-flow respirometry. Following Johnston et al. [58], fish were not fed for 10 days prior to the respiration experiments. After the acclimation period, each fish was placed in a 3500 to 4400 ml non-transparent, cylindrical respirometer placed within a 150 liter tank under acclimation conditions. Individuals were allowed to recover within the respiration chamber for 24 hours, a period considered appropriate to overcome the effect of any handling stress [58]. A constant, circulating water flow in the respirometer was generated by an aquarium pump. In the intermittent-flow system, water exchange between chamber and ambient water was interrupted every 30 min for 15 or 30 min to measure oxygen depletion (max. 10% O_2) by the fish within the chamber, then oxygen concentration was replenished to 100% by flush pumps. Oxygen concentration within the chamber was detected once per minute using a FiBox2 (PreSens – Precision Sensing GmbH, Germany) oxygen meter. The device was calibrated before each measurement in well-aerated seawater at the respective acclimation temperature, calibration at zero oxygen was conducted in nitrogen-bubbled seawater.

In three individuals of *N. rossii,* oxygen consumption was measured before and after acute temperature increase. The same experimental setup as described above was used. After 24 hours of recovery, RMR was recorded for 24 hours under control conditions (1°C), then temperature was increased continuously by 1°C per hour up to 7°C. RMR was recorded at the beginning and at the end (7°C) of the acute warming period. Mean RMR were calculated over 24 hours, and thus represent resting metabolism including spontaneous activity. Blank measurements of bacterial respiration in the respirometer were carried out for each acclimation group, values for RMR were corrected accordingly.

Table 1 Seawater physiochemical conditions of the control conditions and different warm/ hypercapnia acclimations of *N. rossii* at Carlini station

Conditions	Control (1°C, 0.04 kPa CO_2)	Warm normocapnia (7°C, 0.04 kPa CO_2)	Cold hypercapnia (1°C, 0.2 kPa CO_2)	Warm hypercapnia (7°C, 0.2 kPa CO_2)
pH	8.250±0.015	7.914±0.016	7.455±0.018	7.493±0.006
P_{CO2} (µatm)	356.72±18.26	461.43±13.69	2179.04±54.50	2018.55±26.93
P_{CO2} (kPa)	0.037±0.002	0.048±0.001	0.225±0.006	0.208±0.003
DIC (mmol/kgSW)	2595.00±118.66	1942.50±26.39	2401.81±5.58	2369.44±2.04
HCO_3- (mmol/kgSW)	2429.78±110.06	1839.93±26.00	2254.02±3.54	2241.68±1.06
T (°C)	1.45±0.09	7.25±0.07	1.36±0.17	6.88±0.063
S (psu)	32.47±0.35	32.75±0.21	32.77±0.29	32.63±0.26
duration (weeks)	1	4	4	4

Animal condition, sampling and haematological parameters

After measurement of RMR and at the end of the acclimation period, specimens of *N. rossii* were anesthesized with 0.5 g/l tricaine methano- sulphonate (MS 222). Blood was taken with a syringe from the caudal vein, the liver was taken for mitochondrial isolation. Parts of liver and muscle samples were immediately freeze-clamped and frozen in liquid nitrogen for pH_i analysis, as described by Pörtner [47]. Afterwards, individuals were killed by a spinal cut behind the head plates. The work was carried out according to the ethics and guidelines of German law. Experiments had been approved according to § 8 animal welfare act (18.05.2006; 8081. I p. 1207) by the veterinary inspection office, Bahnhofsplatz 29, 28195 Bremen, Germany, under the permit number Az.: 522-27-11/02-00 (93) on January 15th, 2008 (permit valid until Jan 14th 2012).

Fulton's condition factor (CF) of *N. rossii* was calculated according to the formula [59]:

$$CF = \text{animal weight [g]} \times 100/\text{standard length [cm]}^3 \quad (1)$$

The hepatosomatic index (HSI) was determined by

$$HSI = \text{liver weight [g]}/\text{total weight [g]} \times 100 \quad (2)$$

The haematocrit of *N. rossii* was estimated in a blood subsample using a haematocrit centrifuge (Compur Microspin, Bayer Diagnostics Mfg. Ltd., Microspin, Ireland). Lactate concentration was measured with an Accutrend® Lactate tester (Roche Diagnostics GmbH, Germany). Osmolarity of the serum was measured after centrifugation of the blood for 10 min at 2000 g. For the measurement, a Vapour Pressure Osmometer 5500 (Wescor Inc., USA) was used.

Isolation of liver mitochondria and mitochondrial oxygen consumption measurements

Immediately after excision, the liver was rinsed and total liver weight was determined before a subsample of liver tissue was taken, weighed and rinsed with 5 ml/g ice-cold isolation buffer containing 80 mM sucrose, 85 mM KCl, 5 mM EGTA, 5 mM EDTA, 50 mM HEPES and 1% w/v bovine serum albumin (BSA, fatty acid free) (pH 7.1 at 20°C). The liver tissue was then finely minced with scissors, suspended in 10 volumes ice-cold isolation buffer, and then put into a 30 ml Potter-Elvehjem glass homogenizer (VWR International, Germany) and slowly homogenised with three strokes at 80 revolutions/ minute. The homogenate was centrifuged (600 g, 10 min., 0°C), the supernatant collected and the pellet vigorously resuspended by vortexing in isolation buffer and centrifuged for a second time. Supernatants were then combined and centrifuged for 10 min at 11.000 g (0°C). The supernatant was discarded, any remaining droplets of fat removed with a cotton swab and the pellet resuspended in isolation buffer and centrifuged again. As a last step, supernatant was discarded again, and the pellet was resuspended in ice-cold mitochondria assay buffer (80 mM sucrose, 85 mM KCl, 5 mM KH_2PO_4, 50 mM HEPES, 1% w/v BSA (fatty acid free), pH 7,1 at 20°C) at 1 ml/g initial liver weight. This mitochondrial preparation was kept on ice away from light and used for mitochondrial oxygen consumption measurements. The mitochondrial protein concentration was determined according to Bradford [60] using a bovine serum albumin (BSA) standard, and considering the protein content of the mitochondrial assay buffer.

Mitochondrial respiration measurements were conducted in two thermostatted perspex respiration chambers of 3 ml volume (World Precision Instruments, Inc., USA), equipped with an adjustable stopper and ports for the injection of metabolites and inhibitors and one for insertion of a TX micro-optode (PreSens – Precision Sensing GmbH, Germany), used for fluoroptic measurement of PO_2. The oxygen traces were recorded with a PowerLab recording unit and Chart v5.5.6 software (ADInstruments GmbH, Germany). Mitochondrial respiration rates were converted to nmol O_2*mg extracted mitochondrial $protein^{-1}$*min^{-1}.

Measurements were carried out in assay buffer with a final volume of 1200 µl with mitochondrial concentrations adjusted to about 3 mg mitochondrial protein per ml, at 0, 6, and 12±0.1°C, respectively. Chamber temperature was maintained with a thermostat (LAUDA, Germany). Respiration was recorded and malate and pyruvate added to a final concentration of 1.3 mM and 1.6 mM, respectively, as substrates for complex I (state II), and ADP (final conc. 0.16 mM) was added to measure state III (max. slope). Then, 2 mM succinate was added as complex II substrate and 0.16 mM ADP for state III respiration.

Enzyme assays

Frozen liver tissue was ground into powder by mortar and pestle under liquid nitrogen and homogenized in a glass homogenizer in 9 vol. buffer containing 20 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 0.1% Triton X-100, pH 7.4, and afterwards with an Ultra Turrax (Silent Crusher M (Heidolph Instruments, Germany), followed by 10 min centrifugation at 1,000 *g* at 4°C. Cytochrome *c* oxidase (COX) activity was determined according to a protocol modified from Moyes et al. [61] in buffer containing 20 mmol l^{-1} Tris–HCl, 0.05% Tween 20 and 0.057 mM reduced cytochrome *c* at pH 8.0. The decrease in extinction at λ = 550 nm through oxidation of cytochrome *c* (ε_{550} = 19.1 $mol^{-1}cm^2$) was monitored in a thermostatted spectrophotometer (Beckman, Fullerton, CA, USA) at 0,

6 and 12°C. Protein concentration of the tissue extract was determined according to Bradford [60], enzyme activity is given in μmol*mg protein^{-1}*min^{-1}.

Acid–base parameters

Intracellular acid–base variables

Measurement of pH_i was carried out according to the homogenization technique developed by Pörtner [47]. A solution of 1 mM nitrilotriacetic acid (NTA) and 160 mM potassium fluoride (KF) was used to keep the NTA concentration as low as possible. Cco_2 was measured by gas chromatography (6890N Network GC System, Agilent Technologies), total CO_2 in cell water was calculated according to Pörtner [47], assuming a fractional tissue water content of 0.78 [62]. Intracellular acid–base parameters were calculated using the following, modified Henderson-Hasselbalch equation.

$$PCO_2 = C_{CO2} \mathrm{x} \left(10^{\mathrm{pH}-\mathrm{pK}'''} \mathrm{x}\, \alpha + \alpha\right)^{-1} \quad (3)$$

$$[HCO_3^-] = Cco_2 - \alpha PCO_2 \quad (4)$$

Intracellular pK''' and α (solubility) values were evaluated according to Heisler [62] using $[Na^+]$ = 0.02 M, [M] = 0.21 mol l^{-1}, I = 0.12 mol l^{-1} and [Protein] = 220 g l^{-1} [47].

The tissue buffer values β_{NB} for liver and muscle were adopted from Van Dijk et al. [63].

Extracellular values

Blood plasma pH (extracellular pH, pH_e) was measured immediately after sampling at the acclimation temperature with a pH meter (WTW 340i, WTW, Germany. Electrode: InLab® Viscous, Mettler Toledo GmbH, Germany). The pH meter was calibrated daily with NBS buffers (WTW, Germany). Measurements were carried out in a closed microcentrifuge tube (0.5 ml) to minimize contact with environmental air. Plasma total CO_2 (C_{CO2}) was measured after centrifugation by means of a carbon dioxide analyser (Corning 965, CIBA, Corning Diagnostics, England). Blood carbonate chemistry was calculated using the modified Henderson-Hasselbalch equation (eqn. 3 and 4). Values for the CO_2-solubility coefficient α and the negative logarithm of the dissociation constant K''' were calculated after Heisler [62]. The values required for the calculation (ionic strength, protein concentration, Na^+ concentration) were adopted from Egginton [64].

Blood non-bicarbonate buffer value (β_{NB})

After heparinization of the blood (100 U/ml), the β_{NB} capacity was determined at 0.5°C in a thermostatted tonometer. 1.5 ml of whole blood was equilibrated with different PCO_2 (1 kPa, 2 kPa, 3 kPa CO_2) for 1 hour before pH (electrode: InLab® Viscous, Mettler Toledo GmbH, Germany) and C_{CO2} were measured (gas chromatography: 6890N Network GC System, Agilent Technologies). The β_{NB} capacity ($-\Delta[HCO_3^-]/\Delta pH$) was determined with values for $\Delta[HCO_3^-]$ calculated after equation 4.

Data analysis and statistics

The temperature coefficient Q_{10} was calculated for routine metabolic rate and mitochondrial respiration (state III) according to the formula

$$Q_{10} = \left(MO_{2(2)}/MO_{2(1)}\right)^{10/(T_2-T_1)} \quad (5)$$

The respiratory control ratio (RCR) was calculated as the ratio between mitochondrial state III (complex I and II) and state IV (after ADP depletion) respiration.

All data were tested for outliers at the 95% significance level using Nalimov's test [65] as well as for normality (Kolmogorov-Smirnov) and homogeneity of variance. Differences in routine metabolic rate, mitochondrial oxygen consumption and COX activity at the assay temperatures 0, 6, 12°C, blood and intracellular acid–base variables, between the different acclimation groups were tested using unpaired, two-tailed t-tests and one-way analysis of variance (ANOVA, with Tukey post-hoc test). $p \leq 0.05$ was considered the significance threshold. All data are presented as means ± standard error of the mean (SEM).

Results

Animal condition and haematological parameters

The relative liver weights (hepatosomatic indices, HSI), condition factors, haematocrits, blood osmolarities and lactate levels, as well as the blood acid–base parameters determined for control and acclimated *N. rossii*, are summarized in Table 2. The HSI showed a non-significant tendency to decrease after warm-acclimation of *N. rossii* in both the normocapnic (7°C, 0.04 kPa CO_2) and the hypercapnic (7°C, 0.2 kPa CO_2) groups. Acclimation to higher PCO_2 at 1°C had no significant effect on the HSI. The condition factor of *N. rossii*, indicating nutritional status, was significantly decreased in the warm hypercapnic group. The haematocrit of *N. rossii* displayed no acclimation effect. Lactate concentrations in the blood, on average, remained below detection limit (<0.8 mmol*l^{-1}). Only in the cold hypercapnic group, lactate was slightly elevated to 1.13±0.11 mmol*l^{-1}. The blood osmolarity in *N. rossii* was significantly reduced in all warm and hypercapnia acclimated animals.

Routine metabolic rate

N. rossii exposed to acute warming (7°C) showed significantly increased, two-fold higher RMR compared to the control group (1.35±0.20 (control at 1°C, n=5) *vs.* 2.71±0.41 (acutely warmed to 7°C, n=3) mmolO_2*kg^{-1}*h^{-1}), as displayed in Figure 1.

Table 2 Animal condition (HSI= hepatosomatic index, CF=condition factor), and blood parameters of the Antarctic fish *N. rossii*

Acclimation							
T	PCO_2	N	HSI	CF	Haematocrit	Lactate	Osmolarity
(°C)	(kPa)					(mmol*l^{-1})	(mOsm*l^{-1})
1	0.04	9	1.83±0.33	1.69±0.03	28±2	<0.8	436.8±9.5
7	0.04	9	0.91±0.04*	1.57±0.07	29±2	<0.8	373.8±15.7*
1	0.2	5	1.22±0.12	1.59±0.03	29±1	1.13±0.11	399.5±13.3
7	0.2	10	0.81±0.06*	1.5±0.03*	31±1	<0.8	390.0±8.6*

Control: 1°C, 0.04 kPa CO_2; warm normocapnia: 7°C, 0.04 kPa CO_2; cold hypercapnia: 1°C, 0.2 kPa CO_2; warm hypercapnia: 7°C, 0.2 kPa CO_2.
All values are given as means ± SEM. * indicate a significant difference to control conditions (*t*-test, $p \leq 0.05$).

Oxygen consumption in the control group and the long-term warm and/ or hypercapnia acclimated *N. rossii* was measured at the end of the 4-weeks acclimation period. In comparison to the control group, the RMR of the long-term warm and normocapnia acclimated fish was significantly increased at 7°C (2.23±0.16 mmol O_2*kg^{-1}*h^{-1} (n=5), Q_{10} of 2.38). However, after long-term warm acclimation RMR was significantly lower compared to that of the acutely warmed *N. rossii* at 7°C (Q_{10} of 3.2). RMR after cold hypercapnia acclimation (1.28±0.10 mmol O_2*kg^{-1}*h^{-1}, n=6) was similar to that in control animals. Warm hypercapnia acclimation resulted in a significantly increased RMR (2.49±0.22 mmol O_2*kg^{-1}*h^{-1}, n=5, measured at 7°C) compared to the control group measured at 1°C. No significant difference in RMR was found between the long-term warm normocapnic and the warm hypercapnic groups.

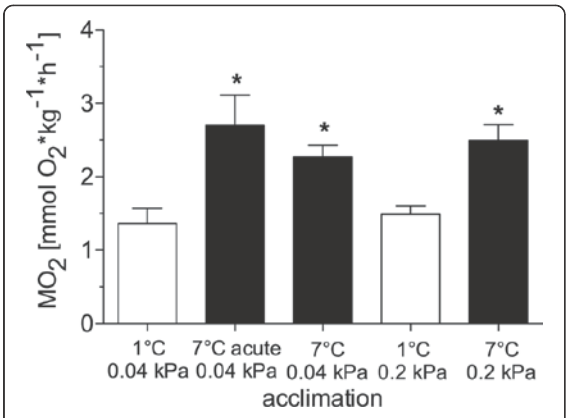

Figure 1 Routine metabolic rate of *N. rossii* at different acclimation conditions. Oxygen consumption of control, cold hypercapnia (white bars), warm normocapnia/ hypercapnia (black bars)) acclimated fish, and after acute warming (1°C/ hour) to 7°C, normocapnia. All data are presented as means ± SEM, n=3-6. * indicate significant difference of warm/ hypercapnia acclimation/ acute warming to control *N. rossii* (*t*-test, $p \leq 0.05$).

Mitochondrial respiration, respiratory control ratio (RCR) and mitochondrial Q_{10}

Liver mitochondrial state III respiration increased significantly when assay temperature was changed acutely from 0°C (2.79±0.18 nmol O_2*mg*min^{-1}) to 6°C (5.64±1.17 nmol O_2*mg*min^{-1}) and 12°C (7.55±1.49 nmol O_2*mg*min^{-1}) in the control group (n=10), with similar rates in the warm normocapnic fish (n=5; 0°C: 3.86±0.19; 6°C: 5.53±0.34; 12°C: 6.55±1.62 nmol O_2*mg*min^{-1}). In cold hypercapnia (n=9; 0°C: 1.73±0.42; 6°C: 2.65±0.30; 12°C: 4.49±0.94 nmol O_2*mg*min^{-1}) and warm hypercapnia acclimated fish, state III respiration was significantly increased only at 12°C (n=9; 0°C: 1.95±0.36; 6°C: 3.89±0.85; 12°C: 6.40±1.50 nmol O_2*mg*min^{-1}) (Figure 2). In the cold hypercapnic animals, state III respiration was significantly reduced (at 6°C and 12°C in the assay) compared to the control group.

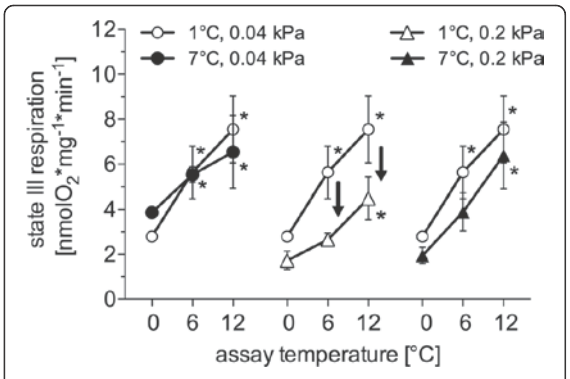

Figure 2 State III respiration rate in liver mitochondria of *N. rossii*. State 3 respiration was measured in the presence of malate, pyruvate, succinate and ADP at the respective assay temperature (0°C, 6°C, 12°C) in four different long-term acclimated groups; open circles: 1°C, 0.04 kPa CO_2 (control); filled circles: 7°C, 0.04 kPa CO_2 (warm normocapnic); open triangles: 1°C 0.2 kPa CO_2 (cold hypercapnic); filled triangles: 7°C 0.2 kPa CO_2 (warm hypercapnic). * indicate significantly higher mitochondrial respiration than at 0°C assay. ↓ depicts significantly reduced mitochondrial respiration in comparison to control acclimation (1°C, 0.04kPa CO_2). All data are presented as means ± SEM, n=5-10.

Within each experimental group, RCR did not vary significantly between the different assay temperatures (0, 6, 12°C), whereas between the experimental groups the mean RCR of the 0, 6 and 12°C assays were significantly reduced in both cold and warm hypercapnia acclimated animals compared to the control group (Table 3). There was no difference in the Q_{10} values for mitochondrial state III respiration in the acute assay temperature range from 0-12°C between the different acclimation groups.

Enzyme activities

In the warm hypercapnic group, COX activities in liver were decreased compared to the control at all temperatures. A similar trend was apparent in the cold hypercapnic animals, but only significant in the assay at 12°C (Figure 3). COX activities in liver extracts of the warm normocapnic group were significantly higher than in the control group at the 6°C assay.

Acid–base parameters of N. rossii

Intracellular

The pH_i of muscle tissue of the control *N. rossii* was 7.33±0.07 (*n*=3) (Table 4). The highest, though not significant deviation from the control group was found in the warm normocapnic animals (0.08 pH units lower). Liver pH_i was lower than the respective muscle pH_i in all treatments, control pH_i of liver tissue was 7.08±0.03 (*n*=3). In parallel, liver PCO_2 in all acclimation groups was significantly higher than muscle PCO_2. All acclimation groups showed muscle tissue [HCO_3^-] elevated above controls ([HCO_3^-] = 3.99±0.41 mM), up to 6.72±0.66 mM (*n*=5) in cold hypercapnic specimens and 6.85±0.37 mM (*n*=10) in warm hypercapnic individuals.

Liver [HCO_3^-] was generally higher than in muscle samples. [HCO_3^-] in liver under control conditions (9.21±0.37 mM, *n*=3) was slightly lower than in the cold hypercapnic group (10.11±1.19 mM, *n*=5) and the warm hypercapnic group (10.35±0.72 mM, *n*=7). These data have to be considered with caution, as due to sample shortage (most liver tissue was needed for isolation of mitochondria) there was only one liver sample available for pH_i estimation in the warm normocapnic group, however, with a value similar to those in the warm hypercapnic group (*n*=7).

Visualisation of the intracellular acid–base parameters in a pH-bicarbonate diagram (Figure 4) illustrates that in liver tissue, changes in acid–base status of the cold hypercapnic group changed in parallel to the assumed non-bicarbonate buffer line of controls; only in the warm normocapnic *N. rossii* was the resulting value located distinctly below the non-bicarbonate buffer line of controls. In white muscle, the CO_2 induced acidosis in both cold and warm hypercapnic groups was compensated for by a significant rise in [HCO_3^-], resulting in

Table 3 Respiratory control ratio (RCR; for each assay temperature and as mean RCR over all three assay temperatures at the respective acclimation) and Q_{10} of *N. rossii* (control: 1°C, 0.04 kPa CO_2; warm normocapnia: 7°C, 0.04 kPa CO_2; cold hypercapnia: 1°C, 0.2 kPa CO_2; warm hypercapnia: 7°C, 0.2 kPa CO_2)

Acclimation			RCR		Q_{10}	
Temperature [°C]	PCO_2 [kPa]	Assay temp. [°C]	CI+CII	mean	0-12°C	N
1	0.04	0	4.9±0,4	4.6±0.8	1.6±0.3	5
		6	5.2±0.8			5
		12	3.6±0.2			5
7	0.04	0	5.0±0.6	4.0±1.0	1.8±0.3	5
		6	3.9±0.6			5
		12	3.0±0.9			5
1	0.2	0	4.6±0.6	3.8±0.8*	2.4±0.4	10
		6	4.0±0.6			10
		12	3.0±0.4			10
7	0.2	0	4.2±0.6	3.5±0.6*	2.1±0.3	9
		6	3.4±0.3			9
		12	2.9±0.2			9

* indicates a significantly different RCR compared to control *N. rossi*.

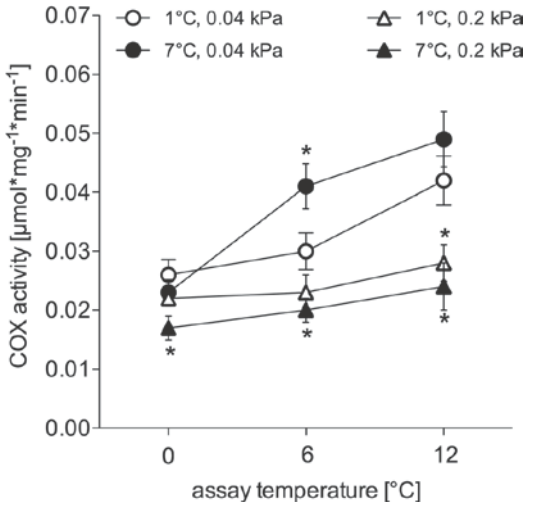

Figure 3 Cytochrome *c* oxidase (COX) activity in liver extracts of long-term acclimated *N. rossii*. Open circles: 1°C, 0.04 kPa CO_2 (control); filled circles: 7°C, 0.04 kPa CO_2 (warm normocapnic); open triangles: 1°C 0.2 kPa CO_2 (cold hypercapnic); filled triangles: 7°C 0.2 kPa CO_2 (warm hypercapnic). * depicts a significantly higher/ lower enzyme activity of the respective acclimation group compared to control, at the given assay temperature. Activity values are given as means ± SEM in µmol*mg^{-1}*min^{-1}, *n*=4-7.

Table 4 Intracellular and extracellular pH ($pH_{i/\ e}$) and acid–base parameters in white muscle/ liver tissue homogenates and blood, respectively, from *N. rossii*. Control: 1°C, 0.04 kPa CO_2; warm normocapnia: 7°C, 0.04 kPa CO_2; cold hypercapnia: 1°C, 0.2 kPa CO_2; warm hypercapnia: 7°C, 0.2 kPa CO_2

Acclimation		White muscle homogenate				Liver homogenate				Blood acid–base parameters			
T (°C)	PCO_2 (kPa)	N	PCO_2 (kPa)	$[HCO_3^-]$ (mmol*l^{-1})	pHi	N	PCO_2 (kPa)	$[HCO_3^-]$ (mmol*l^{-1})	pHi	N	PCO_2 (kPa)	$[HCO_3^-]$ (mmol*l^{-1})	pHe
1	0.04	3	0.640±0.126	3.994±0.410	7.325±0.065	3	2.555±0.270	9.213±0.379	7.080±0.027	9	1.18±0.19	8.05±0.56	7.438±0.055
7	0.04	5	1.071±0.123	5.294±0.335	7.242±0.037	1	3.616	8.020	6.890	9	1.16±0.05	6.31±0.28[a,b]	7.315±0.045[b]
1	0.2	5	0.936±0.117	6.724±0.664[a]	7.383±0.027	5	3.031±0.484	10.107±1.197	7.053±0.066	5	1.46±0.09	11.28±0.32[a,b,c]	7.508±0.028
7	0.2	10	1.296±0.167	6.849±0.373[a]	7.290±0.038	7	4.495±0.521	10.347±0.724	6.920±0.039	10	1.41±0.11	10.08±0.57[a]	7.507±0.037

Data are presented as means ± SEM. Superscript letters indicate following significant differences: a – sign. different to control conditions, b – sign. different to temperature & CO_2 acclimation. c –sign. different to temperature acclimation (*t*-test, ANOVA, $p \leq 0.05$).

values close to the PCO_2 isobar of ~ 0.95 and 1.15 kPa CO_2, respectively.

Extracellular

Cold hypercapnia acclimation did not significantly affect pH_e, which was 7.44±0.06 (n=9) under control conditions (Table 4). pH_e was significantly higher in the warm hypercapnic group (7.51±0.04, n=10) than in the warm normocapnic (7.32±0.05, n=9) group. None of the acclimations significantly affected extracellular PCO_2, however, changes in bicarbonate result as a consequence of CO_2 enrichment from 1.2 to 1.4 kPa in warm and from 1.2 to 1.5 kPa in cold acclimated animals. Cold hypercapnia acclimation was in fact associated with a significant increase in bicarbonate levels ($[HCO_3^-]$ control: 8.05 mmol*l^{-1}, cold hypercapnic 11.28 mmol*l^{-1}). In contrast, blood $[HCO_3^-]$ of the warm normocapnic animals was significantly decreased (6.31 mmol*l^{-1}). When displayed in a pH-bicarbonate diagram (Figure 5), together with the blood buffer line β_{NB} of 30.3 mmol*pH^{-1}*l^{-1} which we measured for *N. rossii* at 0°C, it becomes obvious that long-term hypercapnia acclimation involves a marked accumulation of $[HCO_3^-]$, resulting in elevated steady state levels.

Discussion

Aerobic energy metabolism

During acute warming, the RMR of *N. rossii* increased as a result of rising metabolic rate with a Q_{10} of 3.1 (Figure 1), which is consistent with previous studies on acute thermal responses of different Antarctic fish species [11,26,66,67]. A part of the increase in metabolic rate can likely be attributed to the high costs of the low capacity cardio-vascular system to meet the increasing metabolic oxygen demand, which has been demonstrated for Antarctic eelpouts [13,68]. Due to limited cardiac scope and increased friction of the vascular system at high blood flow rates, sufficient oxygen delivery at warm temperatures result in a relatively

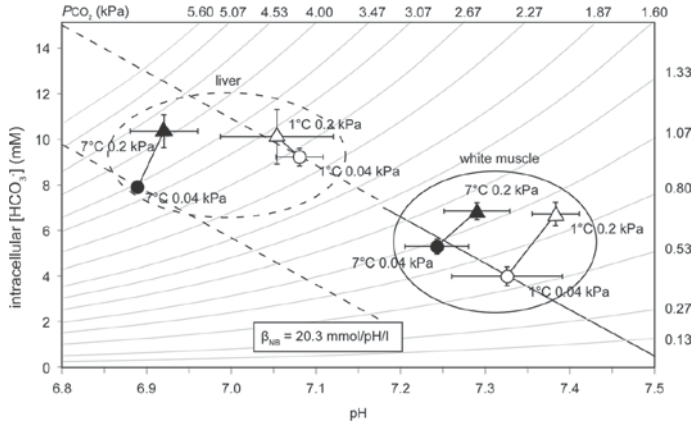

Figure 4 pH_i/ $[HCO_3^-]$ (Davenport) diagram of intracellular pH (pH_i) and acid–base parameters. Values of liver and white muscle homogenates of control (1°C, 0.04 kPa CO_2) and warm normocapnia (7°C 0.04 kPa CO_2), cold hypercapnia (1°C, 0.2 kPa CO_2) and warm hypercapnia (7°C, 0.2 kPa CO_2) acclimated *N. rossii* (c.f. Table 4). All data are given as means ± SEM, n=1-7. The solid line marks the non-bicarbonate buffer line (β_{NB}) for white muscle, the dashed line for liver. β_{NB} value was adopted from VanDijk et al. [63] for the Antarctic eelpout *P. brachycephalum*.

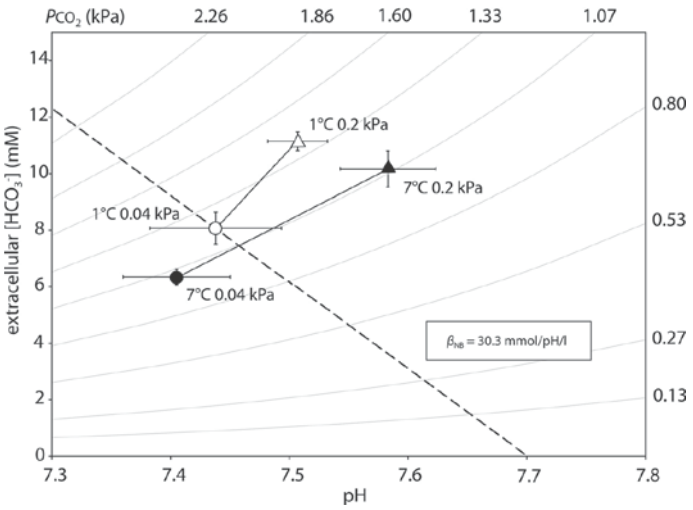

Figure 5 pH_e/ [HCO_3^-] (Davenport) diagram of the extracellular acid–base variables. Acid–base parameters are depicted for control (1°C 0.04 kPa CO_2) and warm normocapnia (7°C 0.04 kPa CO_2), cold hypercapnia (1°C, 0.2 kPa CO_2) and warm hypercapnia (7°C, 0.2 kPa CO_2) acclimated *N. rossii* (c.f. Table 4). The dashed line marks the *in vitro* non-bicarbonate buffer line (β_{NB}), curved lines represent PCO_2 isopleths. All data are presented as means ± SEM, *n*=5-10.

higher workload for the heart. A compensation of cardiac scope during long-term acclimation may alleviate this to some extent, contributing to a lower RMR and Q_{10} of 2.3 after warm acclimation (Figure 1), which indicate a partial, incomplete compensation of RMR in *N. rossii* (type 3 after Precht [69]). The same effect occurred in the long-term warm hypercapnic acclimated *N. rossii*, providing evidence that the partially compensated RMR in the warm hypercapnic fish was exclusively induced by temperature and not by elevated PCO_2.

Following long-term acclimation of *N. rossii* to higher temperatures and PCO_2, animal condition displayed pronounced changes. While control values of HSI and condition factor were within the same range reported recently for *N. rossii* caught in Potter Cove, King George Island [28], they were reduced in warm and hypercapnia acclimated *N. rossii*, although the fish were fed to satiation (control HSI 1.83, condition factor 1.69, warm hypercapnic HSI 0.81, condition factor 1.5; see Table 2). This reduction may be attributed to a reduced aerobic scope caused by elevated RMR (see above) at the high acclimation temperatures chosen.

The fact that the warm-acclimated fish could not completely compensate their RMR to a level comparable to the control animals could indicate beginning limitations in the circulatory system of *N. rossii* and in oxygen supply to tissues. As a result, the aerobic scope for the SDA response (specific dynamic action) [70,71] might be limited at warmer temperatures. Consequently, fish may not be capable to ingest sufficient food over time to meet the required energy demand and to sustain basal metabolic rate, even if fed *ad libitum*. To maintain RMR elevated in warmer water, energy stores such as liver fat may be mobilized [72], resulting in the observed lower HSI and condition factor (see above).

The paradigm that Antarctic fish have limited acclimation capacity because of their thermal specialization has been challenged by several studies reporting compensatory adjustments of whole animal respiration, cardiovascular response and blood viscosity at elevated temperatures [11,16,24,66,73]. Most of these studies focused on the cryo-pelagic fish *P. borchgrevinki* or on several *Trematomus* species. In *N. coriiceps*, the congener of *N. rossii*, an acclimation-induced shift in critical thermal maxima (CT_{max}) was observed, but the increase was small compared to the shifts observed in other Antarctic species (e.g. *P. brachycephalum, Gobionotothen gibberifrons, T. pennellii and T. hansoni,* see Bilyk and DeVries [66] for further details), further corroborating our conclusions for *N. rossii*.

In general, the measurement of RMR provides a suitable indicator of a species' thermal tolerance, as limits in oxygen consumption can reflect the onset of whole animal oxygen limitation and associated limitations in circulatory capacity [74]. Nevertheless, the precise determination of aerobic limits under increased temperature and PCO_2 benefits from the combined study of several indicators including enzyme and mitochondrial capacities, the limiting factors in ATP supply.

The mitochondrial state III respiration of the control group rose continuously with rising experimental assay

temperatures of 0, 6 and 12°C. The RCR values were stable between 0 and 12°C (see Table 3), indicating efficient mitochondrial coupling up to 12°C. A decrease in Q_{10} from 2.4 (range 0-6°C) to 1.6 (range 6-12°C), indicates that state III respiration became less responsive to temperature at higher assay temperatures, and led to a similar decrease in mitochondrial scope as reported for *N. rossii* [28] and *Lepidonotothen nudifrons* [12] beyond 9°C.

In contrast to the elevation of RMR in the warm normocapnia acclimated *N. rossii*, maximum mitochondrial respiration rates were not significantly higher than in the control group (Figure 2). This was reflected in similar values of RCR (control: 4.6±0.8, warm normocapnia: 4.0±1.0) and Q_{10} between these two groups over the whole range of assay temperatures (Q_{10} from 0 to 12°C, control: 1.6; warm normocapnia: 1.8) (see Table 3). Only the trend towards a reduced thermal slope of mitochondrial state III respiration of the warm normocapnic fish (linear regression analysis of state III respiration from 0 to 12°C assay temperature; warm normocapnic: slope 0.22 [(nmol O_2*min^{-1}*mg^{-1})*1°C], control group: 0.39) could point towards a beginning compensation of mitochondrial respiration. The partial (type III) compensation at the whole animal level (RMR, Figure 1) could therefore originate at the mitochondrial level, possibly underpinning relevant adjustments of the cardiovascular system.

Interestingly, cold hypercapnia acclimation led to significantly reduced state III respiration at acute assay temperatures of 6°C and 12°C compared to the control group, accompanied by a significantly reduced mean RCR over the whole range of assay temperatures (Table 2). Similarly, state III respiration was depressed in the warm hypercapnia acclimated *N. rossii*, below that of the control group (Figure 2), and showed significantly reduced RCRs, indicating a clear effect of elevated ambient PCO_2 on mitochondrial metabolism. This effect did not translate into a change in whole animal RMR but may reflect a decrease in tissue and whole animal aerobic and functional scope. In contrast, a down-regulation in resting aerobic metabolic rate occurred under acute hypercapnic acidosis in muscle tissue of the invertebrate *Sipunculus nudus*, reflecting a reduction in ATP consuming processes of maintenance metabolism (e.g. anabolic/ catabolic protein metabolism) [75,76]. Such energy savings might also occur in fishes and affect proteins involved in mitochondrial respiration (e.g. reduced citrate synthase activities in hypercapnia acclimated *Sparus aurata* [40]), thereby causing a lower state III respiration. Since state IV respiration remained unchanged after cold or warm hypercapnia acclimation (data not shown), the reduced coupling capacities were likely caused by the reduced state III respiration (per mg mitochondrial protein) and not by increased proton leak rates. The reduced COX activities (per mg cellular protein) (Figure 3) in the liver of both cold and warm hypercapnia acclimated *N. rossii* support this hypothesis and are in line with the projected changes in protein activity, including possible modifications in the mitochondrial membrane.

The differences observed at the mitochondrial level only partially reflected the whole animal level (see Figure 1), specifically in that the mitochondrial studies exclusively concentrated on liver tissue, which only constitutes a fraction of whole animal metabolism. A possible whole organism consequence of such capacity limits in mitochondrial metabolism under conditions of elevated energy demand (e.g. activity, reproduction) may be shifts in metabolic pathways [40] and a decrease in aerobic scope under long-term elevated PCO_2. Further alterations may include a reduction in growth or behavioural capacities under long-term increased PCO_2, as observed in coral reef fish (*Amphiprion percula* & *Neopomacentrus azysron*) [77-79].

Acid–base regulation

The changes in mitochondrial capacities may be related to shifts in extra- and particularly intracellular acid–base status. The liver pH_i in control *N. rossii* of this study (pH_i 7.08, Table 4) were similar to values recorded for the eelpout *Z. viviparus* (pH_i 7.06, [63]). The pH_i values of the white muscle samples (e.g. control group; pH_i 7.325) were close to values reported for Antarctic and non-Antarctic fish in other studies (e.g. *G. morhua* 7.34, [80]; *P. brachycephalum* 7.42-7.43 [81], *Harpagifer antarcticus* pH_i 7.36 at 1°C [49], 7.33 *N. coriiceps* [82]). In the warm normocapnia acclimated group, white muscle values followed the α-stat pattern [83], with a lowering of pH with increasing temperature by −0.014 pH units/°C. Such a rise in body temperature also caused a linear drop of pH_i in white muscle of the North Sea eelpout *Z. viviparus* (−0.016 pH units/°C) [63]. The illustration of intracellular acid–base parameters in the pH-bicarbonate diagram (Figure 4) emphasizes a defence of liver pH_i by the non-bicarbonate buffer system (such as proteins or amino acid residues) in the cold hypercapnia acclimated fish, in similar ways as recorded e.g. for *G. morhua* [80] or freshwater catfish *Liposarcus pardalis* [43].

In the liver of the warm hypercapnia acclimated *N. rossii* (Figure 4), pH_i was compensated by intracellular HCO_3^- accumulation, in parallel to the findings in the blood (pH_e) and muscle pH_i. This compensation of chronically increased PCO_2 of both cold and warm groups may have contributed to the observed shifts in metabolic steady state towards slightly alkaline pH values. The long-term reaction to acute changes in acid–base status may include shifts in the use of metabolic substrates by favouring oxidative decarboxylation of dicarboxylic acids (malate, glutamate/ aspartate) [75,84]. These reactions could help to reduce the elevated

proton load under chronic elevated PCO_2, thereby playing an important role in the buffering of changes in the acid–base status. Nevertheless, such modifications appear to be insufficient to maintain full mitochondrial capacities in *N. rossii*, paralleled by the observed reduced COX activities and RCR of the cold/ warm hypercapnic mitochondria.

During long-term elevated ambient PCO_2, CO_2 enters the mitochondria by diffusion, yielding an increase in proton and $[HCO_3^-]$ levels. Taking into account the pH and total CO_2 gradient maintained between mitochondria and the cytoplasm under control conditions, liver mitochondrial $[HCO_3^-]$ of the warm hypercapnic animals were up to 10 mmol/l higher than in the warm normocapnic group (or 4 mmol/l in the cold hypercapnic group compared to their controls); calculated after [47,85]. Earlier studies of the acute effects of alkaline pH and increased $[HCO_3^-]$ on liver mitochondria revealed inhibitions in the TCA-cycle [86], thereby lowering mitochondrial respiratory capacities and their capacity to supply ATP. In trout (*O. mykiss*) hepatocytes, acutely increased $[HCO_3^-]$ at 1 kPa PCO_2 also depresses mitochondrial metabolism via interruptions in the TCA-cycle, possibly caused by alterations in citrate and phosphate transport [87].

Intracellular acid–base regulation is supported by the respective adjustments in extracellular acid–base status, e.g. the accumulation of extracellular bicarbonate during compensation for the respiratory acidosis [88]. The same shift in 'set points' towards alkaline values observed at the intracellular level occurred in the blood. In contrast to other pH_e values recorded for temperate marine fish (e.g. cod 7.95 [80], flounder 7.78 [89], seabream 7.65 [40]), the extracellular pH of *N. rossii* was quite low in the present study (pH 7.44 in the control group). A study by Egginton [90] revealed a low blood pH of 7.5 for *N. coriiceps* directly after capture, which increased to 7.7 over 96 hours during recovery from landing stress. In cannulated *N. coriiceps* pH_e increased from 7.5 to 8.0 during recovery, a value consistent with the blood pH of 8.01 measured for *N. rossii* by Egginton et al. [64]. pH values measured in the present study may be lower than these values due to 'grab and stab' effects, as the cannulation of animals was experimentally not possibly. Nevertheless, these handling effects should have affected measurements in all experimental groups in similar ways and thereby still allow for comparison between the different acclimation groups.

As expected for marine teleost fish (e.g. *Conger conger* [91], *G. morhua* [80] or *Sparus aurata* [40]), the acute acidosis evoked by higher environmental PCO_2 was compensated for by a significant increase in plasma $[HCO_3^-]$ in both the cold and warm hypercapnic groups. The depiction in the pH-bicarbonate diagram (Figure 5) shows that the increase in plasma $[HCO_3^-]$ cannot solely be attributed to extracellular non-bicarbonate buffering. Instead, combined acid–base parameters were positioned above the non-bicarbonate buffer line, likely due to the involvement of proton equivalent ion transfer processes [92]. Although the pattern of compensation is similar for many teleost fish, the $[HCO_3^-]$ reached differ between species: e.g. levels reached 22 mM in *C. conger* [91], and 32 mM in cod, respectively [80] when exposed to 1 kPa CO_2. The exposure to a moderate PCO_2 of 0.2 kPa led to lower but still significantly elevated $[HCO_3^-]$ of 11.3 mM in cold hypercapnic *N. rossii*.

The compensation of higher ambient PCO_2 via elevated $[HCO_3^-]_e$ and $[HCO_3^-]_i$ can lead to an increase in ATP demand for ion exchanging processes to maintain $[HCO_3^-]$ at this higher level, as it was reported for long-term hypercapnia acclimated eelpout (*Z. viviparus*) [93]. The reaction to this constantly higher ATP demand could be a shift in energy budget with reduced ATP consuming processes, e.g. protein turnover or anabolism [93]. This new metabolic equilibrium under increased metabolic demands for acid–base regulation could result in shifted 'set points', as we observed in the warm or cold hypercapnia acclimated *N. rossii*, with pH shifted towards alkaline values and thus a constant, slight metabolic alkalosis in both groups.

Both temperature and hypercapnia influence blood parameters in Antarctic fish, which has been demonstrated for blood osmolarity after thermal acclimation in notothenioids [94]. This explains the observed decreased serum osmolarities in our warm acclimated animals. The unaffected osmolarities in cold hypercapnia acclimated *N. rossii* are in line with earlier findings by Larsen et al. [80] for cod (*G. morhua*) exposed to 1 kPa CO_2. Although we observed hypercapnia induced changes in ion regulation, the higher $[HCO_3^-]_e$ in the blood are too small to significantly alter total osmolarity. Hence, the changes in osmolarity can exclusively be attributed to long-term warm acclimation.

The haematocrit levels of *N. rossii* were unaffected by warm and/ or hypercapnia acclimation, and within the range reported for its sympatric sister species *N. coriiceps* [26,95] (Table 2). While acute warming causes an elevation of haematocrit in red-blooded notothenioids [26], long-term warm acclimation leaves haematocrit levels constant, consistent with results from other studies on Antarctic notothenioids [94]. Thus, the oxygen carrying capacities of the blood of warm and/ or hypercapnia acclimated *N. rossii* do not seem to be limiting under these conditions.

It has been assumed that the extracellular non-bicarbonate buffering is mostly accomplished by proteins in the blood [96], and thus strongly depends on haematocrit [97], which varies greatly between fish species, also among Antarctic fish species [26,28,98,99]. The

haematocrit levels measured in *N. rossii* (28–31, see Table 2) thus result in high blood β_{NB} values (30.3 mmol/l pH). Similarly, the red-blooded Antarctic fish *Dissostichus mawsoni* and *P. borchgrevinki* showed higher β_{NB} values (~28 and 18 mmol/l pH, respectively) than the haemoglobinless icefish *Pagetopsis macropterus* (β_{NB} ~3 mmol/l pH) [100].

Some possible limitations in oxygen availability may have occurred at the intracellular level in the warm normocapnia acclimated fish, where liver pH_i was lower than in the control group. This pH difference cannot be exclusively attributed to α-stat regulation [83], as pH_i changed by −0.032 pH units/°C. Possibly, the high acclimation temperature of 7°C led to limiting oxygen supply to the liver tissue as a consequence of elevated metabolic demand,, resulting in a slight contribution of anaerobic metabolism and thereby lactate production, thus shifting the pH_i of the warm normocapnic group to acidic values. Nevertheless, other tissues with lower metabolic loads than liver, such as white muscle, may still be able to metabolise anaerobic end products to some degree, allowing the animals to survive at these warmer temperatures (4–6 weeks acclimation time in this study).

We did, however, not observe elevated lactate values in the blood of warm normocapnic/ hypercapnic fish, and the generally low lactate values of *N. rossii* were similar to those measured in *N. rossii* and *N. coriiceps* earlier [28,101]. Only in the cold hypercapnia acclimated animals, lactate levels were slightly elevated, but are likely the result of minor handling stress and do not originate from a beginning anaerobic metabolism in the liver, as they are still close to the levels of 1 mM reported for *N. coriiceps* under natural conditions [26,90].

A higher ATP demand under conditions of elevated temperature in combination with an intracellular acidosis might shift or even impair liver functionality over a longer time-scale in the warm-acclimated animals (normocapnia/ hypercapnia), which could relate to the reduced HSI in the animals of the present study (see Table 2).

Conclusion

This study investigated the thermal plasticity and acclimation abilities to higher temperature and PCO_2 levels of the Antarctic teleost fish *N. rossii*, by studying metabolic responses at different organisation levels (whole animal, blood, cellular and mitochondrial level).

At the whole animal level, our findings reveal partial compensation of RMR in the long-term warm normocapnia and hypercapnia acclimated fish in comparison to acute warm (7°C) exposed *N. rossii*. Long-term acclimation to 0.2 kPa CO_2 had no effect on RMR.

In the mitochondria, we observed only limited compensation of state III respiration following normocapnic warm acclimation. In contrast, both warm and cold hypercapnia acclimation led to reduced mitochondrial capacities, possibly mediated by changes in the TCA-cycle or the whole mitochondria, as indicated by reduced enzyme capacities.

In cold and warm hypercapnia acclimated fish, we observed shifts in the 'set points' of acid–base regulation to more alkaline values at both extra- and intracellular levels, mediated by actively accumulated [HCO_3^-]. These shifts may be involved in the hypercapnia-induced changes in cellular and mitochondrial energy demand. During long-term hypercapnia, shifts towards oxidative decarboxylation processes may maintain new acid–base equilibria. As the reduced mitochondrial capacities of the cold and warm hypercapnia acclimated fish were not visible in whole animal respiration, *N. rossii* might be limited in energy supply and aerobic scope for e.g. activity, growth and reproduction.

In the context of other data available for other high-Antarctic notothenioids [16,73], our data suggest that among the notothenioids the cold-adapted *N. rossii* will have only a moderate scope for acclimation and tolerance towards ocean acidification and warming of the Southern Ocean. At the chosen temperature of warm acclimation (7°C), the liver function of *N. rossii* may shift or become disturbed, thereby likely reducing whole animal performance over longer time-scales.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS and FCM designed the study, carried out the animal capture, acclimation, mitochondrial respiration experiments and extracellular acid–base determination and drafted the manuscript. AS performed the data analyses and interpretation. SB carried out the intracellular acid–base determination and contributed to writing the manuscript. EL performed the enzyme measurements. KM participated in the coordination of the study and contributed to revising the manuscript. HOP participated in the study design and substantially contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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References

1. Ferron FA, Simones JC, Aquino FE, Setzer AW: **Air temperature series for King George Island, Antarcitca.** *Pesquisa Ant Brasil* 2004, **4:**155–169.
2. Cook AJ, Fox AJ, Vaughan DG, Ferrigno JG: **Retreating Glacier Fronts on the Antarctic Peninsula over the Past Half-Century.** *Science* 2005, **308:**541–544.

3. Turner J, Colwell SR, Marshall GJ, Lachlan-Cope TA, Carleton AM, Jones PD, Lagun V, Reid PA, Iagovkina S: **Antarctic climate change during the last 50 years.** *Int J Climatol* 2005, **25:**279–294.
4. Meredith PM, King CJ: *Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century*. Washington, DC, USA: American Geophysical Union; 2005.
5. Turner J, Lachlan-Cope TA, Colwell S, Marshall GJ, Connolley WM: **Significant Warming of the Antarctic Winter Troposphere.** *Science* 2006, **311:**1914–1917.
6. Clarke A, Murphy EJ, Meredith MP, King JC, Peck LS, Barnes DKA, Smith RC: **Climate change and the marine ecosystem of the western Antarctic Peninsula.** *Philos Trans R Soc Lond B Biol Sci* 2007, **362:**149–166.
7. Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL, Wanninkhof R, Wong CS, Wallace DW, Tilbrook B, *et al*: **The oceanic sink for anthropogenic CO_2.** *Science* 2004, **305:**367–371.
8. Caldeira K, Wickett ME: **Anthropogenic carbon and ocean pH.** *Nature* 2003, **425:**365.
9. Caldeira K, Wickett ME: *Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean: The ocean in a high-CO2 world*. Washington, DC, USA: American Geophysical Union; 2005.
10. Cao L, Caldeira K: **Atmospheric CO_2 stabilization and ocean acidification.** *Geophys Res Lett* 2008, **35:**L19609.
11. Somero GN, DeVries AL: **Temperature tolerance of some Antarctic fishes.** *Science* 1967, **156:**257–258.
12. Hardewig I, Peck LS, Pörtner HO: **Thermal Sensitivity of Mitochondrial Function in the Antarctic Notothenioid *Lepidonotothen nudifrons*.** *J Comp Physiol B* 1999, **169:**597–604.
13. Mark FC, Bock C, Pörtner HO: **Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS.** *Am J Physiol Regul Integr Comp Physiol* 2002, **283:**R1254–R1262.
14. Peck LS: **Prospects for survival in the Southern Ocean: vulnerability of benthic species to temperature change.** *Antarct Sci* 2005, **17:**497.
15. Verde C, Parisi E, di Prisco G: **The evolution of thermal adaptation in polar fish.** *Gene* 2006, **385:**137–145.
16. Franklin CE, Davison W, Seebacher F: **Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinki*.** *J Exp Biol* 2007, **210:**3068–3074.
17. Pörtner HO: **Climate-dependent evolution of Antarctic ectotherms: An integrative analysis.** *Deep Sea Res Part 2 Top Stud Oceanogr* 2006, **53:**1071–1104.
18. Pörtner HO: **Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals.** *Naturwissenschaften* 2001, **88:**137–146.
19. Mark FC, Hirse T, Pörtner HO: **Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes.** *Polar Biol* 2005, **28:**805–814.
20. Clarke A: **What is cold adaptation and how should we measure it?** *Amer Zool* 1991, **31:**81–92.
21. Peck LS: **Prospects for survival in the Southern Ocean: vulnerability of benthic species to temperature change.** *Antarctic Science-Institutional Subscription* 2005, **17:**497–508.
22. Pörtner HO, Lannig G: **Oxygen and capacity limited thermal tolerance**. In *Fish Physiology, Vol 27: Hypoxia*. Edited by Richards JG, Farrell AP, Brauner C. Burlington: Academic; 2009:143–191.
23. Lowe CJ, Davison W: **Thermal sensitivity of scope for activity in *Pagothenia borchgrevinki*, a cryopelagic Antarctic nototheniid fish.** *Polar Biol* 2006, **29:**971–977.
24. Seebacher F, Davison W, Lowe CJ, Franklin CE: **A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes.** *Biol Lett* 2005, **1:**151–154.
25. Podrabsky J, Somero G: **Inducible heat tolerance in Antarctic notothenioid fishes.** *Polar Biol* 2006, **30:**39–43.
26. Beers JM, Sidell BD: **Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin.** *Physiol Biochem Zool* 2011, **84:**353–362.
27. Pörtner HO, Lucassen M, Storch D: **Metabolic biochemistry: its role in thermal tolerance and in the capacities of physiological and ecological function**. In *The Physiology of Polar Fishes. Volume 21*. Edited by Farrell AP, Steffensen JF. San Diego: Elsevier Academic Press; 2005:79–154 [Hoar WS, Randall DR, Farrell AP (Series Editor): *Fish Physiology*].
28. Mark FC, Lucassen M, Strobel A, Barrera-Oro E, Koschnick N, Zane L, Patarnello T, Pörtner HO, Papetti C: **Mitochondrial Function in Antarctic Nototheniids with ND6 Translocation.** *PLoS One* 2012, **7:**e31860.
29. Pörtner HO, Hardewig I, Peck LS: **Mitochondrial Function and Critical Temperature in the Antarctic Bivalve, *Laternula elliptica*.** *Comp Biochem Physiol A* 1999, **124:**179–189.
30. Heise K, Puntarulo S, Pörtner HO, Abele D: **Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress.** *Comp Biochem Physiol C Toxicol Pharmacol* 2003, **134:**79–90.
31. Johnston I, Guderley H, Franklin C, Crockford T, Kamunde C: **Are mitochondria subject to evolutionary temperature adaptation?** *J Exp Biol* 1994, **195:**293.
32. Urschel M, O'Brien K: **Mitochondrial function in Antarctic notothenioid fishes that differ in the expression of oxygen-binding proteins.** *Polar Biol* 2009, **32:**1323–1330.
33. Lemieux H, Tardif J, Dutil J, Blier P: **Thermal sensitivity of cardiac mitochondrial metabolism in an ectothermic species from a cold environment, Atlantic wolffish (*Anarhichas lupus*).** *J Exp Mar Bio Ecol* 2010, **384:**113–118.
34. Walther K, Sartoris F, Bock C, Pörtner HO: **Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*.** *Biogeosciences Discussions* 2009, **6:**2837–2861.
35. Pörtner HO, Farrell AP: **Ecology. Physiology and climate change.** *Science* 2008, **322:**690–692.
36. Metzger R, Sartoris F, Langenbuch M, Pörtner HO: **Influence of elevated CO_2 concentrations on thermal tolerance of the edible crab *Cancer pagurus*.** *J Therm Biol* 2007, **32:**144–151.
37. Foss A, Rųsnes B, Ųiestad V: **Graded environmental hypercapnia in juvenile spotted wolffish (*Anarhichas minor* Olafsen): effects on growth, food conversion efficiency and nephrocalcinosis.** *Aquaculture* 2003, **220:**607–617.
38. Fivelstad S, Olsen AB, Asgård T, Baeverfjord G, Rasmussen T, Vindheim T, Stefansson S: **Long-term sublethal effects of carbon dioxide on Atlantic salmon smolts (*Salmo salar* L.): ion regulation, haematology, element composition, nephrocalcinosis and growth parameters.** *Aquaculture* 2003, **215:**301–319.
39. Melzner F, Gutowska M, Hu M, Stumpp M: **Acid–base regulatory capacity and associated proton extrusion mechanisms in marine invertebrates: An overview.** *Comp Biochem Physiol A Mol Integr Physiol* 2009, **153:**S80.
40. Michaelidis B, Spring A, Pörtner HO: **Effects of long-term acclimation to environmental hypercapnia on extracellular acid–base status and metabolic capacity in Mediterranean fish *Sparus aurata*.** *Mar Biol* 2007, **150:**1417–1429.
41. Pörtner HO, Bock C, Reipschläger A: **Modulation of the cost of pHi regulation during metabolic depression: A P-31-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle.** *J Exp Biol* 2000, **203:**2417–2428.
42. Bock C, Sartoris FJ, Wittig RM, Pörtner HO: **Temperature-dependent pH regulation in stenothermal Antarctic and eurythermal temperate eelpout (Zoarcidae): an *in-vivo* NMR study.** *Polar Biol* 2001, **24:**869–874.
43. Brauner CJ, Wang T, Wang Y, Richards JG, Gonzalez RJ, Bernier NJ, Xi W, Patrick M, Val AL: **Limited extracellular but complete intracellular acid–base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*.** *J Exp Biol* 2004, **207:**3381–3390.
44. Lannig G, Eilers S, Pörtner HO, Sokolova IM, Bock C: **Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas* - changes in metabolic pathways and thermal response.** *Mar Drugs* 2010, **8:**2318–2339.
45. Abe H: **Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle.** *Biochemistry c/c of Biokhimiya* 2000, **65:**757–765.
46. Sartoris FJ, Bock C, Pörtner HO: **Temperature-dependent pH regulation in eurythermal and stenothermal marine fish: an interspecies comparison using P-31-NMR.** *J Therm Biol* 2003, **28:**363–371.
47. Pörtner HO: **Determination of Intracellular Buffer Values After Metabolic Inhibition by Fluoride and Nitrilotriacetic Acid.** *Respir Physiol* 1990, **81:**275–288.

48. Young S, Egginton S: **Allometry of skeletal muscle fine structure allows maintenance of aerobic capacity during ontogenetic growth.** *J Exp Biol* 2009, **212:**3564–3575.
49. Moerland TS, Egginton S: **Intracellular pH of muscle and temperature: insight from in vivo ^{31}P NMR measurements in a stenothermal Antarctic teleost (*Harpagifer antarcticus*).** *J Therm Biol*1998, **23:**275–282.
50. Sosinski JSK: **Biomass estimate of commercial fish on the shelf of South Georgia by the swept area method.** *Bull Sea Fish Inst* 1987, **5–6:**8–14.
51. Duhamel G: *Distribution and abundance of fish on the Kerguelen Island shelf*. 1985th edition. Stockholm: Proc V Congr europ Ichthyol; 1987.
52. Casaux R, Mazzotta A, Barrera-Oro E: **Seasonal aspects of the biology and diet of nearshore nototheniid fish at Potter Cove, South Shetland Islands, Antarctica.** *Polar Biol* 1990, **11:**63–72.
53. Gon O, Heemstra P: *Fishes of the Southern Ocean*. Grahamstown, South Africa: J.L.B. Smith Institute for Ichthyology; 1990.
54. Everson I: **Inshore fishes from the South Orkney and South Shetland Islands, the Antarctic Peninsula and South Georgia.** *Br Ant Surv Bull* 1969, **19:**89–96.
55. Schloss I, Ferreyra GA, González O, Atencio A, Fuentes V, Tosonotto G, Mercuri G, Sahade R, Tatián M, Abele D: **Long term hydrographic conditions and climate trends**. In *The Potter Cove coastal ecosystem, Antarctica Synopsis of research performed 1999–2006 at the Dallmann Laboratory and Jubany Station, King George Island (Isla 25 de Mayo) Ber Polarforsch Meeres*. Edited by Potter C, Wiencke C, Ferreyra G, Abele D, Marenssi S. 2008.
56. Caldeira K, Wickett M: **Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean.** *J Geophys Res* 2005, **110:**1–12.
57. Pierrot D, Lewis E, Wallace DWR: **MS Excel Program Developed for CO_2 System Calculations**. In *Book MS Excel Program Developed for CO2 System Calculations*. ORNL/CDIAC-105a editionth edition. City: Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy; 2006.
58. Johnston IA, Clarke A, Ward P: **Temperature and metabolic rate in sedentary fish from the Antarctic, North Sea and Indo-West Pacific Ocean.** *Mar Biol* 1991, **109:**191–195.
59. Ricker WE: **Computation and interpretation of the biological statistics of fish populations.** *Bull Fish Res Board Can* 1975, **191:**382.
60. Bradford M: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Anal Biochem* 1976, **72:**248–254.
61. Moyes C, Mathieu-Costello O, Tsuchiya N, Filburn C, Hansford R: **Mitochondrial biogenesis during cellular differentiation.** *Am J Physiol Cell Physiol* 1997, **272:**C1345.
62. Heisler N: *Acid–base Regulation in Animals*. Amsterdam: Elsevier Science Publisher B.V. (Biomedical Division); 1986.
63. Van Dijk PL, Hardewig I, Pörtner HO: **Temperature-dependent shift of pHi in fish white muscle: contributions of passive and active processes.** *Am J Physiol* 1997, **272:**R84–R89.
64. Egginton S, Taylor E, Wilson R, Johnston I, Moon T: **Stress response in the Antarctic teleosts (*Notothenia neglecta* Nybelin and *N. rossii* Richardson).** *J Fish Biol* 1991, **38:**225–235.
65. Noack S: *Statistische Auswertung von Menü-und Versuchsdaten mit Taschenrechner und Tischcomputer*. Berlin-New York: De Gruyter; 1980.
66. Bilyk KT, DeVries AL: **Heat tolerance and its plasticity in Antarctic fishes.** *Comp Biochem Physiol A Mol Integr Physiol* 2011, **158:**382–390.
67. Vanella FA, Calvo J: **Influence of temperature, habitat and body mass on routine metabolic rates of Subantarctic teleosts.** *Sci* Mar 2005, **69:**317–323.
68. Pörtner HO, Mark FC, Bock C: **Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques.** *Respir Physiol Neurobiol* 2004, **141:**243–260.
69. Precht H, Christophersen J, Hensel H, Larcher W: *Temperature and life*. Berlin, Heidelberg: Springer; 1973.
70. Fry F: **The Effect of Environmental Factors on the Physiology of Fish.** *Fish Physiol* 1971, **6:**1–98.
71. Jobling M: **The influences of feeding on the metabolic rate of fishes: a short review.** *J Fish Biol* 1981, **18:**385–400.
72. Lannig G, Storch D, Pörtner HO: **Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation.** *Polar Biol* 2005, **28:**575–584.
73. Robinson E, Davison W: **The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption.** *Polar Biol* 2008, **31:**317–326.
74. Melzner F, Bock C, Pörtner HO: **Temperature-dependent oxygen extraction from the ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*.** *J Comp Physiol B* 2006, **176:**607–621.
75. Langenbuch M, Pörtner HO: **Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid–base variables.** *J Exp Biol* 2002, **205:**1153–1160.
76. Langenbuch M, Bock C, Leibfritz D, Pörtner HO: **Effects of environmental hypercapnia on animal physiology: A C-13 NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*.** *Comp Biochem Physiol A Mol Integr Physiol* 2006, **144:**479–484.
77. Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MC, Chivers DP: **Replenishment of fish populations is threatened by ocean acidification.** *Proc Natl Acad Sci U S A* 2010, **107:**12930–12934.
78. Domenici P, Allan B, McCormick MI, Munday PL: **Elevated carbon dioxide affects behavioural lateralization in a coral reef fish.** *Biol Lett* 2012, **8:**78–81.
79. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sørensen C, Watson SA, Munday PL: **Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function.** *Nat Clim Chang* 2012, **2**(3):201–204.
80. Larsen BK, Pörtner HO, Jensen FB: **Extra- and Intracellular Acid–base Balance and Ionic Regulation in cod (*Gadus Morhua*) during Combined and Isolated Exposures to Hypercapnia and Copper.** *Mar Biol* 1997, **128:**337–346.
81. Van Dijk PLM, Tesch C, Hardewig I, Pörtner HO: **Physiological disturbances at critically high temperatures: A comparison between stenothermal Antarctic and eurythermal temperate eelpouts (Zoarcidae).** *J Exp Biol* 1999, **202:**3611–3621.
82. Taylor S, Egginton S, Taylor E, Franklin C, Johnston I: **Estimation of intracellular pH in muscle of fishes from different thermal environments.** *J Therm Biol* 1999, **24:**199–208.
83. Reeves RB: **An imidazole alphastat hypothesis for vertebrate acid–base regulation: tissue carbon dioxide content and body temperature in bullfrogs.** *Respir Physiol* 1972, **14:**219–236.
84. Pörtner HO: **Contributions of anaerobic metabolism to pH regulation in animal tissues: theory.** *J Exp Biol* 1987, **131:**69–87.
85. Pörtner HO, MacLatchy LM, Toews DP: **Acid–base regulation in the toad *Bufo marinus* during environmental hypoxia.** *Respir Physiol* 1991, **85:**217–230.
86. Simpson DP: **Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria.** *J Clin Invest* 1967, **46:**225.
87. Walsh PJ, Mommsen TP, Moon T, Perry S: **Effects of acid–base variables on in vitro hepatic metabolism in rainbow trout.** *J Exp Biol* 1988, **135:**231–241.
88. Pörtner HO, Reipschläger A, Heisler N: **Acid–base Regulation, Metabolism and Energetics in *Sipunculus nudus* as a Function of Ambient Carbon Dioxide Level.** *J Exp Biol* 1998, **201:**43–55.
89. Nonnotte G, Truchot JP: **Time course of extracellular acid–base adjustments under hypo- or hyperosmotic conditions in the euryhaline fish *Platichthys flesus*.** *J Fish Biol* 1990, **36:**181–190.
90. Egginton S: **Stress response in two Antarctic teleosts (*Notothenia coriiceps* Richardson and *Chaenocephalus aceratus* Lönnberg) following capture and surgery.** *J Comp Physiol B* 1994, **164:**482–491.
91. Toews D, Holeton G, Heisler N: **Regulation of the acid–base status during environmental hypercapnia in the marine teleost fish *Conger conger*.** *J Exp Biol* 1983, **107:**9–20.
92. Pörtner HO: **Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view.** *Mar Ecol Prog Ser* 2008, **373:**203–217.
93. Deigweiher K, Koschnick N, Pörtner HO, Lucassen M: **Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia.** *Am J Physiol Regul Integr Comp Physiol* 2008, **295:**R1660–R1670.
94. Hudson H, Brauer P, Scofield M, Petzel D: **Effects of warm acclimation on serum osmolality, cortisol and hematocrit levels in the Antarctic fish, *Trematomus bernacchii*.** *Polar Biol* 2008, **31:**991–997.
95. Heise K, Estevez M, Puntarulo S, Galleano M, Nikinmaa M, Pörtner HO, Abele D: **Effects of seasonal and latitudinal cold on oxidative stress**

parameters and activation of hypoxia inducible factor (HIF-1) in zoarcid fish. *J Comp Physiol B* 2007, **177:**765–777.
96. Cameron JN: **Acid–base homeostasis: past and present perspectives.** *Physiol Zool* 1989, **62:**845–865.
97. Wood CM, McDonald D, McMahon B: **The influence of experimental anaemia on blood acid–base regulation in vivo and in vitro in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*).** *J Exp Biol* 1982, **96:**221.
98. Smit G, Hattingh J, Burger A: **Haematological assessment of the effects of the anaesthetic MS 222 in natural and neutralized form in three freshwater fish species: interspecies differences.** *J Fish Biol* 1979, **15:**633–643.
99. Kunzmann A: ***Blood Physiology and Ecological Consequences in Weddell Sea Fishes (Antarctica)*** *PhD.* 1991.
100. Wells RMG: **Respiration of Antarctic fish from McMurdo Sound.** *Comp Biochem Physiol* 1987, **88**(A):417–424.
101. Egginton S: **A comparison of the response to induced exercise in red- and white-blooded Antarctic fishes.** *J Comp Physiol B* 1997, **167:**129–134.

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PUBLICATION III

Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*

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Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*

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Abstract

Mitochondrial plasticity plays a central role in acclimation capacities of aerobic metabolism in ectotherms to changing temperatures or $P\mathrm{CO_2}$. Activities of key mitochondrial enzymes (citrate synthase (CS), cytochrome *c* oxidase (COX)) from heart, red muscle, white muscle and liver in the Antarctic fish *Notothenia rossii*, were measured after warm- (7°C) and hypercapnia- (0.2 kPa CO_2) acclimation *vs.* control conditions (1°C, 0.04 kPa CO_2).

In heart, enzymes showed elevated activities after cold-hypercapnia acclimation, and a warm-acclimation-induced upward shift in thermal optima. Red muscle displayed the highest plasticity towards hypercapnia by increased enzyme activities. In white muscle, enzyme activities were temperature-compensated. CS activity in liver decreased after warm-normocapnia acclimation (temperature-compensation), while COX activities were lower after cold- and warm-hypercapnia exposure, but increased after warm-normocapnia acclimation.

In conclusion, warm-acclimated *N. rossii* display low thermal compensation in response to rising energy demand in highly aerobic tissues, such as heart and red muscle. Chronic environmental hypercapnia also elicits increased enzyme activities in these tissues, possibly to compensate for an elevated energy demand for acid-base regulation or a compromised mitochondrial metabolism. This might be supported by enhanced metabolisation of liver energy stores. These patterns reflect a limited capacity of *N. rossii* to reorganise energy metabolism in response to rising temperature and $P\mathrm{CO_2}$.

Key words

Hypercapnia acclimation, warm acclimation, citrate synthase (CS), cytochrome c oxidase (COX), Antarctic fish, aerobic energy metabolism, ocean acidification

1. Introduction

Metabolic capacities of mitochondria are strongly connected to the aerobic scope of ectothermic animals, as these ATP producing organelles contribute the greatest part to oxygen consumption and are thus directly affected by limitations in oxygen supply close to an animal's upper thermal limit (Pörtner, 2002). Due to their central role in aerobic energy metabolism, they are a key factor in defining acclimation- and adaptation capacities of ectothermal animals to changes in abiotic, environmental conditions, such as increasing seawater temperature and PCO_2.

Mitochondrial responses to these environmental changes include for example changes in functional capacity, content, volume or cristae surface (Guderley, 1990; Michaelidis et al., 2007; Strobel et al., 2012; Windisch et al., 2011). Amongst others, they become visible in activities of the mitochondrial matrix enzyme citrate synthase (CS). This enzyme plays a central role in several metabolic pathways as it catalyses the first step of the tricarbonic acid cycle (TCA-cycle) and interlinks the TCA-cycle with glycolysis and fatty acid metabolism via the utilization of acetyl-CoA. The mitochondrial transmembrane protein cytochrome *c* oxidase (COX) is a substantial part of the electron transport system, and both enzymes are frequently used as indicators of tissue specific aerobic capacity (Cai and Adelman, 1990). Furthermore, changes in COX activity may be related to alterations in mitochondrial membrane structure (O'Brien and Mueller, 2010; Wodtke, 1981), and in CS activity to changes in mitochondrial matrix volume (e.g. Guderley, 2004; Guderley and St-Pierre, 2002; Hardewig et al., 1999b).

Prolonged elevations in temperature usually cause an increase in physiological rates and associated energy demand, which then need an adjustment of energy production. Similarly, the maintenance of acid-base equilibria, i.e. elevated bicarbonate concentrations to compensate for increases in extra- and intracellular PCO_2 under chronic hypercapnia, may cause an increase in energy demand (Melzner et al., 2009). In several fish species, adjustments of aerobic capacity involve shifts in the activities of individual enzymes or even between metabolic pathways (Lucassen, 2006; Melzner et al., 2009; Michaelidis et al., 2007; Windisch et al., 2011).

A recent study measuring mitochondrial respiration in *N. rossii* acclimated to elevated temperatures and PCO_2 revealed reductions in mitochondrial oxidative phosphorylation capacities in response to hypercapnia, which is mainly due to an inhibited flux through complex I (succinate-dehydrogenase) of the electron transport system. The authors propose an

increased metabolisation of NADH-linked substrates in order to enhance the energetic efficiency of mitochondrial oxidative capacity in *N. rossii* exposed to chronic warmth and or hypercapnia (Strobel et al., 2012). Thus, elevated PCO_2 is suggested to induce shifts in mitochondrial substrate preferences in order to compensate for the detrimental effect of environmental hypercapnia.

Mitochondrial proliferation has been found in cold-adapted polar species and in cold-acclimated eurythermal species, which frequently comes along with enhanced enzyme activities in the cold-adapted eurytherms (Crockett and Sidell; Johnston et al., 1998; Sommer and Pörtner, 2002). However, various Antarctic fish studied so far do not show cold-compensated up-regulation of mitochondrial oxidative capacities or routine metabolic rates; aerobic capacities are proposed to be rather increased by mitochondrial proliferation and increased organ size in cold-water species (Hardewig et al., 1999a; Johnston et al., 1998; Lannig et al., 2003; Van Dijk et al., 1999).

In contrast to recent findings of reduced mitochondrial capacities in the Antarctic eelpout *Pachycara brachycephalum* in the warmth (Lannig et al., 2005), the nototheniid *Pagothenia borchgrevinki* responds to warm-acclimation mainly by increasing muscle COX activities, while glycolytic and TCA enzyme levels remain unchanged. The authors suggest that high COX activities come along with increased oxidative capacities, which may support elevated metabolic costs at warmer temperatures in Antarctic fish (Seebacher et al., 2005). However, these activities could also solely relate to large excess capacities of COX, which provides a mechanism to sustain high oxygen affinity of mitochondria (Gnaiger et al., 1998). Therefore, it appears difficult to conclude from high COX activities to a general activity-elevation in all components of the electron transport system.

Similar to *P. borchgrevinci*, COX activities were increased in heart and liver of carp (*Cyprinus carpio*) after warm-acclimation (Cai and Adelman, 1990). In contrast, a lack of change or even a loss of specific COX activities had been shown in liver of cold-acclimated cod *Gadus morhua* and eelpout *Zoarces viviparus*, while CS activity showed a strong thermal response, increasing in the cold and decreasing in the warmth (Lucassen, 2006; Lucassen et al., 2003). In cold-acclimated channel catfish, *Ictalurus puncatus*, a positive compensation of total liver CS activities were found, whereas total COX activities remained similar between fish acclimated to 25°C and 15°C (Kent et al., 1988). Taken together, these studies emphasize strong differences between tissue types and the difficulty to compare non-related species or such originating from different climate zones. Only few studies exist that report enzymatic responses to long-term elevated PCO_2 in fish, for example, a decrease in CS activity in heart,

red muscle and white muscle (0.5 kPa CO_2, sea bass *Sparus aurata* (Michaelidis et al., 2007)).

Most studies on mitochondrial aerobic enzyme capacities have been conducted on temperate zone fish (e.g. Dalziel et al., 2005; Grim et al., 2010; Hulbert et al., 2006; Martin-Perez et al., 2012), while hardly any study analysed the role of mitochondrial aerobic enzymes in warm- acclimation of Antarctic fish (Lannig et al., 2005). Yet, the adjustment of aerobic capacities by setting mitochondrial content and capacities mark a crucial step in defining thermal tolerance windows and acclimation capacities of ectothermal animals (Pörtner, 2002). However, aerobic mitochondrial capacities strongly depend on tissue-specific metabolic functions and energy demand, which leads to a wide variety of tissue aerobic capacities and mitochondrial densities (Dalziel et al., 2005).

The present study was therefore designed to investigate enzymatic responses in tissues of different metabolic activity (liver, heart, red muscle and white muscle) of Antarctic notothenioid fish to ocean warming and acidification. We used the demersal notothenioid *Notothenia rossii*, which displays a circum-polar distribution at habitat temperatures between -1.9 and 2°C (Everson, 1969; Gon and Heemstra, 1990; Schloss et al., 2008), as a representative for Antarctic stenotherms.

We studied the acclimation capacities of two enzymes involved in aerobic mitochondrial metabolism, namely citrate synthase (CS) and cytochrome c oxidase (COX), in *N. rossii* acclimated for four to six weeks to the warmth (7°C) and/ or elevated PCO_2 of 0.2 kPa (2000 µatm). We analysed CS and COX activities per mg cellular protein and per g tissue fresh weight at acute rising assay temperatures of 0, 6, 9 and 12°C. The enzyme activities measured were also used as markers of possible shifts in the usage of metabolic pathways (COX/CS ratio) in response to changes in metabolic demands.

2. Material and Methods

2.1 Animal capture and acclimation

Antarctic, demersal marbled rockcod, *N. rossii*, were caught using baited traps in December 2009 in Potter Cove, King George Island, Antarctic Peninsula (62°14'S; 058°41'W) at a seawater temperature of 0.8±0.9°C, salinity 33.5±0.2 psu. Fish were collected

with baited traps (length 124 cm, width 64 cm, height 56 cm, mesh size 25 mm) and trammel nets (length 15 m, inner mesh 25 mm).

The fish were reared and acclimated in the aquaria facilities at Dallmann Laboratory, Carlini Station (formerly Jubany Station, King George Island) under natural light conditions. Animals were fed to satiation every other day with chopped fish muscle and snails. For acclimation, animals were randomly selected and acclimated to 1°C, 0.04 kPa CO_2 (control group, *n*=9, mass 155-804 g; total length 25-39.4 cm), 1°C, 0.2 kPa CO_2 (cold hypercapnic group, *n*=10, mass 144-510 g; total length 23.8-32.8 cm), 7°C, 0.04 kPa CO_2 (warm normocapnic group, *n*=5, mass 151-412 g; total length 23.6-33.9 cm) and 7°C, 0.2 kPa CO_2 (warm hypercapnic group, *n*=10, mass 137-504 g; total length 21.4-31.3 cm). For detailed acclimation conditions, specific seawater conditions and seawater carbonate chemistry see (Strobel et al., 2012).

2.2 Sampling

At the end of the acclimation period, specimens of *N. rossii* were anesthetized with 0.5 g/l tricaine methano-sulphonate (MS 222) and killed by a spinal cut behind the head plates. Liver (L), heart (H), red muscle (RM, pectoral muscle) and white muscle (WM, lateral muscle) samples were removed and immediately freeze-clamped and shock-frozen in liquid nitrogen and stored at -80°C for later analysis. The work was carried out according to the ethics and guidelines of German law. Experiments had been approved according to § 8 animal welfare act (18.05.2006; 8081. I p. 1207) by the veterinary inspection office, Bahnhofsplatz 29, 28195 Bremen, Germany, under the permit number Az.: 522-27-11/02-00 (93) on January 15th, 2008 (permit valid until Jan 14th 2013).

2.3 Enzyme assays

Frozen liver tissue was ground into powder by mortar and pestle under liquid nitrogen and homogenized in a glass homogenizer in 9 vol. buffer containing 20 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 0.1% Triton X-100 (pH 7.4) and afterwards with an Ultra Turrax (Silent Crusher M (Heidolph Instruments, Schwabach, Germany), followed by 10min centrifugation at 1,000 *g* at 4°C. Enzyme activities of each sample extract were measured at 0, 6, 9 and 12°C in a UV/VIS spectrophotometer (Beckman, Fullerton, CA, USA) equipped with a

thermostatted cell holder. To check the efficiency of the extraction procedure, enzymatic activity was estimated in re-extracted pellets.

Citrate Synthase (CS; EC 4.1.3.7) activity was detected according to Sidell et al (1987) in a buffer containing 75 mmol l^{-1} Tris-HCl, 0.25 mmol l^{-1} DTNB, 0.4 mmol l^{-1} acetyl-CoA, 0.5 mmol l^{-1} oxaloacetate. The activity was determined from the increase in absorbance at λ =412 nm, caused by the transfer of sulfhydryl groups from coenzyme A to 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), quantified by use of the extinction coefficient (ε_{412}) of DTNB of 13.61 $mMol^{-1}cm^{-1}$.

Cytochrome *c* oxidase (COX; 1.9.3.1) activity was determined according to a protocol modified from Moyes et al. (1997) in buffer containing 20 mmol l^{-1} Tris-HCl, 0.05% Tween 20 and 0.057 mM reduced cytochrome *c* at pH 8.0. The activity was determined from the decrease in extinction at λ = 550 nm through oxidation of cytochrome *c,* using the extinction coefficient ε_{550} = 19.1 mol^{-1} cm^2.

Protein concentration of the tissue extract was determined according to Bradford (Bradford, 1976) by measuring the absorbance at λ=595 nm and 20°C in a spectrophotometer (Pharmacia LKB Biochrom 4060, Pharmacia, UK). The enzyme activity (CS and COX) was calculated per mg tissue fresh-weight (nmol min^{-1} mg FW^{-1}) as well as per mg cellular protein (nmol min^{-1} mg $protein^{-1}$) to account for changes in mitochondrial density and composition on the one hand (normalized to FW), and on the other hand, to overcome the effect of changing amounts of tissue lipids in fish acclimated at different temperatures (normalized to protein).

2.4 Calculations and statistics

The COX/CS ratio (mean across all assay temperatures as a measure for the enzyme-activity ratio between individual tissues) is given for the four tissue types of every acclimation group. The temperature coefficient (Q_{10}) was calculated for the temperature ranges 0-6°C, 6-12°C, 0-9°C and 0-12°C. All data were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance. For all tissues, mean enzyme activities per acclimation group were compared to controls using unpaired t-test or analysis of variance (ANOVA) followed by a Tukey-test. All data are presented as means ± standard error of the mean (SEM) per mg cellular protein or mg tissue fresh-weight. Differences were considered significant if $p \leq 0.05$.

3. Results

3.1 Effect of warm-acclimation on enzyme activity

Generally, CS and COX activities per mg protein in control *N. rossii,* were lowest in liver (CS: 10±1.5, COX: 32.3±2.7 nmol min^{-1} mg $protein^{-1}$), followed by similarly low levels in white muscle (CS: 17.3±2.7, COX: 18.0±4.3 nmol min^{-1} mg $protein^{-1}$). In heart and red muscle, they where about three-fold higher than in liver and white muscle (heart CS: 74.8±7.8, COX: 83.8±15.2 nmol min^{-1} mg $protein^{-1}$; red muscle CS: 101±14.9, COX: 41.0±2.7 nmol min^{-1} mg $protein^{-1}$, Table 1).

Mean CS activities per milligram FW in control *N. rossii* were similar in heart and red muscle (heart CS: 9.98±1.05, COX: 13.96±2.07 nmol min^{-1} mg FW^{-1}; red muscle CS: 8.36±1.23, COX: 3.31±0.25 nmol min^{-1} mg FW^{-1}) and up to six-fold higher than in liver and white muscle (liver CS: 1.61±0.19, COX: 4.89±0.41 nmol min^{-1} mg FW^{-1}; white muscle CS: 1.72±0.37, COX: 1.55±0.36 nmol min^{-1} mg FW^{-1}, Table 1).

In comparison to the control group of *N. rossii*, warm-normocapnia acclimation (7°C, 0.04 kPa CO_2) caused a significant increase in CS and COX activity in the heart, most prominently visible at 12°C assay temperature. CS and COX activities measured in white muscle were significantly reduced, while in liver CS activities were lower at 12°C and COX activities were elevated at 9°C following warm-acclimation (Figure 1).

Oxidative capacities depicted per mg FW generally showed a similar trend to those given per mg total protein after warm-acclimation in all tissues (Figure 2). Only the heart did not show the same significant increase in COX activity at 12°C that occurred per mg cellular protein.

3.2 Effect of hypercapnia acclimation on enzyme activity

Cold-hypercapnia acclimation caused a higher mean CS activity across all assay temperatures in heart (Table 1) and a significantly higher activity at 12°C when expressed in nmol min^{-1} mg FW^{-1}, while no effect was visible in COX activity. In red muscle, CS and COX activities (per mg protein and FW) were significantly elevated at all assay temperatures. In liver, COX activities at 9°C and the mean across all temperatures were reduced after - hypercapnia acclimation compared to the control (Table 1, Figure 1 and Figure 2).

In the warm-hypercapnia acclimated fish, COX activity (per mg protein and per mg FW) in heart was reduced in assays at 6 and 9°C, and also at 12°C when depicted per mg tissue weigh. CS activity was reduced as well, but the effect was more pronounced when plotted per mg FW (Figure 2). Both COX and CS enzyme activities (expressed per mg protein and per mg FW) were elevated in red muscle after warm-hypercapnia acclimation.

In white muscle, COX activities showed only an elevation after warm-normocapnia acclimation when depicted per mg cellular protein (Figure 1). In liver of the warm-hypercapnic group, COX activity (nmol min^{-1} mg $protein^{-1}$) was significantly decreased below control values (Figure 1), while CS activity remained unchanged in relation to mitochondrial protein content and even displayed elevated activities when plotted against mg FW (Figure 2).

3.3 COX to CS ratio, tissue protein content

In the *N. rossii* control, the COX/CS ratio was similar in heart (1.4±0.3) and white muscle (1.0±0.1), lowest in red muscle (0.5±0.1), and highest in liver (3.1±0.0; Figure 3). The COX/CS ratio in the heart fell significantly below control values during warm-acclimation (0.5±0.0), but was not affected by cold- or warm-hypercapnia acclimation. In contrast, it was slightly elevated in red muscle in the cold- and warm-ypercapnia acclimated fish, but not in the warm-normocapnia acclimated group. Similar to the heart, warm-normocapnia acclimation also caused a reduction in the COX/CS ratio in white muscle by about 50% (0.4±0.1), while the warm-hypercapnic group showed a significantly higher ratio (2.0±0.3) than the white muscle of the control fish (1.0±0.1). In liver, the ratio was significantly increased after warm-normocapnia acclimation (4.6±0.2), and reduced below control values (3.1±0.0) in the warm-hypercapnia group (1.7±0.3; Figure 3).

Following warm-hypercapnia acclimation, the amount of cellular protein per gram tissue fresh weight was significantly decreased only in heart (control: 161.1±23.7 mg g^{-1}; warm hypercapnic: 89.9±17.3 mg g^{-1}) and white muscle (control: 74.1±8.1 mg g^{-1}; warm hypercapnic: 52.7±5.5 mg g^{-1}) in comparison to the respective control (Figure 4).

4. Discussion

Measurements of maximal CS and COX activities are frequently used to determine changes in aerobic metabolic capacity in response to changes in environmental abiotic

conditions (O'Brien and Mueller, 2010). In this study, we assessed the different responses in CS and COX activity to long-term elevated warming and/ or hypercapnia in tissues characterized by high rates of aerobic energy generation (heart, red muscle), compared to the metabolically less active white muscle as well as to liver, which represents the hub of intermediary metabolism. The enzyme activities we measured in heart, red muscle, white muscle and liver fibres were consistent with values reported previously for cold-adapted fish (Crockett and Sidell, 1990; Hardewig et al., 1999b; Lucassen, 2006; Mark et al.). Heart and red muscle had up to six-fold higher enzymatic activities than white muscle and liver, indicating a higher mitochondrial density and metabolic capacity in the former tissues, which is often found in fish (Dalziel et al., 2005). Our findings thus confirm the higher oxidative capacity (and consequently oxygen demand) of the heart reflected by a two-fold higher COX activity than in red muscle (Walesby and Johnston, 1980) and of red muscle compared to white muscle. Many nototheniids including *N. rossii* primarily rely on a labriform swimming style by using the pectoral fins (Johnston, 2003), explaining the high aerobic capacity of the pectoral red muscle.

4.1 Effect of warm-acclimation

In many temperate fish species, a positive cold compensation likely involves mitochondrial proliferation or enhanced aerobic capacities of individual mitochondria, as for example shown by enhanced total enzyme activities in muscle of cold-acclimated cod (*G. morhua*), stickleback (*Gasterosteus aculeatus*) and trout (*Oncorhynchus mykiss*) (Egginton et al., 2000; Guderley et al., 2001; Lannig et al., 2003). Conversely, one would expect a reduction of mitochondrial content and enzyme activities towards the warmth in order to reduce mitochondrial maintenance costs and mitochondrial proton leakage (Pörtner, 2002).

For the sake of clarity, Table 2 gives a simplified overview on the trends of enzyme activities in response to warm and/ or hypercapnia acclimation in all four tissue types of *N. rossii* investigated in the present study. It depicts that a typical warm-compensation as seen in temperate zone species (e.g. Lannig et al., 2005; Lucassen, 2006) was not observed in the heart of warm normocapnia acclimated *N. rossii*. Instead, mean CS activities were significantly higher than in controls, and also COX activities per mg protein were elevated at 12°C.

In control animals, the activities of both enzymes were already thermally limited beyond 9°C assay temperature, which was also mirrored in a drop of acute Q_{10} values from a

Q_{10} of 1.8±0.2 (range 0-9°C) to 1.3±0.1 (range 9-12°C, Table SI in supplement). In an earlier study that measured mitochondrial respiration in a control group of *N. rossii* at various assay temperatures, patterns of limited mitochondrial oxidative capacities beyond 9°C were found to correspond to limitations in mitochondrial enzyme capacities as well (Mark et al., 2012).

Warm-acclimation of *N. rossii* induced a shift in the thermal limit of both CS and COX activities, which suggests a capacity to adjust enzyme characteristics in heart towards warmer temperatures. A similar pattern was observed in heart of the common carp (*C. carpio*) and in red muscle of the Antarctic notothenioid *P. borchgrevinki,* where enzyme activities increased in the warmth (Cai and Adelman, 1990; Seebacher et al., 2005).

Thus, our data suggest that heart tissue of *N. rossii* does not compensate for warming to 7°C in the conventional sense, as this would imply a down-regulation of mitochondrial capacities. Instead, COX activities per mg tissue measured in *N. rossii* were hardly reduced compared to the control, and CS activities were even increased towards the warmth (Figure 2, Table 2). Although higher oxidative capacities may be needed to meet the energy demand of the heart at warmer ambient temperature, uncompensated mitochondrial capacities or densities also imply elevated maintenance costs of heart mitochondria. Such a coherence was also observed in warm-acclimated *P. brachycephalum*, where survival at warmer temperatures came along with elevated metabolic costs (Mark et al., 2006) due to uncompensated mitochondrial densities. The authors suggested a partial warm-compensation mediated by controlled uncoupling to counterbalance for elevated mitochondrial oxygen demand (Mark et al., 2006), a mechanism that could also apply for *N. rossii*. However, the involvement of this process remains to be investigated.

In the heart, we observed a stronger increase in CS than COX activity in the warmth, which was also reflected in a significantly lower COX/CS ratio in the warm-acclimated compared to the control group (Figure 3). Similar to findings in red muscle of striped bass (*Morone saxatilis*) (Egginton and Sidell, 1989) or in liver of temperate and Antarctic eelpout (*Z. viviparus* & *P. brachycephalum* (Lucassen et al., 2003)), the membrane enzyme COX was less sensitive to temperature changes in warm-acclimated *N. rossii,* which suggests a relative increase in matrix enzymes at more-or-less constant mitochondrial size and cristae surface density.

CS plays a central role in mitochondria by interlinking several metabolic pathways. For example, it catalyses the first reaction of the TCA-cycle, namely the condensation of oxaloacetate with acetyl-CoA to citrate, and thus reflects the entrance of acetyl-CoA into the TCA after final oxidation of carbohydrates and fatty acids. A lower COX/CS ratio in heart

due to the strong thermal stimulation of CS may indicate enhanced TCA-activity from anaplerotic pathways (Lucassen et al., 2003).

Seasonal cold- or warm-acclimation in muscle of temperate zone fish and evolutionary cold-adaptation in polar fishes, are well known to cause compensatory changes in mitochondrial content including tissue enzyme contents and activities (see above and Egginton and Sidell, 1989; Johnston et al., 1998; Lannig et al., 2003). Warm-acclimation of the cold-adapted *N. rossii* did not reverse this pattern: Red muscle CS (mean Q_{10} 1.7±0.1) or COX activities of warm-normocapnia acclimated *N. rossii* remained similar to the enzyme activities in the control group (Table 2), indicating no enzymatic temperature compensation or inverse mitochondrial proliferation in red muscle. The present findings are similar to those in the high-Antarctic notothenioid *P. borchgrevinki*, which even increases oxidative phosphorylation capacities in red muscle to cover elevated metabolic costs for labriform locomotion in the warmth (Seebacher et al., 2005). In line with findings of high capacities to increase critical swimming speed in *P. borchgrevinki* following warm-acclimation (Seebacher et al., 2005), the uncompensated, high enzymatic activities observed in red muscle of *N. rossii* imply elevated metabolic rates but also costs in the warmth, similar to the results described for the heart tissue.

In liver, the CS activity per mg protein of the warm-acclimated group was reduced by warmer assay temperatures, leading to activity levels at 9°C assay temperature similar to those of the control group assayed at 0°C. This indicates partial temperature compensation after long-term warm acclimation in this tissue, and corroborates earlier findings of reduced CS activity in liver of warm-acclimated Antarctic eelpout, *P. brachycephalum* (Lannig et al., 2005). In contrast, the COX activity was stimulated by warm-normocapnia acclimation of *N. rossii* (Figure 1, Table 2), in line with elevated COX activities reported in liver of warm-acclimated carp (Cai and Adelman, 1990). This picture contradicts the general pattern of reversed mitochondrial proliferation observed in muscle tissue of temperate species in the warmth (Guderley and St-Pierre, 2002; Johnston et al., 1998) and indicates metabolic reorganisation or shunting of metabolic pathways in the liver.

The different COX/CS ratios observed in liver compared to the other tissues in this study are mainly due to low CS activities and may reflect the different metabolic duties of this tissue. The TCA-cycle in liver is a metabolic sink for succinyl-CoA from the oxidation of odd chain fatty acids and supports biosynthetic processes, e.g. lipid-biosynthesis from excess citrate or gluconeogenesis from malate (Owen et al., 2002; Windisch et al., 2011). The elevated COX/CS ratio in liver after warm-acclimation (Figure 3) is a result of increased

COX activities that can serve to enhance oxygen affinity (Gnaiger et al., 1998). This also may entail a shift from high energy substrates (fatty acids) to carbohydrate fuels (pyruvate entry from carbohydrate oxidation) and glycogen catabolism, which are energy generating pathways that consume less oxygen (Sidell et al., 1987; Windisch et al., 2011). As a result, the higher aerobic capacities might be accompanied by a concomitant degradation of the liver energy stores at warmer temperatures, in line with observations of a lower hepatosomatic index of warm-acclimated *P. brachycephalum* (Lannig et al., 2005) and *N. rossii* (Strobel et al., 2012).

Furthermore, the tissue protein content in liver was slightly higher in the warm-acclimated *N. rossii* compared to control (Figure 4), which mirrors a reduced lipid or glycogen content and concomitantly higher cellular protein fraction per mg liver tissue following warm-exposure. The energy reserves in the liver may be used to support enhanced aerobic capacities in the heart of warm-acclimated *N. rossii* (see above), but in the long run reduced ATP supply by the liver will contribute to limit the performance of highly aerobic tissues like heart or red muscle.

In the white muscle, the activities of both enzymes, CS and COX, were reduced after warm acclimation, similar to warm-acclimated white sucker *Castotomus commersoni* (Hardewig et al., 1999), and to reduced COX activities in skeletal muscle of warm- *vs*. cold-acclimated killifish *Fundulus heteroclitus* (Grim et al., 2010). The reduced CS and COX activities are consistent with predicted patterns of reduced mitochondrial enzyme activities in warm-acclimated individuals (Guderley, 1998; Lannig et al., 2003), and furthermore propose a decreased number of matrix and membrane molecules (Egginton and Sidell, 1989). The generally low enzyme activity could be an energy saving effect in order to adjust oxygen demand in this specific tissue of low aerobic activity, as already proposed for mitochondria of Antarctic fish (Pörtner, 2002).

4.2 Hypercapnia effects on enzyme activity

In the hearts of cold-hypercapnia acclimated *N. rossii*, mean CS activities were significantly elevated above control values (Table 1 and 2, Figure 2), while COX activities were not affected by elevated PCO_2. Thus, cold-hypercapnia acclimation seems to elicit a slight increase in mitochondrial aerobic capacities in the heart. This may be a general response of aerobically active tissues to higher metabolic demands, which are supposed to

support acid-base regulation during chronic hypercapnia (Deigweiher et al., 2009; Strobel et al., 2012).

In the heart tissue of the warm-hypercapnic group, however, particularly the enzyme activities per mg FW were lower than in the control group and did not show the same stimulation displayed by the hearts of warm-normocapnic fishes. A lower protein content per mg heart tissue suggests a decrease in mitochondrial enzyme content after warm-hypercapnia acclimation (Figure 4), and in combination with the reduced CS and COX activities this indicates a parallel decrease in mitochondrial size and cristae surface density (Egginton and Sidell, 1989; Johnston et al., 1998).

As the COX/CS ratio did not change in the heart of warm-hypercapnia acclimated fish, there seemed to be no shifts in metabolic pathways. The combination of the two stressors, elevated temperature and PCO_2, may thus trigger a reduction of mitochondrial capacities in this tissue and induce energy conserving processes in the heart, which go beyond the simple effect of inverse mitochondrial proliferation. In contrast, elevated seawater PCO_2 or higher temperature alone appeared to have a less severe impact on heart mitochondria in *N. rossii*. This is a first indicator for a reduced performance of Antarctic fish due to the synergistic effect of ocean warming and acidification and also identifies the heart as one of the most sensitive organs with the least capacities to acclimate (Somero, 2002).

Of all tissue types investigated under hypercapnia acclimation, the red muscle was most responsive. In both cold- and warm-hypercapnic groups, enzyme activities and COX/CS ratios were significantly higher compared to control- and warm-normocapnic *N. rossii* (Table 2 and Figure 3). Thus, the findings in cold- and warm-hypercapnic fish were clearly an acclimation response triggered by 0.2 kPa CO_2, which caused a 2- to 2.5-fold increase in enzyme activity, independent of temperature. Furthermore, tissue protein content was not different between the control and both hypercapnia acclimated groups (Figure 4). A change in mitochondrial structure or abundance (as shown for muscle of several temperate species (Egginton and Sidell, 1989; Johnston et al., 1998) seems therefore unlikely in red muscle of warm-normocapnia or -hypercapnia acclimated *N. rossii.* Instead, *N. rossii* appears to increase mitochondrial aerobic capacities in red muscle in response to chronic hypercapnia. This could be a reaction to higher metabolic demands, as also seen in the heart of warm-normocapnic *N. rossii* or in red muscle of warm-acclimated *P. borchgrevinki* (Seebacher et al., 2005).

Alternatively, higher enzymatic capacities can also be the result of a compensation of the inhibitory effects of hypercapnia on the mitochondrial TCA-cycle or electron transport system: elevated intracellular bicarbonate concentrations may suppress the TCA-cycle

through competitive inhibition of the citrate synthase function under acute and chronic hypercapnia (Simpson, 1967). We have postulated that this inhibitory effect of bicarbonate is in part compensated for by shifts towards anaplerotic mechanisms, which additionally fuel the TCA-cycle and thereby displace the competitive inhibitor bicarbonate in order to maintain TCA-cycle function (Langenbuch and Pörtner, 2003; Strobel et al., 2013). The high mitochondrial enzyme activities in red muscle of cold- and warm-hypercapnia acclimated fish propose that this tissue's mitochondria may be highly compromised by elevated PCO_2, possibly causing a compensation by elevations in CS and COX capacities.

In liver tissue, the results depict a different situation than in heart and red muscle. In the present study, we found that COX activities per mg protein were significantly reduced in the livers of both cold- and warm-hypercapnia acclimated fish. A parallel study revealed patterns of reduced mitochondrial state III respiration (maximum oxidative phosphorylation with substrates related to mitochondrial Complex I and II and ADP) in liver following cold- and warm-hypercapnia acclimation of *N. rossii* (Strobel et al., 2013), which is illustrated in Figure 5. Together with the data on reduced mitochondrial oxidative phosphorylation capacities in isolated liver mitochondria, this suggests that chronic hypercapnia exposure exerts an inhibitory effect on the complexes of the mitochondrial electron transport system. As a result, oxidative phosphorylation capacities and likely also mitochondrial oxygen affinities are reduced in liver following cold- and warm-hypercapnia acclimation.

Yet, CS activities were not affected by chronic hypercapnia, and also total protein contents per g liver tissue were not altered in *N. rossii* in response to cold- and warm-hypercapnia acclimation. Decreased COX activities at unchanged CS activities led to a lower COX/CS ratio in liver of cold- and warm-hypercapnic fish. Such a picture indicates shifts in metabolic pathways in liver, e.g. shunting TCA-cycle intermediates away from the electron transport system towards gluconeogenesis to support other tissues. Nevertheless, hypercapnia compensation in liver may be time-limited, as indicated by significantly reduced hepatosomatic indices in cold and warm-hypercapnia acclimated *N. rossii* already after five weeks acclimation time (Strobel et al., 2012). Thus it remains unclear whether a shift in metabolic pathways in response to warm- and hypercapnia acclimation is sufficient to support an elevated energy demand of other, highly oxidative tissues in the long run.

In white muscle, hypercapnia acclimation (cold/ warm) had no effect on CS activity per mg protein, but led to reduced total thermal capacities in white muscle tissue, which corresponds to reduced CS activity in white muscle in the warm temperate seabream *S. aurata* exposed to 0.5 kPa CO_2 (Michaelidis et al., 2007). The authors postulate a shift from aerobic

to anaerobic metabolism in hypercapnia acclimated *S. aurata*, which is unlikely for *N. rossii* due to a different preference of metabolic pathways in white muscle of these two species: in warm temperate *S. aurata,* glycolytic pathways dominate (Bone et al., 1978), while in Antarctic *N. rossii* ATP synthesis of white muscle strictly depends on aerobic mitochondrial energy metabolism (Walesby and Johnston, 1979) and thus possesses generally low anaerobic capacities. Furthermore, *S. aurata* is using the white trunk musculature for (anaerobic) locomotion, while nototheniid fish mainly use labriform swimming with the red musculature of the pectoral fins (Walesby and Johnston, 1979). Thus, their white muscle is not used intensively and is therefore energetically not very demanding. In white muscle of *N. rossii,* COX activities per mg protein and consequently COX/CS ratio, were elevated in the warm-hypercapnic group, an effect that was not visible when enzyme activities were related to tissue weight. This difference occurred due to a significantly reduced amount of protein per g tissue after warm-hypercapnia acclimation (Figure 4). Although white muscle plays a minor role in whole animal energy metabolism, the reduced CS activities and lower protein content after hypercapnia acclimation indicate a slight compensation or even catabolism of white muscle protein, possibly in response to an elevated energy demand under elevated seawater PCO_2.

Overall, the different enzymatic responses of the four tissues analysed in this study appear to be connected to the different metabolic duties and, consequently, different metabolic regulation in each tissue.

5. Conclusions

In summary, CS and COX activities in hearts of warm-normocapnia acclimated *N. rossii* showed a shift in thermal optimum towards warmer temperatures, but generally very low temperature-compensation abilities. A shift towards enhanced TCA-activity and fatty acid oxidation may warrant enough energy to maintain heart activity and oxygen delivery to tissues by the circulatory system even at 7°C, but at the expense of elevated metabolic costs for heart mitochondria.

Red muscle, the most important muscle for locomotion in *N. rossii*, showed no temperature compensation but increased capacities following Q_{10}, which may be necessary to sustain swimming performance at warmer temperatures. The elevated energy demand

resulting from the high enzymatic activities in heart and red muscle may be supported by using fatty acids or glucose from liver energy stores.

In white muscle of warm-acclimated fish, reduced CS and COX activities indicate a decreased number of matrix and membrane enzymes, which could save energy in order to adjust oxygen demand in this specific tissue.

During cold-hypercapnia acclimation, *N. rossii* seems to initiate a slight increase in mitochondrial aerobic capacities in the heart by increased CS capacities. In the heart tissue of the warm hypercapnic group, enzyme activities (particularly per mg FW) were lower than in the control group. This suggests a critical synergy of temperature and hypercapnia for the heart, which has the least plasticity for acclimation due to its design for utmost energetic efficiency (Moyes, 1996), and may not have the abilities for further capacity increase.

The highly active red muscle appeared to respond most to both cold- and warm-hypercapnia, as it showed a large increase in aerobic capacities. These high enzyme activities are suggested to be either a response to elevated maintenance costs for acid-base regulation or a compensation for disturbances in mitochondrial metabolism by elevated PCO_2 and bicarbonate.

In liver, elevated CO_2 had the opposite effect, in that it caused decreased COX activities, while CS activities were maintained at control levels. Furthermore, a reduced COX/CS ratio in liver of hypercapnia acclimated *N. rossii* reflect shifts in metabolic pathways in liver, e.g. towards gluconeogenesis. This appears to support elevated enzyme capacities in very active tissues such as red muscle, which may shift their metabolism towards enhanced use of carbohydrates (Windisch et al., 2011).

Thus, *N. rossii* displays (in part limited) capacities to adjust mitochondrial aerobic metabolism to ocean warming and acidification, which are clearly related to tissue type and function. Central aerobic tissues of high metabolic demand like heart and red muscle need to augment mitochondrial metabolism to meet the increased energy demand of perfusion and locomotion in the warmth. Tissues of little metabolic activity and duties like white muscle can down regulate their capacities to compensate for increased capacities of other tissues to keep routine metabolic rate constant. Tissues such as liver, which are involved in metabolic regulation and ATP supply may be able to shunt metabolic pathways to match energy metabolism to metabolic demands under specific environmental conditions.

These compensating mechanisms involved may not be complete and entail a net depletion of energy stores, which in the long run may reduce the capacities of other energy-demanding processes such as reproduction or growth, as observed in reduced hepatosomatic

indices in cold- and warm-hypercapnia acclimated *Notothenia rossii.* This species will therefore only have a limited ability to compensate the effects of ocean acidification and warming.

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References

Bone, Q., Kiceniuk, J., Jones, D., 1978. On the role of the different fibre types in fish myotomes at intermediate swimming speeds. Fish. Bull. 76, 691-699.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Cai, Y., Adelman, I., 1990. Temperature acclimation in respiratory and cytochrome *c* oxidase activity in common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. A 95, 139-144.

Crockett, E., Sidell, B., 1990. Some pathways of energy metabolism are cold adapted in Antarctic fishes. Physiol. Zool. 63, 472-488.

Dalziel, A.C., Moore, S.E., Moyes, C.D., 2005. Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types. Am. J. Physiol. 288, R163-R172.

Deigweiher, K., Koschnick, N., Portner, H.O., Lucassen, M., 2008. Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am. J. Physiol. 295, R1660-R1670.

Egginton, S., Cordiner, S., Skilbeck, C., 2000. Thermal compensation of peripheral oxygen transport in skeletal muscle of seasonally acclimatized trout. Am. J. Physiol. 279, R375-388.

Egginton, S., Sidell, B.D., 1989. Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. Am. J. Physiol. 256, 1-9.

Everson, I., 1969. Inshore fishes from the South Orkney and South Shetland Islands, the Antarctic Peninsula and South Georgia. Br. Ant. Surv. Bull 19, 89-96.

Gnaiger, E., Lassnig, B., Kuznetsov, A., Rieger, G., Margreiter, R., 1998. Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome *c* oxidase. J. Exp. Biol. 201, 1129.

Gon, O., Heemstra, P., 1990. Fishes of the Southern Ocean. J.L.B. Smith Institute for Ichthyology, Grahamstown, South Africa.

Grim, J., Miles, D., Crockett, E., 2010. Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. J. Exp. Biol. 213, 445-452.

Guderley, H., 1990. Functional significance of metabolic responses to thermal acclimation in fish muscle. Am. J. Physiol. 259, R245-252.

Guderley, H., 1998. Temperature and growth rates as modulators of the metabolic capacities of fish muscle. In: Pörtner, H.O., Playle, R.C. (Eds.), Seminar Series, vol. 66. Cold ocean physiology, Cambridge University Press, Cambridge, pp. 58-87.

Guderley, H., 2004. Metabolic responses to low temperature in fish muscle. Biol. Rev. 79, 409-427.

Guderley, H., Leroy, P.H., Gagné, A., 2001. Thermal acclimation, growth, and burst swimming of threespine stickleback: enzymatic correlates and influence of photoperiod. Physiol. Biochem. Zool. 74, 66-74.

Guderley, H., St-Pierre, J., 2002. Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. J. Exp. Biol. 205, 2237.

Hardewig, I., Dijk, P.L.M., Leary, S., Moyes, C., 2000. Temporal changes in enzyme activity and mRNA levels during thermal challenge in white sucker. J. Fish Biol. 56, 196-207.

Hardewig, I., Peck, L.S., Pörtner, H.O., 1999a. Thermal sensitivity of mitochondrial function in the Antarctic notothenioid *Lepidonotothen nudifrons*. J. Comp. Physiol. B 169, 597-604.

Hardewig, I., van Dijk, P.L., Moyes, C.D., Portner, H.O., 1999b. Temperature-dependent expression of cytochrome *c* oxidase in Antarctic and temperate fish. Am. J. Physiol. 277, R508-516.

Hulbert, A., Turner, N., Hinde, J., Else, P., Guderley, H., 2006. How might you compare mitochondria from different tissues and different species? J. Comp. Physiol. B 176, 93-105.

Johnston, I., 2003. Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. Comp. Biochem. Physiol. B 136, 701-713.

Johnston, I.A., Calvo, J., Guderley, H., Fernandez, D., Palmer, L., 1998. Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. J. Exp. Biol. 201, 1-12.

Kent, J., Koban, M., Prosser, C., 1988. Cold-acclimation-induced protein hypertrophy in channel catfish and green sunfish. J. Comp. Physiol. B 158, 185-198.

Langenbuch, M., Pörtner, H.O., 2003. Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthesis? J. Exp. Biol. 206, 3895-3903.

Lannig, G., Eckerle, L.G., Serendero, I., Sartoris, F.J., Fischer, T., Knust, R., Johansen, T., Pörtner, H.O., 2003. Temperature adaptation in eurythermal cod (*Gadus morhua*): a comparison of mitochondrial enzyme capacities in boreal and Arctic populations. Mar. Biol. 142, 589-599.

Lannig, G., Storch, D., Pörtner, H.O., 2005. Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. Polar Biol. 28, 575-584.

Lucassen, M., Koschnick, N., Eckerle, L.G., Pörtner, H.O., 2006. Mitochondiral mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. J. Exp. Biol. 209, 1410-1420.

Lucassen, M., Schmidt, A., Eckerle, L.G., Pörtner, H.O., 2003. Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. Am. J. Physiol. 285, R1410-1420.

Mark, F., Lucassen, M., Pörtner, H.O., 2006. Thermal sensitivity of uncoupling protein expression in polar and temperate fish. Comp. Biochem. Physiol. D 1, 365-374.

Mark, F.C., Lucassen, M., Strobel, A., Barrera-Oro, E., Koschnick, N., Zane, L., Patarnello, T., Pörtner, H.O., Papetti, C., 2012. Mitochondrial Function in Antarctic Nototheniids with ND6 Translocation. PLoS ONE 7, e31860.

Martin-Perez, M., Fernandez-Borras, J., Ibarz, A., Millan-Cubillo, A., Felip, O., de Oliveira, E., Blasco, J., 2012. New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream. J. Proteome Res 11, 3577-3547.

Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313-2331.

Michaelidis, B., Spring, A., Pörtner, H.O., 2007. Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. Mar. Biol. 150, 1417-1429.

Moyes, C., Mathieu-Costello, O., Tsuchiya, N., Filburn, C., Hansford, R., 1997. Mitochondrial biogenesis during cellular differentiation. Am. J. Physiol. 272, C1345.

Moyes, C.D., 1996. Cardiac metabolism in high performance fish. Comp. Biochem. Physiol. A 113, 69-75.

O'Brien, K.M., Mueller, I.A., 2010. The unique mitochondrial form and function of Antarctic Channichthyid icefishes. Integr. Comp. Biol. 50, 993-1008.

Owen, O.E., Kalhan, S.C., Hanson, R.W., 2002. The key role of anaplerosis and cataplerosis for citric acid cycle function. J Biol. Chem. 277, 30409-30412.

Pörtner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Biochem. Physiol. A 132, 739-761.

Schloss, I., Ferreyra, G.A., González, O., Atencio, A., Fuentes, V., Tosonotto, G., Mercuri, G., Sahade, R., Tatián, M., Abele, D., 2008. Long term hydrographic conditions and climate trends in Potter Cove. Wiencke, C., Ferreyra, G., Abele, D., Marenssi, S. (Eds): The Potter Cove coastal ecosystem, Antarctica. Synopsis of research performed 1999-2006 at the Dallmann Laboratory and Jubany Station, King George Island (Isla 25 de Mayo). Ber. Polarforsch. Meeres.

Seebacher, F., Davison, W., Lowe, C.J., Franklin, C.E., 2005. A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. Biol. Lett. 1, 151-154.

Sidell, B., Driedzic, W., Stowe, D., Johnston, I., 1987. Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. Physiol. Zool. 60, 221-232.

Simpson, D.P., 1967. Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. J. Clin. Invest. 46, 225.

Somero, G.N., 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. Integr. Comp. Biol. 42, 780-789.

Sommer, A., Pörtner, H.O., 2002. Metabolic cold adaptation in the lugworm *Arenicola marina*: comparison of a North Sea and a White Sea population. Mar. Ecol. Prog. Ser. 240, 171-182.

Strobel, A., Bennecke, S., Leo, E., Mintenbeck, K., Pörtner, H.O., Mark, F., 2012. Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO2. Front. Zool. 9, 28.

Strobel, A., Graeve, M., Poertner, H., Mark, F., 2013. Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. PLoS ONE *in revision*.

Van Dijk, P.L.M., Tesch, C., Hardewig, I., Pörtner, H.O., 1999. Physiological disturbances at critically high temperatures: A comparison between stenothermal Antarctic and eurythermal temperate eelpouts (Zoarcidae). J. Exp. Biol. 202, 3611-3621.

Walesby, N., Johnston, I., 1980. Fibre types in the locomotory muscles of an antarctic teleost, *Notothenia rossii*. Cell Tissue Res. 208, 143-164.

Walesby, N.J., Johnston, I.A., 1979. Activities of some enzymes of energy metabolism in the fast and slow muscles of an antarctic teleost fish (*Notothenia rossii*). Biochem. Soc. Trans. 7, 659-671.

Windisch, H.S., Kathöver, R., Pörtner, H.O., Frickenhaus, S., Lucassen, M., 2011. Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways. Am. J. Physiol. 301, R1453-R1466.

Wodtke, E., 1981. Temperature adaptation of biological membranes: Compensation of the molar activity of cytochrome *c* oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). Biochim. Biophys. Acta 640, 710-720.

Figures

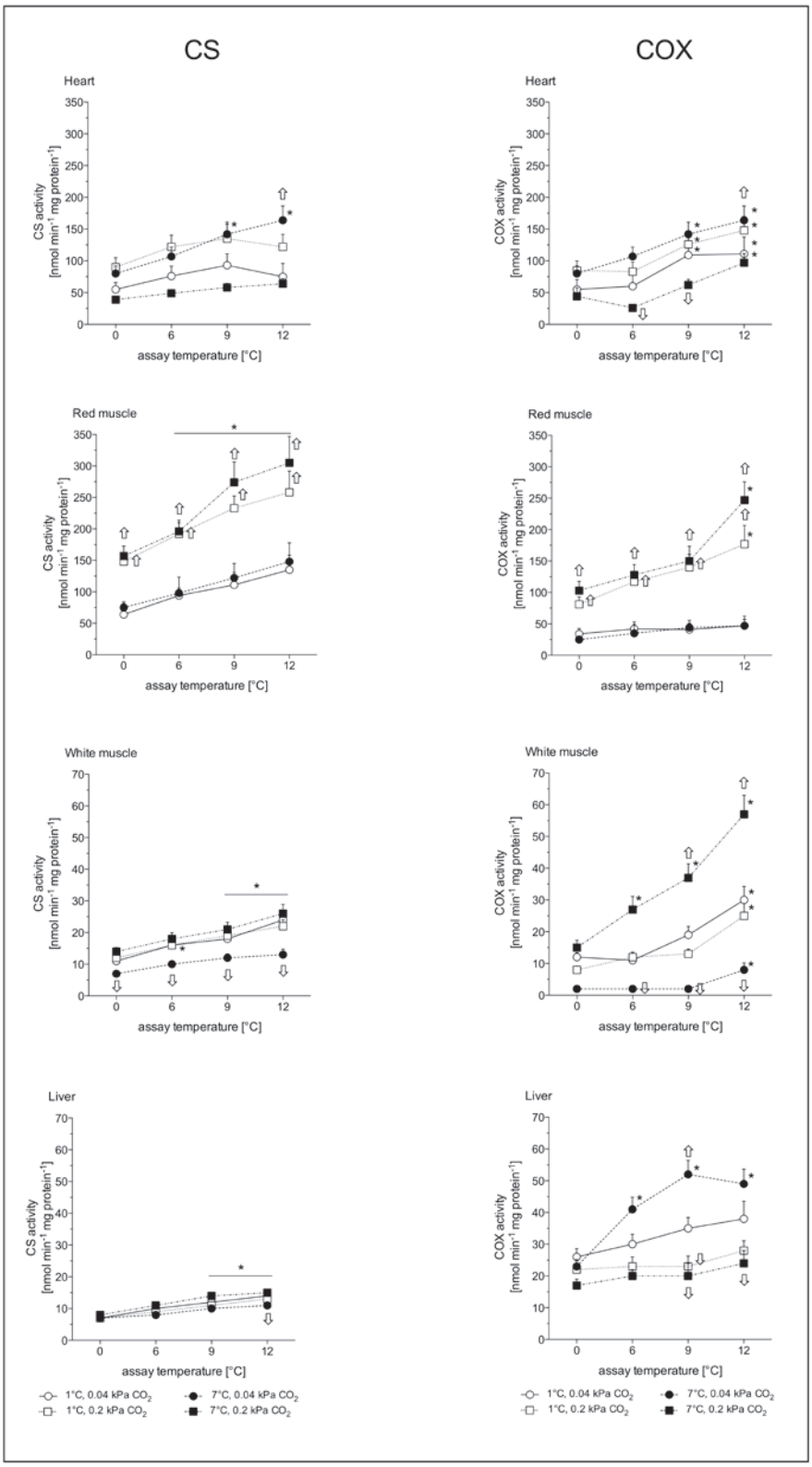

Figure 1: Maximum activities of citrate synthase (CS) and cytochrome *c* oxidase (COX) per mg protein in heart, liver, white muscle and red muscle of *N. rossii*. White circles represent the control group (cold normocapnia: 1°C, 0.04 kPa CO_2, *n*=6), black circles the warm

normocapnia (7°C, 0.04 kPa CO_2, n=3-4), white squares the cold hypercapnia (1°C, 0.2 kPa CO_2, n=7-8) and black squares the warm hypercapnia (7°C, 0.2 kPa CO_2, n=6-8) acclimated *N. rossii.* Values are given as means ± SEM. Activities were assayed at 0, 6, 9 and 12°C. Arrows depict a significant ($p \leq 0.05$) increase/ decrease in enzyme activity of a given treatment compared to the control group at the respective assay temperature. * denotes a significant ($p \leq 0.05$) increase in enzyme activity within an acclimation group compared to the 0°C assay.

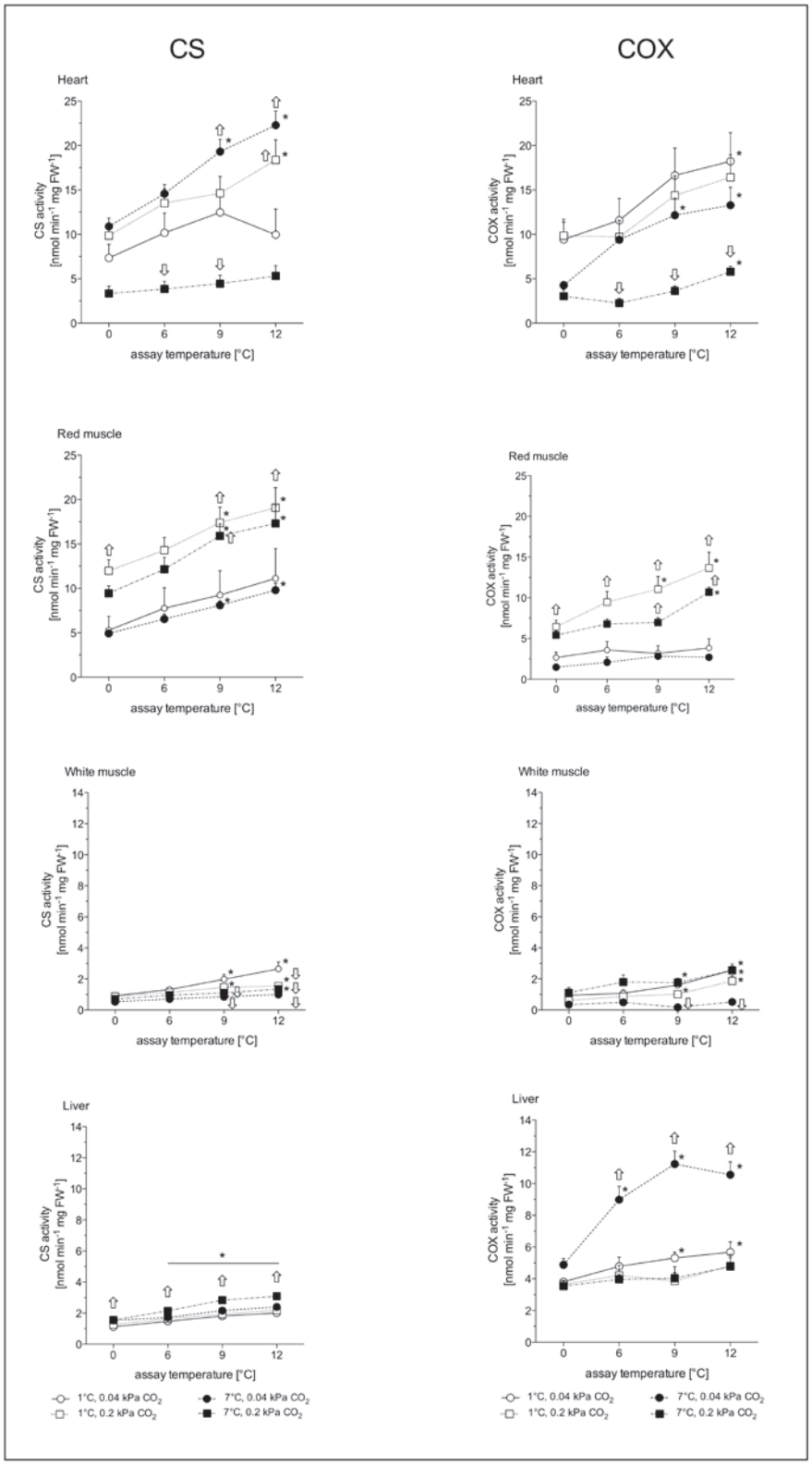

Figure 2: Effects of warm and hypercapnia acclimation on activities of citrate synthase (CS) and cytochrome *c* oxidase (COX) per mg tissue fresh weight (FW) in heart, liver, white muscle and red muscle of *N. rossii.* White circles represent the control group (cold normocapnia: 1°C, 0.04 kPa CO_2, *n*=6), black circles the warm normocapnia (7°C, 0.04 kPa CO_2, *n*=3-4), white squares the cold hypercapnia (1°C, 0.2 kPa CO_2, *n*=7-8) and black squares

the warm hypercapnia (7°C, 0.2 kPa CO_2, *n*=6-8) acclimated *N. rossii.* Enzyme activities are given as means ± SEM at 0, 6, 9 and 12°C. Arrows depict a significant ($p \leq 0.05$) increase/ decrease in enzyme activity of a given treatment compared to the control group at the respective assay temperature. * depicts a significant ($p \leq 0.05$) increase in enzyme activity within an acclimation group compared to the 0°C assay.

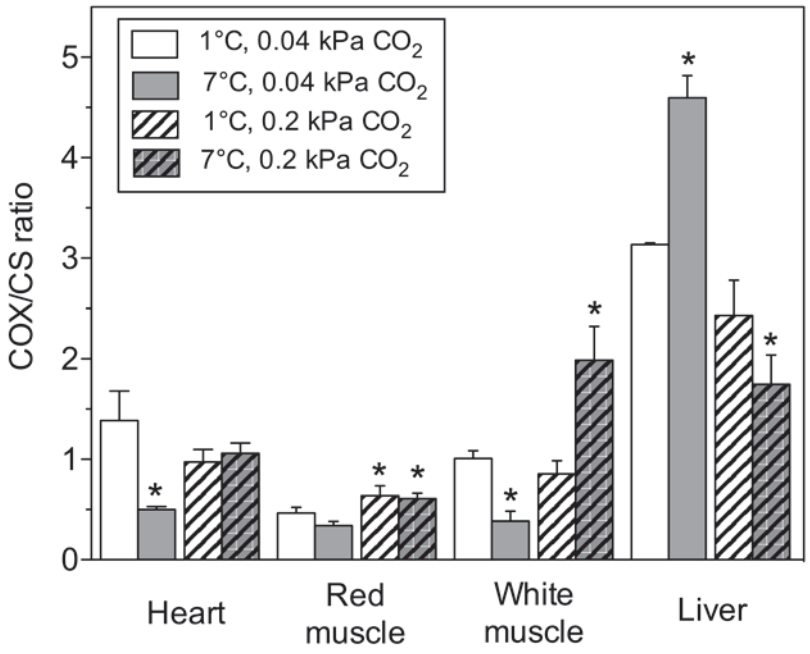

Figure 3: Effect of warm and hypercapnia acclimation on the COX/CS ratio in *N. rossii.* COX/CS ratio in heart, liver, white muscle and red muscle of control (white bars; 1°C, 0.04 kPa CO_2, *n*=6), warm normocapnia (grey bars; 7°C, 0.04 kPa CO_2, *n*=3-4), cold hypercapnia (white hatched bars; 1°C, 0.2 kPa CO_2, *n*=7-8) and warm hypercapnia (grey hatched bars; 7°C, 0.2 kPa CO_2, *n*=6-8) acclimated *N. rossii.* Values are given as means ± SEM. * shows a significantly ($p \leq 0.05$) increased/ decreased ratio compared to the control group within a respective tissue.

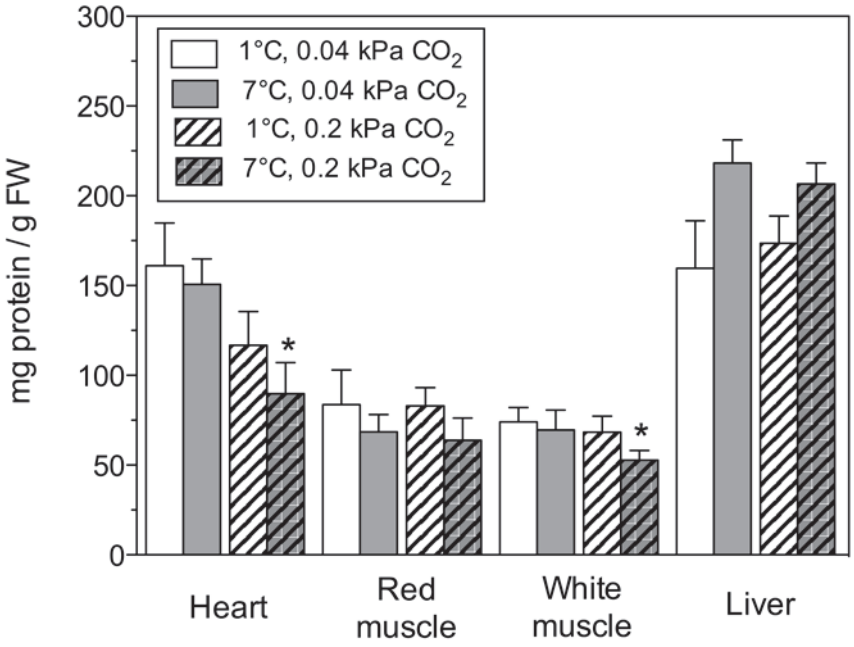

Figure 4: Total protein content in heart, red muscle, white muscle and liver of control, warm and hypercapnia acclimated *N. rossii*. White bars: control - 1°C, 0.04 kPa CO_2, n=6; Grey bars: warm normocapnic - 7°C, 0.04 kPa CO_2, n=3-4; White hatched bars: cold hypercapnic - 1°C, 0.2 kPa CO_2, n=7-8; Grey hatched bars: warm hypercapnic, 7°C, 0.2 kPa CO_2, n=6-8. * indicates data significantly ($p \leq 0.05$) decreased compared to the control group within a respective tissue. Values given as means ± SEM.

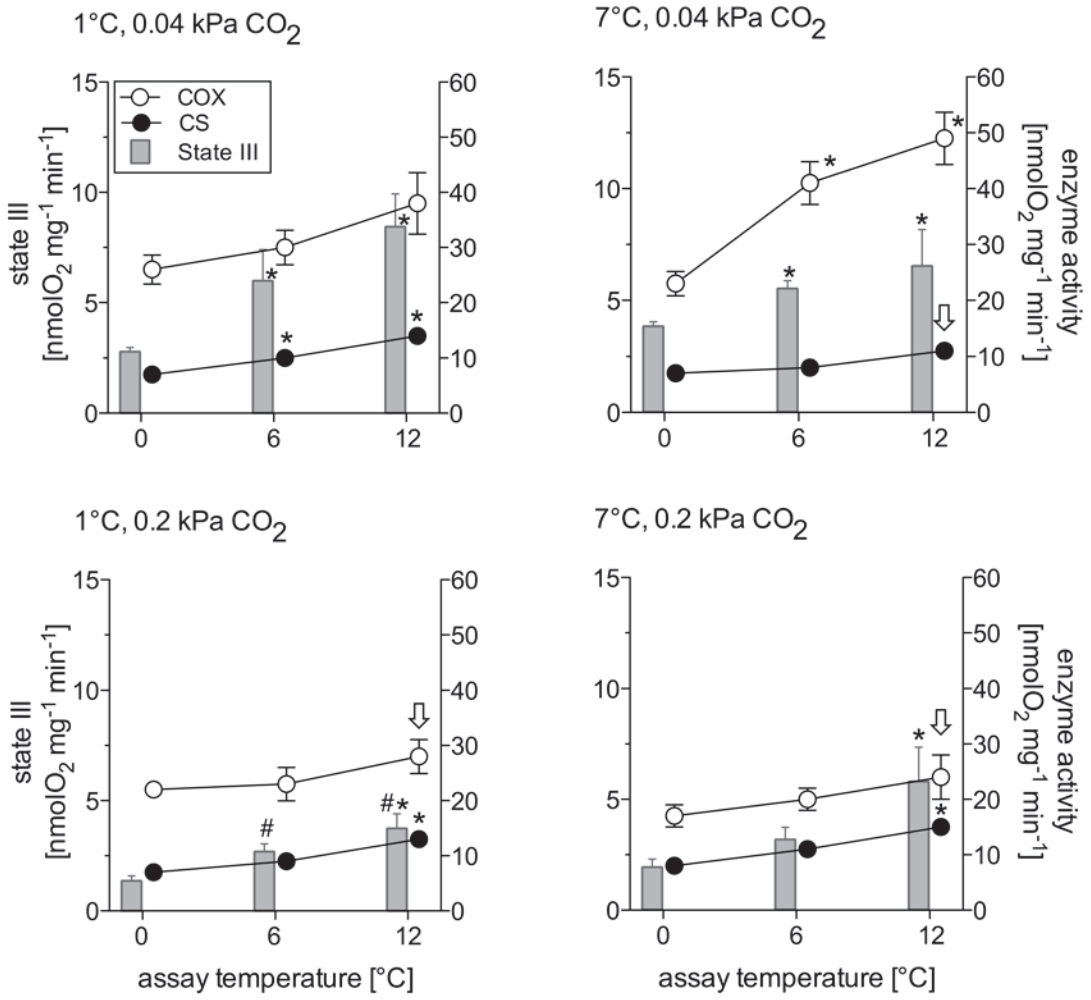

Figure 5: Mitochondrial state III respiration (grey bars), citrate synthase (CS, black circles) and cytochrome *c* oxidase (COX, white circles) activity per mg protein in liver mitochondria/ tissue of *N. rossii*, respectively. The four treatment groups are: control (cold normocapnia: 1°C, 0.04 kPa CO_2, n=6), warm normocapnic (7°C, 0.04 kPa CO_2, n=3-4), cold hypercapnic (1°C, 0.2 kPa CO_2, n=7-8) and warm hypercapnic (7°C, 0.2 kPa CO_2, n=6-8). State III respiration was measured in the presence of malate, pyruvate, succinate and ADP. Data are presented as means ± SEM relative to assay temperature (0, 6 and 12°C). * depicts a significant ($p \leq 0.05$) increase in state III respiration or enzyme activity within an acclimation group compared to the 0°C assay. # indicates significantly reduced mitochondrial respiration in comparison to control group. Arrows depict a significant ($p \leq 0.05$) decrease in enzyme activity of a given treatment compared to the control group at the respective assay temperature.

Tables

Table 1: Mean Citrate Synthase (CS) and Cytochrome *c* oxidase (COX) activities in nmol per minute and mg protein of *N. rossii* in all tissues investigated and all acclimation conditions.

Acclimation	Tissue	CS activity nmol min^{-1} mg $protein^{-1}$	COX activity nmol min^{-1} mg $protein^{-1}$	CS activity nmol min^{-1} mg FW^{-1}	COX activity nmol min^{-1} mg FW^{-1}	N
N. rossii						
1°C 0.04 kPa CO_2	Heart	74.8±7.8	83.8±15.2	10.0±1.1	14.0±2.1	6
1°C 0.2 kPa CO_2		117.3±9.6*	110.5±15.9	14.1±1.8	12.6±1.7	7
7°C 0.04 kPa CO_2		123.3±18.6*	123.3±18.6	16.8±2.5*	9.8±2.0	3
7°C 0.2 kPa CO_2		52.5±5.4	57.3±15.2*	4.2±0.4*	3.7±0.8*	7
1°C 0.04 kPa CO_2	Red muscle	101±14.9	41.0±2.7	8.4±1.2	3.3 ±0.2	6
1°C 0.2 kPa CO_2		207.8±24.1*	128.8±20.2*	15.7±1.6*	10.2±1.5*	7
7°C 0.04 kPa CO_2		110.8±15.7	37.8±4.9	7.3±1.0	2.3±0.3*	3
7°C 0.2 kPa CO_2		233.0±34.1*	157.0±31.5*	13.7±1.8*	7.5±1.1*	8
1°C 0.04 kPa CO_2	White muscle	17.3±2.7	18.0±4.4	1.7±0.4	1.5±0.4	6
1°C 0.2 kPa CO_2		17.3±2.1	14.5±3.7	1.3±0.2	1.1±0.3	8
7°C 0.04 kPa CO_2		10.5±1.3*	3.5±1.5*	0.8±0.1	0.4±0.1*	4
7°C 0.2 kPa CO_2		19.8±2.5	34.0±8.9*	1.0±0.1	1.8±0.3	8
1°C 0.04 kPa CO_2	Liver	10.8±1.5	32.3±2.7	1.6±0.2	4.9±0.4	5
1°C 0.2 kPa CO_2		10.0±1.3	24.0±1.4*	1.8±0.2	4.1±0.3	7
7°C 0.04 kPa CO_2		9.0±0.9	41.3±6.5	2.0±0.2	8.9±1.4*	3
7°C 0.2 kPa CO_2		12.0±1.6	20.3±1.4*	2.4±0.3	4.1±0.3	6

* Significant differences in enzyme activity compared to the control group within the respective tissue. Acclimations are: control (1°C, 0.04 kPa CO_2), warm normocapnic (7°C, 0.04 kPa CO_2), cold normocapnic (1°C, 0.2 kPa CO_2) and warm normocapnic (7°C, 0.04 kPa CO_2).

Table 2: Simplified trends of Citrate Synthase (CS) and Cytochrome *c* oxidase (COX) Q_{10} values of *N. rossii* in all tissues investigated and all acclimation conditions compared to control

Acclimation	Enzyme	Heart		Red Muscle		Liver		White Muscle	
		prot	FW	prot	FW	prot	FW	prot	FW
Warm	CS	↗	↗	→	→	→	→	↓	↓
Normocapnia	COX	↗	→	→	→	↑	↑	↓	↓
Cold	CS	→	↗	↑	↑	→	→	→	→
Hypercapnia	COX	→	→	↑	↑	↘	→	→	→
Warm	CS	→	↓	↑	↑	→	→	→	→
hypercapnia	COX	↓	↓	↑	↑	↘	↑	↑	→

→ indicates similar enzyme activities compared to control. ↑/↓ displays elevated/ decreased enzyme activities compared to control, respectively. ↗/↘ depicts a slight trend of increasing/ decreasing enzyme activities towards warmer assay temperatures. Trends of enzyme activities are given per mg protein (prot) and

per mg fresh weight (FW). Differences in enzyme activity trends per mg protein and per g fresh weight are highlighted in grey.

Supplementary

Table S.1: Citrate Synthase (CS) and Cytochrome *c* oxidase (COX) Q_{10} values of *N. rossii* in all tissues investigated and all acclimation conditions.

Tissue	Acclimation	CS			COX			N
		0-12°C	0-9°C	9-12°C	0-12°C	0-9°C	9-12°C	
Heart	1°C 0.04 kPa CO2	1.79±0.17	1.99±0.25	1.35±0.13	1.81±0.16	1.98±0.21	1.61±0.23	5
	1°C 0.2 kPa CO2	1.83±0.08	1.79±0.08	1.97±0.29	1.86±0.19	1.97±0.37	1.94±0.31	7
	7°C 0.04 kPa CO2	1.81±0.05	1.92±0.06	1.61±0.09	1.99±0.19	2.34±0.00	1.28±0.38	3
	7°C 0.2 kPa CO2	1.62±0.15	1.72±0.24	1.52±0.19	2.28±0.43	2.16±0.54	2.70±0.60	6
Red muscle	1°C 0.04 kPa CO2	1.89±0.10	1.98±0.18	1.70±0.20	1.65±0.49	1.10±0-36	1.78±0.21	6
	1°C 0.2 kPa CO2	1.72±0.08	1.74±0.06	1.75±0.23	1.79±0.20	1.70±0.18	2.32±0.48	5
	7°C 0.04 kPa CO2	1.67±0.05	1.67±0.04	1.89±0.10	1.99±0.14	1.93±0.21	2.64±0.63	3
	7°C 0.2 kPa CO2	1.66±0.10	1.81±0.10	1.42±0.10	1.91±0.16	1.42±0.14	2.96±0.36	7
White muscle	1°C 0.04 kPa CO2	1.78±0.6	1.81±0.09	1.56±0.15	2.01±0.19	1.69±0.19	3.34±1.03	5
	1°C 0.2 kPa CO2	1.59±0.02	1.63±0.03	1.51±0.08	2.65±0.20	2.06±0.39	2.35±0.18	8
	7°C 0.04 kPa CO2	1.66±0.10	1.70±0.02	1.59±0.32	2.75±0.54	1.98±0.57	5.83±1.01	4
	7°C 0.2 kPa CO2	1.83±0.17	1.63±0.07	1.85±0.47	2.92±0.67	2.58±0.23	1.12±0.29	8
Liver	1°C 0.04 kPa CO2	1.73±0.08	1.71±0.04	1.85±0.26	1.39±0.06	1.43±0.06	1.31±0.21	3
	1°C 0.2 kPa CO2	1.60±0.02	1.65±0.01	1.48±0.07	1.28±0.14	1.24±0.22	3.21±0.89	7
	7°C 0.04 kPa CO2	1.47±0.07	1.48±0.12	1.47±0.07	1.92±0.11	2.55±0.17	0.82±0.08	3
	7°C 0.2 kPa CO2	1.77±0.04	1.96±0.09*	1.36±0.15	1.27±0.15	1.38±0.12	1.78±0.67	6

[a] Significant differences in Q_{10} within a temperature range compared to the control group within the respective tissue.
[b] Significant difference in Q_{10} compared to the 0-9°C range within a given tissue and acclimation condition.
Acclimations are: control (1°C, 0.04 kPa CO_2), warm normocapnic (7°C, 0.04 kPa CO_2), cold normocapnic (1°C, 0.2 kPa CO_2) and warm normocapnic (7°C, 0.04 kPa CO_2).

PUBLICATION IV

Compensation capacities for ocean acidification in the Austral nototheniid *N. angustata*

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Compensation capacities for ocean acidification in the Austral notothenid *N. angustata*

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Abstract

In this study, we investigated mitochondrial and whole animal tolerance to temperature and hypercapnia in the Austral nototheniid *Notothenia angustata* as a comparison to its Antarctic congeners. To this end, one group of animals was acclimated under hypercarbic conditions (0.2 kPa CO_2, 15 d, n=6), the control group was kept under normocarbic conditions (0.04 kPa CO_2, n=10). Permeabilised heart mitochondria of both groups were assayed in normocapnic (0.04 kPa CO_2) and hypercapnic (3.0 kPa CO_2) respiration buffers at 9, 15 and 21°C. Furthermore, routine metabolic rates (RMR) were estimated in the control group under acutely rising temperature (3°C/d) and normocarbic and hypercarbic conditions, respectively. Results indicate that *N. angustata* has a limited thermal tolerance with whole animal critical temperatures at or below 19°C, which is mirrored in very low capacity increases with rising temperature at the mitochondrial level. Acute hypercarbia led to slightly, but not significantly increased RMR, and to higher critical temperatures. Chronic hypercarbia-acclimation induced a compensatory upregulation of mitochondrial capacities, presumably by bicarbonate-mediated intracellular signalling pathways. These enhanced mitochondrial capacities not only result in increased hypercapnia tolerance, but also bring about a higher thermal tolerance at the mitochondrial and whole animal level, which has not been observed in Antarctic notothenioids.

Key words

Mitochondria, heart fibres, routine metabolic rate, bicarbonate, acclimation

Introduction

Hand in hand with ocean warming, ocean acidification is increasingly threatening life in the world's oceans (IPCC, 2007). This has sparked an increasing number of studies of the effects of ocean acidification and warming on water breathing, ectotherm marine animals throughout the last decade (Kroeker et al., 2010). Amongst those species, fish have generally been regarded as reasonably tolerant towards ocean acidification, due to their well-developed capacities for ion-regulation (Melzner et al., 2009;Deigweiher et al., 2010).

This may be the reason that there is only a handful of physiological studies on ocean acidification effects in fish (Deigweiher et al., 2008;Melzner et al., 2009;Moran and Stottrup, 2010;Frommel et al., 2012;Enzor et al., 2013), and only very few studies have dealt with metabolic regulation at the cellular level (Michaelidis et al., 2007;Deigweiher et al., 2010;Strobel et al., 2012;Nilsson et al., 2012;Strobel et al., in revision).

Several authors have argued that increased costs of acid-base regulation and metabolic rearrangements will either entail adjustments of the animal's energy budget, leading to reduced growth and fecundity, or a higher total aerobic energy demand for the whole animal, which would reduce aerobic scope. In this line of thought, increased partial pressure of carbon dioxide ($P\mathrm{CO}_2$) in seawater would act in synergy with ocean warming, and critical temperatures, which mark the onset of anaerobic metabolism, may be reached earlier (Pörtner, 2010).

Specifically Antarctic notothenioid fish may be among the first fish species to suffer from these synergistic restrictions of ocean acidification and warming. They have evolved an energy saving mode of life in the cold and stable waters of the Southern Ocean by reduction of several physiological traits and capacities, and as a result possess narrow thermal tolerance ranges (Somero and DeVries, 1967) and very limited capacities to acclimate to environmental fluctuations (Strobel et al., 2012;Enzor et al., 2013;Strobel et al., in revision). Some notothenioids have wider thermal tolerance ranges, either because they belong to the three families within the suborder Notothenioidei that have never become strictly Antarctic (Pseudaphritidae, Bovichtidae, Eleginopidae) and reside in Austral cold-temperate to sub-Antarctic habitats or they diverged from their Antarctic ancestors during the expansion of the Antarctic convergence to the shelves of South America, Australia, and New Zealand in the last glacial maximum about 11 mya (Cheng et al., 2003;Matschiner et al., 2011).

One of the latter species is the New Zealand black cod *Notothenia angustata*, closely related to its Antarctic sister species within the family Nototheniidae, one of the five members

of the 'Antarctic clade' of the Notothenioidei (Eastman, 2005). The bentho-pelagic *N. angustata* inhabits the cool temperate shallow waters around the southern coast of New Zealand's South Island at depths down to 100 m. Its environmental temperature ranges from 2°C in winter to 18°C during summer, and like its Antarctic relatives, it feeds on a variety of invertebrates, small fish and seaweed (Guynn et al., 2002;Cheng et al., 2003). It still shares several physiological similarities with its Antarctic relatives and closely resembles them in terms of habitus, activity patterns and choice of habitat.

Due to its higher degree of eurythermality, *N. angustata* may carry larger capacities for acclimation to environmental changes and therefore makes it an ideal eurythermal comparative model for Antarctic notothenioids in studying their physiological response to ocean acidification and warming.

Seawater hypercarbia leads to diffusive entry of CO_2 into the bloodstream of water breathing marine organisms, creating blood hypercapnia, which is associated with a drop in extracellular (i.e. blood) pH (pH_e). Teleost fish generally compensate for elevated ambient PCO_2 by an active extracellular- and intracellular accumulation of bicarbonate (HCO_3^-) to buffer pH_e (e.g. Heisler, 1984;Brauner et al., 2004;Melzner et al., 2009;Brauner and Baker, 2009;Esbaugh et al., 2012;Strobel et al., 2012). In some cases, this can lead to an increased energy demand to maintain new steady states of ion gradients for acid-base regulation (Deigweiher et al., 2008;Strobel et al., in revision). Antarctic notothenioids have been reported to either compensate their whole animal respiration under chronic hypercapnia (e.g. N. rossii, Strobel et al., submitted) or display increased routine metabolic rates (Enzor et al., 2013).

In Antarctic *Notothenia rossii*, long-term acclimated to 0.2 kPa CO_2, both extracellular pH and intracellular pH (pH_i) were compensated by uptake of HCO_3^-, resulting in an extracellular PCO_2 of up to 1.6 kPa (Strobel et al., 2012). Taking into account the PCO_2 gradients between blood, cytosol and mitochondrial matrix (Pörtner and Sartoris, 1999;Pörtner, 2012), the mitochondrial PCO_2 of fish chronically exposed to elevated PCO_2 can be expected to range between 2 and 3 kPa, along with significantly increased [HCO_3^-]. This is indeed what we found in a previous study on Antarctic *Notothenia rossii*, after long-term acclimation to 0.2 kPa CO_2, intracellular PCO_2 was around 1 kPa in white muscle and around 3 kPa in liver (Strobel et al., 2012).

Specifically bicarbonate has the potential to hamper cellular and specifically mitochondrial metabolism, as it is an intermediate of several metabolic pathways and also has a putative role as a neurotransmitter (Nilsson et al., 2012). Acute hypercapnia has thus been

shown to decrease capacities of the tricarbonic acid (TCA) cycle and the mitochondrial electron transport system (ETS) in mammals (Simpson, 1967) and trout (Walsh et al., 1988), namely by the competitive inhibition of citrate synthase (CS) and succinate dehydrogenase (SDH) by bicarbonate.

While the acclimatory response to hypercapnia may be diverse in tissues of different metabolic duties (Strobel et al., 2012;Strobel et al., in revision), a general trend in active aerobic tissues like heart and red muscle appears to be an increase in enzymatic activities and mitochondrial capacities, possibly to overcome reduced TCA turnover due to competitive inhibition by bicarbonate (Strobel et al., submitted). Further anaplerotic enhancement of the TC cycle capacities to compensate the inhibitory effects of elevated intracellular HCO_3^- may include shifts in metabolic pathways to oxidative decarboxylation reactions (Langenbuch and Pörtner, 2003;Strobel et al., in revision).

Accordingly, enzymes of the aerobic metabolism (CS and cytochrome *c* oxidase (COX)) were elevated in red muscle and heart of hypercarbia-acclimated *N. rossii* (Strobel et al., submitted). An increase in COX/CS ratio in combination with generally increased substrate levels could overcome the limitation of the TCA induced by HCO_3^- to maintain aerobic capacities in red muscle of *N. rossii* under long-term elevated PCO_2 (Strobel et al., submitted).

Yet, these acclimation responses at the mitochondrial level are hard to quantify, as conventionally, mitochondrial respiration is measured in normocapnic (0.04 kPa CO_2) respiration medium (MiRO6, MiP2 Oroboros protocols, Gnaiger, 2012). This PCO_2 does not reflect the true intracellular PCO_2, as there is a gradient from the seawater via blood and tissues towards the mitochondria (Pörtner and Sartoris, 1999). Under hypercapnia, this gradient is even increased, probably leading to a higher PCO_2 within the mitochondria under future conditions of ocean acidification (Strobel et al., 2012). Therefore, the mitochondrial capacities in teleost fish need to be analysed in respiration media with a higher PCO_2 to allow for drawing conclusions on mitochondrial capacities under physiological conditions.

The aim of this paper was to compare mitochondrial respiration in permeabilized heart fibres in the Austral notothenioid *N. angustata* in normocapnic (0.04 kPa CO_2) and acute hypercapnic (3 kPa CO_2) respiration media. Furthermore, we aimed to analyse the acclimation capacities of the mitochondrial energy metabolism to ocean acidification of this species in comparison to Antarctic notothenioids. To this end, we acclimated *N. angustata* to 0.2 kPa CO_2 for 15 days at their habitat temperature of 15°C, and measured heart fibre respiration in

normocapnic and acute hypercapnic respiration buffer in hypercarbia-acclimated (0.2 kPa CO_2, 15°C) and control fish (0.04 kPa CO_2, 15°C).

Methods

Animal collection and acclimation

Collection. N. angustata were caught close to Otago harbour, New Zealand (45°52.26'S; 170°30.12'E) using baited traps at depths of 8 m in November 2011 at a local temperature of 13°C. They were maintained for three weeks in large outside tanks (approx. 2,000 l) at the Portobello Marine Station, Otago, New Zealand, with direct supply of seawater at a water-temperature of 14.5°C and a salinity of 34.4 psu, until used for the experiments.

Acclimation. Fish were acclimated to 0.2 kPa CO_2 in two well-aerated, 200 liter aquarium tanks equipped with three individuals each (n = 6, mass 158 – 1470 g, standard length 20.0 – 40.5 cm), with seawater (14°C) directly pumped from Portobello Bay. A PCO_2 of 0.2 kPa corresponds to the predicted seawater PCO_2 for the year 2200 according to the Intergovernmental Panel on Climate Change (IPCC) ‚business-as-usual' scenario (IPCC, 2007). During the 15-day acclimation period, the animals were kept at dim light during the days and fed to satiation with chopped fish fillet on day 7. The acclimation tanks were continuously equilibrated with CO_2 by means of a pH-feedback system (iks ComputerSysteme GmbH, Germany) that maintained the desired seawater pH (stability ±0.05 pH units) by opening/ closing a solenoid valve (Aqua Medic GmbH, Germany), which was connected to a bottle of pure CO_2.

pH was measured with a WTW 340i pH meter and WTW SenTix 81 electrode calibrated daily with IUPAC precision pH buffers (WTW Germany). Seawater carbonate chemistry parameters were calculated from measured seawater pH and alkalinity values adopted from Clarke (Gaston et al., 2009) with the software CO2SYS (Pierrot et al., 2006) using the dissociation constants of Mehrbach (Mehrbach et al., 1973).

The control group (14°C, 0.04 kPa CO_2) constituted six individuals kept in the outside aquaria tanks of Portobello marine laboratory (see above) with masses of 1905 – 3360 g and standard length from 33.0 – 51.0 cm.

Whole animal respiration experiments

With four *N. angustata*, routine metabolic rate (RMR) was measured in an intermittent-flow system similar as described in Strobel *et al.* (2012) under the following conditions:

a) control conditions (14°C, 0.04 kPa CO_2) (n = 4, mass 733 – 1427 g; standard length 30 – 40 cm)
b) during acutely increasing temperatures (3°C per 24 hours) from 15 to 21°C (n = 4, mass 733 – 1427 g; standard length 30 – 40 cm)
c) after 15 days acclimation to 0.2 kPa CO_2 (n = 4, mass 158 – 1468 g, standard length 20 – 40.5 g)
d) during acute hypercarbia (0.2 kPa CO_2) and acute increasing temperatures (3°C per 24 hours) from 14 to 21°C (n = 3, mass 261.2 – 1046 g, standard length 23 – 40 cm)

Four transparent respiration chambers of 4800 to 5800 ml were placed in a 300 l experimental tank supplied several small aquaria pumps and air supply to ensure sufficient mixing of the water. The seawater was heated by four 250 W heating element (Jaeger, EHEIM GmbH, Germany), controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany), controlled by a thermostat (LAUDA Proline RP845, LAUDA, Germany)). Acute elevated seawater P_{CO_2} was regulated by the iks aquastar pH-feetbacksystem (iks ComputerSysteme GmbH, Germany). Before starting the experiments, fish were not fed for three days and allowed to recover from handling stress inside the chambers for 24 hours. In the intermittent-flow system, water exchange between chamber and ambient water was interrupted every 15 min to measure oxygen depletion of the fish within the chamber over a period of 10 min. In each cycle, the animal depleted the oxygen within the chamber by maximum 10 - 15%, then oxygen concentration was replenished to 100% by flushing pumps. Oxygen concentration within the chamber was detected once per minute using a temperature-compensated FiBox2 (PreSens – Precision Sensing GmbH, Germany) oxygen meter and flouroptic sensors (optodes}. The optodes were calibrated before each measurement in well-aerated seawater at the respective acclimation temperature, zero calibration was conducted with nitrogen-bubbled seawater. Blank measurements for determination of bacterial respiration within the respirometers were carried out for each fish, and oxygen consumption was corrected accordingly.

Sampling

Fish were anaesthetized with 0.5 g/l tricaine methano-sulphonate (MS 222) and then killed by a spinal cut behind the head plates. Blood was taken with a syringe from the caudal vein, the heart (ventricle) was excised and placed immediately into 1 ml ice-cold biopsy buffer (modified after Kutznetsov et al. 2008). The biopsy buffer contained (in mM): 2.77 Ca_2EGTA, 7.23 K_2EGTA, 0.32 Na_2ATP, 0.13 $MgCl_2*6H_2O$, 0.25 Taurine, 0.38 Na_2Phosphocreatine, 0.14 Imidazole, 0.001 DTT, 1.0 MES and 8.9 Sucrose (435mOsm, pH 7.02 at 24°C). Further samples of all relevant tissues were shock-frozen in liquid nitrogen and stored at -80°C.

Mitochondrial respiration

The heart tissue was mechanically dissected in ice-cold biopsy buffer using scissors and forceps and stored on ice in the biopsy buffer until used for the different respiration assays. For each respiration experiments, a subsample of the heart fibre bundles was permeabilized for 30min with 10 µg/ml saponin by gentle mixing on ice for 30 min. Afterwards, the fibres were removed and washed three times for 10 min on ice in 2 ml of modified assay medium (MiRO5) containing (in mM) 20 Na^+HEPES, 10 KH_2PO_4, 1% w/v BSA (fatty acid free), 215 sucrose, 0.5 EGTA, 3 $MgCl_2*6\ H_2O$, 20 Taurine and 60 Lactobionate (435mOsm, pH 7.02 at 24°C). Then, the subsample was blotted, weighed, and immediately used for respirometric analysis. Respiration of each subsample was measured at 9, 15 and 21°C in 2 ml assay medium + 300U/ml catalase (for reoxygenation of the respiration medium with hydrogen-peroxide during the assays) in glass-chambers of an Oroboros Oxygraph-2k^{TM} respirometer (Oroboros Instruments, Innsbruck, Austria). The heart fibre respiration was converted to nmol O_2 min^{-1} $mg_{fresh\ weight}^{-1}$.

Resting respiration (state II) was measured with Complex I (CI) substrates, 10mM glutamate, 2mM malate, and 10mM pyruvate. State III respiration of CI was induced by 1mM ADP, state III respiration of Complex I and II (CII) by adding 10mM succinate and 1mM ADP. By further titrating with 0.5mM ADP until maximum respiration was reached, maximum capacity of the phosphorylation system (max. OXPHOS) was determined. Leak respiration (state IV^+) was evaluated by adding 4µg/ml oligomycin, stepwise titration with of the uncoupler carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone (FCCP) (2mM stock) revealed maximum capacity of the electron transport system (uncoupled flux (*u*), maximum

ETS capacity *'E'*). After inhibition of CI with 5 µM rotenone (E_{Rot}, state III*u* of CII), non-mitochondrial respiration (ROX) was detected by adding 2.5 µM antimycin A, followed by addition of the artificial substrates for Complex IV (CIV, cytochrome *c* oxidase, COX), 2mM ascorbate and 0.5mM *N,N,N',N'*-tetramethyl-*p*-phenylendiamine dihydrochloride (TMPD).

Heart fibre respiration was assayed in the standard, normocapnic respiration buffer (MiRO5) of 0.04 kPa CO_2 (pH 7.02 at 24°C), and in acute hypercapnic respiration buffer with 3 kPa CO_2 (pH 7.02 at 24°C). A mitochondrial PCO_2 of 3 kPa in an aerobically active tissue like heart corresponds to a seawater PCO_2 of 0.2 kPa, which was used for the animal incubation (Strobel et al., 2012). The hypercapnic MiRO5 was equilibrated in a closed vial on a magnetic stirrer under an atmosphere of 3kPa CO_2, which was mixed from pressurized air and pure CO_2 by means of a Multi-Channel Flow Ratio/Pressure Controller (MKS Instruments GmbH, Germany).

Statistical analysis

Differences in heart fibre oxygen consumption at the assay temperatures 9, 15, 21°C, between the different acclimation groups were tested using unpaired, two-tailed t-test and one-way analysis of variance (ANOVA, with Tukey post-hoc test). A $p \leq 0.05$ was considered the significance threshold. All values were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance and are given in means ± SEM.

Results

In the control group of *N. angustata*, OXPHOS CI, OXPHOS CI+CII, Leak and ETS flux were comparable for all assay temperatures. In contrast, the 0.2 kPa CO_2 acclimated fish displayed elevated OXPHOS CI and OXPHOS CI+CII with increasing assay temperature, which was significantly higher at 15 and 21°C compared to the control group ($P<0.05$, Figure 1 and 2). Also Leak and ETS flux were significantly higher at equivalent temperatures in the CO_2 acclimated fish compared to the control group. The addition of cytochrome *c* had no effect on OXPHOS CI+CII rates, indicating that the outer mitochondrial membrane remained intact after fibre preparation in both fish groups (data not shown).

In the CO_2 acclimated group, ETS flux and E_{Rot} at 21°C were significantly higher than those at 9 and 15°C, and also in the control group, E_{Rot} was significantly elevated at 21°C.

Between the two acclimation groups, the flux through CIV was significantly higher at all temperatures in the CO_2 acclimated fish, with the highest flux at 21°C. In the control fish, CIV flux was significantly elevated at the highest assay temperature, resulting in generally higher flux through CIV relative to OXPHOS than in the hypercarbia-acclimated group (Figure 1).

Hypercapnic respiration medium (3 kPa CO_2) had no acute effect on the flux in all respiratory states compared to normocapnic medium (0.04 kPa CO_2) in the normocarbia-acclimated fish, with similar results for the hypercarbia-acclimated fish. Only OXPHOS flux on CI and CII substrates (OXPHOS CI+CII, Figure 2) and CIV flux assayed in the hypercapnic respiration buffer at 21°C were significantly lower than those in the normocapnic buffer.

The respiratory control ratio, RCR^+, (OXPHOS CI+CII/ Leak) describes the ratio between phosphorylating (state III) and non-phosphorylating respiration (state IV^+) respiration and thus provides a measure for coupling efficiency (Gnaiger, 2009). The CO_2 acclimated fish had significantly higher RCR^+ with CI and CII substrates (14.0±2.4 at 9°C, 11.8±3.3 at 15°C, normocapnic buffer) compared to the control group at 9 and 15°C (7.8±0.9 at 9°C, 5.7±0.5 at 15°C, normocapnic buffer). RCR^+ decreased with temperatures in both acclimation groups, and were comparable at 21°C (control: 5.5±1.3, CO_2 acclimated: 6.3±0.4, normocapnic buffer). The RCR^+ calculated for the hypercapnic buffer were within a similar range.

In both groups and respiration buffers, the P/E coupling control ratio, calculated as OXPHOS CI+CII/ ETS, were similar and had values close to 1, indicating no limitations by the phosphorylation system at any temperature (data not shown).

The L/E coupling control ratio (calculated as Leak/ ETS flux) is an index of uncoupling at constant ETS capacity. In the control group, it rose with temperature assayed in normo- and hypercapnic respiration buffer (normocapnic buffer: 9°C: 0.1±0.0, 15°C 0.2±0.0, 21°C: 0.3±0.1; hypercapnic buffer: 9°C 0.1±0.0, 15°C 0.2±0.0, 21°C: 0.4±0.1). In the CO_2 acclimated fish, the L/E was less impacted by rising temperature, it rose from 0.1±0.2 at 9°C to a maximum of 0.2±0.0 at 21°C in both normocapnic and hypercapnic respiration buffers (Figure 3). Since P/E ratios (OXPHOS CI+CII / maximal ETS capacities) were around 1.0 in all assays, maximum leak capacities were the same in relation to OXPHOS (data not shown).
In both the control and CO_2 acclimated groups, the contribution of CI linked respiration towards total CI and CII linked respiration was in the range of 67.8 – 86.4 %. Overall, neither assay temperature, nor respiration buffer or CO_2 acclimation did significantly influence CI

contribution, only in the control group it was significantly lower at 21°C compared to the percentual contribution of CI at 9°C in the normocapnic buffer (P<0.05, Table 1).

Routine metabolic rate (RMR) of *N. angustata* followed a typical exponential function under normocarbia when acutely warmed from 13 to 20 °C (figure 5), but dropped abruptly from beyond 20°C. The other group of *N. angustata* which was exposed to acute hypercapnic conditions displayed a continuous increase in RMR up to 21°C. In these acute warming experiments, RMR of the normocapnic *N. angustata* at 21°C (3045±313.2 µmol O_2*kg^{-1}*h^{-1}) differed significantly from the acute hypercapnic *N. angustata* at 21°C (4591±727.4 µmol O_2*kg^{-1}*h^{-1}).

RMR of *N. angustata* acclimated to 0.2 kPa CO_2, 14°C (2099±352.9 µmol O_2*kg^{-1}*h^{-1}) was similar to RMR of *N. angustata* measured at 14°C under normocarbic conditions (2502±651.2 µmol O_2*kg^{-1}*h^{-1}).

Discussion

Thermal response

The control *N. angustata* displayed no capacity to increase mitochondrial respiratory flux with rising assay temperatures. Rising assay temperatures were further paralleled by rising proton leak capacities, as indicated by an increase (though non-significant) in the L/E ratio from 9 to 21°C. The L/E ratio calculated for control *N. angustata* indicates that up to 10% of their ETS capacities can be lost due to proton leakage at 9°C, while this increases up to 30% of their maximum capacities at 21°C. Thus, proton leak capacities seemed to rise disproportionally to their capacities to increase flux through the ETS indicating decreased mitochondrial energy efficiency in the warmth (Pörtner, 2006).

Furthermore, Complex I has been postulated to play an important role in increasing OXPHOS capacities to support the thermal tolerance window in temperate fish (Hilton et al., 2010). According to TCA cycle stoichiometry, oxidation of 1 pyruvate yields 1 succinate and 4 NADPH. This results in a maximum contribution of Complex I to OXPHOS of 80%, if only NADH from the TCA cycle is considered as substrate for Complex I. This ratio is not always observed in fish, and Complex I contribution is often found to be lower than that, but tends to increase with temperature (Hilton et al., 2010;Mark et al., 2012;Strobel et al., in revision). In *N. angustata*, however, Complex I contribution to total OXPHOS (CI+CII) was found to

close to 80% at 9 and 15°C, but was significantly reduced at 21°C (60%, table 1), indicating reduced thermal capacities of their heart mitochondria at increased temperatures.

Notably, *N. angustata* experiences temperatures above 15°C in their natural habitat off the coast of New Zealand. Therefore, it was quite surprising to find such low thermal flexibilities in the heart mitochondria, i.e. no significant rise in OXPHOS from 9 to 15°C. A seawater temperature of 15°C may therefore be already close to the thermal limit of aerobic heart performance in this species, despite a continuously rising whole animal routine metabolic rate that was similar to other notothenioids at elevated temperature (Strobel et al., submitted). Critical temperatures have never been determined for *N. angustata*, but the data on routine metabolic rate, measured at acute rising temperatures (1.5°C rise in temperature per 12 hours, range 14 – 21°C, figure 5), suggest critical thermal limits around 18-20°C (drop in oxygen consumption beyond 19°C). As the capacities to increase metabolic rates during acute thermal challenge normally cover a wider range than their actual long-term acclimation capacities (Bilyk and DeVries, 2011), the temperatures which are tolerated by *N. angustata* over a longer period are probably below 19°C. This would explain their limited mitochondrial thermal responsiveness and consequently, why they do not occur at lower latitudes and warmer water temperatures.

Hypercapnia response

In contrast, acclimation of *N. angustata* to elevated seawater PCO_2 caused significantly higher OXPHOS and ETS capacities in heart mitochondria compared to the controls (normocapnic respiration buffer). Additionally, they had the capacities to increase flux through ETS significantly with temperature. In fact, the L/E ratio rose only from 0.08 at 9°C to 0.15 at 21°C, which is an explanation for the high ETS capacities in the hearts of hypercarbia-acclimated *N. angustata*. Furthermore, their Complex I-dependent flux remained close to 80% of total OXPHOS (CI & CII) at all temperatures, another factor reflecting enhanced thermal capacities in the hypercarbia-acclimated group.

Despite being generally good acid-base regulators, some fish still demonstrate a high vulnerability even towards only slightly elevated seawater PCO_2 (Munday et al., 2009;Munday et al., 2012). Amongst others, tropical reef fish display reduced calcification rates, partly uncompensated extracellular pH and neuronal disturbances, evidenced by impaired predator avoidance, prey selection and learning (Nilsson et al., 2012;Ferrari et al., 2012). Accordingly, chronic exposure to elevated seawater PCO_2 may not only come along

with elevated metabolic costs, e.g. uptake of HCO_3^- for pH compensation and maintenance of shifts in acid-base equilibria (Deigweiher et al., 2008), which would demand increased ATP synthesis. Another, equally important factor of changes in acid-base steady-state are the elevated bicarbonate levels, which may exert their particular effect on cellular and mitochondrial metabolism and signalling pathways.

For instance, bicarbonate levels are involved in intracellular pH sensing (Tresguerres et al., 2010a). Within mammalian and fish mitochondria, bicarbonate is known to stimulate the soluble adenylyl cyclase (sAC), with a half-maximal effect dosis well within the physiological range (in dogfish: 5mM, Tresguerres et al., 2010b). sAC forms cAMP, which induces phosphorylation of protein kinase A (PKA). This protein in the end phosphorylates/ activates several components (i.e. proteins) of the electron transfer system and also related processes. For example, Complex I and particularly Complex IV are phosphorylated by PKA, and thereby OXPHOS activity and ATP synthesis are increased (Zippin et al., 2001;Tresguerres et al., 2010b). Interestingly, Complex II (Succinate-dehydrogenase) has not been reported to be activated by PKA and neither did the present study find hypercapnia related effects on complex II, therefore it may be rather Complex I which defines ATP synthesis capacities under chronic hypercapnia. This is further supported by the finding that cAMP increased the membrane potential in mouse liver mitochondria, probably via Complex I (Acin-Perez et al., 2009).

Complex IV usually possesses excess capacities and serves as a so-called 'electron sink', which matched the sum of the partial capacities of all contributory branches converging at the Q-junction (e.g. Complex I and Complex II, Gnaiger, 2009). cAMP also stimulates mitochondrial dehydrogenases, and in combination with enhanced Complex IV capacities to handle electrons, the reducing equivalents of the TCA-cycle can be efficiently utilized in the ETS (Buck and Levin, 2011). Thereby, the mitochondrial sAC-signalling cascade links substrates flux through TCA with rate of OXPHOS activity, and this linkage minimizes electron leakage from the ETS, which is in line with the low L/E ratios, high COX activities and increased RCR^+ measured in the CO_2 acclimated *N. angustata* (figures 1, 3 and 4).

Furthermore, cAMP and PKA regulate metabolic key enzymes, such as phosphofructokinase (PFK) (glycolytic control enzyme) or PEPCK, by direct post-translational modification or alterations in gene transcription, i.e. activation of transcription factors through PKA (Zippin et al., 2001). As such, there may be a close link between cAMP levels and the levels of metabolism-controlling enzymes, and chronic hypercapnia exposure is

suggested to alter mitochondrial complex activities (particularly COX) via transcriptional or post-translational modification (Acin-Perez et al., 2009).

On the other hand, acutely increased physiological levels of bicarbonate have been reported to inhibit the TCA-cycle at several points in mammalian mitochondria, amongst others the enzymes citrate synthase and succinate dehydrogenase (CII) (Simpson, 1967;Wanders et al., 1983). Such acute inhibitions may cause compensatory changes during hypercarbia-acclimation. One example is enhancement of mitochondrial anaplerotic pathways feeding into the TCA-cycle, such as increased glutamate decarboxylation (Langenbuch and Pörtner, 2003;Strobel et al., in revision), which also provides additional, non-TCA related, NADH as Complex I substrate.

Therefore, CO_2 acclimation of *N. angustata* may involve compensation of acute inhibitory bicarbonate effects by changes in gene expression and translation. In comparison to the control group, this may have lead to the observed elevated ETS activities, which reflect a new metabolic steady state in *N. angustata* under high CO_2 conditions. Slightly lower COX/OXPHOS ratios in the hypercarbia-acclimated group indicate a remodelling of the components of the ETS (table 1), resulting in stronger contribution of Complexes I to III in comparison to Complex IV. The different respiration buffers in which the heart fibre assays were carried out (normocapnic, hypercapnic) reveal the acute inhibitory effect of bicarbonate, specifically at warm temperatures (figure 2). Once taken away in the normocapnic buffer, OXPHOS capacities increase even more in this group, revealing the true capacity increase compared with the control group. Furthermore, this increase in the normocapnic buffer seems only possible in the new mitochondrial steady state after hypercarbia acclimation, as it was not observed in the control group.

Conclusion

Presumably as a remnant of its notothenioid heritage, *N. angustata* possess only a marginally larger thermal tolerance window of their mitochondrial metabolism than their Antarctic relatives. Yet, they appear to have adapted to warmer waters by exploiting the maximum mitochondrial capacities in their habitat, including full usage of Complex I capacities and a reduced proton leak. This is in line with our observations in its Antarctic congener *N. rossii*, in which an elevated Complex I-dependent flux after warm-acclimation was interpreted as a response to increase mitochondrial aerobic capacities (Strobel et al., in revision). CO_2 acclimation seems to elicit mitochondrial remodelling by a number of

regulative processes, resulting in higher capacities that match a high bicarbonate background, which also improve thermal tolerance.

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References

Acin-Perez, R., Salazar, E., Kamenetsky, M., Buck, J., Levin, L. R., and Manfredi, G.: Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation, Cell metabolism, 9, 265-276, 10.1016/j.cmet.2009.01.012, 2009.

Bilyk, K. T., and DeVries, A. L.: Heat tolerance and its plasticity in Antarctic fishes, Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 158, 382-390, 10.1016/j.cbpa.2010.12.010, 2011.

Brauner, C. J., Wang, T., Wang, Y., Richards, J. G., Gonzalez, R. J., Bernier, N. J., Xi, W., Patrick, M., and Val, A. L.: Limited extracellular but complete intracellular acid-base regulation during short-term environmental hypercapnia in the armoured catfish, Liposarcus pardalis, The Journal of experimental Biology, 207, 3381-3390, 10.1242/jeb.01144, 2004.

Brauner, C. J., and Baker, D. W.: Patterns of acid-base regulation during exposure to hypercarbia in fishes, in: Cardio-Respiratory Control in Vertebrates, edited by: Glass, M. L., and Wood, S. C., Berlin, 43-63, 2009.

Buck, J., and Levin, L. R.: Physiological sensing of carbon dioxide/bicarbonate/pH via cyclic nucleotide signaling, Sensors, 11, 2112-2128, 10.3390/s110202112, 2011.

Cheng, C. H. C., Chen, L., Near, T. J., and Jin, Y.: Functional antifreeze glycoprotein genes in temperate-water New Zealand nototheniid fish infer an Antarctic evolutionary origin, Molecular biology and evolution, 20, 1897-1908, 2003.

Deigweiher, K., Koschnick, N., Pörtner, H. O., and Lucassen, M.: Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia, American Journal of Physiology- Regulatory, Integrative and Comparative Physiology, 295, R1660-R1670, 2008.

Deigweiher, K., Hirse, T., Bock, C., Lucassen, M., and Pörtner, H. O.: Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids, Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology, 180, 347-359, 10.1007/s00360-009-0413-x, 2010.

Eastman, J. T.: The nature of the diversity of Antarctic fishes, Polar Biol., 28, 93, 2005.

Enzor, L. A., Zippay, M. L., and Place, S. P.: High latitude fish in a high CO2 world: Synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids, Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 164, 154-161, 10.1016/j.cbpa.2012.07.016, 2013.

Esbaugh, A., Heuer, R., and Grosell, M.: Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, Opsanus beta, Journal of Comparative Physiology B, 1-14, 10.1007/s00360-012-0668-5, 2012.

Ferrari, M. C., Manassa, R. P., Dixson, D. L., Munday, P. L., McCormick, M. I., Meekan, M. G., Sih, A., and Chivers, D. P.: Effects of ocean acidification on learning in coral reef fishes, PLoS One, 7, e31478, 10.1371/journal.pone.0031478, 2012.

Frommel, A., Maneja, R., Lowe, D., Malzahn, A., Geffen, A., Folkvord, A., Piatkowski, U., Reusch, T., and Clemmesen, C.: Severe tissue damage in Atlantic cod larvae under increasing ocean acidification, Nature Climate Change, 2, 42–46, doi:10.1038/nclimate1324, 2012.

Gaston, K. J., Chown, S. L., Calosi, P., Bernardo, J., Bilton, D. T., Clarke, A., Clusella-Trullas, S., Ghalambor, C. K., Konarzewski, M., Peck, L., Porter, W. P., Pörtner, H. O., Rezende, E. L., Schulte, P., Spicer, J. I., Stillman, J. H., Terblanche, J. S., and van Kleunen, M.: Macrophysiology: a conceptual reunification, American Naturalist, 174, 595-612, 2009.

Gnaiger, E.: Capacity of oxidative phosphorylation in human skeletal muscle: New perspectives of mitochondrial physiology, The International Journal of Biochemistry & Cell Biology, 41, 1837-1845, 10.1016/j.biocel.2009.03.013, 2009.

Gnaiger, E.: Mitochondrial Pathways and Respiratory Control. An Introduction to OXPHOS Analysis., 3rd Edition ed., Mitochondr Physiol Network 17.18, OROBOROS MiPNet Publications, Innsbruck, 64 pp., 2012.

Guynn, S., Dowd, F., and Petzel, D.: Characterization of gill Na/K-ATPase activity and ouabain binding in Antarctic and New Zealand nototheniid fishes, Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology , 131, 363-374, 2002.

Heisler, N.: Acid-Base Regulation in Fishes, Fish Physiology, 10, 315-401, 1984.

Hilton, Z., Clements, K. D., and Hickey, A. J.: Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes, Journal of comparative physiology. B, Biochemical, Systemic, and Environmental Physiology, 180, 979-990, 10.1007/s00360-010-0477-7, 2010.

IPCC: Fourth Assessment Report of the Intergovernmental Panel on Climate Change, University Press, Cambridge, Cambridge, 2007.

Kroeker, K. J., Kordas, R. L., Crim, R. N., and Singh, G. G.: Meta‐analysis reveals negative yet variable effects of ocean acidification on marine organisms, Ecology letters, 13, 1419-1434, 2010.

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R., and Kunz, W. S.: Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells, Nature Protocols, 3, 965-976, 2008.

Langenbuch, M., and Pörtner, H. O.: Energy budget of hepatocytes from Antarctic fish (Pachycara brachycephalum and Lepidonotothen kempi) as a function of ambient CO2: pH-dependent limitations of cellular protein biosynthesis?, The Journal of Experimental Biology, 206, 3895-3903, 10.1242/jeb.00620, 2003.

Mark, F. C., Lucassen, M., Strobel, A., Barrera-Oro, E., Koschnick, N., Zane, L., Patarnello, T., Pörtner, H. O., and Papetti, C.: Mitochondrial Function in Antarctic Nototheniids with *ND6* Translocation, PLoS ONE, 7, e31860, 10.1371/journal.pone.0031860, 2012.

Matschiner, M., Hanel, R., and Salzburger, W.: On the Origin and Trigger of the Notothenioid Adaptive Radiation, PLoS ONE, 6, e18911, 10.1371/journal.pone.0018911, 2011.

Mehrbach, C., Culberso.Ch, Hawley, J. E., and Pytkowic, R. M.: Measurement of Apparent Dissociation-Constants of Carbonic-Acid in Seawater at Atmospheric-Pressure, Limnology and Oceanography, 18, 897-907, 1973.

Melzner, F., Göbel, S., Langenbuch, M., Gutowska, M. A., Pörtner, H. O., and Lucassen, M.: Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4-12 months) acclimation to elevated seawater pCO_2, Aquatic Toxicology, 92, 30-37, 2009.

Michaelidis, B., Spring, A., and Pörtner, H. O.: Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*, Marine Biology 150, 1417-1429, 2007.

Moran, D., and Stottrup, J.: The effect of carbon dioxide on growth of juvenile Atlantic cod *Gadus morhua* L, Aquatic Toxicology, 102, 24-30, 2010.

Munday, P. L., Crawley, N. E., and Nilsson, G. E.: Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes, Marine Ecology Progress Series, 388, 235-242, 2009.

Munday, P. L., McCormick, M. I., and Nilsson, G. E.: Impact of global warming and rising CO2 levels on coral reef fishes: what hope for the future?, The Journal of Experimental Biology, 215, 3865-3873, 10.1242/jeb.074765, 2012.

Nilsson, G. E., Dixson, D. L., Domenici, P., McCormick, M. I., Sorensen, C., Watson, S. A., and Munday, P. L.: Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function, Nature Climate Change, 2, 201-204, Doi 10.1038/Nclimate1352, 2012.

Pörtner, H. O., and Sartoris, F. J.: Invasive studies of intracellular acid-base parameters: environmental and functional aspects., in: Regulation of tissue pH in plants and animals: A reappraisal of current techniques., edited by: Taylor, E. W., Egginton, S., and Raven, J. A., Cambridge University Press, Cambridge, England, 69-98, 1999.

Pörtner, H. O.: Climate-dependent evolution of Antarctic ectotherms: An integrative analysis, Deep Sea Research Part II: Topical Studies in Oceanography, 53, 1071-1104, 10.1016/j.dsr2.2006.02.015, 2006.

Pörtner, H. O.: Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems, The Journal of Experimental Biology, 213, 881-893, 10.1242/jeb.037523, 2010.

Pörtner, H. O.: Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes, Marine Ecology Progress Series, 470, 273-290, 2012.

Simpson, D. P.: Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria, Journal of Clinical Investigation, 46, 225, 1967.

Somero, G. N., and DeVries, A. L.: Temperature tolerance of some Antarctic fishes., Science, 156, 257-258, 1967.

Strobel, A., Bennecke, S., Leo, E., Mintenbeck, K., Pörtner, H. O., and Mark, F. C.: Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2, Front Zool, 9, 28, 10.1186/1742-9994-9-28, 2012.

Strobel, A., Graeve, M., Pörtner, H. O., and Mark, F. C.: Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic nototheniid fish, *Lepidonotothen squamifrons* vs. *Notothenia rossii*, PLoS One, in revision.

Strobel, A., Leo, E., Pörtner, H. O., and Mark, F. C.: Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*, Comparative Biochemistry & Physiology, Part B, Biochemistry & Molecular Biology, submitted.

Tresguerres, M., Buck, J., and Levin, L. R.: Physiological carbon dioxide, bicarbonate, and pH sensing, Pflugers Archiv European Journal of Physiology, 460, 953-964, 10.1007/s00424-010-0865-6, 2010a.

Tresguerres, M., Parks, S. K., Salazar, E., Levin, L. R., Goss, G. G., and Buck, J.: Bicarbonate-sensing soluble adenylyl cyclase is an essential sensor for acid/base homeostasis, Proceedings of the National Academy of Sciences of the United States of America, 107, 442-447, Doi 10.1073/Pnas.0911790107, 2010b.

Walsh, P. J., Mommsen, T. P., Moon, T., and Perry, S.: Effects of acid-base variables on in vitro hepatic metabolism in rainbow trout, The Journal of Experimental Biology, 135, 231, 1988.

Wanders, R. J. A., Meijer, A. J., Groen, A. K., and Tager, J. M.: Bicarbonate and the Pathway of Glutamate Oxidation in Isolated Rat-Liver Mitochondria, European Journal of Biochemistry, 133, 245-254, Doi 10.1111/J.1432-1033.1983.Tb07455.X, 1983.

Zippin, J. H., Levin, L. R., and Buck, J.: CO2/HCO3−-responsive soluble adenylyl cyclase as a putative metabolic sensor, Trends in Endocrinology & Metabolism, 12, 366-370, http://dx.doi.org/10.1016/S1043-2760(01)00454-4, 2001.

Figures

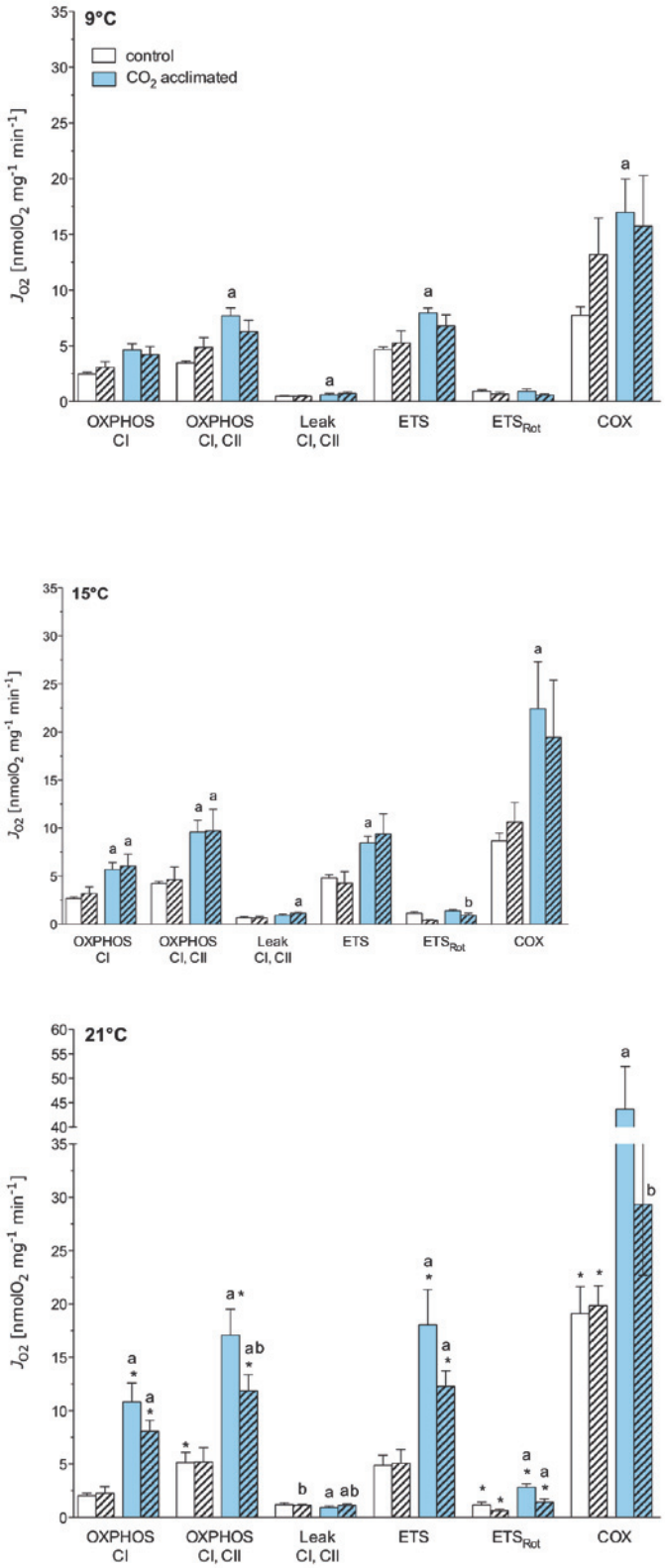

Figure 1 Respiration of permeabilised heart fibres of control (14°C, 0.04 kPa CO_2, white bars) and CO_2 acclimated (14°C, 0.2 kPa CO_2, blue bars) *N. angustata.* Assays were

conducted at 9, 15 and 21°C. Plain bars represent fibre respiration measured in normocapnic (0.04 kPa CO_2, 0.04 kPa CO_2) respiration buffer, hatched bars in acute hypercapnic (3 kPa CO_2) buffer. The following respiratory states are presented: CI state III maximum flux (OXPHOS CI); CI and CII state III maximum flux (OXPHOS CI, CII); ATPase inhibited flux - State IV^+ (Leak CI, CII); CI and CII uncoupled flux (ETS); CII uncoupled flux (E_{Rot}); Complex IV – Cytochrome *c* oxidase (COX). J_{O2} = mean±SEM oxygen flux (nmol O_2 mg^{-1} min^{-1}). 'a' depicts significant difference (t-test, P<0.05) between CO_2 acclimation and control within a respiratory state and temperature; 'b' denotes significant difference (t-test, P<0.05) of acute hypercapnic to normocapnic buffer; * significant difference (t-test, P<0.05) to 15°C assay.

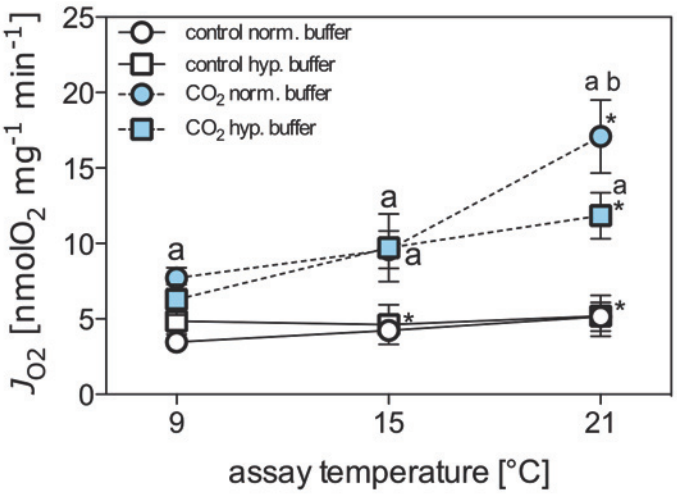

Figure 2 State III respiration of Complex I and II (OXPHOS CI, CII) in permeablilised heart fibres of control (14°C, 0.04 kPa CO_2) and CO_2 (14°C, 0.2 kPa CO_2) acclimated *N. angustata*. White dots: control group, normocapnic (0.04 kPa CO_2) respiration buffer (MiRO5), white squares: control group, acute hypercapnic (3 kPa CO_2) MiRO5; Blue dots: CO_2 acclimated fish, normocapnic MiRO5, blue squares: CO_2 acclimated fish, acute hypercapnic MiRO5. J_{O2} = mean±SEM oxygen flux (nmol O_2 mg^{-1} min^{-1}). 'a' depicts significant difference (t-test, P<0.05) between CO_2 acclimation and control within a respiratory state and temperature; 'b' denotes significant difference (t-test, P<0.05) of acute hypercapnic to normocapnic buffer.

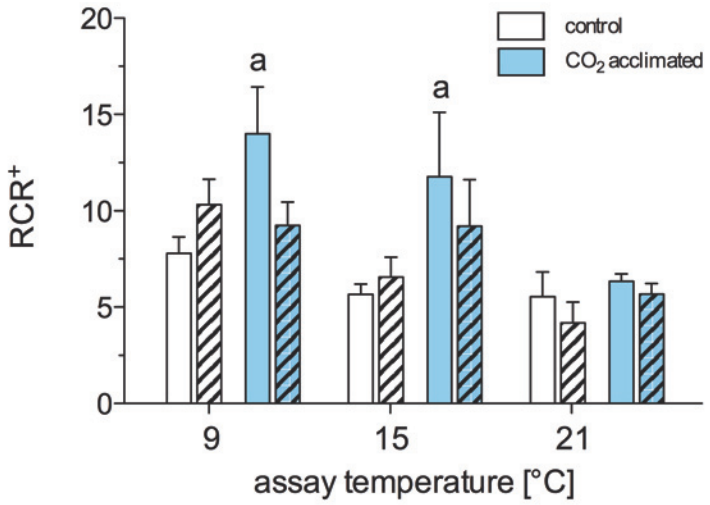

Figure 3 L/E ratio relative to assay temperature. L/E ratio was calculated in permeabilised heart fibres from control (14°C, 0.04 kPa CO_2, white bars) and 0.2 kPa CO_2 acclimated (blue bars) *N. angustata* in normocapnic (0.04 kPa CO_2, plain bars) and acute hypercapnic (3 kPa CO_2, hatched bars) respiration buffer.

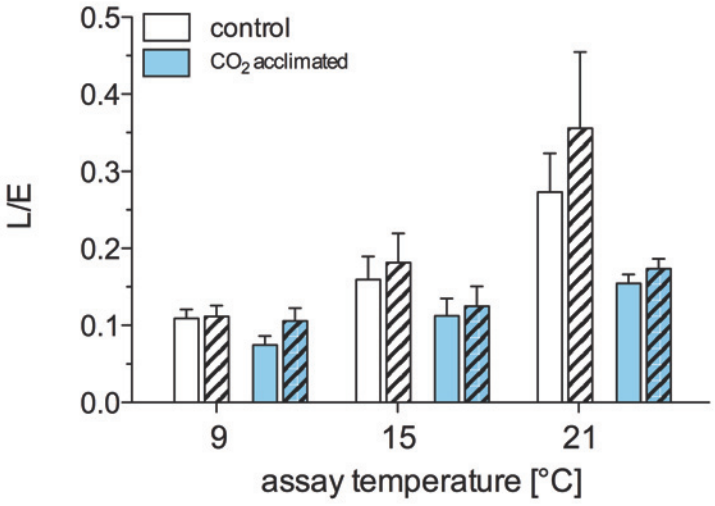

Figure 4 Respiratory control ratio (RCR^+) in permeabilised heart fibres of control (14°C, 0.04 kPa CO_2, white bars) and CO_2 acclimated (14°C, 0.2 kPa CO_2, blue bars) *N. angustata*. RCR^+ was calculated as OXPHOS CI, CII/ Leak CI, CII (state IV^+) respiration in heart fibres measured in normocapnic (0.04 kPa CO_2, plain bars) and acute hypercapnic (3 kPa CO_2, hatched bars), at 9, 15 and 21°C. 'a' shows a significant difference (t-test, $P<0.05$) between CO_2 acclimation and control.

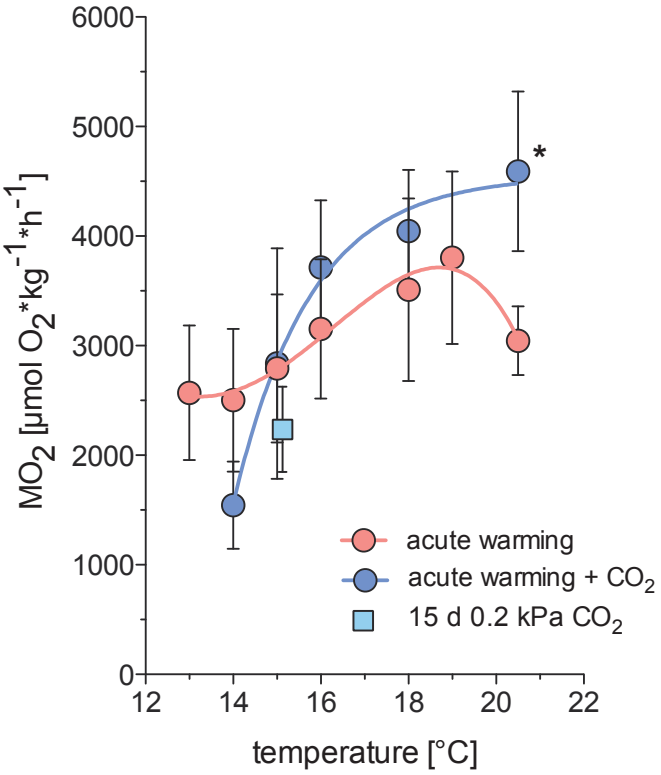

Figure 5 Routine metabolic rate (RMR) of *N. angustata* under acute warming (3°C/day) in normocarbic (red symbols, n=4) and hypercarbic seawater (0.2 kPa CO_2; blue symbols, n=3). The light blue square symbol depicts RMR after 15 days of hypercarbia acclimation (0.2 kPa CO_2; n=4). The asterisk indicates a significant difference in RMR between the normocarbic and the hypercarbic group at the given temperature.

Tables

Table 1 Percentage contribution of Complex I to total OXPHOS and COX excess in control and CO_2 (0.2 kPa CO_2) acclimated *N. angustata*.

	Temperature [°C]	Control		CO_2 acclimated	
		0.04 kPa CO_2	3 kPa CO_2	0.04 kPa CO_2	3 kPa CO_2
% CI	**9**	79.7±1.3	80.6±1.8	75.5±2.4	85.0±1.3
	15	78.1±2.2	80.9±3.0	75.4±3.3	80.3±1.9
	21	60.3±4.5#	67.8±6.2	79.6±2.2	86.4±1.9
COX/OXPHOS	**9**	2.1±0.2	2.3±0.5	2.2±0.3	2.4±0.3
	15	2.0±0.1	2.8±0.5	2.3±0.3	1.9±0.2
	21	4.2±0.7	5.7±1.7#	2.5±0.2	2.4±0.2*

COX/OXPHOS provides a comparative measure of cytochrome *c* oxidase capacity relative to oxidative phosphorylation. The percentage contribution of complex I (% CI) was calculated as OXPHOS CI, CII/ OXOHOS CI. It indicates the capacity of CI compared to total phosphorylation with CI and CII substrates. Both ratios were determined for permeabilised heart fibres in normocapnic (0.04 kPa CO_2) and acute hypercapnic (3 kPa CO_2) respiration buffer (MiRO6) at 9, 15 and 21°C. * indicates significant differences between control and CO_2 acclimation (ANOVA, $P < 0.05$). # depicts significant differences to 9°C assay (ANOVA, $P < 0.05$). Means ± SEM.

PUBLICATION V

Metabolic capacities in relation to temperature and hypercapnia in cephalopods from various climate zones

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Metabolic capacities in relation to temperature and hypercapnia in cephalopods from various climate zones

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Abstract

Coleoid cephalopods are the largest, metabolically most active invertebrates, and have adapted to habitats from the poles to the tropics. Yet, their sensitivity and acclimation capacity to ongoing ocean warming and acidification is poorly explored. This study compares Antarctic (*Pareledone charcoti*) and sub-tropical (*Eledone moschata*) octopods to metabolically more active, well-studied cuttlefish (*Sepia officinalis*). We acclimated *E. moschata* to 16 and 21°C (0.04 kPa CO_2) and *S. officinalis* to 0.112 kPa CO_2, and measured routine metabolic rate (RMR) and mitochondrial respiration (heart) under normocapnic and hypercapnic conditions.

Mitochondria of *S. officinalis* were better coupled and had higher respiration rates with higher acute thermal flexibilities than the octopods. Fibre respiration in Antarctic octopods was elevated compared to the sub-tropical species, indicating cold-compensation at cellular level. Warm-acclimated *E. moschata* showed uncompensated RMR and heart fibre respiration, indicating low thermal acclimation capacities. After long-term hypercapnia acclimation, the acid-base status in the haemolymph of *S. officinalis* was incompletely compensated with an extracellular pH remaining 0.1 pH units below control levels. Hypercapnia did not affect the RMR of *S. officinalis*, but led to increased mitochondrial capacities. In all three species, acute hypercapnia had no effect on the hearts' oxidative phosphorylation capacities, but depressed COX capacities in the octopods.

Our data suggest that *S. officinalis* compensate elevated PCO_2 by increasing mitochondrial turnover through enhanced amino acid catabolism. Higher mitochondrial COX excess capacities, which can increase mitochondrial oxygen affinity, and lower acute thermal sensitivities could render *S. officinalis* less vulnerable to ocean acidification and warming than octopods.

Key words

Pareledone charcoti, *Eledone moschata*, *Sepia officinalis*, ocean acidification/ warming, respiration, mitochondria

Introduction

Coleoid cephalopods are the largest, most active invertebrates, found in all marine habitats from the poles to the tropics (35). Sharing a similar lifestyle with fish, such as predation behavior and habitat distribution, a supposedly convergent evolution has led to similar performances and sophisticated respiratory and circulatory systems in cephalopods. The closed circulatory system, driven by two branchial hearts and a powerful systemic heart, allows a high cardiac output and oxygen transport at comparable or higher rates than in comparatively active, similar-sized fish. Constraints of their molluscan heritage include for example low blood oxygen-carrying capacities and minor efficient locomotion by jet propulsion (56, 66, 68).

With rising ambient temperature, an increase in oxygen demand exceeds the capacity of oxygen supply by the circulatory and ventilatory system and causes an uncompensated decrease in oxygen levels in the body fluids (42, 45). In order to meet elevated oxygen demands, cephalopods typically increase ventilation, heart rate and stroke volume (66). The capacity of mitochondria to produce energy aerobically is one of the mechanisms supporting and constraining the performance of any tissue, as their aerobic capacities contribute to shaping the aerobic energy metabolism. In oxygen supply systems, the key tissue is the systemic heart (44). A compensatory long-term acclimation response to warmer temperatures is therefore based on changes in mitochondrial capacities. To date, little is known about mitochondrial oxidative capacities and limits to oxidative metabolism in cephalopods (but see Mommsen and Hochachka (34) and Oellermann *et al.* (38)).

Seibel and Walsh (57) propose an inverse relationship between vulnerability to decreasing seawater pH and metabolic rate, with less active cephalopods, such as deep-living species being more susceptible to ocean acidification than more active (e.g. shallow-living) ones. This vulnerability is founded in the ability to buffer metabolic end products, such as CO_2 and protons, and the concentration of respiratory proteins buffering extracellular fluids against pH changes, which declines with metabolic rate and depth (67). In contrast, highly active animals such as squid are also extremely impacted by decreases in seawater pH (51). This is due to the fact that blood-oxygen binding at the gills and release at the tissues in cephalopods is highly pH sensitive (5). In squids like *Illex illecebrosus,* who possess only little venous oxygen reserves and highly pH-sensitive oxygen transport characteristics, a blood pH change of as little as 0.25 units can be lethal (51).

As anthropogenic CO_2 emissions are expected to cause a rise in atmospheric PCO_2 to above 0.1 kPa by the year 2100 in realistic scenarios ('business-as-usual') of the Intergovernmental Panel on Climate Change (21), an efficient pH_e-compensation is crucial for cephalopods to tolerate increases in seawater PCO_2 (32). However, an efficient acid-base balance might occur on the expenses of ionic homeostasis by ion-transporters, which may include higher costs by ATP-dependent pumps (12, 20). If under chronically elevated seawater PCO_2 acid-base imbalances in extra- and intracellular body fluids cannot be compensated, this may lead to metabolic depression (a condition expected to retard growth and reproduction), reduced activity and disruption of oxygen-transport mechanisms, and – if persistent – death (53, 58).

Furthermore, combined ocean acidification and warming might lead to additional energetic requirements, which would further reduce the thermal tolerance of marine ectotherms (41). In line with this, cephalopod sensitivity to ocean warming and acidification may vary, depending on the one hand on pH sensitivities (and concentration) of their respiratory proteins, and on the other hand on metabolic rate and mitochondrial compensation for a changing energy demand, e.g. for acid-base homeostasis.

Among cephalopods, the Octopoda have been able to adapt during their radiation to water temperatures ranging from > 30°C in tropical to -2°C in Antarctic waters (36). To date, some studies have measured respiration rates during acute thermal challenge in temperate octopods such as *Octopus vulgaris* (7, 23), and in the Antarctic octopod *Pareledone charcoti* (10), for which upper critical temperatures between 8 and 10°C have been determined (51). Despite, long-term warm-acclimation capacities of octopods have poorly been studied so far. Particularly in light global climate change, acclimation capacities towards chronically elevated temperatures become increasingly important (21).

In contrast to octopods, acute thermal limits (25°C, (16, 31)) and long-term warm-acclimation capacities (29) have intensively been analyzed in the cuttlefish *S. officinalis*. However, the mitochondrial background of thermal acclimation has not been comprehensively studied in cephalopods. In fish, mitochondrial compensation to temperature includes e.g. changes in mitochondrial respiration, densities or enzyme capacities (9), or shifts from carbohydrate to fatty acid oxidation (59). In contrast, *S. officinalis* shows only limited temperature compensation abilities of mitochondrial capacities. Nevertheless, thermal acclimation can induce shifts in mitochondrial substrate preferences in cuttlefish (38), similar to fish.

Next to these studies on thermal sensitivities, *S. officinalis* is amongst the few cephalopods for which CO_2 sensitivities have been previously assessed. In the highly active Humboldt squid *Dosidicus gigas*, a species living in association with hypercapnic oxygen minimum zones, an acute PCO_2 of 0.1 kPa has been reported to depress metabolic rates by 31% (54). Similarly, acute severe hypercapnia (1 kPa CO_2) reduces oxygen consumption in the temperate cephalopod *S. officinalis* (Schmidt *et al.*, unpublished), but not so during acute exposure to 0.6 kPa CO_2 (18). At such intermediately elevated PCO_2 of 0.6 kPa, *S. officinalis* shows a partially compensated pH_e (leading to significantly elevated extracellular bicarbonate levels) and efficient intracellular pH (pH_i) regulation during acute exposure to 0.6 kPa CO_2 (18), without any metabolic disturbances or depression, which probably relates to their (for invertebrates) relatively efficient acid-base regulatory system (18). However, all these studies only investigated short-term responses to elevated ambient PCO_2, and no comparable studies exist for octopods so far.

To date, nobody has provided an overview over acute or long-term hypercapnia effects on mitochondrial metabolism in cephalopods. In this study, we aim to compare the susceptibility of cephalopods from different climate zones towards global warming and ocean acidification in. We investigated the difference in aerobic metabolic capacities in the benthic, Antarctic octopod *Pareledone charcoti* and the sub-tropical *Eledone moschata,* and compare these findings to those in a well-studied reference organism, the bentho-pelagic cuttlefish *Sepia officinalis*. In the light of ongoing global change, we measured the acclimation capacities of sub-tropical octopods towards warmer temperatures, as well as those of temperate cuttlefish towards elevated PCO_2. We assessed routine oxygen consumption rates of the Antarctic octopods at their habitat temperature, of the sub-tropical octopods at their mean habitat temperature of 16°C and after five month acclimation to 21°C. Furthermore, we determined oxygen consumption of the cuttlefish at their habitat temperature of 16°C under normocapnic conditions (0.039 kPa CO_2) and after five months of hypercapnia acclimation (0.112 kPa CO_2; seawater PCO_2 predicted for the year 2100 (21)). By measurement of their mitochondrial respiration (in heart fibres of the systemic heart) at different, rising assay temperatures in normocapnic (0.039 kPa CO_2) and hypercapnic (1.6 kPa CO_2) respiration medium, the thermal flexibility and potential warm/ hypercapnia acclimation capacities of cellular aerobic metabolism were investigated.

The benthic, Antarctic octopus *P. charcoti* is distributed on continental shelves around the Antarctic Peninsula and in the Ross Sea at temperatures between -2 and 2°C (2). The

benthic, sub-tropical (musky) octopus *E. moschata*, is abundant throughout the Mediterranean Sea and off the Portuguese coast (8, 39). The more active, bottom-dwelling common cuttlefish *S. officinalis* is one of the most abundant cephalopods and of great commercial interest. It occurs from the temperate North Atlantic to the sub-tropical Mediterranean and in Atlantic coastal waters off Senegal (3). In the Mediterranean Sea, both *S. officinalis* and *E. moschata* face water temperatures from 10 up to 25°C (4).

Material and Methods

Animal capture, maintenance and acclimation

Specimens of adult *P. charcoti* were caught during the expedition ANTXXVII/3 with the German research vessel 'RV Polarstern' in February 2011 by means of bottom trawls off King George Island (62°18'S 58°41'W) at 350 m water depth at a local water temperature of 0.1°C and a salinity of 34.4 psu. Animals were kept in well-aerated aquaria on board of RV Polarstern with permanent seawater supply under dim light (12 h light/ dark). Logistic implications impeded any warm- or CO_2- acclimations on board. Animals were not fed prior to the experiments and measurements of RMR, which were conducted one week after capture.

Adult specimens of *E. moschata* from the Adriatic Sea were collected in May 2010 at a water temperature of 16°C by local fisherman in Chioggia, Italy, and transported to the Alfred Wegener Institute, Bremerhaven, Germany, immediately.

Four specimens were kept under control conditions at 16°C, 0.04 kPa CO_2. Another four octopods were warm-acclimated to 21°C, 0.04 kPa CO_2, for a time-period of five months. The animals were acclimated in separate seawater recirculation systems in individual closed incubation boxes with a volume of 8.5 l. Each box was perfused from a header tank containing well-aerated seawater at the desired temperature (16°C/ 21°C), which was kept constant by several 250 W heating elements (Jaeger, EHEIM GmbH, Germany) controlled by a Temperature Controller TMP1380 (iSiTEC GmbH, Germany). They were fed every other day with brown shrimp (*Crangon crangon*).

Eggs of sub-tropical *S. officinalis* were collected by local fisherman in the Venice Lagoon, Chioggia, Italy, in May 2009 at a local temperature of 16°C. They were transported to and raised in a closed, recirculating aquarium system at the Alfred Wegener Institute, Bremerhaven, Germany, on a diet of mysids (*Neomysis integer*) and brown shrimp (*C. crangon*) at constant temperature (16.0 ± 0.1°C), salinity (30-32 psu) and pH (>8.0).

Adult cuttlefish were either kept under control conditions (16°C, 0.04 kPa CO_2) in the general aquaria facilities of the Alfred Wegener Institute (30-32 psu), or acclimated to 16°C and 0.112 kPa CO_2 for five months, respectively (N = 8). Cuttlefish were kept in a recirculating seawater system in individual closed incubation boxes (volume 8.5 l), fed by a header tank containing well-aerated, hypercarbic seawater. To adjust seawater PCO_2, the water was injected with gas mixed from compressed air and CO_2 using a mass flow controlling gas mixing system (HTK Hamburg GmbH, Germany). Cuttlefish were fed every other day with live shrimp (*Palaemon sp.* or *C. crangon*).

The pH was monitored daily with a NIST (National Institute of Standards and Technology) buffer calibrated WTW 3310 pH meter (WTW, Germany) and a glass electrode (InLab Routine Pt1000®, Mettler Toledo GmbH, Germany). Dissolved inorganic carbon (DIC) was measured by gas chromatography (Agilent 6980 N, Agilent Technologies, Germany), following a protocol modified from Lenfant and Aucutt (27), and Pörtner *et al.* (40). Seawater carbonate chemistry was calculated with the CO2SYS software (28), using the measured pH and dissolved inorganic carbon values.

Routine metabolic rate

Routine metabolic rate of *E. moschata* (control/ after long-term warm-acclimation), *P. charcoti* and *S. officinalis* (control/ after long-term acclimation to 0.112 kPa) was measured via intermittent-flow respirometry. Cephalopods were not fed for five days prior to the respiration experiments to ensure complete digestion of the last meal (17). RMR in this study is defined as the oxygen consumption during rest in unfed (not starving) animals, including spontaneous activity. The respiration chambers were covered with black cloth in order to minimize external disturbance and spontaneous activity of the animal. Each animal was placed in a 870 ml cylindrical respirometer under acclimation conditions (*E. moschata:* 16°C, 0.04 kPa, N=4 54-76g; 21°C, 0.04 kPa N=4, 25-31g. *P. charcoti* 0°C 0.04 kPa, N=4, 43.6–91.5g. *S. officinalis* 16°C, 0.04 kPa, N=6, 88-146g; 16°C, 0.112 kPa, N=7, 33-46g).

Individuals were allowed to recover within the respiration chambers for 24 hours, an appropriate period to overcome the effect of any handling stress (10, 69). Constant seawater-mixing within the respirometer was generated by an aquarium pump. In the intermittent-flow system, water exchange between chamber and ambient water was interrupted every 15 min for 15 min to measure oxygen depletion (max. 10% O_2 depletion) by the animal within the chamber, then oxygen concentration was replenished to 100% by flush pumps. Oxygen

concentration within the chamber was detected once per minute using a fluoroptic sensor connected to a FiBox2 (PreSens – Precision Sensing GmbH, Germany) oxygen meter. Oxygen consumption of the animal was calculated by using the linear declining rate of oxygen content within the respiration chamber for each 15 min measurement interval. The oxygen meter was calibrated before each measurement in well-aerated seawater at the respective acclimation-temperature, calibration at zero oxygen was conducted in nitrogen-bubbled seawater.

Blank measurements of bacterial respiration in the respirometer were carried out for all species and each acclimation group, values for RMR were corrected accordingly.

Dissection and heart fibre preparation

After anaesthetization of the animals in 2.5% ethanol, the mantle cavity was opened and samples of blood were taken from the anterior vena cava for further experiments. The animals were then sacrificed by decapitation. Branchial hearts were excised, immediately freeze-clamped and stored in liquid nitrogen. After dissection, a piece of systemic heart tissue was transferred into ice-cold modified biopsy medium (in $mmol*l^{-1}$: 2.77 Ca_2EGTA, 7.23 K_2EGTA, 14.46 KOH, 5.77 Na_2ATP, 6.56 $MgCl_2*6H_2O$, 20 taurine, 20 imidazole, 0.5 dithiothreitol (DTT), 50 MES, 588 Sucrose and 252 glycine, 1000 mOsm, pH 7.4 at 24°C). Fibre preparation followed the description by Kuznetsov *et al.* (25). The heart tissue was mechanically dissected in ice-cold medium by scissors and forceps in small fibre bundles and stored on ice in respiration medium. For each respiration experiment, a subsample of the heart fibre bundles was permeabilized for 30 min with 50 µg/ml saponin by gentle mixing on ice. Afterwards, the fibres were washed three times for 10 min in 2 ml ice-cold, modified assay medium (mitochondrial respiration medium, MiRO5), modified from (25, 34)) containing (in $mmol*l^{-1}$) 50 Na^+Hepes, 25 KH_2PO_4, 50 KCl, 50 NaCl, 350 sucrose, 0.5 EGTA, 10 $MgCl_2*6$ H_2O, 20 taurine, 50 lactobionate, 150 glycine and $10g*l^{-1}$ freshly added BSA (fatty acid free) (1000 mOsm, pH 7.4 at 24°C). Then, the subsample was blotted dry, divided in two and weighed, and immediately transferred into 2 ml air saturated assay medium (MiRO5) + 300U/ml catalase (for reoxygenation with hydrogen-peroxide) in glass-chambers of an Oroboros Oxygraph-2k^{TM} respirometer (Oroboros Instruments, Austria) at the respective assay temperature for respirometric analysis.

Heart fibre respiration assays were measured in the standard, normocapnic respiration buffer (MiRO5) of 0.04 kPa CO_2, and in acute hypercapnic respiration buffer with 1.6 kPa

CO_2. Considering a maximum gradient of about 0.4 kPa between the intracellular and extracellular space (46), 1.6 kPa would be the corresponding intracellular PCO_2 to the extracellular PCO_2 in *S. officinalis* long-term acclimated to 0.112 kPa CO_2. Although we did not measure intracellular acid-base parameters in systemic heart due to sample shortage, the calculated PCO_2 of 1.6 is well within a range that can be expected for cuttlefish heart, as intracellular PCO_2 values of 0.4 kPa was measured in control *S. officinalis* (Häfker 2012, *unpublished*) and 0.7 kPa in *Lolliguncula brevis* (47) mantle tissue.

Heart fibre respiration assays

Respiration of each subsample was measured at 0, 6 and 12°C (*P. charcoti*) or 16, 21 and 26°C (*E. moschata* and *S. officinalis*) in randomized order. The heart fibre respiration was converted to nmol O_2 min^{-1} mg fresh weight $(fw)^{-1}$. For measurement of heart mitochondrial capacities, the concentration and choice of substrates for the combined substrate-inhibitor protocol was made in accordance to previous experiments on systemic heart fibre respiration of *S. officinalis* by Oellermann *et al.* (38). Resting respiration (state II) was measured with complex I (CI) substrates, 5mM proline, 5mM malate, and 5mM pyruvate. State III respiration (maximum coupled oxidative phosphorylation) of complex I (OXPHOS CI) was induced by 1mM ADP, state III respiration of complex I and complex II (CII) by adding 5mM succinate (OXPHOS CI, CII). Integrity of the mitochondrial membranes was tested wit 0.01mM cytochrome c. Leak capacity (state IV^{+}) was evaluated by adding 4 µg/ml oligomycin, followed by uncoupling of the respiration induced by titration of up to 2.5 µM carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone (FCCP). After inhibition of complex I with 0.005mM rotenone (state III*u* of complex II), non-mitochondrial respiration (ROX) was detected by adding 0.0025mM antimycin A, followed by addition of the artificial substrates for complex IV (cytochrome C oxidase, COX), 2mM ascorbate and 0.5mM *N,N,N',N'*-tetramethyl-*p*-phenylendiamine dihydrochloride (TMPD). To avoid low oxygen tensions within the chambers during the respiration experiments, oxygen concentrations were restored to air saturation when reaching 100nmol O_2 ml^{-1} by re-oxygenation with 1% hydrogen-peroxide.

Extracellular acid-base parameters.

Hemolymph pH (extracellular pH, pH_e) from *S. officinalis* was measured immediately after sampling at the acclimation temperature with a pH meter (WTW 340i, WTW, Germany. Electrode: In Lab® Viscous, Mettler Toledo GmbH, Germany). The pH meter was calibrated daily with NIST buffers (WTW, Germany). Measurements were carried out in a closed microcentrifuge tube (0.5 ml) to minimize contact with environmental air. Plasma total CO_2 (C_{CO_2}) was measured after centrifugation by means of a Gas Chromatography (Agilent 6980 N, Agilent Technologies, Germany). Blood carbonate chemistry was calculated using the following, modified Henderson-Hasselbalch equation:

$$PCO_2 = C_{CO_2} \times (10^{pH-pK'''} \times \alpha + \alpha)^{-1} \quad (1)$$

$$\left[HCO_3^-\right] = C_{CO_2} - \alpha \; PCO_2 \quad (2)$$

$[HCO_3^-]$ represents bicarbonate concentration. Values for the CO_2-solubility coefficient α and pK''' (first apparent dissociation constant of carbonic acid) were calculated for 16°C and 32 psu from values for *Carcinus maenas* hemolymph (64), which possesses a similar extracellular hemocyanin concentration and ionic composition (18).

Data analysis and statistics

Using the heart fibre respiration data of all species, the following indicators for mitochondrial capacities were calculated:

1) Capacity of complex I relative to total coupled oxidative phosphorylation (OXPHOS), calculated as OXPHOS CI, CII/ OXPHOS CI (%).

2) Percentage proton leak fraction of OXPHOS I, II (% Leak): State IV^+/ OXPHOS CI, CII, and respiratory control ratio (RCR^+) as State IV^+/ OXPHOS CI, CII

3) Capacity of cytochrome *c* oxidase (COX) relative to OXPHOS CI, CII (COX/ OXPHOS CI, CII)

4) Temperature coefficient Q_{10} was calculated for RMR and heart fibre respiration (only for complex IV/ COX) according to the formula:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2-T_1)} \quad (3)$$

R denotes the respiratory rate (of the heart fibres/ RMR) at a higher (T_2) or lower (T_1) temperature. Differences in heart fibre oxygen consumption at the assay temperatures 0, 6 and 12, or 16, 21 and 26°C, and between the different acclimation groups were tested using unpaired, two-tailed t-test and one-way analysis of variance (ANOVA, with Tukey post-hoc test). A $p \leq 0.05$ was considered the significance threshold. All values were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance and are given in means ± SEM.

Results

Cuttlefish

Whole-animal oxygen consumption. The respiration rate of hypercapnia-acclimated *S. officinalis* (16°C, 0.112 kPa CO_2; 0.10±0.0 µmol O_2 g^{-1} min^{-1}) was not different to the RMR of control *S. officinalis* (0.04 kPa CO_2; 0.11±0.0 µmol O_2 g^{-1} min^{-1}) at 16°C. Both were similar compared to the RMR of *E. moschata* at 16°C (0.09±0.1 µmol O_2 g^{-1} min^{-1}).

Permeabilized heart fibre respiration. After five months of acclimation to 0.112 kPa CO_2, the heart fibres of *S. officinalis* showed a higher state III respiration than the control group at the warmest assay temperature of 26°C in the normocapnic respiration buffer. In the same 26°C assay, state III respiration of the hypercapnia acclimated cuttlefish measured in the hypercapnic (1.6 kPa CO_2) mitochondrial respiration medium was significantly lower than in the normocapnic medium (Figure 3).

State IV^+ respiration rose significantly with assay temperature only in normocapnic mitochondrial respiration medium in the control cuttlefish. The percentage contribution of state IV^+ to state III respiration (% Leak) was lower in the cuttlefish acclimated to elevated PCO_2, while their heart fibres exposed to acute hypercapnia had an elevated COX/ OXPHOS CI, CII ratio.

Extracellular acid-base parameters. Control extracellular pH (pH_e) of *S. officinalis* was 7.49±0.03, HCO_3^- 5.3±1.6 mM and PCO_2 0.7±0.2kPa. After long-term hypercapnia acclimation, a new extracellular steady-state HCO_3^- of 9.9±1.7 mM and PCO_2 of 1.1±0.3 kPa was calculated at a significantly lower pH_e of 7.40±0.01.

Octopods

Whole-animal oxygen consumption. Respiration rates were mass corrected (gramm wet weight) for all species using the weight exponent 0.75 (37). As respiration rate of *P. charcoti* was only measured at 0°C (0.03±0.0 µmol O_2 g^{-1} min^{-1}), a combined Q_{10} value was calculated using the respiration rates of *P. charcoti* at 0°C and *E. moschata* at 16°C (0.09±0.1 µmol O_2 g^{-1} min^{-1}), a procedure already used for a comparison between Antarctic and temperate octopus species (10). The resulting Q_{10} of 2.2 is also depicted in Figure 1, and demonstrates an uncompensated shift from *P. charcoti* respiration rates at its habitat temperature to the respiration rates of *E. moschata* at their habitat temperature of 16°C.

RMR of the warm-acclimated *E. moschata* (21°C, 0.04 kPa CO_2) measured at 21°C (0.13±0.0 µmol O_2 g^{-1} min^{-1}) was only non-significantly higher than the RMR of control *E. moschata* (16°C, 0.04 kPa CO_2) measured at 16°C, with a Q_{10} of 2.7 between the two groups (Figure 1).

Permeabilized heart fibre respiration. In *E. moschata*, thermal acclimation did not affect complex I or II activity (state III respiration; data of complex I respiration not shown), nor net state IV^+ respiration (proton leak capacities). Only complex IV (COX) assayed at 21°C displayed increased activity following warm-acclimation to 21°C (Figure 2). A shift was also visible in the Q_{10} for COX activity over the whole thermal range: Q_{10} of control *E. moschata* was 1.8, and 2.2 in warm-acclimated *E. moschata* (Table 2). Furthermore, the COX/state III ratio measured in the 26°C assay was higher in heart fibres of the warm-acclimated octopus compared to their control.

In *P. charcoti*, state III respiration (CI, CII) rose from 0.44±0.0 µmol O_2 g^{-1} min^{-1} at 0°C, to 0.53±0.0 µmol O_2 g^{-1} min^{-1} at 6°C and was significantly higher at 12°C compared to the 0°C assay with values of 0.73±0.1 µmol O_2 g^{-1} min^{-1} in normocapnic buffer. The respiration rates of all other respiratory states investigated also rose acutely with temperature, and were significantly elevated at the warmest assay temperature of 12°C (data not shown).

In both octopus species, *P. charcoti* and *E. moschata,* heart fibre respiration measured in the hypercapnic (1.6 kPa CO_2) respiration medium was not significantly affected when compared to the respiration rates in normocapnic mitochondrial respiration medium (MiRO5). Only state IV^+ respiration in the warm-acclimated *E. moschata* assayed at 16°C was lower in the hypercapnic respiration medium compared to the normocapnic respiration medium (Figure 2). Nevertheless, a trend to lower COX Q_{10} values and lower COX/OXPHOS CI, CII

ratios was visible at the highest assay temperatures in the hypercapnic *vs.* normocapnic respiration medium in *E. moschata* and *P. charcoti* (Table 2).

Comparison between species

State III respiration of *P. charcoti* at 0°C was 0.44±0.0 µmol O_2 g^{-1} min^{-1}, of *E. moschata* at 16°C 0.79±0.2 µmol O_2 g^{-1} min^{-1} and 1.11±0.2 µmol O_2 g^{-1} min^{-1} in the warm-acclimated *E. moschata* at 21°C. In control *S. officinalis*, state III respiration at 16°C was 1.40±0.5 µmol O_2 g^{-1} min^{-1} and 2.66±0.4 µmol O_2 g^{-1} min^{-1} in the hypercapnia-acclimated group (Figure 4). The values of *E. moschata* and *S. officinalis* were not significantly different, only the fibre respiration of *P. charcoti* at 0°C was significantly lower than the one of *E. moschata* and *S. officinalis* at 16°C.

The respiratory control ratio (RCR^+) and percentage of OXPHOS CI to total OXPHOS flux of *S. officinalis* (control and hypercapnia acclimated) was significantly higher than in both octopus species. Consequently, control *S. officinalis* showed a lower contribution of state IV^+ to OXPHOS CI, CII (% Leak) than *P. charcoti,* and hypercapnia acclimated *S. officinalis* had the lowest state IV^+ contribution (% Leak) of all species investigated (Table 2).

Discussion

Hypercapnia sensitivity of S. officinalis

A number of studies report reduced metabolic rates (i.e. metabolic depression) during acute and long-term severe hypercapnia, e.g. in mussels or sipunculid worms (33, 49), usually accompanied by an uncompensated decrease in pH_e, independent of reduced or restored pH_i levels. Several studies show that pH_e can be already altered at low to intermediate hypercapnia levels in invertebrates, however, they are not necessarily accompanied by depressed metabolic rates (e.g. burrowing shrimp (11) or spider crabs (71)).

In our study, the cuttlefish *S. officinalis* also showed no sign of metabolic depression in their oxygen consumption rates after five months of exposure to 0.112 kPa CO_2 (RMR: 0.1 µmol O_2 g^{-1} min^{-1}), and also another study demonstrated that *S. officinalis* is able to maintain stable RMRs around 0.09 µmol O_2 g^{-1} min^{-1} during acute exposure to an intermediate PCO_2 of 0.6 kPa (18). In contrast, the Humboldt squid *D. gigas* showed considerably reduced RMRs

during acute exposure to 0.1 kPa CO_2 (54), which supports the findings on a relatively lower hypercapnia sensitivity of *S. officinalis* compared to other cephalopods.

When *S. officinalis* was exposed to acute hypercapnia in another study, it displayed rapid bicarbonate accumulation, which nevertheless was insufficient for complete compensation of pH_e (18). Similarly, pH_e of the long-term hypercapnia-acclimated cuttlefish remained significantly below control levels (0.09 pH units), paralleled by slightly elevated HCO_3^- and PCO_2 levels. However, it is postulated that such incomplete compensation (up to 0.2 pH units below control) is within the tolerance limit to maintain proper functioning of the respiratory protein, hemocyanin, due to very large Bohr factors below -1 in this species (18). Other studies on the very active squids *I. illecebrosus* and *L. pealei* suggest an even tighter regulation of blood parameters (i.e. pH_e) in order to optimize hemocyanin function when blood PCO_2 rises, as a drop in arterial pH by only 0.15 pH units could hamper oxygen saturation of their pigment (43, 50). Furthermore, active cephalopods like squid (50) or *S. officinalis* probably possess quite efficient intracellular acid-base compensatory mechanisms. Acute exposure to 0.6 kPa CO_2 only caused a very minor decrease in pH_i by 0.03 units in *Sepia* mantle tissue, without involving anaerobic metabolic pathways in order to provide extra energy for acid-base regulation (18).

In line with these findings, Hu *et al.* (20) report expression patterns of ion-transporters compensated back to control levels after 42 days exposure to 0.3 kPa CO_2. As a consequence, metabolic costs largely remain constant during hypercapnia acclimation in *S. officinalis*, which is also reflected in our RMR data of hypercapnia *vs.* control cuttlefish.

Nevertheless, the study by Hu *et al*. (20) revealed an increase in ATP-synthase and COX mRNA expression during 42 days exposure of juvenile *S. officinalis* to 0.3 kPa CO_2, which gives a first hint of slight alterations in mitochondrial energy metabolism.

Indeed, state III respiration of the control group measured in normocapnic respiration buffer showed the same pattern as the heart fibres of the hypercapnia acclimated group measured in the hypercapnic buffer, which can be taken as a first indicator for compensatory mechanisms in mitochondrial metabolism towards chronic hypercapnia. Following a hypothesis for mammalian and fish mitochondria, acutely elevated bicarbonate concentrations can competitively inhibit citrate synthase and succinate-dehydrogenase within the TCA-cycle (60, 65), (Strobel *et al.* 2013, *under review*). This acute effect might then be compensated by shifts in altered activities and/ or quantities of mitochondrial complexes and shifts in metabolic pathways, such as enhanced glutamate oxidation feeding into the TCA-cycle via 2-oxoglutarate (Strobel *et al.* 2013, *under review*).

Furthermore, the intra-mitochondrial soluble adenylyl cyclase (sAC) is being discussed to be directly stimulated by bicarbonate, and then produces the second messengers cyclic adenosine monophosphate (cAMP) in mammals. In the course of this, it activates protein kinase A (PKA), which in the end phosphorylates Complex IV of the electron transfer system (ETS), and possibly Complex I as well (63). As a long-term response, the sAC and cAMP signaling cascade may directly influence the expression or activity of metabolic enzymes and mitochondrial complexes via transcription factors and post-translational modifications, and thereby elicit constantly elevated levels of e.g. COX (70). In line with these hypotheses, transcriptional or post-translational regulation of slightly elevated ATP synthase and COX expression patterns has been demonstrated in hypercapnia exposed, juvenile *S. officinalis* (20). In consequence, elevated intracellular bicarbonate is postulated to increase OXPHOS efficiency and ATP synthesis due to a high flux through the ETS (1, 6, 63).

In fact, mitochondrial state III respiration of the hypercapnia acclimated *S. officinalis* in this study was significantly elevated when assayed at 26°C in normocapnic respiration buffer (Figure 3), which indicates a shift towards compensated mitochondrial capacities compared to the control group via an increase in ATP synthesis capacities to overcome the inhibitory effect of elevated PCO_2. Due to acclimation to new intracellular conditions, i.e. slightly lower pH_i and higher bicarbonate levels, mitochondrial capacities of the hypercapnia acclimated *S. officinalis* then show similar respiration patterns (state III) in the hypercapnic buffer, as the control group in the normocapnic buffer (Figure 3). This corresponds to their 'new' intracellular environment with increased bicarbonate levels. Accordingly, shifts towards enhanced mitochondrial complex and enzyme activities, induced by sAC/ cAMP, seem to bring mitochondrial capacities back to normal levels.

When the heart fibres of the hypercapnia acclimated cuttlefish are measured in normocapnic buffer, their excessive capacities (i.e. higher enzyme/ ETS activities) become visible (Figure 3, lower panel). Thus, compensation of mitochondrial capacities as a response to bicarbonate induced mitochondrial inhibition seems likely.

In the control animals, the relative proton leak increased with assay temperature, similar to findings in Antarctic bivalves (*Laternula elliptica*) (48) and in the lugworm *Arenicola marina* (61). In the hypercapnia acclimated cuttlefish, state IV^+ respiration did not increase with acute assay temperature. Reduced proton leak capacities can be mediated by structural changes of mitochondrial membranes or by an increase in ATP synthase activity relative to electron flux through the ETS, thereby reducing mitochondrial membrane potential

(38). For mammals, it has been postulated that the bicarbonate-stimulated phosphorylation of COX (see above) is keeping mitochondrial membrane potential low while complex I of the ETS is fully activated (1), a hypothesis which corresponds to our findings of a low relative proton leak capacity in hypercapnia acclimated cuttlefish. The mechanisms for changes in proton leakage remain unclear for cephalopods at the moment, nevertheless a reduced state IV^+ respiration seems to support shifts in mitochondrial capacities and a high mitochondrial coupling ratio (RCR^+ 3.9 in the hypercapnic animals, RCR^+ 3.1 in the control group, Table 2).

Thermal sensitivities of octopods

The RMR of long-term warm-acclimated *E. moschata* measured at 21°C was higher than in the control group measured at 16°C. Therefore, this species appears to lack the possibility for temperature compensation towards chronically elevated temperatures as high as 21°C (Figure 1). A Q_{10} of 2.7 between these two groups strengthens their inability for warm-compensation, as complete compensation would occur with a Q_{10} of 1 (equal metabolic rates at the respective acclimation temperatures, type 2 adaptation, (52)).

In cuttlefish, as in other ectotherms, maintenance of the aerobic metabolism with rising temperatures is mainly limited by oxygen supply by the circulatory system (30). Thus, the observed uncompensated RMR of *E. moschata* in the warmth, which goes beyond a simple Q_{10} effect, could be a result of high energetic demands of the circulatory system, i.e. their systemic heart. The systemic heart possesses a generally high metabolic activity, and thus a high aerobic demand, due to its role in pumping oxygen rich blood from the gills into systemic tissues (66).

Also the oxidative phosphorylation capacity (state III respiration) of *E. moschata* showed no sign of temperature compensation, as indicated by a Q_{10} of 1.9 between control and warm-acclimated octopods (Figure 4). Furthermore, warm-acclimation led to reduced capacities to increase coupled oxidative phosphorylation rates with acutely rising assay temperature (Q_{10} 16-26°C 1.0 in warm-acclimated animals, Q_{10} 1.2 in control animals; Figure 2), although RCR^+ and proton leak capacities were the same in both acclimation groups.

However, a slight compensatory response to the higher energy demand was visible by increased COX capacities of *E. moschata* cardiac fibres following long-term warm-exposure (Figure 2). Such an up-regulation of COX capacity could enhance the mitochondrial oxygen affinity and oxygen diffusion into the mitochondria, likely to increase aerobic respiration capacities in the warmth (15). A similar picture has been observed in cuttlefish originating

from the English Channel after 21°C acclimation (38), which indicates that sub-tropical octopods also possess at least a minor flexibility to respond to ocean warming. However, it remains questionable if octopods can modify mitochondrial substrate preferences and oxygen efficiency in the same way as cuttlefish do (38), and if the uncompensated elevation of mitochondrial and whole animal oxygen consumption can be sustained or might impact long-term survival or population performance of these octopods.

All these data, and particularly the low acute Q_{10} of 1.2 from 16 to 26°C of the state III respiration in the control animals suggest that the sub-tropical *E. moschata* possesses generally low capacities to adjust their mitochondrial capacities with rising temperatures. Thus, their heart mitochondria may not be able to cover rising metabolic demands at warmer temperatures.

Surprisingly, the Antarctic octopus, *P. charcoti,* could increase state III respiration from 0 to 12°C with a Q_{10} of 2.3, which means that their mitochondria likely possess the capacity to increase their metabolism with temperature. For this species, an acute whole-animal critical temperature between 8 and 10°C has been determined (51), and we also measured an exponential rise in RMR of *P. charcoti* during acute warming (warming by 1.5°C/ 12h; data not shown) up to 9°C.

Comparison of metabolic capacities between Antarctic and sub-tropical cephalopods

A comparison of heart fibre respiration between *P. charcoti* at 0°C and *E. moschata* at 16°C revealed that *P. charcoti* possesses state III respiration rates slightly higher than expected by extrapolation of *E. moschata* respiration rates down to 0°C (Q_{10} 1.4, Figure 4). This pattern would suggest a slight metabolic cold adaptation by increased aerobic capacities in the cold at the cellular level. In contrast, at the whole animal level, the calculated Q_{10} between *P. charcoti* at 0°C and *E. moschata* at 16°C is around 2, well within the normal range of a biological system (Figure 1 and 4). Similarly, Daly and Peck (10) postulated a lack of metabolic cold adaptation at whole animal level for Antarctic octopods. Elevated heart mitochondrial capacities in the cold might be an adaptation to facilitate tissue function and to overcome decelerating processes on e.g. enzyme activity, which occur at constant close-to-zero temperatures (9).

A similar dichotomy is seen in fish locomotory muscle, where MCA occurs in some tissues, but not at whole-animal level. Particularly at the level of whole organism metabolism, MCA studies are limited due to the fact that muscle tissue, which is the dominant contributor

to body mass, possesses very different levels of aerobic and anaerobic energy generation and thus enzyme activities in tropical *vs*. polar species, and is strongly dependent on a species' mode of life and level of activity (24).

The heart fibres of both octopus species had similar RCR^+ at their respective habitat temperature, and state IV^+ respiration contributed to coupled state III respiration by almost 50%. In addition to the slightly higher coupled phosphorylation capacities of the Antarctic *P. charcoti* compared to *E. moschata* at their respective habitat temperature, COX activities of the Antarctic species assayed at 0, 6 or 12°C were at the same level as COX activities in control *E. moschata* observed at 16, 21 and 26°C (data for *P. charcoti* COX activities not shown). This clearly indicates elevated COX activities in the Antarctic octopods, which probably relate to higher mitochondrial densities (proliferation) or a higher cristae surface density within the mitochondria, which support higher aerobic capacities in the cold-adapted than in the sub-tropical octopods at their respective habitat temperatures.

Indeed, mitochondrial proliferation is commonly referred to reflect cold-adaptation to overcome reduced cytosolic metabolite diffusion capacities between mitochondria in polar ectotherms (14, 22). Furthermore, it compensates for the elevated energy demand for the maintenance of ion gradients across cell membranes in the cold (19). Similarly, mitochondrial proliferation and concomitantly higher COX activities in red muscle compensate for reduced mitochondrial oxidative capacity per mg protein at low temperature in some Antarctic fish species (22).

Comparing *E. moschata* with *S. officinalis* at 16°C, net state III respiration was slightly higher in the cuttlefish (Figure 4). This probably relates to generally higher aerobic capacities of the systemic heart in the more active cuttlefish. Although not a high performance cephalopod, *S. officinalis* uses undulatory swimming, e.g. for predation, while octopods commonly are sit-and-wait predators with lower metabolic activities (55).

Higher mitochondrial capacities are also reflected in the significantly higher RCR^+ (3.1 in control *S. officinalis,* 2.0 in control *E. moschata*) or lower state IV^+ respiratory flux of *S. officinalis* (up to 35%) compared to both Antarctic and Sub-tropical octopus species (up to 55%; Table 2), respectively. Oellermann *et al.* (38) calculated an even lower relative proton leak around 18% in cuttlefish originating from the English Channel. These findings of tightly coupled mitochondria in cuttlefish underline the relationship between whole-animal activity level and high mitochondrial phosphorylation capacity, and thus further attributes *S. officinalis* higher aerobic capacities than the less active *E. moschata.*

Furthermore, *S. officinalis* displayed a higher contribution of complex I to total coupled oxidative phosphorylation capacities compared to the octopods. The basis for aerobic energy production in cephalopods are the coupled catabolism of carbohydrates (pyruvate) and amino acids (proline), both delivering electrons to complex I. Proline is oxidized in squid heart mitochondria at high rates, and is therefore considered as the most effective amino acid substrate in cephalopods (34). Proline provides an input of carbon into the TCA at the level of 2-oxoglutarate after oxidation to glutamate, a reaction of glutamate dehydrogenase which yields an additional reduction equivalent (NADH) for complex I, but also consumes ½ mole O_2 per mol proline. Full oxidation of both pyruvate (which enters the TCA via acetyl-CoA) and glutamate yields 4 NADH and 1 $FADH_2$ (62). Thus, combined amino acid and carbohydrate catabolism increases the ATP production per mol O_2 by 50% (26). In contrast, succinate as substrate is much less efficient (38). High complex I capacities therefore might enhance mitochondrial ATP yield and oxygen efficiency, in this case for *S. officinalis*.

In line with our findings on higher aerobic cardiac capacities in *S. officinalis vs. E. moschata*, higher citrate synthase and ATPase activities have been recorded in *S. officinalis* compared to the octopods *E. cirrhosa* and *O. vulgaris*, which also reflect higher maximum capacities of cardiac metabolism (13). On the one hand, they may be a precondition for the more active lifestyle (i.e. greater sustainable swimming capacity) of *S. officinalis,* compared to the more sedentary octopods. On the other hand, higher aerobic capacities and more oxygen efficient mitochondria could support the systemic heart of *S. officinalis* during warming or rising seawater PCO_2, thus making them less vulnerable to climate change than octopods. In line with this, a full temperature compensation after acclimation to 20°C has been demonstrated for *S. officinalis* (29), while we measured no warm-acclimation capacities in *E. moschata.*

Furthermore, a comparison of acute Q_{10} values of state III respiration from 16–26°C of control *S. officinalis* and *E. moschata* and for the Antarctic octopus *P. charcoti* between 0 and 12°C served as another indicator for thermal sensitivities of their heart mitochondria. *S. officinalis* state III respiration rose with a Q_{10} of 1.4, which indicates that heart mitochondria of cuttlefish have insufficient capacities to follow acute rising energy demand of the cellular metabolism. The acute Q_{10} of *E. moschata* were even lower with values around 1.2, which supports the previous hypotheses on lower thermal capacities in the sub-tropical octopods compared to *S. officinalis*. Interestingly, the Antarctic octopus, *P. charcoti*, displayed Q_{10} values of 2.3 between 0 and 12°C, which certifies them capacities to adjust mitochondrial metabolism during acute rising temperature along with increasing chemical and physical

reaction speeds due to a simple Q_{10} effect. In contrast, the sub-tropical octopods and cuttlefish may need to adjust their mitochondrial capacities when seawater temperatures chronically rise above 16°C.

In terms of acute hypercapnia tolerance, no difference in state III respiration between normocapnic *vs.* hypercapnic buffer was visible in control *S. officinalis*. Also in the Antarctic octopus *P. charcoti* and the Sub-tropical octopus *E. moschata*, heart fibre respiration was not significantly reduced by acute exposure to hypercapnia of 1.6 kPa CO_2 in the respiration buffer, indicating sufficient mitochondrial functioning and no metabolic suppression during these acute hypercapnic conditions. It is possible that the chosen PCO_2 of 1.6 was not high enough to trigger extensive mitochondrial responses to acute hypercapnia, so that they only became visible after long-term hypercapnia exposure. Nevertheless, we noticed a trend towards a reduced scope for increasing COX respiration with rising assay temperatures leading to a lower acute Q_{10} (range 16-26°C or 0-12°C for the sub-tropical or Antarctic octopods, respectively) for COX in the heart fibres assayed in acute hypercapnic *vs.* normocapnic buffer (Table 2). Such limitations in COX capacities may reduce the mitochondrial oxygen affinity and flux through the ETS (15). This is a first indication that oxygen diffusion to mitochondria and their aerobic capacities could become limited due to elevated PCO_2, which particularly may affect the octopods *P. charcoti* and *E. moschata*.

Conclusions

This study compared the metabolic capacities of Antarctic and Sub-tropical octopods and cuttlefish to assess their vulnerability to future ocean warming and acidification. After five months of exposure of *S. officinalis* to 0.112 kPa CO_2, their oxygen consumption rates were not affected despite somewhat incomplete pH_e compensation. This suggests sufficient oxygen supply to tissues, probably supported by increased mitochondrial capacities in response to CO_2 acclimation.

In warm-acclimated *E. moschata*, RMR and heart fibre respiration were not temperature compensated. In response to a higher energy demand in the warmth, COX activities were increased, possibly to enhance mitochondrial oxygen affinity and thus aerobic respiration capacities. However, this appears insufficient to support full temperature compensation of the whole animal.

In both the Antarctic and sub-tropical octopods, acute hypercapnia exposure of heart fibres did not reduce respiration (oxidative phosphorylation), but impaired COX activities.

This gives a first hint at reduced mitochondrial oxygen affinities and flux through the electron transport system in octopods under acute hypercapnia. However, this was only visible in the octopods and not in the cuttlefish, emphasizing that octopods may be less tolerant to acute hypercapnia than cuttlefish, and further supports mitochondrial compensation capacities in *Sepia* to chronic hypercapnia.

The comparison of mitochondrial capacities between Antarctic and sub-tropical octopod species revealed similar COX activities and elevated state III respiration rates of the Antarctic species at their respective habitat temperatures, likely the result of mitochondrial proliferation and cold-compensated mitochondrial capacities in the Antarctic octopods. Such elevated mitochondrial capacities in the Antarctic animals probably compensate for decelerating effects on e.g. enzyme activities or metabolite diffusion in the permanent cold and thereby facilitate similar cellular metabolic activities of *P. charcoti* and *E. moschata* at 0°C or 16°C, respectively. The heart fibres of Antarctic octopods showed lower thermal sensitivities to acute rising temperatures than the sub-tropical *Eledone.* Thus, *P. charcoti* appears to have mitochondrial capacities to respond to temperature changes up to 12°C, while the sub-tropical *E. moschata* may need to compensate for chronic warmer temperatures above 16°C.

In a comparison of the octopods with *S. officinalis,* net state III respiration was higher in the cuttlefish. Together with highly coupled, oxygen efficient mitochondria and lower proton leak rates, their systemic heart probably possesses higher aerobic capacities than the octopods, which is in line with their more active lifestyle compared to sedentary octopods. Considering the generally different mitochondrial capacities and acute thermal responses, both octopod species may be more put at risk by future ocean warming and acidification than the cuttlefish *S. officinalis.*

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References

1. **Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR, and Manfredi G**. Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell Metab* 9: 265-276, 2009.

2. **Allcock A**. On the confusion surrounding *Pareledone charcoti* (Joubin, 1905) (Cephalopoda: Octopodidae): endemic radiation in the Southern Ocean. *Zool J Linn Soc* 143: 75-108, 2005.

3. **Boletzky Sv**. *Sepia officinalis*. In: *Cephalopod Life Cycles: Species Accounts*, edited by Boyle PR1983, p. 31-52.

4. **Boucaud-Camou E, and Boismery J**. The migrations of the cuttlefish (*Sepia officinalis L*) in the English Channel. In: *The Cuttlefish, first International Symposium on the cuttlefish Sepia*, edited by Boucaud-Camou E. Caen, France: Centre de Publications de l'Université de Caen, 1991, p. 179-189.

5. **Brix O, Lykkeboe G, and Johansen K**. The significance of the linkage between the Bohr and Haldane effects in cephalopod bloods. *Respir Physiol* 44: 177-186, 1981.

6. **Buck J, and Levin LR**. Physiological sensing of carbon dioxide/bicarbonate/pH via cyclic nucleotide signaling. *Sensors* 11: 2112-2128, 2011.

7. **Cerezo Valverde J, and García García B**. Suitable dissolved oxygen levels for common octopus (*Octopus vulgaris* cuvier, 1797) at different weights and temperatures: analysis of respiratory behaviour. *Aquaculture* 244: 303-314, 2005.

8. **Costello MJ, Emblow C, and White RJ**. European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their identification. In: *Collection Patrimoines Naturels*, edited by Costello MJ, Emblow C, and White RJ. France: Muséum national d'Histoire naturelle, 2001.

9. **Crockett E, and Sidell B**. Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol Zool* 63: 472-488, 1990.

10. **Daly H, and Peck L**. Energy balance and cold adaptation in the octopus *Pareledone charcoti*. *J Exp Mar Biol Ecol* 245: 197-214, 2000.

11. **Donohue P, Calosi P, Bates AH, Laverock B, Rastrick S, Mark FC, Strobel A, and Widdicombe S**. Impact of exposure to elevated PCO_2 on the physiology and behaviour of an important ecosystem engineer, the burrowing shrimp *Upogebia deltaura*. *Aquatic Biol* 15: 73-86, 2012.

12. **Dorey N, Melzner F, Martin S, Oberhänsli F, Teyssié JL, Bustamante P, Gattuso JP, and Lacoue-Labarthe T**. Ocean acidification and temperature rise: effects on calcification during early development of the cuttlefish *Sepia officinalis*. *Mar Biol* 1-16, 2012.

13. **Driedzic W, Sidell B, Stewart J, and Johnston I**. Maximal activities of enzymes of energy metabolism in cephalopod systemic and branchial hearts. *Physiol Zool* 63: 615-629, 1990.

14. **Egginton S, and Sidell BD**. Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 256: 1-9, 1989.

15. **Gnaiger E**. Mitochondrial pathways through complexes I+II: Convergent electron transport at the Q-Junction and additive effect of substrate combinations. In: *Mitochondrial Pathways and Respiratory Control*, Austria: OROBOROS MiPNet Publications, 2008.

16. **Grigoriou P, and Richardson C**. The effect of ration size, temperature and body weight on specific dynamic action of the common cuttlefish *Sepia officinalis*. *Mar Biol* 154: 1085-1095, 2008.

17. **Grigoriou P, and Richardson CA**. Aspects of the growth of cultured cuttlefish *Sepia officinalis* (Linnaeus 1758). *Aquacult Res* 35: 1141-1148, 2004.

18. **Gutowska MA, Melzner F, Langenbuch M, Bock C, Claireaux G, and Pörtner HO**. Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. *J Comp Physiol* 180: 323-335, 2009.

19. **Hochachka PW**. Channels and pumps—determinants of metabolic cold adaptation strategies. *Comp Biochem Physiol **B** Biochem Mol Biol* 90: 515-519, 1988.

20. **Hu MY, Tseng Y-C, Stumpp M, Gutowska MA, Kiko R, Lucassen M, and Melzner F**. Elevated seawater PCO_2 differentially affects branchial acid-base transporters over the course of development in the cephalopod *Sepia officinalis*. *Am J Physiol Regul Integr Comp Physiol* 300: R1100-R1114, 2011.

21. **IPCC**. Fourth Assessment Report of the Intergovernmental Panel on Climate Change In: *Climate Change 2007: The Physical Science Basis*, edited by Solomon, SD, Manning, M, Chen, Z, Marquis M, Averyt KB, Tignor M and Miller HL. Cambridge, UK: Cambridge University Press, 2007.

22. **Johnston IA, Calvo J, Guderley H, Fernandez D, and Palmer L**. Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J Exp Biol* 201: 1-12, 1998.

23. **Katsanevakis S, Stephanopoulou S, Miliou H, Moraitou-Apostolopoulou M, and Verriopoulos G**. Oxygen consumption and ammonia excretion of *Octopus vulgaris* (Cephalopoda) in relation to body mass and temperature. *Mar Biol* 146: 725-732, 2005.

24. **Kawall H, Torres J, Sidell B, and Somero G**. Metabolic cold adaptation in Antarctic fishes: evidence from enzymatic activities of brain. *Mar Biol* 140: 279-286, 2002.

25. **Kuznetsov A, Veksler V, Gellerich F, Saks V, Margreiter R, and Kunz W**. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* 3: 965-976, 2008.

26. **Lee P**. Nutrition of cephalopods: fueling the system. *Mar Freshw Behav Physiol* 25: 35-51, 1994.

27. **Lenfant C, and Aucutt C**. Measurement of blood gases by gas chromatography 1. *Respir Physiol* 1: 398-407, 1966.

28. **Lewis E, and Wallace DWR**. MS Excel Program Developed for CO2 System calculations. In: *MS Excel Program developed for CO2 system calculations*. Oak Ridge, Tennessee: ORNL/CDIAC-105 Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy: 2006.

29. **Melzner F**. Systemic investigations on the physiology of temperature tolerance in the common cuttlefish *Sepia officinalis*. In: *PhD Thesis*. Bremen: University of Bremen, 2005, p. 242.

30. **Melzner F, Bock C, and Pörtner HO**. Temperature-dependent oxygen extraction from the ventilatory current and the costs of ventilation in the cephalopod *Sepia officinalis*. *J Comp Physiol B Biochem Sys. Environ Physiol* 176: 607-621, 2006.

31. **Melzner F, Bock C, and Pörtner HO**. Allometry of thermal limitation in the cephalopod *Sepia officinalis*. *Comp Biochem Physiol A Mol Integr Physiol* 146: 149-154, 2007.

32. **Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, and Pörtner HO**. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* 6: 2313-2331, 2009.

33. **Michaelidis B, Ouzounis C, Paleras A, and Pörtner HO**. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar Ecol Prog Ser* 293: 109-118, 2005.

34. **Mommsen TP, and Hochachka PW**. Respiratory and enzymatic properties of squid heart mitochondria. *Eur J Biochem* 120: 345-350, 1981.

35. **O'Dor RK, and Webber DM**. The constraints on cephalopods: Why squid aren't fish. *Can J Zool* 64: 1591-1605, 1986.

36. **O'Dor RK, and Webber DM**. Invertebrate athletes: Trade-offs between transport efficiency and power density in cephalopod evolution. *J Exp Biol* 160: 93-112, 1991.

37. **O'Dor R, and Wells M**. Energy and nutrient flow. In: *Cephalopod Life Cycles 2*, edited by Boyle, PR. New York: Academic Press: 109-133, 1987.

38. **Oellermann M, Pörtner HO, and Mark FC**. Mitochondrial dynamics underlying thermal plasticity of cuttlefish (*Sepia officinalis*) hearts. *J Exp Biol* 215: 2992-3000, 2012.

39. **Orsi Relini L, Mannini A, Fiorentino F, Palandri G, and Relini G**. Biology and fishery of *Eledone cirrhosa* in the Ligurian Sea. *Fish Res* 78: 72-88, 2006.

40. **Pörtner HO**. An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus, Loligo pealei*) hemocyanin. *J Exp Biol* 150: 407, 1990.

41. **Pörtner HO**. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar Ecol Prog Ser* 373: 203-217, 2008.

42. **Pörtner HO**. Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* 88: 137-146, 2001.

43. **Pörtner HO**. Coordination of metabolism, acid-base regulation and hemocyanin function in cephalopods. In: *Physiology of cephalopod molluscs: lifestyle and performance adaptations*, edited by Pörtner HO, O'Dor RK, and Macmillan DL. Switzerland: Gordon and Breach Science Publishers, 1994.

44. **Pörtner HO**. Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. *Comp Biochem Physiol A Mol Integr Physiol* 133A: 303-321, 2002.

45. **Pörtner HO**. Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. *J Exp Biol* 205: 2217-2230, 2002.

46. **Pörtner HO, Branco LG, Malvin GM, and Wood SC**. A new function for lactate in the toad *Bufo marinus*. *J Appl Physiol* 76: 2405-2410, 1994.

47. **Pörtner HO, Finke E, and Lee PG**. Metabolic and energy correlates of intracellular pH in progressive fatigue of squid (*L. brevis*) mantle muscle. *Am J Physiol Regul Integr Comp Physiol* 271: R1403-1414, 1996.

48. **Pörtner HO, Hardewig I, and Peck LS**. Mitochondrial function and critical temperature in the antarctic bivalve, *Laternula elliptica*. *Comp Biochem Physiol A Mol Integr Physiol* 124: 179-189, 1999.

49. **Pörtner HO, Reipschlager A, and Heisler N**. Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *J Exp Biol* 201: 43-55, 1998.

50. **Pörtner HO, Webber DM, Boutilier RG, and O'Dor RK**. Acid-base regulation in exercising squid (*Illex illecebrosus, Loligo pealei*). *Am J Physiol Regul Integr Comp Physiol* 261: R239-246, 1991.

51. **Pörtner HO, and Zielinski S**. Environmental constraints and the physiology of performance in squid. *S Afr J mar Sci* 20: 207-221, 1998.

52. **Precht H**. Concepts of the temperature adaptation of unchanging reaction systems of cold-blooded animals. In: *Physiological adaptation*, edited by Prosser CL. New York: Ronald Press, 1958, p. 50-78.

53. **Reipschläger A, and Pörtner HO**. Metabolic depression during environmental stress: The role of extracellular versus intracellular ph in *Sipunculus nudus*. *J Exp Biol* 199: 1801-1807, 1996.

54. **Rosa R, and Seibel BA**. Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proc Natl Acad Sci* 105: 20776-20780, 2008.

55. **Seibel BA**. On the depth and scale of metabolic rate variation: scaling of oxygen consumption rates and enzymatic activity in the Class Cephalopoda (Mollusca). *J Exp Biol* 210: 1-11, 2007.

56. **Seibel BA, Robison BH, and Haddock SH**. Post-spawning egg care by a squid. *Nature* 438: 929, 2005.

57. **Seibel BA, and Walsh JE**. Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. *J Exp Biol* 206: 641-650, 2003.

58. **Seibel BA, and Walsh PJ**. Potential Impacts of CO_2 Injection on Deep-Sea Biota. *Science* 294: 319-320, 2001.

59. **Sidell B, Crockett E, and Driedzic W**. Antarctic fish tissues preferentially catabolize monoenoic fatty acids. *J Exp Biol* 271: 73-81, 1995.

60. **Simpson DP**. Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. *J Clin Inves* 46: 225, 1967.

61. **Sommer A, and Pörtner HO**. Metabolic cold adaptation in the lugworm *Arenicola marina*: comparison of a North Sea and a White Sea population. *Mar Ecol Prog Ser* 240: 171-182, 2002.

62. **Storey KB, and Storey JM**. Carbohydrate metabolism in cephalopod molluscs. In: *The Mollusca Vol. 1*, edited by Hochachka PW. New York: Academic Press, 1983.

63. **Tresguerres M, Buck J, and Levin LR**. Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflugers Arch* 460: 953-964, 2010.

64. **Truchot J**. Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *J Exp Biol* 64: 45-57, 1976.

65. **Wanders RJA, Mejer AJ, Groen AK, and Tager JM**. Bicarbonate and the pathway of glutamate oxidation in isolated rat ‐ liver mitochondria. *Eur J Biochem* 133: 245-254, 1983.

66. **Wells M**. The cephalopod heart: The evolution of a high-performance invertebrate pump. *Cell Mol Lif Sci* 48: 800-808, 1992.

67. **Wells M, Hanlon R, Lee P, and Dimarco F**. Respiratory and cardiac performance in Lolliguncula brevis (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. *J Exp Biol* 138: 17, 1988.

68. **Wells M, and Smith P**. The performance of the octopus circulatory system: a triumph of engineering over design. *Cell Mol Life Sci* 43: 487-499, 1987.

69. **Wells MJ, O'Dor RK, Mangold K, and Wells J**. Feeding and metabolic rate in Octopus. *Mar Behav Physiol* 9: 275–287, 1983.

70. **Zippin JH, Levin LR, and Buck J**. CO_2/HCO_3^--responsive soluble adenylyl cyclase as a putative metabolic sensor. *Trends Endocrinol Metab* 12: 366-370, 2001.

71. **Zittier Z, Bock C, and Pörtner HO**. Synergistic impacts of increasing temperature and CO_2 levels on the physiology of *Mytilus edulis* from the White Sea. *Mar Biol* (September 7, 2012). doi: 10.1007/s00227-012-2073-8.

Figures

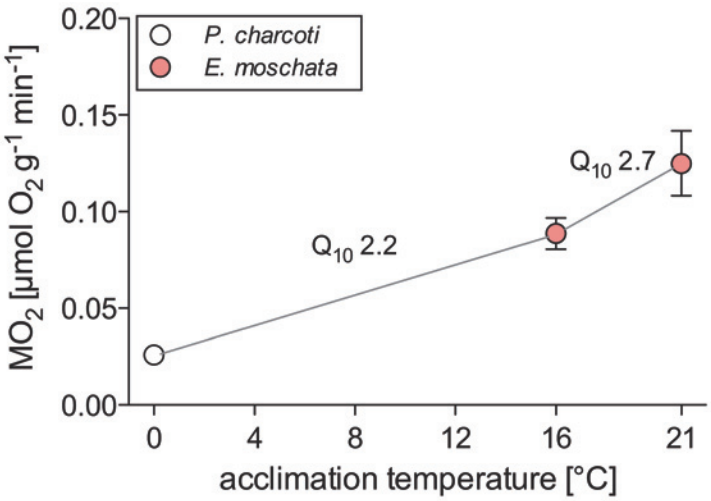


Figure 1 Respiration rate (routine metabolic rate, RMR) of *P. charcoti* at 0°C (white dot, *N*=4), *E. moschata* at 16°C (red dot, *N*=4) and after five months of warm-acclimation at 21°C (red dot, *N*=4). The Q_{10} of 2.2 was calculated using the mean respiration rate of *P. charcoti* at 0°C and *E. moschata* at 16°C. Values are given as means ± SEM.

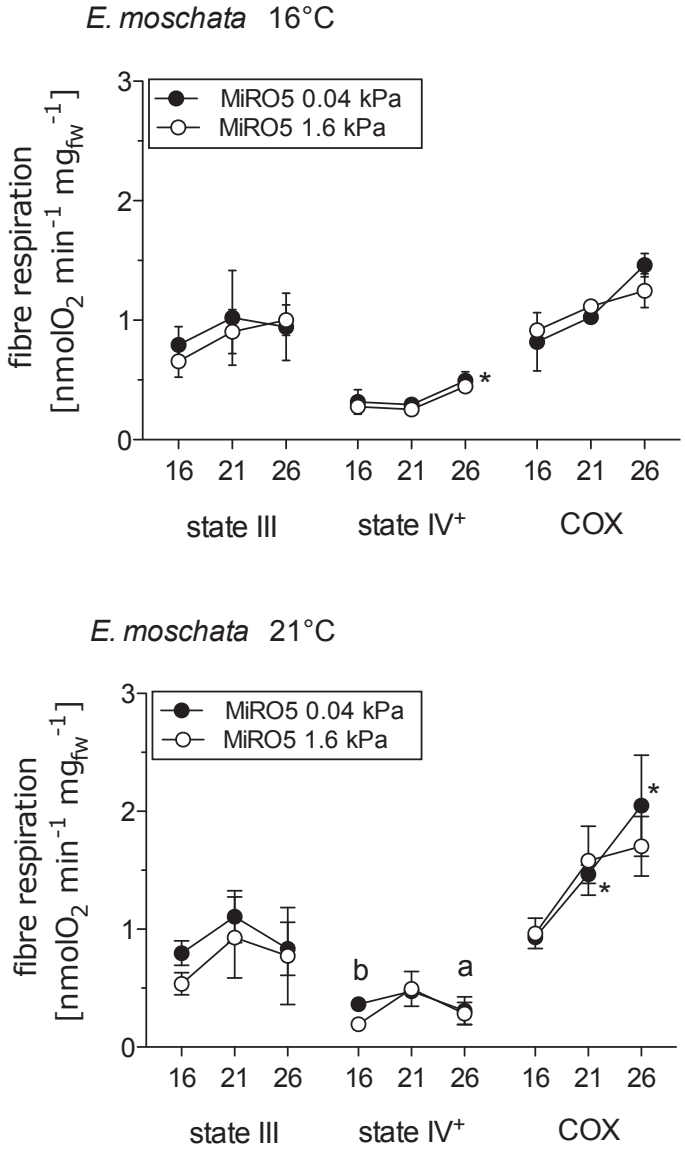


Figure 2 Respiration in permeabilized systemic heart fibres of control (16°C, 0.04 kPa CO_2; *N*=3) and warm-acclimated *E. moschata* (21°C, 0.04 kPa CO_2; *N*=3). State III, state IV^+ and COX respiration were assayed at 16, 21 and 26°C in normocapnic (0.04 kPa CO_2; black dots) mitochondrial respiration buffer (MiRO5), white dots represent respiration in hypercapnic (1.6 kPa CO_2) MiRO5. * Indicate a significant rise with assay temperature compared to the 16°C assay. [a] Depicts a significant difference to the fibre respiration rate of the control group at the given respiratory state and assay temperature, [b] indicates a significantly lower respiration rate compared to the one in normocapnic MiRO5 at the given respiratory state and assay temperature ($p \leq 0.05$). Fibre respiration is presented in [nmol O_2 min^{-1} $mg_{fresh\ weight\ (fw)}$]. All data are given as means ± SEM.

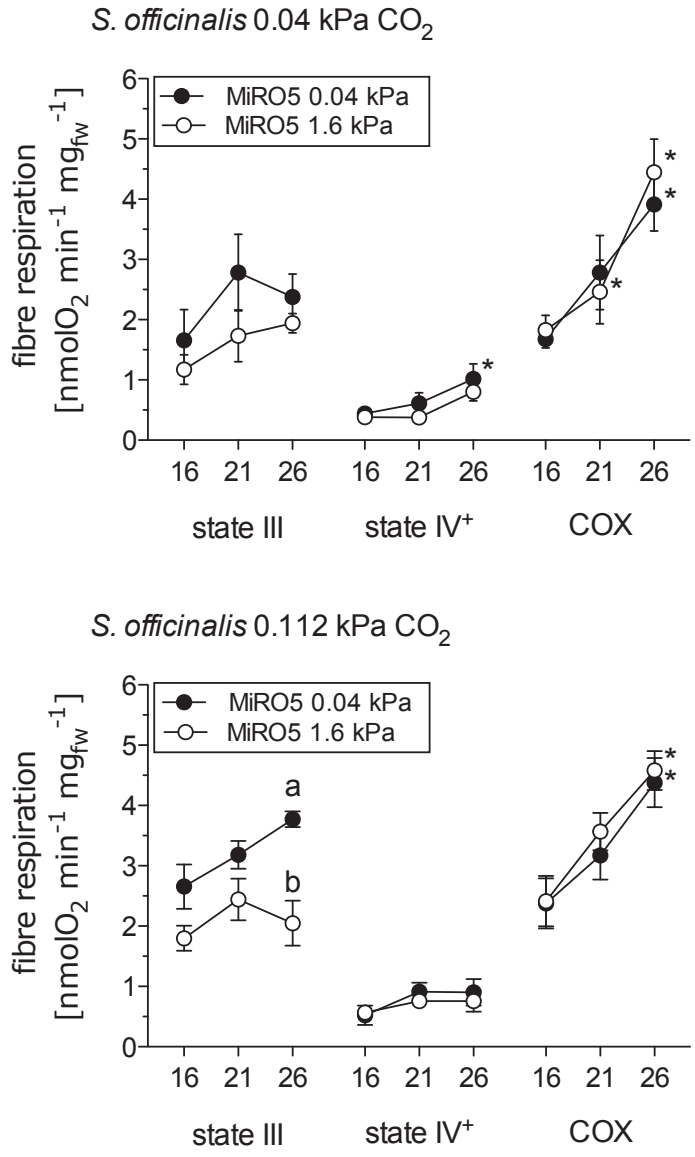


Figure 3 Systemic heart fibre respiration of control (16°C, 0.04 kPa CO_2; *N*=6) and hypercapnia acclimated (16°C, 0.112 kPa CO_2; *N*=8) *S. officinalis*. State III, state IV^+ and COX respiration were assayed at 16, 21 and 26°C. Black dots represent the fibre respiration in normocapnic (0.04 kPa CO_2) respiration medium (MiRO5), white dots in hypercapnic (1.6 kPa CO_2) respiration medium (MiRO5). * Indicate a significant rise with assay temperature compared to 16°C assay. [a] Depicts a significant difference to the fibre respiration rate of the control group, [b] indicates a significantly lower respiration rate compared to the one in normocapnic MiRO5 at the given respiratory state and assay temperature ($p \leq 0.05$). Fibre respiration is presented in [nmol O_2 min^{-1} $mg_{\text{fresh weight (fw)}}{}^{-1}$], the values are means ± SEM.

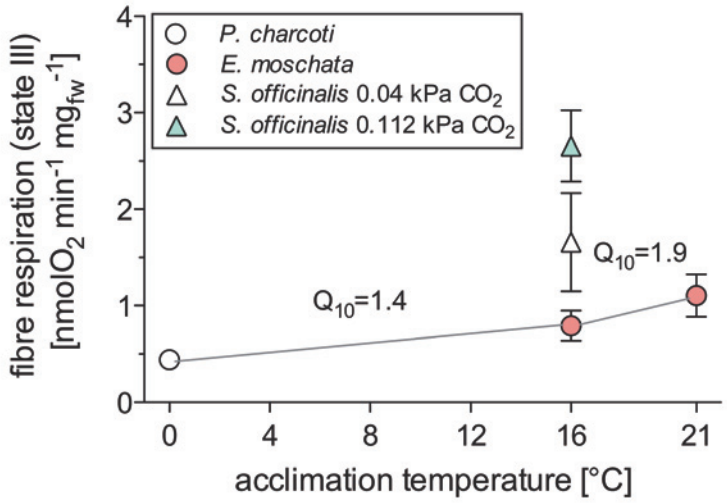


Figure 4 Mean state III respiration rate in permeabilized heart fibres of *P. charcoti* at 0°C (white dot, *N*=4), *E. moschata* at 16°C (red dot, *N*=4) and after 5 month warm-acclimation at 21°C (red dot, *N*=4), and *S. officinalis* at 16°C, 0.04 kPa CO_2 (white triangle, *N*=6) and after 5 month hypercapnia acclimation at 16°C, 0.112 kPa CO_2 (blue triangle, *N*=8). The Q_{10} was calculated between *P. charcoti* at 0°C and *E. moschata* at 16°C, and for control *vs.* warm-acclimated *E. moschata*. Fibre respiration is presented as [nmol O_2 min^{-1} $mg_{fresh\ weight\ (fw)}^{-1}$]. Data are means ± SEM.

Tables

Table 1 Seawater physicochemistry during acclimation of Adriatic *E. moschata* and *S. officinalis*

	E. moschata		*S. officinalis*	
	Control	Warm normocapnic	Control*	Hypercapnic
pH_{TOTAL}	8.01±0.04	7.99±0.05	8.0±0.1	7.76±0.05
PCO_2 [kPa]	0.04	0.04	-	0.114
PCO_2 [ppm]	409.5±52.0	387.9±71.9	-	1143.8±89.4
DIC [mmol*kg^{-1}SW]	2.20±0.03	2.19±0.03	-	2.27±0.04
HCO_3^- [mmol*kg^{-1}SW]	2018.5±28.2	1979.3±32.3	-	2132.3±8.0
T [°C]	16.0±0.4	20.6±0.6	15.4±0.1	16.1±0.4
S [psu]	31.2±1.5	31.2±1.4	31.5±0.3	31.2±1.3
duration [weeks]	19	20	>54	21

Data are expressed as means±SEM. * Control *S. officinalis* were raised from eggs and kept in the general aquaria facilities of the Alfred Wegener Institute, Bremerhaven, Germany, until the experiments. In these aquaria systems, Dissolved Inorganic Carbon (DIC) was not recorded. HCO_3^- = Bicarbonate, T = Temperature, S = Salinity.

Table 2 Ratios based on the mean respiratory flux in *P. charcoti* (0°C, 0.04 kPa CO_2), *E. moschata* (control and 21°C-acclimated) and normocapnia (0.04 kPa CO_2) or hypercapnia (0.112 kPa CO_2) acclimated *S. officinalis* at 16°C.

Specimen & acclimation	*N*	RCR^+	OXPHOS I [%]	Leak [%]	Leak [%]	Q_{10} COX		COX/OXPHOS	COX/OXPHOS
		0.4 kPa CO_2	0.4 kPa CO_2	0.4 kPa CO_2	1.6 kPa CO_2	0.4 kPa CO_2	1.6 kPa CO_2	0.4 kPa CO_2	1.6 kPa CO_2
P. charcoti 0°C 0.04 kPa CO_2	4-9	1.8±0.1	57.9±2.6	53.9±3.1	57.5±5.8	2.1	1.4	1.99±0.1	1.55±0.24
E. moschata 16°C 0.04 kPa CO_2	4	2.0±0.2	54.1±5.3	49.3±6.3	40.2±3.7 [b]	1.8	1.4	1.58±0.3	1.40±0.2
E. moschata 21°C 0.04 kPa CO_2	4	2.1±0.2	53.3±5.4	47.2±7.3	47.7±8.3	2.2	1.8	1.47±0.2	1.74±0.2
S. officinalis 16°C 0.04 kPa CO_2	6	3.1±0.4 [a]	68.8±2.5 [a]	34.5±3.9 [b]	36.8±5.1 [b]	2.3	2.4	1.37±0.2	1.95±0.2
S. officinalis 16°C 0.112 kPa CO_2	8	3.9±0.3 [a]	71.5±2.7 [a]	24.0±2.8 [a,c]	35.2±3.7 [b*]	1.8	1.9	1.01±0.1	1.85±0.2*

[a] Significantly different to *E. moschata* 16°C, 21°C
[b] Significant difference to *P. charcoti*
[c] Significant difference to *S. officinalis* control
* Significant difference hypercapnic *vs*. normocapnic buffer
OXPHOS I [%] was calculated as OXPHOS CI, CII/ OXPHOS CI (%). It indicates the capacity of complex I relative to the total coupled oxidative phosphorylation. % Leak: relative proton leak gives the fraction of state IV^+ relative to coupled state III respiration. Q_{10} was calculated over the whole range of acute assay temperatures. COX/OXPHOS provides a measure for the capacity of cytochrome c oxidase (COX) relative to the maximum coupled phosphorylation. Values are means ± SEM calculated over the whole range of acute assay temperatures.

4 Discussion

This thesis addresses the physiological response of Antarctic and temperate fish and cephalopods towards elevated environmental temperature and PCO_2. It aims to elucidate the responses and acclimation capacities of marine ectothermal fish and cephalopods towards a changing environment by considering the functional integration of various organisational levels, such as mitochondrial, cellular and systemic levels.

Even in species that are considered to possess plasticity for temperature-acclimation, acclimation does not necessarily include full compensation for changes by temperature. The range of acclimatory changes shown by ectothermic organisms in response to acute changes in temperature have been classified by Precht et al. (1973) and are described in Table 4.1. With reference to this classification system, Antarctic ectotherms often fit with the 'no compensation' or 'partial compensation' definitions (Somero and DeVries, 1967; Seebacher et al., 2005; Lowe and Davison, 2006; Franklin et al., 2007; Bilyk and DeVries, 2011), and only few cases report 'perfect compensation' for Antarctic fish (e.g. Brodte et al., 2006).

Table 4.1 The range of responses to acute temperature change demonstrated by ectothermic animals, as classified by Precht et al. (1973).

Type 1	Overcompensation	Physiological rates change to new levels after temperature change but then rebound beyond the initial physiological rate. $Q_{10} < 1$.
Type 2	Perfect compensation	Physiological rates change to new levels as a result of temperature change but then return to original levels. Q_{10} close to 1.
Type 3	Partial compensation	Physiological rates rise after temperature change but then return part of the way to initial levels. $Q_{10} >1$ but < 2.
Type 4	No compensation	Animal shows no acclimatory or compensatory response to a temperature change. Physiological rate functions follow a simple Q_{10} relationship. Q_{10} 2-3.
Type 5	Inverse compensation	Physiological rates change to new levels after temperature change and then continue to move away from initial rates. $Q_{10} > 3$.

The first section (4.1) in this chapter will focus on the response of the Antarctic fish *N. rossii* to ocean acidification and warming acclimation. By combining the most important results of publications I-III, such as mitochondrial capacities, extra- and intracellular acid-base parameters and routine metabolic rates of warm and/ or hypercapnia acclimated *N.*

rossii, this section aims to answer the question on the effect of increased seawater PCO_2 on the thermal acclimation capacity of Antarctic fish (see Introduction, section 1.7, question 'a'), and their acclimation capacities towards them.

Paragraph 4.2 aims to elaborate if high-Antarctic, sub-Antarctic and Austral notothenioids display mitochondrial capacities of different thermal sensitivity, how they relate to possible habitat or lifestyle adaptations of these fish, and if mitochondrial capacities may be a precondition for acclimation capacities to global climate change (see Introduction, section 1.7, question 'b'). To answer these questions, some additional data on mitochondrial capacities in various Antarctic fish will be considered, and compared to the data in publication II and IV. Moreover, the second part of paragraph 4.2 will focus on mitochondrial responses to chronic hypercapnia exposure in Antarctic *vs.* Austral notothenioids (publication IV).

In the last section (4.3), mitochondrial capacities of Antarctic and Austral fish, as presented in publications II and IV, will be compared to those of temperate and Antarctic cephalopods (publication V). These data will be discussed with respect to their preference of similar ecological niches, their physiological constraints and metabolic characteristics (see Introduction section 1.7, question 'c').

4.1 Metabolic responses of the Antarctic fish, *Notothenia rossii*

In a pilote study, the physiological function and acute thermal sensitivity of Complex I and II of the ETS was elaborated in the Antarctic fish *N. rossii* and *N. coriiceps*. This study demonstrated a higher thermal responsiveness of Complex I in *N. rossii* than in *N. coriiceps* and general differences in mitochondrial respiration between the two notothenioids during acute thermal challenge, which was taken as a first indicator for differences in mitochondrial thermal tolerances between fish species (publication AI). However, this study leaves the question open, whether the mitochondria of *N. rossii* and *N. coriiceps* possess acclimation capacities to long-term environmental changes, and if they are different between notothenioids.

Against this background, the effects of ocean warming and acidification on the aerobic energy metabolism of Antarctic fish were analysed in *N. rossii* acclimated to 7°C (warm normocapnic), 0.2 kPa CO_2 (1°C, cold hypercapnic) and to the combination of both (7°C & CO_2, warm hypercapnic) against a control group (1°C, normocapnic).

4.1.1 Mitochondrial capacities of warm- and hypercapnia-acclimated N. rossii

4.1.1.1 Chronic warm exposure of N. rossii

Only few studies, mostly performed on non-Antarctic organisms, have demonstrated full compensation for an increased oxygen demand at the mitochondrial level after warm-acclimation, and these compensation abilities frequently correspond to a higher thermal tolerance (e.g. eastern Pacific abalone (genus *Haliotis*) (Dahlhoff and Somero, 1993), sculpin *Oligocottus maculosus* (Sloman et al., 2008) and eelpout *Zoarces viviparus* (Lannig et al., 2005)).

After warm-acclimation of *N. rossii*, the measurement of isolated liver mitochondrial respiration revealed that OXPHOS capacities (Complex I and II, state III maximum flux) were not significantly different to OXPHOS capacities of control *N. rossii* kept at habitat conditions. Only a reduced slope of increasing state III respiration with acute rising assay temperature pointed towards a marginal compensation of individual mitochondrial capacities after warm-acclimation (publication I, figure 1, and publication II, figure 2).

As pointed out in the introductory chapter and publication AI, single complexes of the ETS may play a fundamental role in the acclimation of mitochondrial function in notothenioids, with a special role for Complex I. Theoretically, oxidation of 1 pyruvate through the TCA-cycle yields 4 $NADH_2$ and thus four pairs of electrons are shuttled down the ETS, while succinate oxidation 'only' delivers 1 $FADH_2$ (one pair of electrons). Following this theoretical stoichiometry, Complex I should respire four times more than Complex II and one would expect OXPHOS respiration to be comprised of 80% Complex I and 20% Complex II-linked flux. Thus it is surprising that liver mitochondrial respiration of control *N. rossii* was constituted by about 70% Complex II respiration and only 30% of Complex I (figure 1, publication I). In isolated mitochondria or permeabilised tissues (*in vitro* system), the TCA-cycle is functionally not 'closed' when using solely Complex I substrates and therefore Complex I can be assayed independent of Complex II. In this case, citrate and 2-oxoglutarate are exchanged rapidly for malate, and only NADH-linked reactions take place; succinate dehydrogenase activity is then fully dependent on a high external succinate concentration (figure 4.1, Gnaiger, 2012). Conversely, the combination of succinate and rotenone, a Complex I inhibitor, yields solely Complex II respiration in isolated mitochondria.

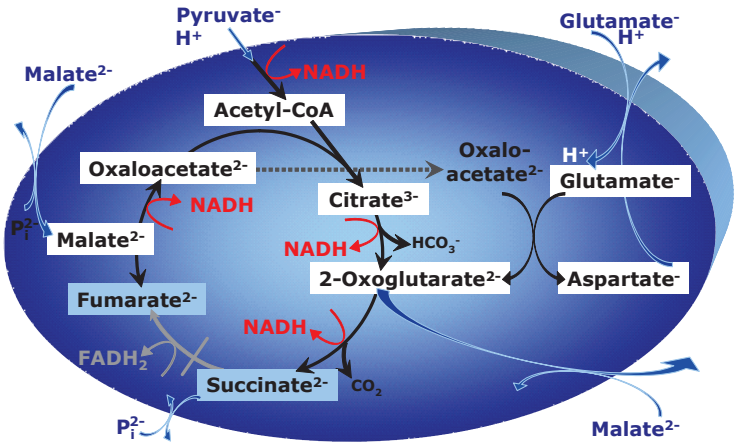


Figure 4.1 Respiratory capacity through Complex I. Substrate combination for CI: Glutamate + Malate + Pyruvate. External succinate is required for securing full operation of the TCA-cycle and convergent CI & CII electron flow at the Q-junction of the ETS in this *in vitro* system of isolated mitochondria or permeabilised tissues. Figure adopted from (Gnaiger, 2012).

Indeed, Complex I and II are functionally coupled via the TCA-cycle under *in vivo* conditions. Thus it is difficult to predict whether the observed Complex I to Complex II ratio (30% to 70%, respectively) in isolated liver mitochondria of *N. rossii* represents the true stoichiometry *in vivo*. Yet, the immense divergence of the measured 30% Complex I contribution in isolated mitochondria of *N. rossii,* and the expected 80% Complex I contribution under 'natural' conditions hints towards general Complex I discrepancy in *N. rossii*. In the mitochondrial respiration experiments with *N. rossii*, Complex I substrates were available in excess, therefore Complex I appeared to be incapable of using its maximum potential. However, the molecular mechanisms for the observed Complex I deficiency must remain unsolved at this point.

In response to chronically elevated temperatures, the substrate turnover of Complex I seemed to be slightly elevated, and this could slightly increase mitochondrial phosphorylation efficiency. These enhanced phosphorylation capacities became also visible in an enhanced P/O ratio and an elevated Complex I/ Complex II ratio due to an increased contribution of Complex I in warm-normocapnia acclimated *N. rossii* (publication I, figure 5). Similarly, the temperate triplefin fish *Bellapiscis medius* also increase the flux through Complex I with warming to meet an elevated (and uncompensated) whole animal energy demand at warmer temperatures (Hilton et al., 2010). A higher flux through Complex I in response to a rising energy demand thus appears to be a common feature in fish, also in cold-adapted notothenioids.

4.1.1.2 Chronic hypercapnia exposure of N. rossii

Five weeks of cold-hypercapnia acclimation of *N. rossii* led to significantly reduced mitochondrial state III respiration, and also in the warm-hypercapnic group state III respiration was depressed below that of the control group (publication I, figure 1, and publication II, figure 2). Furthermore, various other parameters indicated that mitochondrial functioning was directly impaired by elevated PCO_2. Briefly, the following constraints were observed in extracted liver mitochondria of 0.2 kPa CO_2 acclimated *N. rossii*: OXPHOS capacities (Complex I, II) were significantly depressed in cold hypercapnia acclimated fish, and similarly in the warm hypercapnic group (publication I and II). Moreover, the relative proton leak capacity of OXPHOS (Complex I, II) tended to be higher in the hypercapnic animals (publication I). Accordingly, the RCR (OXPHOS/ state IV) was significantly reduced and mitochondria of both cold- and warm hypercapnic *N. rossii* less coupled than their control group. RCR also serves as a simple proxy for inner mitochondrial membrane permeability (publication I and II). In line with these reduced RCR-values, there was a clear trend towards an enrichment of poly-unsaturated fatty acids in liver mitochondria towards the hypercapnic conditions (publication I, Table 3).

Besides this correlation of membrane unsaturation and proton leakage, another important implication of a change in poly-unsaturated fatty acids towards the hypercapnic conditions is their effect on membrane bound enzymes such as COX. There is a certain debate about whether lipid remodelling does or does not enhance catalytic rates of membrane-associated proteins such as COX. Although many examples report enhanced COX activities with rising unsaturation of membrane phospholipids (e.g. carp *Cyprinus carpio* (Wodtke, 1981) or trout *Oncorhynchus mykiss* (Kraffe et al., 2007), c.f. Guderley and St-Pierre, 2002, for review), a similar number of studies found no effect of unsaturation on enzyme activities (e. g. sea bass *Dicentrarchus labrax* (Trigari et al., 1992), c.f. Lee, 2003, for review), and in many cases it must remain unclear if the changes in poly-unsaturated fatty acids are responsible for higher COX capacities (Grim et al., 2010). Also in *N. rossii,* mitochondrial membrane unsaturation could not directly be related to COX-activities, and the mechanisms which are more likely involved in altered COX activities in warm- or hypercapnia acclimated *N. rossii* will be explained in detail in section 4.1.2 and 4.1.3.

Lastly, a higher Complex I contribution to state III respiration was observed in both cold and warm hypercapnia acclimated *N. rossii*, accompanied by elevated Complex I P/O ratios and Complex I/ Complex II ratios (publication I, figure 3 and 5). In warm-acclimated *N.*

rossii (see 4.1.1.1) and cold-temperate crabs (Iftikar et al., 2010), a high contribution of Complex I appeared to be a response to alter total OXPHOS efficiencies. Similarly, a higher flux through Complex I may serve as a partial compensation for reduced mitochondrial capacities in both cold and warm-hypercapnia acclimated *N. rossii*. Thus, the phenomenon of elevated Complex I-dependent respiration seems to be a general feature in response to increasing metabolic energy demand. Instead of remodelling the mitochondrial inner membrane, a shift in metabolic preferences towards Complex I-linked substrates may be the preferred reaction to increase the efficiencies of individual mitochondria, as it could be energetically less costly.

4.1.2 Comparison of tissues of different metabolic function

The mitochondrial responses to chronic warm- and/ or hypercapnia-exposure discussed so far only reflect changes in liver tissue, which constitutes a small (yet significant) fraction of whole animal metabolism (Lannig et al., 2003). Furthermore, mitochondrial capacities are most likely related to the metabolic role and energy demand of each specific tissue (e.g. Dalziel et al., 2005; Hulbert et al., 2006), such as the involvement of red muscle in swimming performance (Johnston, 2003) and the concomitantly high aerobic demand of this tissue, or the important role of liver in lipid storage.

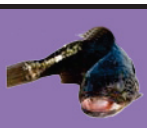
For example, in liver mitochondria of control *N. rossii*, Complex I-linked flux contributed 24.3±2.6% to total OXPHOS. In heart fibres of control *N. rossii*, flux through Complex I constituted 45.9±6.2% of OXPHOS, while both tissues were similarly well coupled (RCR liver: 4.6±0.8, RCR^+ heart 3.7±0.5). Another striking difference between the two tissues was mirrored in the thermal sensitivity of their mitochondria to acute thermal challenge: in the temperature range from 0 to 12°C, liver mitochondria had Q_{10} values of 1.6±0.3, while the heart reached considerably higher values of 3.6±0.7. This indicates a much greater response of the heart to rising temperatures. Due to the important role of heart in active aerobic energy generation for driving the circulatory system, a strong thermal response of mitochondrial respiration may reflect a precondition to maintain functioning cardiac work during acute thermal challenge, while liver plays a more ‘inactive’ role mainly as storage organ, e.g. for glycogen or lipids.

For the sake of clarity, Table 4.2 gives a simplified overview on the trends of enzyme activities in response to warm and/ or hypercapnia acclimation in all tissue types of *N. rossii*

presented herein. For example, Table 4.2 demonstrates that particularly CS activities in heart of warm normocapnia acclimated *N. rossii* were increased towards the warmth. Paralleled by a mitochondrial state III respiration at similar levels as the control group, this indicates that phosphorylation and total ETS capacities may not be limiting for mitochondrial phosphorylation capacities at warmer temperatures.

Furthermore, high mitochondrial oxygen affinities could be facilitated by COX (Complex IV) capacities, which are frequently in large excess compared to maximum coupled oxygen flux through the ETS (e.g. 150 – 200% of state III respiration in mammals; Gnaiger et al., 1998). An excess capacity of COX is necessary to sustain a high oxygen affinity of mitochondria in order to supply the respiratory system with sufficient oxygen, especially when oxygen demand raises, e.g. at warmer temperatures (Gnaiger et al., 1998).

Table 4.2 Overview on metabolic responses of warm normocapnia and cold/ warm hypercapnia acclimated *N. rossii* compared to control.

	Warm normo-capnia	Cold hyper-capnia	Warm hyper-capnia
Liver OXPHOS	→	↓	↓
Complex I	↗	↗	↗
Heart	↗	→	→
CS	↗	→	↓
COX			
Red muscle	→	↑	↑
CS	→	↑	↑
COX			
Liver	→	→	→
CS	↑	↘	↘
COX			
RMR	↑	→	↑

'Liver OXPHOS' represents state III respiration (Complex I and II) of isolated liver mitochondria. 'Complex I' stands for the relative contribution of Complex I-dependent flux to total OXPHOS (Complex I and II). Citrate synthase (CS) and cytochrome *c* oxidase (COX) activities are given per gram tissue fresh weight. RMR = routine metabolic rate. → indicates similar rates/ activities as the control group. ↓ ↑ depict significantly higher or lower rates/ activities compared to control, respectively. ↗/ ↘ indicate a slight increase/ decrease in activity/ contribution towards warmer assay temperatures.

However, similar enzyme activities and state III respiration rates as the control animals suggest that heart tissue of *N. rossii* does not compensate for warming to 7°C in the conventional sense, as this would imply a down-regulation of mitochondrial capacities (Guderley and Johnston, 1996; Lannig et al., 2005; Lucassen et al., 2006). Although heart

mitochondria of *N. rossii* appear to possess capacities to ensure cardiac output for sufficient blood circulation under warm normocapnic conditions (i.e. elevated RMR), uncompensated mitochondrial capacities or densities also imply elevated maintenance costs of heart mitochondria (Pörtner, 2002c) that may need support by the energy stores of other tissues or shifts in metabolic pathways. In fact, a strong thermal stimulation of CS indicates an enhanced TCA-activity from anaplerotic pathways in heart of acclimated *N. rossii* (publication III, Table 4.2 and figure 4.2).

In contrast, the heart tissue of the warm hypercapnic *N. rossii* showed lower CS and COX activities compared to all other treatments. The combination of the two stressors 'warming' and 'elevated PCO_2' appears to exceed the capacities of heart mitochondria and may trigger a reduction of mitochondrial capacities. This is a first indicator for a reduced performance of Antarctic fish due to the synergistic effect of ocean warming and acidification and also identifies the heart as one of the most sensitive organs with the least capacities to acclimate due to its design for utmost energetic efficiency (Moyes, 1996; Somero, 2002). As a result, this could hamper oxygen supply to other highly oxygen-consuming tissues, such as red muscle. A high aerobic demand of red muscle was actually mirrored by enhanced enzyme capacities in warm and hypercapnia acclimated *N. rossii*. This enhancement may be the result of a compensation for inhibitory effects by elevated bicarbonate (see 4.1.3) or elevated costs for acid-base balance under elevated environmental CO_2 levels, which consequently involves mobilisation of energy stores, such as liver fat or protein.

Indeed, different COX to CS ratios were observed in liver compared to the other tissues investigated in this study, which reflect special metabolic duties of this tissue. The COX to CS ratio can be used to reflect preferred metabolic pathways and relative metabolic adjustments in response to warming and hypercapnia in a tissue (c.f. publication III; figure 4.2).

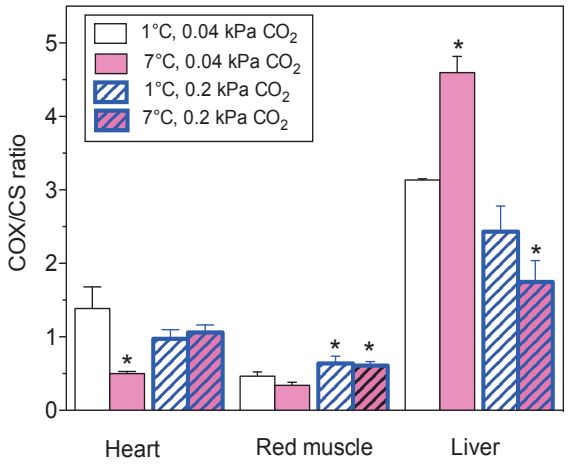


Figure 4.2 Effect of warm and hypercapnia acclimation on the COX to CS ratio in *N. rossii*.
COX/CS ratio in heart, red muscle and liver of control (white bars; 1°C, 0.04 kPa CO_2, n=6), warm normocapnia (grey bars; 7°C, 0.04 kPa CO_2, n=3-4), cold hypercapnia (white hatched bars; 1°C, 0.2 kPa CO_2, n=7-8) and warm hypercapnia (grey hatched bars; 7°C, 0.2 kPa CO_2, n=6-8) acclimated *N. rossii*. Values are given as means ± SEM. *Asterisks* depict a significantly ($p \leq 0.05$) increased/ decreased ratio compared to the control group within a specific tissue. (Figure adopted and modified after publication III).

In liver, the elevated COX to CS ratio after warm acclimation of *N. rossii* (figure 4.2) is a result of increased COX activities that can serve to enhance oxygen affinity (Gnaiger et al., 1998). CS catalyses the first reaction of the TCA-cycle, namely the condensation of oxaloacetate with acetyl-CoA to citrate, and thus ideally CS capacities would reflect the entrance of acetyl-CoA into the TCA-cycle after final oxidation of fatty acids and carbohydrates. If acetyl-CoA is present in excess, it can be shuttled via citrate into the cytosol for fatty acid synthesis. Thus, the TCA-cycle in liver also supports biosynthetic processes, such as the lipid-biosynthesis or gluconeogenesis from malate (figure 4.3; Owen et al., 2002; Windisch et al., 2011). A high COX to CS ratio therefore may entail a shift from high energy substrates (fatty acids) to carbohydrate fuels (pyruvate entry from carbohydrate oxidation) and glycogen catabolism. Also in the liver of cold- and warm-hypercapnic fish, decreased COX activities at unchanged CS activities indicate shifts in metabolic pathways, e.g. shunting TCA-cycle intermediates away from the ETS towards gluconeogenesis to support other tissues.

As a result, the liver energy stores of warm and/ or hypercapnia acclimated *N. rossii* are concomitantly degraded, which is in agreement with lower hepatosomatic indices in warm-normocapnia and -hypercapnia acclimated fish compared to the control group (publication II). The energy reserves in the liver may be used to support enhanced aerobic capacities in the heart of warm-acclimated *N. rossii* (see above), but in the long run reduced ATP supply by the liver will contribute to limit the performance of highly aerobic tissues like heart or red muscle.

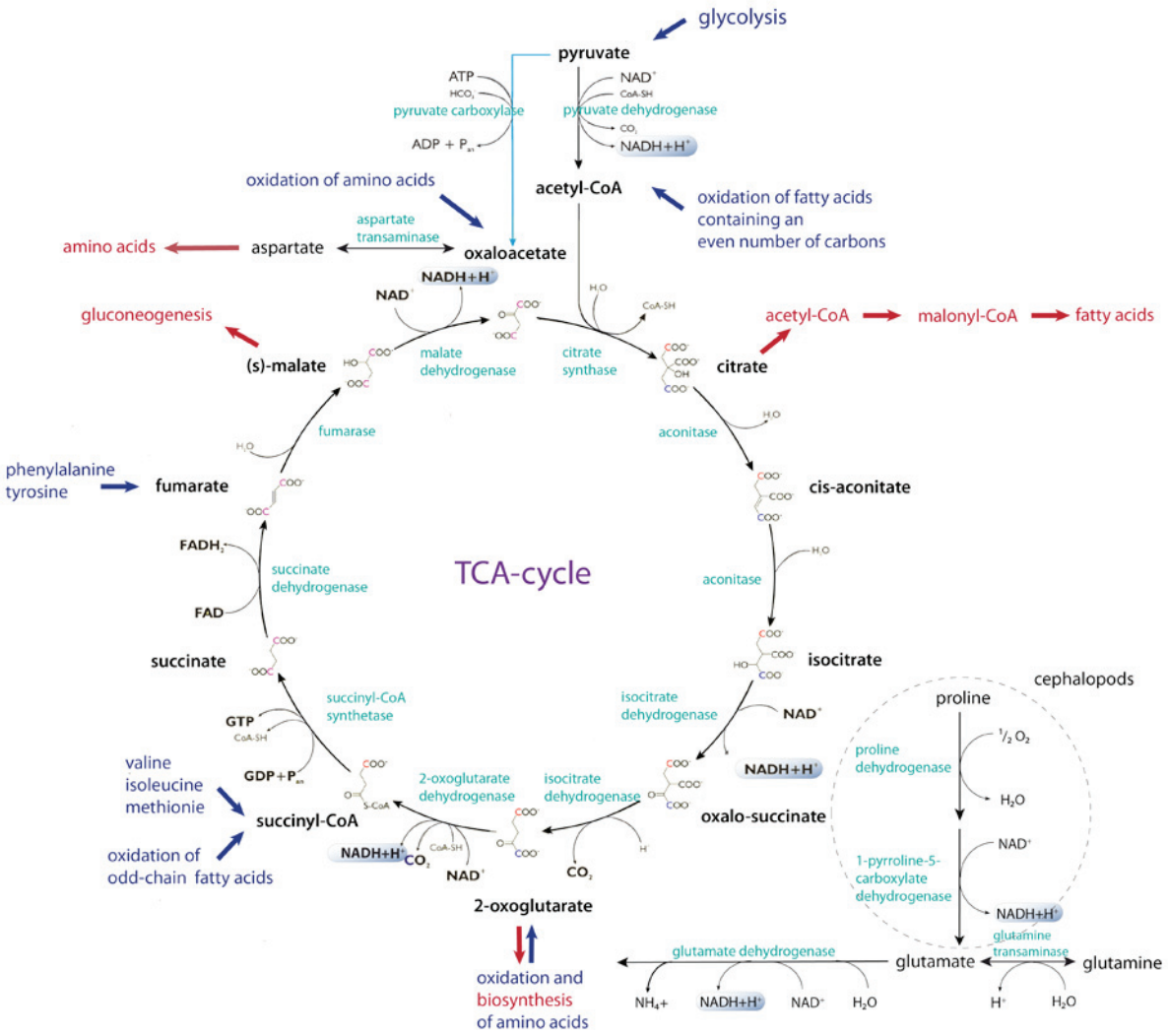


Figure 4.3 TCA-cycle (tricarboxylic acid cycle) with major anaplerotic and cataplerotic reactions. Anaplerosis (blue) is the generation of oxaloacetate from pyruvate via pyruvate carboxylase, the entry of amino acids or the oxidation of fatty acids, which regenerate TCA-cycle intermediates. Cataplerosis (red) is the disposal of TCA-cycle intermediates and is linked to biosynthesis, e.g. during gluconeogenesis and lipogenesis when malate (gluconeogenesis) or citrate (lipogenesis) leaves the mitochondria and is further metabolised to form glucose or fatty acids. Acetyl-CoA for the TCA-cycle can originate e.g. from decarboxylation of pyruvate or from β-oxidation of fatty acids. Cataplerotic reaction of 2-oxoglutarate is the reductive transamination to glutamate (amino acid biosynthesis), the anaplerotic reaction is the deamination of glutamate (amino acid oxidation). Figure extended according to (Mommsen and Hochachka, 1981; Owen et al., 2002).

Taken together, these data emphasize that liver, which is involved in metabolic regulation and ATP supply may be able to shunt metabolic pathways to match its energy metabolism to metabolic demands of other tissues or the organism under specific environmental conditions. Central aerobic tissues of high metabolic demand like heart and red muscle may need to augment mitochondrial metabolism to meet the increased energy demand of perfusion and locomotion in the warmth.

Nevertheless, mitochondrial OXPHOS capacities were reduced in liver of both cold and warm hypercapnia acclimated *N. rossii*. The molecular mechanisms behind these inhibiting effects of chronic hypercapnia and possible compensation mechanisms are presented in the following.

4.1.3 The mechanisms behind hypercapnia compensation

The mitochondrial responses to chronic hypercapnia (as discussed in section 4.1.1.2 and 4.1.2) led to the development of a hypothesis, which aims to explain the interplay between elevated ambient PCO_2 and the observed shifts in flux through different mitochondrial complexes and in metabolic pathways (c.f. publication I, publication III).

Briefly, the background of the influence of hypercapnia on mitochondria is the following (see figure 4.4): Elevated environmental PCO_2 leads to diffusive entry of CO_2 into the blood stream and the gill cells, where it is hydrated to HCO_3^- and H^+, moderated by carbonic anhydrase (CA). Fish then (partially) compensate for the resulting acidosis by increasing blood [HCO_3^-] through active transport mechanisms involving the sodium potassium pump (Na^+/K^+-ATPase, NKA) (Henry and Wheatly, 1992; Evans et al., 2005). In an ATP dependent process, the NKA builds up an electro-chemical [Na^+] gradient across the basolateral gill membranes, which is mainly used by secondary active transporters, such as the apical Na^+/H^+ exchangers and Cl^-/HCO_3^- exchangers. For example, an increased expression of Na^+/ HCO_3^- cotransporter (NBC) was described in the gill of eelpout *Z. viviparus* in response to elevated seawater PCO_2 (Deigweiher et al., 2008), which indicates an interaction of the NKA and NBC in order to actively compensate for acid-base disturbances in body fluids and tissues. Thus, long-term compensation by net-accumulation of $[HCO_3^-]_e$ and a new steady state is likely provided by elevated NKA activities in the basolateral membrane (figure 4.4; Deigweiher et al., 2008).

HCO_3^- can enter mitochondria via an electrogenic uniport (Selwyn and Walker, 1977), and CO_2 also diffusively enters cells and mitochondria and causes higher levels of H^+ and HCO_3^- via CA. While PCO_2 is equal in cytosol and mitochondrial matrix, a pH gradient of 0.6 is maintained between mitochondria and the cytoplasm under environmental conditions. Accordingly, total CO_2 concentrations are also higher in the mitochondria than in the cytosol. Following the calculations by Pörtner (Pörtner et al., 1991; Pörtner et al., 2010) for matrix bicarbonate levels, liver mitochondrial [HCO_3^-] of warm hypercapnic *N. rossii* ([HCO_3^-] 41.2

mmol l^{-1}, publication II) have reached values up to 10 mmol l^{-1} higher than in warm-normocapnia acclimated *N. rossii* ([HCO_3^-] 31.9 mmol l^{-1}).

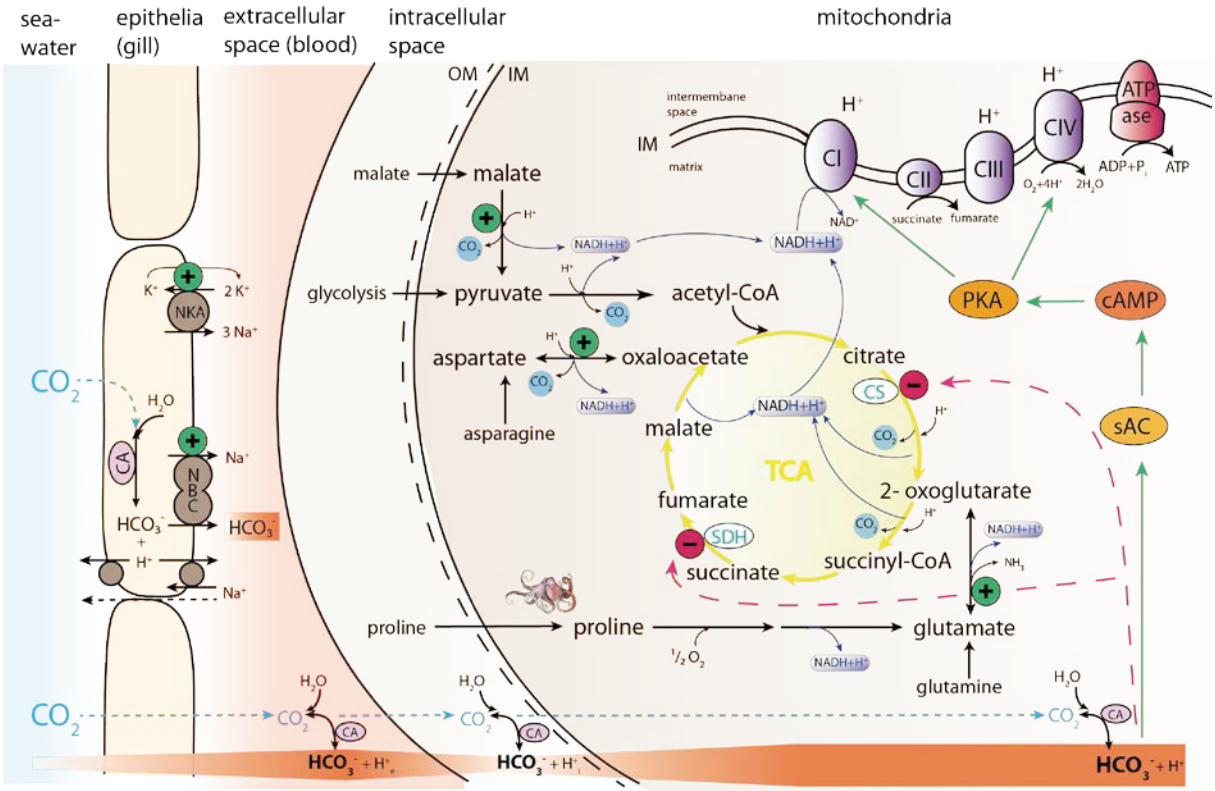


Figure 4.4 Overview of the effect of long-term elevated ambient PCO_2 on the different organisational levels in fish.
The overview is based on the proposed model in publication I and extended after the hypotheses in publication IV and V. Activated (green) and inhibited (red) reactions/ enzyme activities in response to accumulation of HCO_3^-. For the sake of clarity, ion-exchanger are only displayed in the gill epithelia. Dotted blue *arrows* depict the diffusive entry of CO_2, green *arrows* represent the activation of the sAC-cAMP-PKA pathway. The orange bar at the bottom of the figure illustrates increasing HCO_3^- concentrations from seawater through the different compartements (Pörtner et al., 2010). The additional entry point of proline into the TCA-cycle, which occurs mainly in cephalopods, is illustrated by the octopus. Abbreviations: OM: outer mitochondrial membrane, IM: inner mitochondrial membrane, CI: Complex I (NADH dehydrogenase), CII: Complex II (succinate dehydrogenase), CIII: Complex III (cytochrome *c* oxidoreductase), CIV: Complex IV (CIV, cytochrome *c* oxidase), TCA: tricarboxylic acid cycle, CA: carbonic anhydrase, NBC: Na^+/HCO_3^- cotransporter, NKA: Na^+/K^+ ATPase, sAC: soluble adenylyl cyclase cAMP: cyclic adenosine-monophosphate, PKA: protein kinase A, CS: citrate synthase, SDH: succinate dehydrogenase.

Inside mitochondria, two different mechanisms are hypothesized to apply in response to elevated [HCO_3^-] (figure 4.4):

1) *'Compensation by NADH-linked substrates'*. High bicarbonate levels competitively inhibit CS and succinate dehydrogenase function, as e.g. demonstrated for rat liver mitochondria (Simpson, 1967; Wanders et al., 1983). As a result, intramitochondrial steady-

state levels of 2-oxoglutarate and oxaloacetate are lowered. This can initiate an enhanced flux through glutamate dehydrogenase, which feeds into the TCA at the level of 2-oxoglutarate, and an enhanced net oxidative decarboxylation of dicarboxylic acids such as malate, both anaplerotic mechanisms to fuel the TCA-cycle. These reactions could on the one hand help to reduce the proton load in mitochondria (by proton consumption during oxidative decarboxylation (Pörtner, 1987) in order to maintain bicarbonate concentrations in the mitochondrial matrix.

On the other hand, during oxidative decarboxylation of malate and during deamination of glutamate via glutamate dehydrogenase, NAD^+ is reduced to $NADH+H^+$, which fuels Complex I. This excess in non-TCA-linked $NADH+H^+$ can support the ETS to build up the proton gradient across the inner mitochondrial membrane. It is proposed that an increased metabolisation of NADH-linked substrates enhances the energetic efficiency of mitochondrial oxidative capacity in *N. rossii* exposed to chronic warmth and or hypercapnia, which is also reflected in an increased Complex I involvement in the CO_2-acclimated fish (publication I, figure 5).

2) *'sAC stimulated mitochondrial complexes and enzymes'*. The intramitochondrial soluble adenylyl cyclase (sAC) is being discussed to be directly stimulated by bicarbonate to produce the second messenger cyclic adenosine-monophosphate (cAMP). In the next step, cAMP activates protein kinase A (PKA), which in the end can phosphorylate the complexes of the ETS. Furthermore, cAMP and PKA regulate metabolic key enzymes, such as phosphofructokinase (PFK) (glycolytic control enzyme) or PEPCK, by direct post-translational modification or alterations in gene transcription, i.e. activation of transcription factors through PKA (Zippin et al., 2001). As such, there may be a close link between cAMP levels and the levels of metabolism-controlling enzymes, and chronic hypercapnia exposure is suggested to alter mitochondrial complex-activities mainly via transcriptional or post-translational modification (Acin-Perez et al., 2009). Thereby, elevated HCO_3^- levels inside the cell may increase the translation efficiency of e.g. CS in order to compensate for a competitive inhibition of CS by HCO_3^- (figure 4.4). This was also in agreement with stable CS activities in liver of cold- and warm-hypercapnia acclimated *N. rossii* (Table 4.2 and publication III).

Furthermore, the sAC-cAMP-PKA way is postulated to stimulate Complex I and IV activity directly, and particularly the capacity of Complex IV to handle electrons (figure 4.4; Tresguerres et al., 2010). Complex IV usually possesses excess capacities and serves as a so-

called ‘electron sink’, matching the sum of the partial capacities of all contributory branches converging at the Q-junction (e.g. Complex I, Complex II, GpDH. See also figure 1.2 in Introduction and Gnaiger, 2009, 2012). It is postulated that via a high Complex IV capacity, OXPHOS efficiency is optimized by keeping the mitochondrial membrane potential low while Complex I is fully activated (Acin-Perez et al., 2009). However, the preconditions for such a high OXPHOS efficiency are high ATPase capacities, which are the limiting factor for maximum phosphorylation capacities of the ETS. At least in notothenioid heart, the phosphorylation system is working close to maximum coupled respiration capacities (personal observation) and is thus sufficient to use full ETS capacities.

Despite, reduced mitochondrial state III respiration rates have been observed in liver of cold- and warm-hypercapnia acclimated *N. rossii* (Table 4.2; publication I and II). These lower rates thus mirror a generally reduced electron-flux through the ETS in the liver mitochondria of hypercapnia acclimated fish. The reduced mitochondrial complex activities appear to be partially compensated by enhanced Complex I-linked flux and stimulation of Complex IV capacity, supported by anaplerotic reactions feeding the TCA (figure 4.3).

Similar to the hypothesized higher flux through Complex I, either by NADH-linked substrates or by enhanced Complex IV activities, a stimulating effect of acute high bicarbonate concentrations on glutamate, pyruvate or palmitoyl carnitine oxidation, and on citrate egress from mitochondria (as a result of high TCA turnover) has been observed in mammalian liver and kidney (Simpson, 1967; Robinson et al., 1977). In context of these studies on mammals, both mechanisms (*‘Compensation by NADH-linked substrates’* and *‘sAC stimulated mitochondrial complexes and enzymes’*) described above are suggested to be a response to elevated ATP demand, e.g. for maintaining a new acid-base equilibrium under chronic hypercapnia, in *N. rossii*. Nevertheless, it remains questionable if these compensation mechanisms are sufficient to maintain aerobic energy metabolism in liver of *N. rossii* during chronically warm temperatures and hypercapnia in the long run.

4.1.4 Systemic level

In this chapter, the connection to the systemic level is drawn in order to elucidate if shifts in mitochondrial capacities have an impact on whole animal performance. This is particularly of interest in the context that whole organism functions are postulated to be

affected first by limited oxygen supply, while enzyme and mitochondrial functions maintain a wider thermal tolerance window (Pörtner et al., 1999; Mark et al., 2002).

Routine metabolic rates of warm-acclimated fish were lower than those of acutely warm-exposed control fish, which reflects a partial compensation for an acute, warm induced rise in energy turnover in *N. rossii* (type 3 - Table 4.1; Table 4.2).

Still, metabolic rates of warm normocapnia acclimated *N. rossii* are higher at 7°C compared to the control group at 1°C, and thus involve a high oxygen- and metabolic demand at the tissues and also a high workload for the heart, contributing further to the metabolic load (Pörtner et al., 2004). Yet, the high aerobic enzyme activities in heart tissue of warm-acclimated *N. rossii* (Table 4.2) and a high electron-flux through the ETS may ensure cardiac output at warmer temperatures.

Under chronic hypercapnia, routine metabolic rates measured at habitat temperature were not different from the control group, although the reduced liver state III respiration indicates a down-regulation in mitochondrial metabolic rate. Together with the data on reduced COX activities in isolated liver mitochondria of cold hypercapnia acclimated *N. rossii*, this suggests that chronic hypercapnia exposure exerts an inhibitory effect on the complexes of the mitochondrial ETS. As a result, oxidative phosphorylation capacities and likely also mitochondrial oxygen affinities are reduced in liver following hypercapnia acclimation. Yet, CS activities may have been compensated by enhanced protein transcription or translation during chronic hypercapnia, mediated by the sAC-cAMP-PKA signalling pathway. Thus, the postulated enhanced turnover of NADH-linked substrates (section 4.1.3) and shunting of TCA-cycle intermediates towards gluconeogenesis (reduced COX to CS ratio in liver of cold hypercapnic fish, figure 4.2 and 4.3) appear to be sufficient to provide energy for acid-base regulation and metabolic rates under cold hypercapnic conditions at the systemic level.

Accordingly, *N. rossii* could compensate acid-base disturbances in their blood by a significant increase in plasma [HCO_3^-] in both the cold and warm hypercapnic groups. However, the acid-base status was overcompensated in response to this process, which resulted in a metabolic alkalosis by a slight shift of pH_e towards alkaline values and possibly a shift in 'set points' of acid-base regulation (publication II). Similarly, pH_i was compensated by intracellular HCO_3^- accumulation in liver and muscle of the hypercapnia acclimated *N. rossii*. In the long run, the maintenance of this new acid-base equilibrium may come with elevated metabolic costs (e.g. ionic homeostasis; Deigweiher et al., 2008), which could in the end also hamper whole animal aerobic and functional scope.

Similar to the warm normocapnia acclimated *N. rossii*, the warm hypercapnic group could not completely compensate their routine metabolic rate (figure 1 in publication II). The extremely depressed CS and COX-activities in the heart of warm hypercapnia acclimated *N. rossii* go beyond inverse mitochondrial proliferation, which would be a 'normal' temperature compensation effect (Table 4.2). These enzyme data indicate a reduced aerobic capacity of the heart despite a routine metabolic rate comparable to the warm-normocapnia acclimated fish. Such lower metabolic capacities of the heart while routine metabolic rates are unchanged suggests a high oxygen demand of other energy-consuming processes, such as acid-base regulation. Thus, synergistic effect of warming and high PCO_2 appears to result in an elevated tissue oxygen demand. In response, the animal's energy stores could be depleted in the first step (reduced hepatosomatic index, publication II), but in the long run even more severe effects on the whole animal performance, such as a reduced aerobic scope and therefore impaired growth or fecundity, could follow (Pörtner, 2010).

4.2 Comparison of mitochondrial capacities and thermal sensitivities in various notothenioids

4.2.1 Thermal sensitivities in notothenioids originating from different environments

This part of the present study strives to elucidate whether heart mitochondria (measured in saponin-permeabilised cardiac fibres) of notothenioids from different thermal regimes differ in their sensitivity to rising temperature by comparing mitochondrial capacities of the Austral notothenioid *N. angustata*, the sub-Antarctic *L. squamifrons*, the stenotherm *N. rossii, N. coriiceps*, and the high-Antarctic *T. nicolai* and the icefish *C. hamatus*.

It has been suggested earlier that the mitochondrial oxidative capacity of Antarctic fish (per mg mitochondrial protein) is low compared to warm water species (figure 4.5; Johnston et al., 1994).

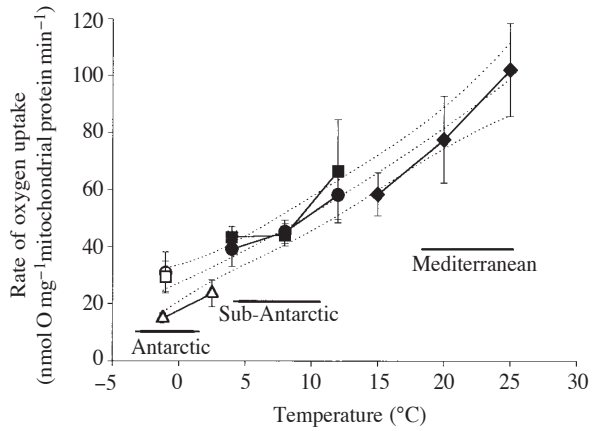


Figure 4.5 Maximal (state III) respiration rates of isolated red muscle mitochondria with pyruvate as substrate.
Values represent means ± SEM (*n*=5 or 6). Open symbols: Antarctic species, filled squares and circles: sub-Antarctic species, filled diamonds: Mediterranean species. The central dotted lines represent a second-order polynomial fitted to the state III respiration data with 95% confidence intervals. Oxygen uptake of isolated mitochondria increases four- to five-fold between -1 and 25°C with a Q_{10} of approximately 2.0 for each species. (adopted from Johnston et al., 1998).

As laid out in the Introduction, cold-adapted or –acclimated fish should increase mitochondrial density or functional activity in order to compensate for decelerating effects of cold temperatures on e.g. diffusion coefficients of cytosolic metabolites or enzyme activities (Tyler and Sidell, 1984). Thus, a proliferation of mitochondria in polar relative to temperate species may serve as a partial compensation for a reduced mitochondrial oxidative capacity per milligram protein at low temperatures (Johnston et al., 1998).

Usually, mitochondria occupy around 16% of the volume of cardiac myocytes in red-blooded notothenioids and up to 50% of the myocyte volume in pelagic, haemoglobin-less icefish (figure 1.3; Johnston et al., 1998; Urschel and O'Brien, 2009).

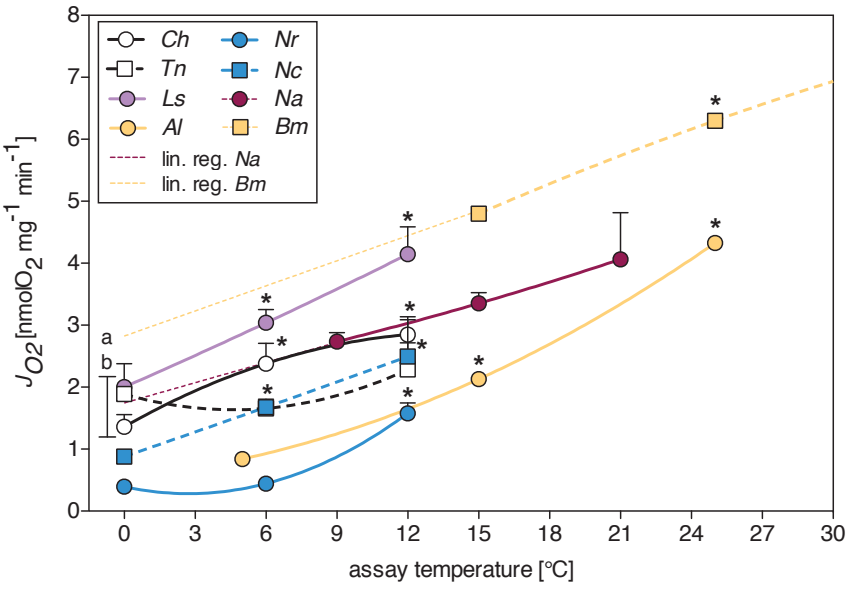


Figure 4.6 State III respiration (J_{O2}) of notothenioids and (cold-) temperate teleost fish.
J_{O2} represents OXPHOS with Complex I and II-linked substrates at acute assay temperatures. J_{O2} of permeabilised heart fibres of *C. hamatus* (*Ch; n*=5), *T. nicolai* (*Tn; n*=4), *N. rossii* (*Nr; n*=4), *N. coriiceps* (*Nr; n*=4), *L. squamifrons* (*Ls; n*=7), and *N. angustata* (*Na; n*=5). For comparison, heart

fibre J_{O2} (Complex I and II) of the temperate eurytherm triplefin *Bellapiscis medius* (*Bm; n*=6-8) (Hilton et al., 2010) and Complex I OXPHOS of isolated heart mitochondria of the cold-temperate, eurytherm wolffish *Anarhichas lupus* (*Al; n*=4) (Lemieux et al., 2010) are displayed. Note: for *A. lupus,* only Complex I-linked respiration is available, thus J_{O2} is relatively lower than in all other fish. *Asterisks* mark a significant rise in J_{O2} compared to the lowest assay temperature; [a] depict a significantly higher J_{O2} compared to *N. coriiceps*, [b] a significantly higher J_{O2} compared to *N. rossii* at 0°C (t-test, $p<0.05$). Values are means ± SEM. Thin, dotted yellow and purple lines represent extrapolation of *B. medius* and *N. angustata* J_{O2} down to 0°C by linear regression analysis.

By extrapolating heart fibre state III respiration (J_{O2}) of the New Zealand black cod, *N. angustata*, down to 0°C via a linear regression analysis, the predicted OXPHOS capacities at 0°C are within the range of the high-Antarctic and sub-Antarctic notothenioids measured in this study (figure 4.6). These extrapolated OXPHOS capacities indicate that the Austral notothenioids still possess aerobic capacities and cold adaptations similar to those of their Antarctic relatives, possibly a heritage of their common Antarctic ancestors. Also OXPHOS (note: only Complex I) capacities of the cold-temperate Atlantic wolffish *Anarhichas lupus* (Lemieux et al., 2010), which experiences habitat temperatures form 0-16°C, are within a similar range as the notothenioids. The OXPHOS capacities of small (body weight about 4 g) blennioid fish (habitat temperature 9-27°C, Hilton et al., 2010) extrapolated down to 0°C are slightly above the ones of the notothenioids, but this effect can be likely attributed to the size difference between the species. These data therefore suggests no difference in tissue oxidative capacities between Antarctic, cold-eurytherm and sub-tropical fish related to MCA, similar as already proposed by Johnston et al (figure 4.5, 4.6; c.f. Johnston et al., 1998).

Notably, the predicted J_{O2} of *N. angustata* at 0°C and also J_{O2} of *C. hamatus, T. nicolai* and *L. squamifrons* at 0°C were significantly higher than the one of *N. rossii* and *N. coriiceps* at 0°C (figure 4.6). A similarly low mitochondrial respiration was reported for liver mitochondria of *N. coriiceps* and *N. rossii* in publication AI, and in isolated red muscle mitochondria of *N. rossii* (Johnston et al., 1998), which can be dedicated to the sedentary behaviour of this species. For a better comparison of relative mitochondrial oxidative capacities in species originating from differently fluctuating thermal regimes, coupling control ratios and the fractional flux through Complex I are presented in Table 4.3.

Table 4.3 Range of habitat temperature fluctuation, ratios and Q_{10} based on heart fibre respirational flux in *C. hamatus* (*n*=5), *T. nicolai* (*n*=4), *N. rossii* (*n*=4), *N. coriiceps* (*n*=4), *L. squamifrons* (*n*=7) and *N. angustata* (*n*=5).

Species	*C. hamatus*	*T. nicolai*	*N. rossii*	*N. coriiceps*	*L. squamifrons*	*N. angustata*
Temp [°C]	-1.5	-1.5	-1.9 to 2	-1.9 to 2	0.7 to 3.5	2 to 15
RCR^+	7.6 ± 0.8^{b}	5.9 ± 0.5^{b}	$3.7\pm0.5^{a,c}$	6.2 ± 1.0^{b}	8.6 ± 1.4^{b}	6.4 ± 0.5^{b}
% CI	$68.7\pm2.4^{b,c}$	$65.3\pm2.6^{a,b,c}$	$45.9\pm6.2^{a,c}$	76.3 ± 2.9^{b}	$63.3\pm3.0^{a,b,c}$	77.4 ± 1.4^{b}
Q_{10}	$2.0\pm0.3^{b,c}$	$1.2\pm0.3^{a,b}$	3.6 ± 0.7^{c}	2.7 ± 0.3	2.1 ± 0.5	$1.4\pm0.1^{a,b}$

Temp [°C] gives the thermal range in the habitat of each species (Ward, 1989; Kock, 1992; Cheng et al., 2003; Pakhomov et al., 2006; Schloss et al., 2008). The respiratory control ratio (RCR^+) provides a measure of coupled state III respiration to the leak respiration state when phosphorylation is inhibited by oligomycin. % CI values indicate the contribution of Complex I to total phosphorylation due to Complex I and Complex II phosphorylation (OXOPHOS CI, CII). Q_{10} (temperature coefficient) was calculated between 0-12°C for the Antarctic fish or between 9-21°C for *N. angustata*. [a] depicts a significant difference to *N. coriiceps* (t-test, $p<0.05$).m[b] depicts a significant difference to *N. rossii* (t-test, $p<0.05$).m[c] indicates a significant differenct to *N. angustata* (t-test, $p<0.05$). Values are means ± SEM over the whole range of assay temperatures.

The capacity to increase mitochondrial respiratory flux between 0 and 12°C was greatest in *L. squamifrons* ($Q_{10\ 0\text{-}12°C}$ 2.1) and *N. coriiceps* ($Q_{10\ 0\text{-}12°C}$ 2.7), and the ones of the wolffish *A. lupus* are within a similar range ($Q_{10\ 5\text{-}15°C}$ 2.5) (Lemieux et al., 2010).

The high-Antarctic *C. hamatus* also showed an initial rise of state III respiration with a $Q_{10\ 0\text{-}6°C}$ of 2.7, however, their state III respiration could not be increased much beyond 6°C ($Q_{10\ 6\text{-}12°C}$ 1.5), and therefore 6°C may already reflect maximum OXPHOS rates of this species.

In contrast, the high-Antarctic *T. nicolai* ($Q_{10\ 0\text{-}12°C}$ 1.2) and the Austral *N. angustata* ($Q_{10\ 0\text{-}12°C}$ 1.4) could not increase state III respiration with assay temperature to the same extent as *L. squamifrons* and *N. coriiceps.* Also in the study by Hilton et al. (2010), heart fibre respiration of the triplefin *B. medius* rose with a Q_{10} of 1.5 from 15 to 30°C (figure 4.6). In their study they found that another triplefin species, *Forsterygion malcolmi,* could not increase state III respiration with temperature (Q_{10} 1.06 between 15 and 30°C), although both species possess similar state III respiration rates at 15°C, the starting point of the experiments. The sub-tidal triplefin *F. malcolmi* does not encounter similar fluctuations in environmental temperature as its interidal congener *B. medius.* Therefore, in case of the triplefins, the scope for adjustment of OXPHOS flux appears to reflect different thermal ranges and temperature fluctuations they encounter in their habitats. Furthermore, this scope of OXPHOS is suggested to serve as a measure for a species' plasticity to respond to rising temperature.

The high-Antarctic species *C. hamatus* and *T. nicolai* do not routinely encounter large fluctuations in their environmental temperature, which usually remains stable around -1.5°C,

whereas *L. squamifrons* may face temperatures up to 3.5°C during summer (D. Gerdes, AWI, unpublished CTD data). Also *N. rossii* and *N. coriiceps* are exposed to larger seasonal thermal fluctuations than the two high-Antarctic species, ranging from -1.9 in winter to 2°C in summer at the Antarctic Peninsula (Schloss et al., 2008).

Accordingly, the flexibility to respond to rising temperature was low in the high-Antarctic *C. hamatus* and *T. nicolai*, and moderately higher in *N. coriiceps* and *L. squamifrons*. Surprisingly, mitochondrial plasticity of the Austral *N. angustata* was comparable to that of the high-Antarctic fish, although *N. angustata* faces habitat temperatures from +2°C in winter up to 15°C during summer (Cheng et al., 2003). Furthermore, the RCR^+ values of *N. angustata* heart fibres decreased with assay temperature, which indicates a limited thermostability of mitochondrial capacities at temperatures beyond 15°C, while all other species (apart form *N. rossii*) displayed high and stable RCR^+ values > 5 over the whole range of assay temperatures (0-12°C; Table 4.3; specific RCR's at the single assay temperatures not shown). This can be taken as a clear sign of *N. angustata's* Antarctic heritage.

Thus, *N. angustata* seems to be an exception for the hypothesis on a simple relation of mitochondrial thermal plasticity to the extent of environmental fluctuations (Table 4.3). Due to their evolutionary origin in the Antarctic region, they still share several physiological similarities to Antarctic notothenioids, such as functional AFGPs.

It has been postulated that the low thermal tolerance range of Antarctic species is related to high activation energies of mitochondrial enzymes that can down-regulate metabolic pathways. Thereby, they can save energy despite high mitochondrial densities (Pörtner, 2002c). A high amount of mitochondria with high activation energies would cause a steep increase in mitochondrial costs with rising temperature and thus only allow a narrow thermal window (Pörtner, 2006), as it is the case in the Antarctic notothenioids analysed in this thesis. In contrast, cold-adapted eurythermal species have comparably lower mitochondrial densities. Their mitochondria possess high ATP synthesis capacities and maintenance costs and are thermally more flexible, and thereby support a broader thermal window (e.g. Sommer and Pörtner, 2002). One could therefore assume that *N. angustata,* which tolerates higher ambient temperature variability (from 2 to 15°C) possesses lower enzymatic activation energies which only cause a moderate increases in mitochondrial costs during warming. When routine metabolic rates (publication IV) and heart fibre respiration rates of *N. angustata* are extrapolated down to 0°C, they show similar respiration rates to high- and sub-Antarctic notothenioids. Therefore, they may have similar mitochondrial

densities or structures, although this must remain speculative at present (figure 4.6). Thus, *N. angustata* may have adjusted its activation energies in order to tolerate the fluctuations in its habitat. Nevertheless, their low capacities to increase mitochondrial respiration beyond 15°C suggests that *N. angustata* may have reached the maximum possible balance between mitochondrial costs and densities and seems to have retained a limited mitochondrial thermal plasticity, which is only slightly larger compared to its Antarctic congeners.

N. rossii displayed the lowest Complex I-linked flux (% CI), coupling ratios (RCR^{+}) and Complex IV (COX) capacity relative to the capacities of oxidative phosphorylation (OXPHOS) (Table 4.3). In the Introduction and in the first section of the Discussion, it has already been pointed out that Complex I is important for a high OXPHOS efficiency and probably involved in acclimation response to warming and elevated PCO_2. Other studies on crustaceans and temperate fish also report limitations in Complex I respiration at higher temperatures in crabs and triplefin fish that show a higher degree of stenothermy than their congeners. Accordingly, stability or increase in Complex I contribution is suggested as an indicator for eurythermy in ectothermal fish and crustaceans (Hilton et al., 2010; Iftikar et al., 2010). Additionally, a high thermal sensitivity of Complex I has already been postulated for *N. rossii.* It was suggested to be the result of different protein (i.e. Complex I) instabilities, and thus thermal stabilities, within different nototheniid and other fish species (publication AI). Furthermore, *N. coriiceps* displayed a greater Complex I coupling efficiency compared to *N. rossii* (publication AI).

The present data revealed a lower fraction of Complex I to OXPHOS in *N. rossii* compared to *N. coriiceps*. Yet, both species showed a stable Complex I fraction during acute rising temperature (data not shown). Therefore, one could assume similar capacities to modulate heart performance with temperature in both species. The extent of adjustment capacities may nevertheless be lower in *N. rossii* due to structural or functional peculiarities of Complex I that may affect protein stability or even translational efficiency in *N. rossii*, which are different in their ecotypically similar congener *N. coriiceps* (publication AI; Zhuang and Cheng, 2010).

In line with these findings, *N. rossii* appears to possess a low mitochondrial performance, particularly when compared to *N. angustata*, which displayed the highest Complex I contribution (Table 4.3). Yet, the percentage of Complex I in *N. angustata* mitochondrial respiration was significantly reduced at 21°C (data not shown), which is another indicator for their limited mitochondrial capacities towards high temperatures.

In summary, the white-blooded icefish *C. hamatus* seems equally sensitive to elevations in temperature as the red-blooded, high-Antarctic *T. nicolai,* and both possess limited aerobic capacities in the warmth. This may relate to the minimal temperature fluctuations these species naturally face in their habitat, and is further in agreement with earlier reports on equal aerobic capacities in hearts of white- and red-blooded Antarctic fish. This is remarkable due to a substantial remodelling of icefish-mitochondria, such as lower cristae density and matrix volume compared to red-blooded species (figure 1.3; O'Brien and Sidell, 2000; Urschel and O'Brien, 2008).

N. coriiceps and *N. rossii* display moderate capacities to respond to rising temperature, but *N. rossii* appears to have lower mitochondrial performances compared to *N. coriiceps*. Due to high heart fibre respiration rates, which rise almost linearly with temperature up to 12°C, the sub-Antarctic *L. squamifrons* may possess higher thermal acclimation capacities than the Antarctic species. The heart of the Austral *N. angustata* likely has moderate capacities to increase its aerobic metabolism under rising temperature, which are not higher than the ones of the Antarctic notothenioids. Reduced RCR^{+} values beyond 15°C and reduced Complex I-linked flux at 21°C suggests that *N. angustata* could also reach a limit in mitochondrial capacities and concomitantly in aerobic heart performance when the temperatures in New Zealand's waters rise dramatically, as predicted for the ocean's all over the globe (IPCC, 2007). Critical temperatures have never been determined for *N. angustata*, but the data on routine metabolic rate in publication IV, measured at acute rising temperatures (1.5°C rise in temperature per 12 hours, range 14 – 21°C), suggest critical thermal limits around 18-20°C (drop in oxygen consumption beyond 19°C). As the capacities to increase metabolic rates during acute thermal challenge normally cover a wider range than their actual long-term acclimation capacities (Bilyk and DeVries, 2011), the temperatures which are tolerated by *N. angustata* over a longer timescale are probably below 19°C.

Based on these findings, heart mitochondrial thermal sensitivities cannot ultimately reflect whether a species has a wide or narrow range of thermal tolerance. Similarly, activation energies of mitochondrial respiration in liver do not differ in Antarctic and temperate eelpout (Lannig et al., 2005), and whole-animal thermal tolerance can therefore not easily be explained by mitochondrial characteristics. Yet, they can indicate if a species possesses high or low capacities to increase heart mitochondrial aerobic metabolism with temperature, which may become a crucial factor for the maintenance of heart performance when environmental conditions change.

4.2.2 Hypercapnia sensitivities in N. rossii and N. angustata

Hypercapnia has been found to be an additional stressor, acting synergistically with temperature (Pörtner, 2010). To elucidate the vulnerability of notothenioids towards elevated PCO_2, the next part of the study compared mitochondrial capacities of the stenotherm *N. rossii* to the cold-eurytherm *N. angustata* under acute and chronic elevated PCO_2. Besides the data of *N. angustata* in publication IV, some additional data for *N. rossii* are presented in figure 4.7. In both species, permeabilised heart fibre respiration was measured in normocapnic and acute hypercapnic (2 kPa CO_2) respiration buffer. In both notothenioids, OXPHOS (maximum flux through Complex I and II) was not affected by acutely hypercapnic conditions.

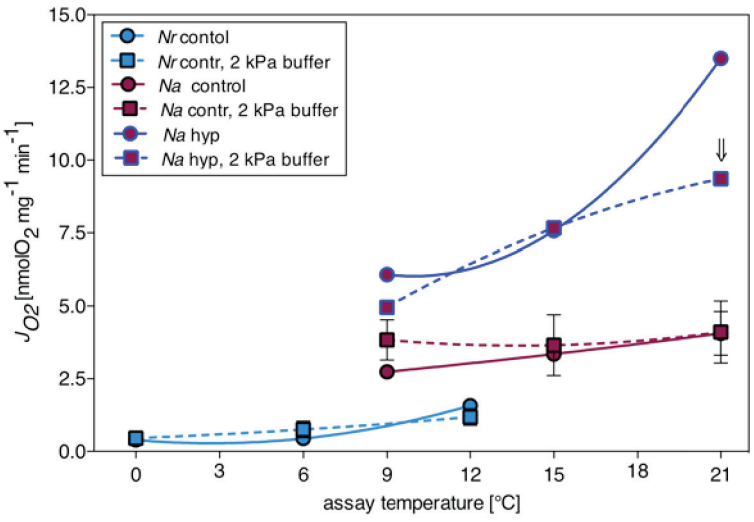


Figure 4.7 State III respiration of *N. rossii* and control or CO_2 acclimated *N. angustata* at acute assay temperatures. OXPHOS (J_{O2}, OXPHOS with Complex I and Complex II-linked substrates) in permeabilised heart fibres of the Antarctic marbled rock cod *N. rossii* (*Nr*) kept at 0°C, 0.04 kPa CO_2 (blue symbols; n=4), and the New Zealand black cod *N. angustata* (*Na*) kept under control conditions (14°C, 0.04 kPa CO_2, purple symbols; n=5) or acclimated to 14°C, 0.2 kPa CO_2 (blue-framed, purple symbols; n=4) for 12 days. In both species, heart fibre respiration was measured in normocapnic (0.04 kPa CO_2, circles and solid lined) and hypercapnic (2 kPa CO_2, squares and dotted lines) mitochondrial respiration buffer. The *arrow* depicts a significantly reduced J_{O2} compared to normocapnic buffer (t-test, p<0.05). Values are means ± SEM.

However, the picture completely changed after acclimation to 0.2 kPa CO_2. Hypercapnia acclimated *N. angustata* had significantly elevated OXPHOS capacities when measured in normocapnic respiration buffer compared to control *N. angustata* (see publication IV and figure 4.7). These data suggest that, in contrast to acute effects, chronic hypercapnia induces compensatory mechanisms in heart mitochondria in *N. angustata*. As already proposed in section 4.1.3 for *N. rossii*, acclimation to chronically elevated [HCO_3^-] and a new acid-base steady state may involve continuously elevated expression and activities of mitochondrial enzymes, such as CS and COX, which are under transcriptional and post-translational control. High HCO_3^- levels may induce the sAC-cAMP-PKA signalling pathway

in order to compensate for a competitive inhibition of CS or succinate-dehydrogenase by HCO_3^- (Simpson, 1967; Wanders et al., 1983) by continuously elevated protein translation (Acin-Perez et al., 2009) or a shift towards a high flux through Complex I of the ETS.

In the heart fibre respiration experiments with hypercapnia acclimated *N. angustata,* these high capacities (due to putative compensation for elevated [HCO_3^-]) were visible by an 'overshoot' in OXPHOS when measured in normocapnic respiration buffer. As the hypercapnic buffer conditions may come closer to the intracellular 'new' acid-base status under chronic hypercapnia, OXPHOS of the CO_2 acclimated *N. angustata* heart fibres was lower in hypercapnic than in normocapnic respiration buffer. This indicates compensation of mitochondrial capacities towards elevated seawater PCO_2, possibly after an initial inhibition of mitochondrial enzymes by elevated HCO_3^- levels (see section 4.1.3).

Although heart fibre respiration was not measured in 0.2 kPa CO_2 acclimated *N. rossii,* their enzymatic responses of CS and COX (c.f. publication III) in heart suggest that this species also possesses slightly stimulated mitochondrial capacities in response to chronic hypercapnia. In combination with *N. rossii's* extremely high CS and COX activities in red muscle, this provides further support that notothenioids respond to hypercapnia acclimation by increased mitochondrial capacities. Nevertheless, the picture was different between various tissue types in *N. rossii*, and their reduced mitochondrial respiration in liver after long-term acclimation to elevated PCO_2 indicates putative shifts in metabolism in other tissues than heart. For *N. angustata*, it remains unclear for now whether their high, hypercapnia-compensated mitochondrial heart capacities come along with restrictions in other tissues. Therefore, it is difficult to make final predictions on the hypercapnia-tolerances of these two species.

4.3 Fish *vs.* cephalopods

In this part of the study, the susceptibility of competing species belonging to two different animal phyla will be discussed by referring to data in publication II, IV and V. In order to gain insight into the acclimation capacities of octopods towards seawater warming, warm-acclimation capacities were assessed for the sub-tropical octopus *E. moschata,* acclimated to 21°C (normocapnia). The experiments on these warm-acclimated octopods revealed that *E. moschata* is not able, or only incompletely so, to compensate its mitochondrial and routine metabolic rates during warm-acclimation to 21°C (control animals: 16°C; figure 1 in publication V). In contrast, a previous study on warm-acclimated *S.*

officinalis revealed that this cuttlefish can fully compensate their routine metabolic rates after long-term acclimation to 20°C (type 2 - Table 4.1; control animals: 15°C; Melzner, 2005). Both species can be found in the same habitat, e.g. the Adriatic Sea, and both display a bottom-dwelling lifestyle, with *S. officinalis* being slightly more active and migratory, while *E. moschata* is essentially benthic throughout their life (Mangold, 1983). Moreover, another study measured fully temperature compensated heart fibre respiration in warm-acclimated *S. officinalis* (Oellermann et al., 2012), while warm-acclimated *E. moschata* was incapable to do so (publication V, figure 2). This indicates that cuttlefish may be more eurythermal than *E. moschata*. Compared to *E. moschata*, *S. officinalis* therefore appears to be more tolerant to changing environmental conditions, and in light of their different thermal acclimation capacities and lifestyle, *S. officinalis* may outcompete *E. moschata* if seawater temperatures continue to rise.

In the attempt to compare temperature and hypercapnia-acclimation capacities between Antarctic fish and cephalopods, the problem arose that *P. charcoti* could not be acclimated to either warmer temperatures or hypercapnia due to a limited amount of experimental individuals onboard *RV Polarstern* in 2011. Therefore, in the following, acclimation capacities for cephalopods must be derived from the analysis of the thermal response of the sub-tropical octopus, *E. moschata*, and the hypercapnia response in *S. officinalis* acclimated to 0.112 kPa CO_2. Particularly when using *S. officinalis* as an example for hypercapnia tolerance, it has to be kept in mind that they are putatively more eurythermal than *E. moschata* and display generally higher mitochondrial capacities to respond to rising temperature and PCO_2 compared to the octopods (publication V). Thus, any acclimation response may be less pronounced in the octopods.

Nevertheless, acute thermal sensitivity of mitochondrial respiration allows a comparison of general mitochondrial capacities of cephalopods and notothenioids. The heart fibre mitochondria of both sub-tropical and Antarctic octopus were less well coupled than the ones of the notothenioids, which hints towards generally lower heart mitochondrial capacities in the octopods. Furthermore, *E. moschata* showed only a low thermal response (Q_{10} 1.2, range 16-26°C) as did *Sepia* (Q_{10} 1.4, range 16-26°C), in contrast to *N. rossii* (Q_{10} 3.6, range 0-12°C). Additionally, *E. moschata* displayed the lowest Complex I capacities, coupling ratios and COX-linked flux, and may therefore display the lowest acclimation capacities of all cephalopod and fish species investigated (Table 2 in publication V).

Yet, mitochondrial respiration rates and acute thermal sensitivities (Q_{10} 2.0, range 0-12°C) of the Antarctic octopods, *P. charcoti*, were found at a level similar to those of *N.*

rossii. Thus, one could assume similar mitochondrial acclimation capacities in members of both phyla that share the same habitat. Moreover, acute critical temperatures between 8 and 10°C have been determined for *P. charcoti* by transition to an anaerobic mode of energy production (Pörtner and Zielinski, 1998). *N. rossii* may have its critical temperatures in a similar range, as a slightly acidic liver pH_i (exceeding α-stat) was measured in 7°C acclimated *N. rossii* compared to liver pH_i of control animals, indicating beginning metabolic limitations (Table 4 in publication II).

In terms of chronic hypercapnia tolerance, more profound differences became visible between cephalopods and (notothenioid) fish. While *N. rossii* could completely restore their blood pH in response to 0.2 kPa CO_2 exposure, the cuttlefish *S. officinalis* acclimated to 0.112 kPa CO_2 only displayed a partial compensation, and their pH_e remained below control levels despite $[HCO_3^-]_e$ accumulation. This is in line with the hypothesis that teleost fish possess a more powerful acid-base regulation machinery than invertebrates (Melzner et al., 2009). However, measurement of routine metabolic rates of hypercapnia acclimated *S. officinalis* (publication V, figure 1; and Gutowska et al., 2009) indicates that such a minor drop in pH_e does not seriously disturb their metabolic equilibria or pH_i, and is still within the tolerance limit to maintain proper functioning in oxygen transport of their respiratory protein, haemocyanin.

Similar to the pattern observed in OXPHOS capacities of *N. angustata* (c.f. publication IV, figure 2, and figure 4.7), bicarbonate accumulation for the maintenance of a new acid-base steady state may have affected mitochondrial functioning in the CO_2-acclimated cuttlefish. When heart fibres of the hypercapnia acclimated *S. officinalis* were measured in normocapnic buffer, they showed slightly higher, putatively hypercapnia-compensated capacities (figure 3 in publication V), as also observed in *N. angustata* (figure 4.7). Following the hypothesis for *N. angustata,* an acute inhibition of enzymes by elevated $[HCO_3^-]_e$ may trigger enhanced enzyme amounts, and thereby activities, in order to compensate for initially reduced enzyme capacities. The sAC-cAMP-PKA signalling pathway may then support the transcriptionally or translationally regulated expression of mitochondrial complexes and enzymes during chronic hypercapnia (Zippin et al., 2001; Acin-Perez et al., 2009). Yet, this effect was less pronounced in the cuttlefish than in *N. angustata*, and together with the incompletely compensated pH_e this suggests lower acid-base compensatory capacities and their limited effect on mitochondrial functions in the invertebrates compared to the vertebrates.

Nonetheless, enhanced mitochondrial energy turnover and maintenance of shifted acid-base equilibria coincide with an elevated metabolic energy demand. In cuttlefish, the TCA-cycle is controlled by the glycolytic pathway and by protein metabolism, which provides anaplerotic 2-oxoglutarate (figure 4.3). Thereby, the coupled catabolism of glycogen and amino acids increases ATP production per mol O_2 by 50% (Lee, 1994) compared to fish, which fuel the cycle with carbohydrate and fat via a single entry point, acetyl-CoA. Cuttlefish have only very limited glycogen stores, therefore, the catabolisation of amino acids (particularly proline) fuels is used to increase aerobic metabolism (figure 4.4; O'Dor and Webber, 1986).

Similar to the enhanced oxidative decarboxylation reactions proposed to occur in fish under chronic hypercapnia, elevated bicarbonate levels and the associated costs of acid-base regulation may trigger an up-regulated TCA-turnover in systemic hearts of cephalopods. Increased oxidation of proline yields 2 $NADH_2$ and glutamate, which then enter the TCA-cycle (Mommsen and Hochachka, 1981). Via this mechanism, mitochondrial efficiency may also be augmented in cuttlefish under chronic hypercapnia (figure 4.4).

Antarctic fishes possess a striking preference for the catabolism of lipid fuels (Crockett and Sidell, 1990), while cephalopods normally fuel aerobic metabolism with dietary carbohydrates and amino acids and have only minimal lipid reserves (Crockett and Sidell, 1990; Lee, 1994). For *N. rossii,* shifts in their enzyme activity ratios suggested enhanced fatty acid oxidation by using their liver lipid stores to sustain aerobic metabolism at elevated seawater PCO_2 (publication III). If cephalopods mobilize their protein and lipid reserves without feeding close to *ad libitum* rates, these reserves would last only one or two weeks. In contrast, fish such as well-fed herring can survive on lipid stores alone for up to 600 days (O'Dor and Webber, 1986).

To overcome this limitation, cephalopods clearly must increase their feeding rates. Such a need would increase competition for prey with other species, such as demersal fish. In addition, particularly Antarctic cephalopods may be threatened by a decrease of unfavorable shifts in prey species composition; in Antarctic food webs, a strong shift towards smaller size primary producers and a drastic decrease in nutritional value of potential prey for higher trophic level consumers, such as cephalopods and fish, has already been reported (see publication AII for review). The response of *P. charcoti* towards changing prey quality is difficult to predict, as the capability to adapt food choice to prey availability is not known for Antarctic octopods. Still, a physiological pressure for higher feeding rates would induce an unfavourable selection pressure in the competition for prey with fish.

Although the present studies on *N. rossii* suggested that these Antarctic fish might be put at risk due to elevated seawater temperature and PCO_2, their generally higher acid-base regulation capacities and larger energy reserves compared to cephalopods will putatively make them 'win' the competition for resources over longer time-scales. As a result, the ecological balance between benthic octopods and notothenioids may shift by future climate changes in the Antarctic.

However, this hypothesis does not include that cephalopods in general have optimized their physiology to 'live fast and die young', with low energy reserves, but fast growth, maturity and generation turnover (O'Dor and Webber, 1986). Yet, the generation turnover of Antarctic octopods is largely unexplored; they have extremely slow growth rates, and also their eggs possess putatively slow development rates (Collins and Rodhouse, 2006). Furthermore, Antarctic octopods display a longevity greater than their temperate counterparts; some species have been observed to survive for at least eight years in captivity (F. Mark, personal observation). Notothenioids are characterized by even slower growth and higher longevity; Antarctic species can reach ages of 15-20 years, and *N. rossii* typically reaches maturity at the age of 8-10 years (publication AII; Mesa and Vacchi, 2001). Due to the faster generation turnover in cephalopods, evolutionary adaptation may occur more rapidly. The rate of seawater warming and acidification may therefore become an important determinant in defining the adaptive potential towards changing abiotic factors in the two competing phyla.

5 Synopsis

This thesis investigated a number of aspects of thermal plasticity and acclimation abilities to higher temperature and PCO_2 levels of Antarctic and Austral fish *N. rossii*, as well as of sub-tropical and Antarctic cephalopods.

At first glance, the Antarctic fish *N. rossii* appeared to possess only little warm-acclimation capacities, but to be capable of dealing with moderately elevated CO_2 concentrations of 0.2 kPa, since routine metabolic rates were incompletely compensated after warm-acclimation, but not altered in response to chronic hypercapnia. Nevertheless, a closer analysis of the fishes' aerobic energy metabolism revealed shifts in metabolic pathways and substrate preferences within mitochondria in response to warm and CO_2 acclimation:

In the long-term 7°C (normocapnic) acclimated *N. rossii,* an elevated flux via Complex I was observed compared to control. Despite a theoretical stoichiometry for mitochondrial respiration of 4:1 for Complex I:Complex II, liver mitochondria of *N. rossii* generally appear not to use its full capacities for substrate oxidation. This Complex I deficiency appeared to be a peculiarity of *N. rossii*, as it also occurred in heart tissue: flux through Complex I was about 45% for *N. rossii,* and about 70% in the other notothenioids analysed in this study, although the same substrate combinations were used in all experiments. The molecular mechanisms for this 'anomaly' of *N. rossii* mitochondria must remain unclear at present. Yet, chronically elevated temperatures seem to increase substrate turnover and flux through Complex I, which could slightly increase mitochondrial phosphorylation efficiency under long-term elevated seawater temperatures.

In cold and warm hypercapnia acclimated *N. rossii,* a shift in 'set points' of acid-base regulation occurred at both extra- and intracellular levels. Such changes in the acid-base equilibrium have been postulated to be maintained by e.g. higher activities of the ATP-dependent NKA (Deigweiher et al., 2008), and therefore occur at the expense of a high cellular and mitochondrial energy demand in order to preserve ionic homeostasis. Indeed, *N. rossii* appeared to increase mitochondrial aerobic capacities in red muscle in response to chronic hypercapnia as a reaction to high metabolic demands of this tissue. In contrast, liver mitochondria of both cold and warm hypercapnia acclimated fish showed reduced capacities (state III respiration), which were also visible in the isolated mitochondrial enzyme activities of the ETS (COX). Such decreased COX activities could come along with shifts in the energy budget away from the ETS towards enhanced gluconeogenesis or fatty acid oxidation in liver, which is in line with reduced hepatosomatic indices in hypercapnia acclimated *N. rossii*.

Negative effects on whole animal performance such as growth are thus conceivable during long-term chronic hypercapnia. Yet, the reduced capacities in the hypercapnia acclimated *N. rossii* appeared to be partially compensated by a higher contribution of Complex I to total OXPHOS capacities and thus shifted substrate preferences of the ETS:

After an acute inhibition of the TCA-cycle by HCO_3^-, e.g. at the level of CS and succinate dehydrogenase, an increased metabolisation of NADH-linked substrates, such as glutamate and malate, could have enhanced TCA-cycle capacities and the energetic efficiency of mitochondrial oxidative capacity, particularly of Complex I. In context of other data available for Complex I-linked flux in heart mitochondria of sub-tropical fish (Hilton et al., 2010), Complex I modulation appears to be a general response to elevations in metabolic energy demand in fish, including *N. rossii*.

Another mechanism that could apply in response to a shifted acid-base equilibrium in hypercapnia acclimated *N. rossii* could be the stimulation of the intracellular and intramitochondrial soluble carrier 'sAC' by HCO_3^-, which in turn activates the second messenger cAMP. This cascade causes PKA to activate metabolic key enzymes by direct phosphorylation or transcriptional and post-translational modification (Zippin et al., 2001), which may lead to continuously elevated levels of mitochondrial enzymes such as CS or COX.

Indeed, analyses of tissue-specific CS and COX-activities in cold hypercapnia acclimated *N. rossii* were well in agreement with these two hypothesis: cold-hypercapnia acclimation seemed to elicit a slight increase in mitochondrial aerobic capacities in the heart by elevated CS activities, and also red muscle increase mitochondrial aerobic capacities in in response to chronic hypercapnia by elevated CS and COX activities. These high mitochondrial enzyme activities in heart of cold hypercapnic fish and red muscle of both cold- and warm-hypercapnia acclimated *N. rossii* propose that these tissue's mitochondria may be highly compromised by elevated PCO_2 (competitive inhibition by HCO_3^-), possibly causing a compensation by elevations in CS and COX capacities. In liver tissue, the results depict a different situation than in heart and red muscle: decreased COX activities at unchanged CS activities indicates shifts in metabolic pathways in liver, e.g. shunting TCA-cycle intermediates away from the ETS towards gluconeogenesis to support the high aerobic demand other tissues, such as heart and red muscle. However, reduced hepatosomatic indices in warm and hypercapnia acclimated fish imply that shifts in metabolic pathways likely are at the expense of liver energy stores, which may be insufficient to support other, highly oxidative tissues in the long run.

In contrast, in the warm hypercapnia acclimated fish, activities of both enzymes were extremely reduced in heart. Moreover, the whole animal respiration measurements demonstrate that the basal metabolism will rise with temperature, and the combination with the stressor 'elevated CO_2' might additionally increase aerobic demand, i.e. for ionic homeostasis (new 'set point' for acid-base regulation) in *N. rossii*. Concomitantly, the fish will require a higher output of their circulatory and ventilatory system. Yet, the extremely low enzymatic capacities in heart of warm-hypercapnia acclimated fish are a first indicator for a putatively imparied oxygen circulation by the heart and therefore possibly reduced performance of Antarctic fish due to the synergistic effect of ocean warming and acidification and also identifies the heart as one of the most sensitive organs with the least capacities to acclimate (Somero, 2002).

Thus, a higher PCO_2 may exacerbate the effect of rising temperature and thereby restrict the aerobic scope of *N. rossii*. According to the OCLTT hypothesis, this may lead to a narrowing of *N. rossii*'s thermal window and reduce the species' individual performance, which could increase the sensitivity of the whole population to climate change (Pörtner, 2010). Also in context of data available for other Antarctic notothenioids (this study; c.f. Urschel and O'Brien, 2009; Beers and Sidell, 2011; Enzor et al., 2013), the results of the present thesis suggest that *N. rossii* will have only a minor scope for acclimation and tolerance towards ocean acidification and warming.

A dramatic rise in temperatures and seawater PCO_2 around the Antarctic by 2°C and 0.03 kPa CO_2, respectively (Murphy and Mitchell, 1995; Ciattaglia et al., 2008), within this century will force *N. rossii* to acclimate to these new conditions within only a few generations. As the present study suggests low warm- and CO_2-acclimation capacities for *N. rossii*, these fish would have to move southwards into high Antarctic shelf areas. This would lead to alterations in community composition in their current habitat and lead to increased competition in high-Antarctic continental shelf areas, as the local species of that area will not have the possibility to move south any further (c.f. publication AII). For example, the present study suggests high thermal sensitivities of heart mitochondria for the high-Antarctic *T. nicolai* and also to a lesser extent for the icefish *C. hamatus*. Particularly the icefish may be threatened by seawater warming and acidification, as the oxygen-carrying capacity of their haemoglobin-less blood is limited, and the present heart fibre respiration experiments also assign them a low potential for physiological acclimation to satisfy an increasing tissue oxygen demand.

The sub-Antarctic *L. squamifrons* displayed a high thermal flexibility towards acute warming and may be able to acclimate to rises in seawater temperature. The austral nototheniid *N. angustata*, appeared to possess low thermal plasticities despite a – on notothenioid standards – broad thermal window. As global average temperature is expected to increase between 1.4°C and 5.8°C over the remainder of this century (IPCC, 2007), one could speculate that *N. angustata* may have to leave its habitat off New Zealand's coast if temperatures there also rise that rapidly, and return to their Antarctic origin.

Thus, a southward move of Austral, sub-Antarctic and Antarctic notothenioids, but also of putatively more competitive non-notothenioid species, may lead to a significant change in the composition of Antarctic communities, with unknown consequences for the Antarctic food web.

In contrast to the minor thermal plasticity of *N. angustata,* chronic acclimation to moderately elevated CO_2-levels might be compensated by enhanced mitochondrial aerobic capacities in these Austral notothenioids. This may be induced by the cAMP signalling pathway, which leads to continuously higher enzyme capacities due to post-translational modifications, similar as proposed for hypercapnia acclimated *N. rossii*. However, such compensations will probably involve shifts in energetic demands and metabolic pathways. Together with a constricted thermal plasticity, additional energy consuming processes such as acid-base maintenance and high enzymatic activities could lead to a reduced aerobic scope in *N. angustata* at higher temperatures and CO_2-levels.

In order to gain insight into the susceptibility of Antarctic octopods to climate change, which are ecotypically similar to Antarctic notothenioids, acute temperature and hypercapnia sensitivity of Antarctic octopods was compared to the ones of Antarctic fish. Furthermore, the well-studied, temperate cuttlefish *Sepia officinalis,* and the sub-tropical octopus *Eledone moschata,* were investigated to gain insight into the vulnerability of cephalopods towards warming and hypercapnia.

After long-term hypercapnia acclimation of the cuttlefish, the acid-base status in the haemolymph of *S. officinalis* was found to be incompletely compensated with an extracellular pH remaining 0.1 pH units below control levels. Elevated PCO_2 did not affect their RMR, but led to increased mitochondrial capacities, possibly mediated by enhanced glutamate oxidation, similar as postulated for fish.

Heart fibre mitochondria of the Antarctic octopus *P. charcoti* appeared to be more flexible towards acute temperature changes than the sub-tropical *E. moschata,* which also did not show any compensation towards long-term elevated temperatures. Under acute

hypercapnia, limitations in COX capacities may reduce the mitochondrial oxygen affinity and flux through the ETS in both octopus species. This is a first indication that oxygen affinity to mitochondria and their aerobic capacities could become limited due to elevated PCO_2, which may affect the octopods *P. charcoti* and *E. moschata* more than the cuttlefish *S. officinalis*.

Antarctic octopods displayed a similar thermal sensitivity to rising temperatures than Antarctic notothenioids, which could indicate similar warm-compensation capacities in the two co-occurring phyla. However, limited COX capacities and thus lower mitochondrial oxygen affinities under acute hypercapnia could make them more sensitive to chronically elevated PCO_2, particularly as they do not possess myoglobin (or a related respiratory protein) for intracellular oxygen-delivery (Hochachka, 1994), which additionally hampers oxygen diffusion in contrast to red-blooded fish. Furthermore, generally higher acid-base regulation capacities and larger energy reserves in fish compared to cephalopods probably will make fish to 'win' the competition for resources over longer time-scales, also depending on the speed of ocean warming and acidification around the Antarctic Peninsula, which exert their specific effect on the energy metabolism of fish and cephalopods.

6 Future perspectives

The analysis of the warm- and hypercapnia-tolerance of the Antarctic fish *N. rossii* in this thesis suggests that this species will be negatively affected by rising seawater temperatures and CO_2 concentrations in the Southern Ocean (Meredith et al., 2005; IPCC, 2007). It further highlights that (sub-) cellular responses of Antarctic and Austral fish to changing environmental conditions are highly dependent on the metabolic function of the respective tissue type. Therefore, the herein hypothesized shifts in metabolic pathways within tissues and mitochondria, anaplerotic reactions feeding the TCA-cycle, and flux through the different components of the mitochondrial ETS in chronically warm and hypercapnia-exposed *N. rossii,* warrant further investigation to obtain a full picture of the differential responses throughout an organism.

For example, studying CO_2 dependent alterations of protein expression patterns could be performed for a classification of stress induced responses in gene expression (mRNA level). Proteomic changes during chronic hypercapnia have rarely been studied in fish. Changes in protein expression could provide a comprehensive picture of the simultaneous changes that occur while an organism is experiencing changes in its acid-base equilibrium, and shed light on the regulatory pathways involved in the acclimation mechanisms of aerobic energy metabolism and energy dependent processes.

However, mRNA gene expression and proteomic studies do not provide full access to the potential shifts between metabolic pathways caused by external drivers such as temperature and CO_2, within cells or tissues. Thus, modern spectroscopic and spectrometric metabolomic (or metabolic) profiling could be used to detect whether metabolic function is altered during exposure of organisms to environmental challenges. Combining proteomic and metabolomic studies can definitely broaden the knowledge on molecular stress responses that modulate tissues-specific shifts in energy metabolism and metabolic pathways.

From the presented results of the mitochondrial respiration experiments, additional specific topics emerged that need further research. The impaired mitochondrial respiration in liver of CO_2 acclimated *N. rossii* was determined in normocapnic (0.04 kPa CO_2) respiration medium, which does not represent the true intracellular PCO_2 before or after compensation for shifts in extra- and intracellular acid-base parameters. Future measurements of mitochondrial respiration should be performed in respiration buffer adjusted to the 'actual' intracellular PCO_2 in control or CO_2-acclimated fish, to better identify possible hypercapnia compensation mechanisms of mitochondria under steady-state PCO_2 conditions that can be

expected *in vivo* in long-term hypercapnia acclimated fish. Furthermore, mitochondrial respiration experiments should also be conducted in buffers set to putative intracellular oxygen partial pressures (1-2 kPa O_2, ca. 5-10% air saturation) in order to compare mitochondrial capacities under *in vivo* PO_2 levels to all previous data measured in the 'conventional' way (at 21% air saturation).

Given the observed lower respiration in isolated liver mitochondria of hypercapnia acclimated *N. rossii*, any energetic limitations (ATP production) that may result in individual mitochondria could be partially compensated for by a higher mitochondrial volume density, cristae surface or proliferation per gram tissue. Although the activity of CS and COX can serve as a measure for changes in mitochondrial volume or cristae density, changes in the activity of these enzymes cannot finally differentiate between changes in individual mitochondrial structure and activity (e.g. through higher/ lower cristae surface density, mitochondrial size or shifts in metabolic pathways) and the effects of mitochondrial proliferation. Therefore, mitochondrial size, structure and abundance should be investigated via Electron or confocal Microscopy to actually visualize mitochondria of control *vs*. warm/ CO_2-acclimated animals. By this analysis, the connection between the measured enzyme activities conducted in the present thesis and mitochondrial shape/ abundance could be drawn for the first time in hypercapnia and warm-acclimated fish.

Another issue that emerged from the hampered mitochondrial function in hypercapnia acclimated *N. rossii* is the role of uncoupling proteins (UCPs) in mitochondrial metabolism and the characterization of their expression during acclimation. As UCPs are capable of increasing proton leak across the inner mitochondrial membrane in stress conditions (Jastroch et al., 2005; Mark et al., 2006), quantitative expression studies or immunological Western Blot analysis could help to determine if UCP expression (gene- and protein-level) is affected by elevated PCO_2 and contributes to the putatively impaired mitochondrial capacities during hypercapnia.

Finally, the differences in mitochondrial capacities between Antarctic fish species showed that one cannot easily conclude from a single parameter such as mitochondrial capacities on a species' vulnerability to future climate change. Nevertheless, an increase in flux through Complex I of the ETS was found in Antarctic and temperate fish (Hilton et al., 2010) under energy-demanding conditions. Furthermore, an increased expression of the mitochondrial Complex I has been shown in CO_2 acclimated, temperate spider crabs (Schiffer et al. *in prep.*, Harms et al. *in prep.*). Thus, high Complex I capacities appear to be a general

feature to increase mitochondrial capacities. Further investigations on this topic, such as mitochondrial respiration experiments or gene expression studies, would need to more precisely characterize the role of the mitochondrial Complex I in acclimation processes to environmental challenges in both vertebrate and invertebrate species.

In addition to these analyses on adult fish or cephalopods, the sensitivity of early life stages should also be taken into account. Previous studies on temperate fish demonstrated a higher sensitivity of eggs and juveniles to chronic hypercapnia than in adults (Moran and Stottrup, 2010). The development period of Antarctic fish eggs and larvae usually takes several months (Mintenbeck et al., 2012), and nothing is known on the effect of changing environmental conditions on their development success. Future analyses should therefore include embryonic and larval development, and mortality data until and after hatching, under conditions of elevated seawater temperature and PCO_2. Such analyses could deepen the understanding on each species' population resilience and effects on the Antarctic fish community.

In summary, the results of this thesis showed that the vulnerability of particular fish or cephalopod species to climate change cannot easily be generalized by measuring single parameters such as mitochondrial capacities, although these organelles play a crucial role in aerobic energy metabolism. Measurements of whole-animal aerobic scope and of thermal limits would be necessary to elucidate the acclimation capacities of fish and cephalopods to long-term elevated temperature and PCO_2. Yet the results of this thesis project have successfully demonstrated that the integration of several physiological organisational levels can contribute to understanding the processes involved in warm- and hypercapnia acclimation processes of marine vertebrates and invertebrates.

7 References

Acin-Perez, R., Salazar, E., Kamenetsky, M., Buck, J., Levin, L. R. and Manfredi, G. (2009). Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation. *Cell Metab* **9**, 265-276.

Allcock, A. (2005). On the confusion surrounding *Pareledone charcoti* (Joubin, 1905) (Cephalopoda: Octopodidae): endemic radiation in the Southern Ocean. *Zool J Linn Soc* **143**, 75-108.

Arntz, W. E. (2005). The Magellan-Antarctic connection: links and frontiers at southern high latitudes. Summary review. *Sci Mar* **69**, 359.

Artegiani, A., Paschini, E., Russo, A., Bregant, D., Raicich, F. and Pinardi, N. (1997). The Adriatic Sea general circulation. Part I: Air–sea interactions and water mass structure. *J Phys Oceanogr* **27**, 1492-1514.

Battersby, B. J. and Moyes, C. D. (1998). Influence of acclimation temperature on mitochondrial DNA, RNA, and enzymes in skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **275**, R905-R912.

Beers, J. M. and Sidell, B. D. (2011). Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. *Physiol Biochem Zool* **84**, 353-362.

Bi, D., Budd, W. F., Hirst, A. C. and Wu, X. (2001). Collapse and reorganisation of the Southern Ocean overturning under global warming in a coupled model. *Geophys Res Lett* **28**, 3927-3930.

Bilyk, K. T. and DeVries, A. L. (2011). Heat tolerance and its plasticity in Antarctic fishes. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 382-390.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Appendix: Physicochemical parameters for use in fish respiratory physiology. *Fish Physiol* **XA**, 403-430.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Brand, M. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol* **35**, 811-820.

Brand, M., Chien, L., Ainscow, E., Rolfe, D. and Porter, R. (1994). The causes and functions of mitochondrial proton leak. *Biochim Biophys Acta* **1187**, 132.

Brand, M. D. (1995). Measurement of mitochondrial protonmotive force. Oxford, UK: Oxford University Press.

Brand, M. D. and Esteves, T. C. (2005). Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab* **2**, 85-93.

Brand, M. D., Harper, M. E. and Taylor, H. C. (1993). Control of the effective P/O ratio of oxidative phosphorylation in liver mitochondria and hepatocytes. *Biochem J* **291**, 739.

Brauner, C. J., Wang, T., Wang, Y., Richards, J. G., Gonzalez, R. J., Bernier, N. J., Xi, W., Patrick, M. and Val, A. L. (2004). Limited extracellular but complete intracellular acid-base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*. *J Exp Biol* **207**, 3381-3390.

Brix, O., Lykkeboe, G. and Johansen, K. (1981). The significance of the linkage between the Bohr and Haldane effects in cephalopod bloods. *Resp Physiol* **44**, 177-186.

Brix, O., Bårdgard, A., Cau, A., Colosimo, A., Condo, S. and Giardina, B. (1989). Oxygen-binding properties of cephalopod blood with special reference to environmental temperatures and ecological distribution. *J Exp Zool* **252**, 34-42.

Brodte, E., Knust, R. and Pörtner, H. O. (2006). Temperature-dependent energy allocation to growth in Antarctic and boreal eelpout (Zoarcidae). *Polar Biol* **30**, 95-107.

Brookes, P. S., Buckingham, J. A., Tenreiro, A. M., Hulbert, A. J. and Brand, M. D. (1998). The proton permeability of the inner membrane of liver mitochondria from ectothermic and endothermic vertebrates and from obese rats: correlations with standard metabolic rate and phospholipid fatty acid composition. *Comp Biochem Physiol B Biochem Mol Biol* **119**, 325-334.

Caldeira, K., Wickett, M. E. (2005). Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J Geophys Res* **110**.

Chamberlin, M. E. (2004). Top-down control analysis of the effect of temperature on ectotherm oxidative phosphorylation. *Am J Physiol Regul Integr Comp Physiol* **287**, R794-R800.

Cheng, C. H. C., Chen, L., Near, T. J. and Jin, Y. (2003). Functional antifreeze glycoprotein genes in temperate-water New Zealand nototheniid fish infer an Antarctic evolutionary origin. *Mol Biol Evol* **20**, 1897-1908.

Choe, K. P. and Evans, D. H. (2003). Compensation for hypercapnia by a euryhaline elasmobranch: effect of salinity and roles of gills and kidneys in fresh water. *J Exp Zool A Comp Exp Biol* **297**, 52-63.

Ciattaglia, L., Rafanelli, C., Araujo, J. and Rodriguez, H. (2008). Long-term measurements of the atmospheric carbon dioxide concentration measured at Jubany Station indicate a relationship with "El Nino". In *The Antarctic Ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo)*, vol. 571 (eds. C. Wiencke G. Ferreyra D. Abele and S. Marenssi), pp. 390-396. Ber. Polarforsch. Meeresforsch.

Clarke, A. (1991). What is cold adaptation and how should we measure it? *Amer Zool* **31**, 81-92.

Clarke, A. (1998). Temperature and energetics: an introduction to cold ocean physiology. In *Cold ocean physiology,* vol. 66 (eds. Pörtner, H. O., Playle, R. C.), pp. 3-32. Cambridge, United Kingdom: Cambridge University Press.

Clarke, A. and Johnston, I. A. (1996). Evolution and adaptive radiation of Antarctic fishes. *Trends Ecol Evol* **11**, 212-218.

Clarke, A., Murphy, E. J., Meredith, M. P., King, J. C., Peck, L. S., Barnes, D. K., Smith, R. C., Clarke, A., Murphy, E. J. and Meredith, M. P. (2007). Climate change and the marine ecosystem of the western Antarctic Peninsula. *Philos Trans R Soc Lond B Biol Sci* **362**, 149-166.

Collins, M. A. and Rodhouse, P. G. (2006). Southern ocean cephalopods. *Adva Mar Biol* **50**, 191-265.

Cook, A. J., Fox, A. J., Vaughan, D. G. and Ferrigno, J. G. (2005). Retreating glacier fronts on the Antarctic Peninsula over the past half-century. *Science* **308**, 541-544.

Costello, M. J., Emblow, C. and White, R. J. (2001). European register of marine species: a check-list of the marine species in Europe and a bibliography of guides to their

identification. In *Collection Patrimoines Naturels*, (eds. Costello M. J., Emblow C., White R. J.), France: Muséum national d'Histoire naturelle

Crockett, E. and Sidell, B. (1990). Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol Zool* **63**, 472-488.

Dahlhoff, E. A. and Somero, G. N. (1993). Effects of temperature on mitochondria from abalone (genus Haliotis): adaptive plasticity and its limits. *J Exp Biol* **185**, 151-168.

Daly, H. and Peck, L. (2000). Energy balance and cold adaptation in the octopus *Pareledone charcoti*. *J Exp Mar Biol Ecol* **245**, 197-214.

Dalziel, A. C., Moore, S. E. and Moyes, C. D. (2005). Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types. *Am J Physiol Regul Integr Comp Physiol* **288**, R163-R172.

Deigweiher, K., Koschnick, N., Pörtner, H. O. and Lucassen, M. (2008). Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. *Am J Physiol Regul Integr Comp Physiol* **295**, R1660-R1670.

Deigweiher, K., Hirse, T., Bock, C., Lucassen, M. and Pörtner, H. O. (2010). Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. *J Comp Physio. B, Biochem Syst Environ Physiol* **180**, 347-359.

DeVries, A. L. (1971). Glycoproteins as biological antifreeze agents in antarctic fishes. *Science* **172**, 1152-1155.

DeWitt, H. H., Heemstra, P. C. and Gon, O. (1990). Nototheniidae. In *Fishes of the Southern Ocean*, (eds. O. Gon and P. C. Heemstra), pp. 279—331. Grahamstown, South Africa: J. L. B. Smith Institute of Ichthyology.

Dickson, A. G. (2010). The carbon dioxide system in seawater: equilibrium chemistry and measurements. In *Guide for Best Practices in Ocean Acidification Researchand Data Reporting*, (eds. U. Riebesell V. J. Fabry and J. P. Gattuso), pp. 17-40.

Driedzic, W., Sidell, B., Stewart, J. and Johnston, I. (1990). Maximal activities of enzymes of energy metabolism in cephalopod systemic and branchial hearts. *Physiol Zool* **63**, 615-629.

Dunn, J. F., Archer, S. D. and Johnston, I. A. (1989). Muscle-fiber types and metabolism in post-larval and adult atages of notothenioid fish. *Polar Biol* **9**, 213-223.

Eastman, J. and DeVries, A. (1985). Adaptations for cryopelagic life in the Antarctic notothenioid fish *Pagothenia borchgrevinki*. *Polar Biol* **4**, 45-52.

Efremov, R. G., Baradaran, R. and Sazanov, L. A. (2010). The architecture of respiratory complex I. *Nature* **465**, 441-445.

Egginton, S. (1997). Control of tissue blood flow at very low temperatures. *J Therm Biol* **22**, 403-407.

Egginton, S. and Sidell, B. D. (1989). Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **256**, 1-9.

Egginton, S., Cordiner, S. and Skilbeck, C. (2000). Thermal compensation of peripheral oxygen transport in skeletal muscle of seasonally acclimatized trout. *Am J Physiol Regul Integr Comp Physiol* **279**, R375-388.

Egginton, S., Skilbeck, C., Hoofd, L., Calvo, J. and Johnston, I. A. (2002). Peripheral oxygen transport in skeletal muscle of Antarctic and sub-Antarctic notothenioid fish. *J Exp Biol* **205**, 769-779.

Enzor, L. A., Zippay, M. L. and Place, S. P. (2013). High latitude fish in a high CO_2 world: Synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids. *Comp Biochem Physiol A Mol Integr Physiol* **164**, 154-161.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation and excretion of nitrogenous waste. *Physiol Rev* **85**, 97-177.

Everson, I. (1969). Inshore fishes from the South Orkney and South Shetland Islands, the Antarctic Peninsula and South Georgia. *Br Ant Surv Bull* **19**, 89-96.

Falco, P. and Zambianchi, E. (2011). Near-surface structure of the Antarctic Circumpolar Current derived from World Ocean Circulation Experiment drifter data. *J Geophys Res* **116**, C05003.

Feely, R. A., Sabine, C. L., Lee, K., Berelson, W., Kleypas, J., Fabry, V. J. and Millero, F. J. (2004). Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science* **305**, 362-366.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497-509.

Franklin, C. E., Davison, W. and Seebacher, F. (2007). Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinki*. *J Exp Biol* **210**, 3068-3074.

Gnaiger, E. (2009). Capacity of oxidative phosphorylation in human skeletal muscle – New perspectives of mitochondrial physiology. *Int J Biochem Cell Biol* **41**, 1837-1845.

Gnaiger, E. (2012). Mitochondrial Pathways and Respiratory Control - An Introduction to OXPHOS Analysis. Innsbruck, Austria: OROBOROS MiPNet Publications

Gnaiger, E., Lassnig, B., Kuznetsov, A., Rieger, G. and Margreiter, R. (1998). Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome *c* oxidase. *J Exp Biol* **201**, 1129.

Gon, O. and Heemstra, P. (1990). Fishes of the Southern Ocean. Grahamstown, South Africa: J.L.B. Smith Institute for Ichthyology.

Gonzalez-Cabrera, P. J., Dowd, F., Pedibhotla, V. K., Rosario, R., Stanley-Samuelson, D. and Petzel, D. (1995). Enhanced hypo-osmoregulation induced by warm-acclimation in antarctic fish is mediated by increased gill and kidney Na^+/K^+-ATPase activities. *J Exp Biol* **198**, 2279.

Greco, G., Martino, G. and Tota, B. (1982). Further characterization of two mitochondrial populations in tuna heart ventricle. *Comp Biochem Physiol B Biochem Mol Biol* **71**, 71-75.

Grim, J., Miles, D. and Crockett, E. (2010). Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. *J Exp Biol* **213**, 445-452.

Guderley, H. (1998). Temperature and growth rates as modulators of the metabolic capacities of fish muscle. In *Cold ocean physiology*, vol. 3 (eds. H. O. Pörtner and R. C. Playle), pp. 58-87. Cambridge, United Kingdom: Cambridge University Press.

Guderley, H. (2004). Metabolic responses to low temperature in fish muscle. *Biol Rev* **79**, 409-427.

Guderley, H. and Johnston, I. (1996). Plasticity of fish muscle mitochondria with thermal acclimation. *J Exp Biol* **199**, 1311.

Guderley, H. and St-Pierre, J. (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *J Exp Biol* **205**, 2237.

Guderley, H., Leroy, P. H. and Gagné, A. (2001). Thermal acclimation, growth, and burst swimming of threespine stickleback: enzymatic correlates and influence of photoperiod. *Physiol Biochem Zool* **74**, 66-74.

Gutowska, M. A., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G. and Pörtner, H. O. (2009). Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. *J Comp Physiol* DOI 10.1007/s00360-009-0412-y.

Guynn, S., Scofield, M. and Petzel, D. (2002). Identification of mRNA and protein expression of the Na/K-ATPase a1-, a2-and a3-subunit isoforms in Antarctic and New Zealand nototheniid fishes. *J Exp Mar Biol Ecol* **273**, 15-32.

Hardewig, I., Peck, L. S. and Pörtner, H. O. (1999a). Thermal sensitivity of mitochondrial function in the Antarctic notothenioid *Lepidonotothen nudifrons*. *J Comp Physiol B* **169**, 597-604.

Hardewig, I., van Dijk, P. L., Moyes, C. D. and Pörtner, H. O. (1999b). Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish. *Am J Physiol* **277**, R508-516.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu Rev Physiol* **57**, 19-42.

Heise, K., Puntarulo, S., Pörtner, H. and Abele, D. (2003). Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. *Comp Biochem Physiol C* **134**, 79-90.

Henry, R. P. and Wheatly, M. G. (1992). Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *Am Zool* **32**, 407.

Hilton, Z., Clements, K. D. and Hickey, A. J. (2010). Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. *J Comp Physiol B, Biochem Syst Environ Physiol* **180**, 979-990.

Hochachka, P. (1994). Oxygen efficient design of cephalopod muscle metabolism. *Mar Freshw Behav Physiol* **25**, 61-67.

Hochachka, P. W. (1988). Channels and pumps—determinants of metabolic cold adaptation strategies. *Comp Biochem Physiol B* **90**, 515-519.

Hoegh-Guldberg, O., Mumby, P., Hooten, A., Steneck, R., Greenfield, P., Gomez, E., Harvell, C., Sale, P., Edwards, A. and Caldeira, K. (2007). Coral reefs under rapid climate change and ocean acidification. *Science* **318**, 1737-1742.

Hofmann, G. E. and Todgham, A. E. (2010). Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Ann Rev Physiol* **72**, 127-145.

Hudson, H., Brauer, P., Scofield, M. and Petzel, D. (2008). Effects of warm acclimation on serum osmolality, cortisol and hematocrit levels in the Antarctic fish, *Trematomus bernacchii*. *Polar Biol* **31**, 991-997.

Hulbert, A., Turner, N., Hinde, J., Else, P. and Guderley, H. (2006). How might you compare mitochondria from different tissues and different species? *J Comp Physiol B, Biochem Syst Environ Physiol* **176**, 93-105.

Iftikar, F. I., MacDonald, J. and Hickey, A. J. R. (2010). Thermal limits of portunid crab heart mitochondria: Could more thermo-stable mitochondria advantage invasive species? *J Exp Mar Biol Ecol* **395**, 232-239.

IPCC. (2007). Fourth Assessment Report of the Intergovernmental Panel on Climate Change
Climate Change 2007: The Physical Science Basis, (eds. Solomon, SD, Manning, M, Chen, Z, Marquis M, Averyt KB, Tignor M and Miller HL). Cambridge, UK: Cambridge University Press.

Jastroch, M., Wuertz, S., Kloas, W. and Klingenspor, M. (2005). Uncoupling protein 1 in fish uncovers an ancient evolutionary history of mammalian nonshivering thermogenesis. *Physiol Genomics* **22**, 150-156.

Jensen, F. B., Koldkjaer, P. and Bach, A. (2000). Anion uptake and acid-base and ionic effects during isolated and combined exposure to hypercapnia and nitrite in the freshwater crayfish, Astacus astacus. *J Comp Physiol B* **170**, 489-495.

Jereb, P. and Roper, C. (2005). Cephalopods of the world, an annotated and illustrated catalogue of cephalopod species known to date. Vol 1: Chambered nautiluses and sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae). *FAO species catalogue for fishery purposes*.

Johnston, I. (1987). Respiratory characteristics of muscle fibres in a fish (*Chaenocephalus aceratus*) that lacks haem pigments. *J Exp Biol* **133**, 415.

Johnston, I. (2003). Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. *Comp Biochem Physiol B Biochem Mol Biol* **136**, 701-713.

Johnston, I., Guderley, H., Franklin, C., Crockford, T. and Kamunde, C. (1994). Are mitochondria subject to evolutionary temperature adaptation? *J Exp Biol* **195**, 293.

Johnston, I. A., Calvo, J., Guderley, H., Fernandez, D. and Palmer, L. (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J Exp Biol* **201**, 1-12.

Kamler, E., Krasicka, B. and Rakusa-Suszczewski, S. (2001). Comparison of lipid content and fatty acid composition in muscle and liver of two notothenioid fishes from Admiralty Bay (Antarctica): an eco-physiological perspective. *Polar Biol* **24**, 735-743.

Keller, M., Sommer, A., Pörtner, H. O. and Abele, D. (2004). Seasonality of energetic functioning and production of reactive oxygen species by lugworm (*Arenicola marina*) mitochondria exposed to acute temperature changes. *J Exp Biol* **207**, 2529.

Kock, K. H. (1992). Antarctic fish and fisheries. Cambridge, UK: Cambridge University Press.

Körtzinger, A. (2010). Der globale Kohlenstoffkreislauf im Anthropozän. Betrachtung aus meereschemischer Perspektive. *Chemie in unserer Zeit* **44**, 118-129.

Kraffe, E., Marty, Y. and Guderley, H. (2007). Changes in mitochondrial oxidative capacities during thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins, phospholipids and their fatty acid compositions. *J Exp Biol* **210**, 149.

Krogh, A. (1914). The quantitative relation between temperature and standard metabolism in animals. *Int Z Phys Chem Biol* **1**, 491-508.

Kunzmann, A. (1991). Blood Physiology and Ecological Consequences in Weddell Sea Fishes (Antarctica) Ber. Polarforsch. 91.

Kurihara, H., Shimode, S. and Shirayama, Y. (2004). Effects of raised CO_2 concentration on the egg production rate and early development of two marine copepods (*Acartia steueri* and *Acartia erythraea*). *Mar Pollut Bull* **49**, 721-727.

Kurihara, H., Kato, S. and Ishimatsu, A. (2007). Effects of increased seawater pCO_2 on early development of the oyster *Crassostrea gigas*. *Aquat Biol* **1**, 91-98.

Kuznetsov, A., Veksler, V., Gellerich, F., Saks, V., Margreiter, R. and Kunz, W. (2008). Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nature Protoc* **3**, 965-976.

Lalli, C. M. and Parsons, T. R. (1997). Biological oceanography: an introduction: Butterworth-Heinemann.

Langenbuch, M. and Pörtner, H. O. (2002). Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid-base variables. *J Exp Biol* **205**, 1153-1160.

Lannig, G., Storch, D. and Pörtner, H. O. (2005). Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. *Polar Biol* **28**, 575-584.

Lannig, G., Eckerle, L. G., Serendero, I., Sartoris, F.-J., Fischer, T., Knust, R., Johansen, T. and Pörtner, H. O. (2003). Temperature adaptation in eurythermal cod (*Gadus morhua*): a comparison of mitochondrial enzyme capacities in boreal and Arctic populations. *Mar Biol* **142**, 589-599.

Larsen, B. K., Pörtner, H. O. and Jensen, F. B. (1997). Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Mar Biol* **128**, 337-346.

Lassnig, B., Stadlmann, S., Rieger, G., Haffner, B., Lemieux, H. and Gnaiger, E. (1998-2008). Selected Media and Chemicals. *Mitochondrial Physiology Network 3.2*, 1-8.

Lee, A. (2003). Lipid–protein interactions in biological membranes: a structural perspective. *Biochim Biophys Acta* **1612**, 1-40.

Lee, A. G. (2004). How lipids affect the activities of integral membrane proteins. *Biochim Biophys Acta* **1666**, 62-87.

Lee, P. (1994). Nutrition of cephalopods: fueling the system. *Mar Freshw Behav Physiol* **25**, 35-51.

Lemieux, H., Tardif, J., Dutil, J. and Blier, P. (2010). Thermal sensitivity of cardiac mitochondrial metabolism in an ectothermic species from a cold environment, Atlantic wolffish (*Anarhichas lupus*). *J Exp Mar Bio Ecol* **384**, 113-118.

Lowe, C. and Davison, W. (2006). Thermal sensitivity of scope for activity in *Pagothenia borchgrevinki*, a cryopelagic Antarctic nototheniid fish. *Polar Biol* **29**, 971-977.

Lucassen, M., Koschnick, N., Eckerle, L. G., Pörtner, H. O. (2006). Mitochondiral mechanisms of cold adaptation in cod (*Gadus morhua* L.) populations from different climatic zones. *J Exp Biol* **209**, 1410-1420.

Lutjeharms, J. (1990). The oceanography and fish distribution of the Southern Ocean. *Fishes of the Southern Ocean*, 6-27.

Mangold, K. (1983). *Eledone moschata*. In *Cephalopod Life Cycles*, vol. 1 (ed. P. Boyle), pp. 387-400. New York: Academic Press.

Mark, F., Lucassen, M. and Pörtner, H. (2006). Thermal sensitivity of uncoupling protein expression in polar and temperate fish. *Comp Biochem Physiol Part D Genomics Proteomics* **1**, 365-374.

Mark, F. C., Bock, C. and Pörtner, H. O. (2002). Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. *Am J Physiol Regul Integr Comp Physiol* **283**, R1254-1262.

Mark, F. C., Hirse, T. and Pörtner, H. O. (2005). Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. *Polar Biol* **28**, 805-814.

Mark, F. C., Lucassen, M., Strobel, A., Barrera-Oro, E., Koschnick, N., Zane, L., Patarnello, T., Pörtner, H. O. and Papetti, C. (2012). Mitochondrial function in Antarctic nototheniids with ND6 translocation. *PLoS ONE* **7**, e31860.

Matschiner, M., Hanel, R. and Salzburger, W. (2011). On the origin and trigger of the notothenioid adaptive radiation. *PLoS ONE* **6**, e18911.

Melzner, F. (2005). Systemic investigations on the physiology of temperature tolerance in the common cuttlefish *Sepia officinalis*. *PhD Thesis*, pp. 242. Bremen: University of Bremen.

Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H. O. (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.

Meredith, P., M., King and C., J. (2005). Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. Washington, DC, ETATS-UNIS: American Geophysical Union.

Mesa, M. L. and Vacchi, M. (2001). Age and growth of high Antarctic notothenioid fish. *Ant Sci* **13**, 227-235.

Metzger, R., Sartoris, F. J., Langenbuch, M. and Pörtner, H. O. (2007). Influence of elevated CO_2 concentrations on thermal tolerance of the edible crab Cancer pagurus. *J Therm Biol* **32**, 144-151.

Michaelidis, B., Spring, A. and Pörtner, H. O. (2007). Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. *Mar Biol* **150**, 1417-1429.

Michaelidis, B., Ouzounis, C., Paleras, A. and Pörtner, H. O. (2005). Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar Ecol Prog Ser* **293**, 109-118.

Mintenbeck, K., Barrera-Oro, E. R., Brey, T., Jacob, U., Knust, R., Mark, F. C., Moreira, E., Strobel, A. and Arntz, W. E. (2012). 5 - Impact of Climate Change on Fishes in Complex Antarctic Ecosystems. In *Advances in Ecological Research*, vol. 46 (eds. J. Ute and W. Guy), pp. 351-426. Burlington: Academic Press.

Mommsen, T. P. and Hochachka, P. W. (1981). Respiratory and enzymatic properties of squid heart mitochondria. *Eur J Biochem* **120**, 345-350.

Moran, D. and Stottrup, J. (2010). The effect of carbon dioxide on growth of juvenile Atlantic cod *Gadus morhua* L. *Aquatic Toxicol* **102**, 24-30-

Moran, O. and Melani, R. (2001). Temperature-dependent conduction properties in Arctic fish peripheral nerve. *Polar Biol* **24**, 9-15.

Moyes, C., Mathieu-Costello, O., Tsuchiya, N., Filburn, C. and Hansford, R. (1997). Mitochondrial biogenesis during cellular differentiation. *Am J Physiol Cell Physiol* **272**, C1345.

Moyes, C. D. (1996). Cardiac metabolism in high performance fish. *Comp Biochem Physiol A Physiol* **113**, 69-75.

Munday, P. L., Crawley, N. E. and Nilsson, G. E. (2009). Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Mar Ecol Prog Ser* **388**, 235-242.

Munday, P. L., McCormick, M. I. and Nilsson, G. E. (2012). Impact of global warming and rising CO2 levels on coral reef fishes: what hope for the future? *J Exp Biol* **215**, 3865-3873.

Murphy, J. and Mitchell, J. (1995). Transient response of the Hadley Centre coupled ocean-atmosphere model to increasing carbon dioxide. Part II: Spatial and temporal structure of response. *J Clim* **8**, 57-80.

Nilsson, G. E., Östlund-Nilsson, S. and Munday, P. L. (2010). Effects of elevated temperature on coral reef fishes: Loss of hypoxia tolerance and inability to acclimate. *Comp Biochem Physiol A Mol Integr Physiol* **156**, 389-393.

North, A. (1996). Locomotory activity and behaviour of the Antarctic teleost Notothenia coriiceps. *Mar Biol* **126**, 125-132.

O'Brien, K. M. (2011). Mitochondrial biogenesis in cold-bodied fishes. *J Exp Biol* **214**, 275-285.

O'Brien, K. M. and Sidell, B. D. (2000). The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. *J Exp Biol* **203**, 1287-1297.

O'Brien, K. M., Skilbeck, C., Sidell, B. D. and Egginton, S. (2002). Muscle fine structure may maintain the function of oxidative fibres in haemoglobinless Antarctic fishes. *J Exp Biol* **206**, 411-421.

O'Brien, K. M. and Mueller, I. A. (2010). The unique mitochondrial form and function of Antarctic channichthyid icefishes. *Integr Comp Biol* **50**, 993-1008.

O'Dor, R. and Webber, D. (1986). The constraints on cephalopods: why squid aren't fish. *Can J Zool* **64**, 1591-1605.

Oellermann, M., Pörtner, H. O. and Mark, F. C. (2012). Mitochondrial dynamics underlying thermal plasticity of cuttlefish (*Sepia officinalis*) hearts. *J Exp Biol* **215**, 2992-3000.

Olbers, D., Gouretski, V., Seiss, G. and Schroeter, J. (1992). Hydrographic Atlas of the Southern Ocean. Bremerhaven, Germany: Alfred Wegener Institute.

Oppenheimer, M. (1998). Global warming and the stability of the West Antarctic Ice Sheet. *Nature* **393**, 325-332.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F. et al. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.

Owen, O. E., Kalhan, S. C. and Hanson, R. W. (2002). The key role of anaplerosis and cataplerosis for citric acid cycle function. *J Biol Chem* **277**, 30409-30412.

Packard, A. (1972). Cephalopods and fish: the limits of convergence. *Biol Rev* **47**, 241-307.

Pakhomov, E., Bushula, T., Kaehler, S., Watkins, B. and Leslie, R. (2006). Structure and distribution of the slope fish community in the vicinity of the sub-Antarctic Prince Edward Archipelago. *J Fish Biol* **68**, 1834-1866.

Peck, L. S. (2005). Prospects for survival in the Southern Ocean: vulnerability of benthic species to temperature change. *Antarctic Science-Institutional Subscription* **17**, 497-508.

Peck, L. S. (2002). Ecophysiology of Antarctic marine ectotherms: limits to life. *Polar Biol* **25**, 31-40.

Peck, L. S., Massey, A., Thorne, M. A. S. and Clark, M. S. (2009). Lack of acclimation in *Ophionotus victoriae*: brittle stars are not fish. *Polar Biol* **32**, 399-402.

Pierrot, D., Lewis, E. and Wallace, D. W. R. (2006). MS Excel Program Developed for CO2 System Calculations. Oak Ridge, Tennessee Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy

Podrabsky, J. and Somero, G. N. (2006). Inducible heat tolerance in Antarctic notothenioid fishes. *Polar Biol* **30**, 39-43.

Porter, R. K., Hulbert, A. and Brand, M. D. (1996). Allometry of mitochondrial proton leak: influence of membrane surface area and fatty acid composition. *Am J Physiol Regul Integr Comp Physiol* **271**, R1550-R1560.

Pörtner, H. O. (1987). Contributions of Anaerobic Metabolism to Ph Regulation in Animal-Tissues - Theory. *J Exp Biol* **131**, 69-87.

Pörtner, H. O. (1990). Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Resp Physiol* **81**, 275-288.

Pörtner, H. O. (2001). Climate change and zemperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* **88**, 137-146.

Pörtner, H. O. (2002a). Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. *Comp Biochem Physiol* **133A**, 303-321.

Pörtner, H. O. (2002b). Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. *J Exp Biol* **205**, 2217-2230.

Pörtner, H. O. (2002c). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp Biochem Physiol* **132A**, 739-761.

Pörtner, H. O. (2006). Climate-dependent evolution of Antarctic ectotherms: An integrative analysis. *Deep Sea Res II* **53**, 1071-1104.

Pörtner, H. O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J Exp Biol* **213**, 881-893.

Pörtner, H. O. (2012). Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Mar Ecol Prog Ser* **470**, 273-290.

Pörtner, H. O. and Zielinski, S. (1998). Environmental constraints and the physiology of performance in squid. *S Afr J mar Sci* **20**, 207-221.

Pörtner, H. O. and Farrell, A. P. (2008). Physiology and climate change. *Science* **322**, 690-692.

Pörtner, H. O. and Lannig, G. (2009). Oxygen and capacity limited thermal tolerance. In *Fish Physiology,* Vol 27: Hypoxia (eds. J. G. Richards A. P. Farrell and C. Brauner), pp. 143-191. Burlington: Academic Press.

Pörtner, H. O., MacLatchy, L. M. and Toews, D. P. (1991). Acid-base regulation in the toad *Bufo marinus* during environmental hypoxia. *Respir Physiol* **85**, 217-230.

Pörtner, H. O., Finke, E. and Lee, P. G. (1996). Metabolic and energy correlates of intracellular pH in progressive fatigue of squid (*L. brevis*) mantle muscle. *Am J Physiol Regul Integr Comp Physiol* **271**, R1403-1414.

Pörtner, H. O., Reipschlager, A. and Heisler, N. (1998). Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *J Exp Biol* **201**, 43-55.

Pörtner, H. O., Hardewig, I. and Peck, L. S. (1999). Mitochondrial function and critical temperature in the Antarctic bivalve, *Laternula elliptica*. *Comp Biochem Physiol A* **124**, 179-189.

Pörtner, H. O., Mark, F. C. and Bock, C. (2004). Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. *Respir Physiol Neurobiol* **141**, 243-260.

Pörtner, H. O.. Langenbuch, M., and Michaelidis, B. (2005). Synergistic effects of temperature extremes, hypoxia, and increases in CO_2 on marine animals: From Earth history to global change. *J Geophys Res* **110**.

Pörtner, H. O., Van Dijk, P., Hardewig, I. and Sommer, A. (2000). Levels of metabolic cold adaptation: trade-offs in eurythermal and stenothermal ectotherms. In *Antarctic Ecosystems: Models for Wider Ecological Understanding* (eds. Davison, W, and Howard Williams, C, E), pp. 109-122. Christchurch: New Zeal. Nat. Sci.

Pörtner, H. O., Bickmeyer, U., Bleich, M., Bock, C., Brownlee, C., Melzner, F., Michaelidis, B., Sartoris, F. J. and Storch, D. (2010). Measurements of CO_2 sensitive processes: Studies of acid-base and regulation. In *Guide for Best Practices in Ocean Acidification Researchand Data Reporting*, (eds. U. Riebesell V. J. Fabry and J. P. Gattuso), pp. 137-166.

Precht, H., Christophersen, J., Hensel, H. and Larcher, W. (1973). Temperature and life. Berlin, Heidelberg: Springer-Verlag.

Pritchard, H., Ligtenberg, S., Fricker, H., Vaughan, D., Van den Broeke, M. and Padman, L. (2012). Antarctic ice-sheet loss driven by basal melting of ice shelves. *Nature* **484**, 502-505.

Reipschläger, A. and Pörtner, H. O. (1996). Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. *J Exp Biol* **199**, 1801-1807.

Robinson, B. H., Oei, J., Cheema-Dhadli, S. and Halperin, M. (1977). Regulation of citrate transport and pyruvate dehydrogenase in rat kidney cortex mitochondria by bicarbonate. *J Biol Chem* **252**, 5661-5665.

Robinson, E. and Davison, W. (2008). The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption. *Polar Biol* **31**, 317-326.

Rolfe, D. F. and Brand, M. D. (1996). Proton leak and control of oxidative phosphorylation in perfused, resting rat skeletal muscle. *Biochim Biophys Acta* **1276**, 45-50.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C. S., Wallace, D. W., Tilbrook, B. et al. (2004). The oceanic sink for anthropogenic CO_2. *Science* **305**, 367-371.

Schloss, I., Ferreyra, G. A., González, O., Atencio, A., Fuentes, V., Tosonotto, G., Mercuri, G., Sahade, R., Tatián, M. and Abele, D. (2008). Long term hydrographic conditions and climate trends in Potter Cove. In *The Potter Cove coastal ecosystem, Antarctica. Synopsis of research performed 1999-2006 at the Dallmann Laboratory and Jubany Station, King George Island (Isla 25 de Mayo)*. (eds. Wiencke, C., Ferreyra, G., Abele, D., Marenssi, S.). Ber. Polarforsch. Meeres.

Scholander, P. F., Flagg, W., Walters, V. and Irving, L. (1953). Climatic adaptation in arctic and tropical poikilotherms. *Physiol Zool* **26**, 67-92.

Seebacher, F., Davison, W., Lowe, C. J. and Franklin, C. E. (2005). A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. *Biol lett* **1**, 151-154.

Seibel, B. A. and Walsh, P. J. (2001). Potential Impacts of CO_2 Injection on Deep-Sea Biota. *Science* **294**, 319-320.

Selwyn, M. J. and Walker, H. A. (1977). Permeability of the mitochondrial membrane to bicarbonate ions. *Biochem J* **166**, 137.

Sidell, B. D., Driedzic, W., Stowe, D. and Johnston, I. (1987). Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. *Physiol Zool* **60**, 221-232.

Sidell, B. D. (1991). Physiological roles of high lipid content in tissues of Antarctic fish species. In *Biology of Antarctic Fish*, (eds. G. di Prisco B. Maresca and B. Tota), pp. 221-231. Berlin, Heidelberg: Springer Verlag.

Sidell, B. D. and O'Brien, K. M. (2006). When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. *J Exp Biol* **209**, 1791-1802.

Simpson, D. P. (1967). Regulation of renal citrate metabolism by bicarbonate ion and pH: observations in tissue slices and mitochondria. *J Clin Invest* **46**, 225.

Sloman, K. A., Mandic, M., Todgham, A. E., Fangue, N. A., Subrt, P. and Richards, J. G. (2008). The response of the tidepool sculpin, *Oligocottus maculosus*, to hypoxia in laboratory, mesocosm and field environments. *Com Biochem Physiol A Mol Integr Physiol* **149**, 284-292.

Smith, C. R., Grange, L. J., Honig, D. L., Naudts, L., Huber, B., Guidi, L. and Domack, E. (2012). A large population of king crabs in Palmer Deep on the west Antarctic Peninsula shelf and potential invasive impacts. *Proce R Soci B* **279**, 1017-1026.

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr Comp Biol* **42**, 780-789.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine ‚winners' and ‚losers'. *J Exp Biol* **213**, 912-920.

Somero, G. N. and DeVries, A. L. (1967). Temperature tolerance of some Antarctic fishes. *Science* **156**, 257-258.

Sommer, A. and Pörtner, H. O. (1999). Exposure of *Arenicola marina* to extreme temperatures: Adaptive flexibility of a boreal and a subpolar population. *Mar Ecol Prog Ser* **181**, 215-226.

Sommer, A. and Pörtner, H. O. (2002). Metabolic cold adaptation in the lugworm *Arenicola marina*: Comparison of a North Sea and a White Sea population. *Mar Ecol Prog Ser* **240**, 171-182.

Spicer, J. I., Raffo, A. and Widdicombe, S. (2007). Influence of CO_2-related seawater acidification on extracellular acid–base balance in the velvet swimming crab *Necora puber*. *Mar Biol* **151**, 1117-1125.

Steffensen, J. F. (2002). Metabolic cold adaptation of polar fish based on measurements of aerobic oxygen consumption: fact or artefact? Artefact! *Comp Biochem Physiol A Mol Integr Physiol* **132**, 789-795.

St-Pierre, J., Pierre-Mathieu, C. and Guderley, H. (1998). Relative contribution of quantitative changes in mitochondria to metabolic compensation during seasonal acclimatisation of rainbow trout *Oncorhynchus mykiss*. *J Exp Biol* **201**, 2961-2970.

Storey, K. and Storey, J. (1979). Octopine metabolism in the cuttlefish, *Sepia officinalis*: octopine production by muscle and its role as an aerobic substrate for non-muscular tissues. *J Comp Physiol B, Biochem Syst Environ Physiol* **131**, 311-319.

Tetens, V., Wells, R. M. G. and Devries, A. L. (1984). Antarctic fish blood: Respiratory properties and the effects of thermal acclimation. *J Exp Biol* **109**, 265-279.

Tresguerres, M., Buck, J. and Levin, L. R. (2010). Physiological carbon dioxide, bicarbonate, and pH sensing. *Pflugers Arch* **460**, 953-964.

Trigari, G., Pirini, M., Ventrella, V., Pagliarani, A., Trombetti, F. and Borgatti, A. (1992). Lipid composition and mitochondrial respiration in warm-and cold-adapted sea bass. *Lipids* **27**, 371-377.

Turner, J., Lachlan-Cope, T. A., Colwell, S., Marshall, G. J. and Connolley, W. M. (2006). Significant warming of the Antarctic winter troposphere. *Science* **311**, 1914-1917.

Turner, N., Hulbert, A. J. and Else, P. (2005). Sodium pump molecular activity and membrane lipid composition in two disparate ectotherms, and comparison with endotherms. *J Comp Physiol B* **175**, 77-85.

Tyler, S. and Sidell, B. D. (1984). Changes in mitochondrial distribution and diffusion distances in muscle of goldfish upon acclimation to warm and cold temperatures. *J Exp Zool* **232**, 1-9.

Urschel, M. and O'Brien, K. M. (2009). Mitochondrial function in Antarctic notothenioid fishes that differ in the expression of oxygen-binding proteins. *Polar Biol* **32**, 1323-1330.

Urschel, M. R. and O'Brien, K. M. (2008). High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis. *J Exp Biol* **211**, 2638.

von Boletzky, S. (1983). *Sepia officinalis*. In *Cephalopod Life Cycles* vol. 1 (ed. Boyle, P. R.). pp. 31-52. London: Academic Press.

Walther, K., Sartoris, F., Bock, C. and Pörtner, H. O. (2009). Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*. *Biogeosciences* **6**, 2837-2861.

Wanders, R. J. A., Mejer, A. J., Groen, A. K. and Tager, J. M. (1983). Bicarbonate and the pathway of glutamate oxidation in isolated rat-liver mitochondria. *European J Biochem* **133**, 245-254.

Ward, P. (1989). The distribution of zooplankton in an Antarctic fjord at South Georgia during summer and winter. *Ant Sci* **1**, 141-150.

Weinstein, R. B. and Somero, G. N. (1998). Effects of temperature on mitochondrial function in the Antarctic fish *Trematomus bernacchii*. *J Comp Physiol B, Biochem Syst Environ Physiol* **168**, 190-196.

Wells, M. (1992). The cephalopod heart: The evolution of a high-performance invertebrate pump. *Cell Mol Life Sci* **48**, 800-808.

Wilson, R., Kuchel, L., Franklin, C. and Davison, W. (2002). Turning up the heat on subzero fish: thermal dependence of sustained swimming in an Antarctic notothenioid. *J Therm Biol* **27**, 381-386.

Windisch, H. S., Kathöver, R., Pörtner, H.-O., Frickenhaus, S. and Lucassen, M. (2011). Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways. *Am J Physiol Regul Integr Comp Physiol* **301**, R1453-R1466.

Wittmann, A. C., Portner, H. O. and Sartoris, F. J. (2012). A role for oxygen delivery and extracellular magnesium in limiting cold tolerance of the sub-antarctic stone crab *Paralomis granulosa*? *Physiol Biochem Zool* **85**, 285-298.

Wodtke, E. (1981). Temperature adaptation of biological membranes: Compensation of the molar activity of cytochrome *c* oxidase in the mitochondrial energy-transducing membrane during thermal acclimation of the carp (*Cyprinus carpio* L.). *Biochim Biophys Acta* **640**, 710-720.

Zhuang, X. and Cheng, C. H. (2010). ND6 gene "lost" and found: evolution of mitochondrial gene rearrangement in Antarctic notothenioids. *Mol Biol Evol* **27**, 1391-1403.

Zippin, J. H., Levin, L. R. and Buck, J. (2001). CO_2/HCO_3^--responsive soluble adenylyl cyclase as a putative metabolic sensor. *Trends Endocrinol Metab* **12**, 366-370.

8 Appendix

8.1 Substrate-inhibitor protocols applied in mitochondrial respiration experiments

Table 8.1 Concentration, meaning and respiratory state induced by substrates and inhibitors in permeabilised heart fibre respiration experiments with notothenioids

Substance	Stock conc. [mM]	Final conc. [mM]	Function	Respiratory state
Glutamate	2000	10	CI substrate	Leak CI, state II
Malate	2000	2	CI substrate	Leak CI, state II
pyruvate	2000	10	CI substrate	Leak CI, state II
ADP	500	2.5	Substrate for ATP generation	OXPHOS, state III
Succinate	2000	10	CII substrate	OXPHOS CI, CII (State III)
Cytochrome C	4	0.01	Electron carrier	Test for membrane integrity
Oligomycin	4 mg/ml	4 µg/ml	Inhibits ATPsynthase, proton leak flux	L_{Omy}, State IV^{+}
FCCP	2	Saturating	Uncoupler, relieves the proton gradient	*E* (State III*u*, ETS)
Rotenone	2000	0.005	CI inhibitor, test for flux via CI	State III$u_{\text{ Rot}}$
Malonate	2000	5	CII inhibitor, test for flux via CII	State III$u_{\text{ Rot Malo}}$
Antimycin A	5	0.0025	CIII inhibitor, test for residual oxygen consumption	ROX
Ascorbate	800	2	CIV substrate	Maximum COX respiration
TMPD	200	0.5	CIV substrate	

FCCP: carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone
TMPD: *N,N,N',N'*-tetramethyl-*p*-phenylendiamine dihydrochloride

Tabel 8.2 Concentration, meaning and respiratory state induced by single substrates and inhibitors in heart fibres respiration experiments on cephalopods

Substance	Stock conc. [mM]	Final conc. [mM]	Function	Respiratory state
Proline	2000	5	CI (amino acid) substrate	Leak CI, state II
Pyruvate	2000	15	CI (carbohydrate) substrate	Leak CI, state II
ADP	500	2.5	Substrate for ATP generation	OXPHOS, state III
Succinate	2000	5	CII substrate	OXPHOS CI, CII (State III)
Cytochrome C	4	0.01	Electron carrier	Test for membrane integrity
Oligomycin	4 mg/ml	4 µg/ml	Inhibits ATPsynthase, proton leak flux	L_{Omy}, State IV^{+}
FCCP	2	Saturating	Uncoupler, relieves the proton gradient	*E* (State III*u*, ETS)
Rotenone	2000	0.005	CI inhibitor, test for flux via CI	State IIIu_{Rot}
Malonate	5	3.5	CII inhibitor, test for flux via CII	State III$u_{\text{Rot Malo}}$
Antimycin A	5	0.0025	CIII inhibitor, test for residual oxygen consumption	ROX
Ascorbate	800	2	CIV substrate	Maximum COX respiration
TMPD	200	0.5	CIV substrate	

FCCP: carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone
TMPD: *N,N,N',N'*-tetramethyl-*p*-phenylendiamine dihydrochloride

Table 8.3 Concentration, function and respiratory state induced by substrates and inhibitors in isolated liver mitochondrial respiration experiments conducted with *N. rossii*

Substance	Stock conc. [mM]	Final conc. [mM]	Function	Respiratory state
Malate	800	1.6	CI (carbohydrate) substrate	Leak CI, state II
Pyruvate	2000	2	CI (carbohydrate) substrate	Leak CI, state II
ADP	100	0.2	Substrate for ATP generation	OXPHOS, state III
Rotenone	5	0.01	CI inhibitor, test for flux via CI	State III u_{Rot}
Succinate	1000	2	CII substrate	OXPHOS CII (State III)
Oligomycin	1 mg/ml	2	Inhibits ATPsynthase, proton leak respiration	State IV
Malonate	100	4x0.05	CII inhibitor, proton leak titration	State IV_{Malo}
FCCP	300	0.05	Uncoupler, relieves the proton gradient	*uncoupled*

FCCP: carbonylcyanide-*p*-(trifluoromethyl) phenylhydrazone

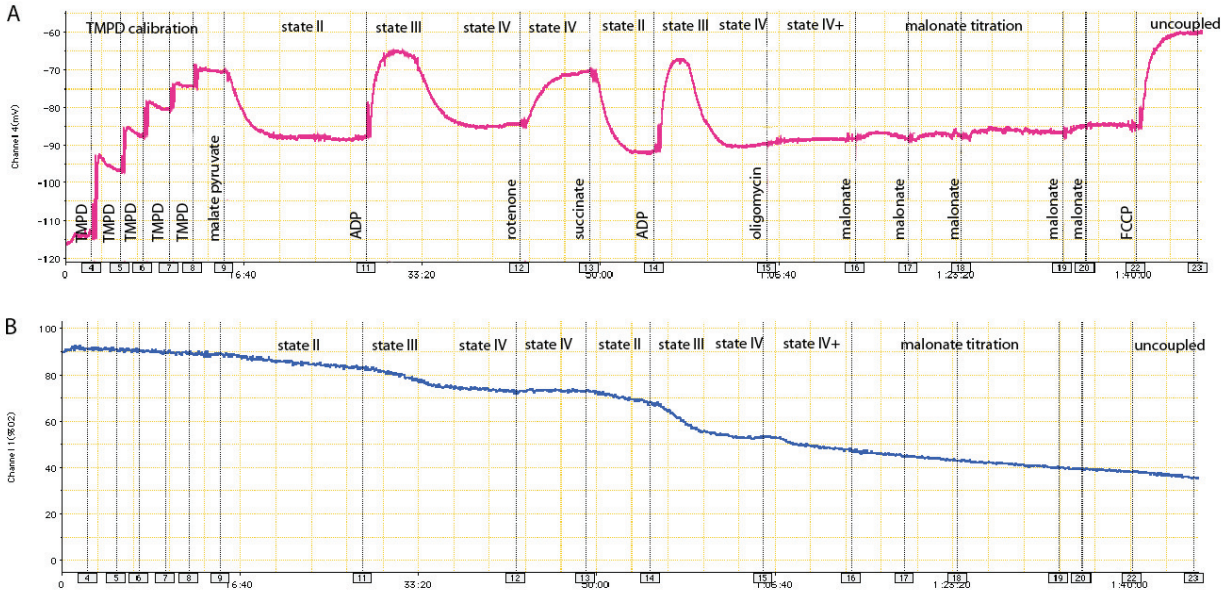


Figure 8.1 Representative mitochondrial respiration trace of isolated fish liver mitochondria. A: Mitochondrial membrane potential (ψ, purple line), B: Slope of declining oxygen concentration (blue line; in percent, starting at 100% O_2 within the respiration chamber) over time (min). The respiratory states that are induced by the respective substrates and inhibitors are displayed in panel A, see Table 8.3 for details. TPMP: triphenylmethylphosphonium. Mitochondrial membrane potential was measured according to Brand (1995) after initial calibration of TPMP reference electrodes by five to six subsequent additions of TPMP (1 mM stock) to a final concentration of 5-6 µM. Note: Data of

the membrane potential measurements are not presented in publications I – V; the mitochondrial respiration data are presented in publication I and II.

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Mitochondrial Function in Antarctic Nototheniids with *ND6* Translocation

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Abstract

Fish of the suborder Notothenioidei have successfully radiated into the Southern Ocean and today comprise the dominant fish sub-order in Antarctic waters in terms of biomass and species abundance. During evolution in the cold and stable Antarctic climate, the Antarctic lineage of notothenioids developed several unique physiological adaptations, which make them extremely vulnerable to the rapid warming of Antarctic waters currently observed. Only recently, a further phenomenon exclusive to notothenioid fish was reported: the translocation of the mitochondrial gene encoding the NADH Dehydrogenase subunit 6 (*ND6*), an indispensable part of complex I in the mitochondrial electron transport system. This study investigated the potential physiological consequences of *ND6* translocation for the function and thermal sensitivity of the electron transport system in isolated liver mitochondria of the two notothenid species *Notothenia coriiceps* and *Notothenia rossii*, with special attention to the contributions of complex I (NADH DH) and complex II (Succinate DH) to oxidative phosphorylation. Furthermore, enzymatic activities of NADH:Cytochrome c Oxidoreductase and Cytochrome C Oxidase were measured in membrane-enriched tissue extracts. During acute thermal challenge (0–15°C), capacities of mitochondrial respiration and enzymatic function in the liver could only be increased until 9°C. Mitochondrial complex I (NADH Dehydrogenase) was fully functional but displayed a higher thermal sensitivity than the other complexes of the electron transport system, which may specifically result from its unique amino acid composition, revealing a lower degree of stability in notothenioids in general. We interpret the translocation of *ND6* as functionally neutral but the change in amino acid sequence as adaptive and supportive of cold stenothermy in Antarctic notothenid. From these findings, an enhanced sensitivity to ocean warming can be deduced for Antarctic notothenioid fish.

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Introduction

Antarctic marine life is broadly associated with successful metabolic adaptation to a cold environment, where low temperature and higher oxygen solubility in water are strong selective factors and are paralleled by a specialisation of animals to limited thermal windows [1]. Fish living in this environment are considered highly stenothermal, and their adaptations have been verified at the molecular, cellular, tissue, and organismal hierarchical organisation levels [2,3,4,5]. In this respect, the Antarctic lineage of the perciform suborder Notothenioidei that radiated from a single ancestral benthic fish group within the Southern Ocean displays the most remarkable adaptations, most notably in the gain of antifreeze glycoproteins [6,7], the loss of haemoglobin and red blood cells in the derived icefish family (Channichthyidae) [8], and the loss of myoglobin in six species within the channichtids [9].

Besides a cold adapted metabolism [10,11,12] and concomitant high stenothermy in this group [13,14,15,16], some metabolic plasticity and level of acclimation is possible: warm acclimation can lead to a shift of heat tolerance limits to higher temperatures [17,18] and involve metabolic compensation [19] at the expense of reduced performance at low temperatures [20]. Similarly, long-term warm acclimation of the Antarctic eelpout *P. brachycephalum* involves metabolic rearrangements [21] and indicates an improvement of hepatic metabolism accompanied by a shift of energy sources [22,23]. In this context, studies of energy allocation in isolated cells of Antarctic notothenioids have suggested that within a thermal range of about −1°C to 12°C, thermal tolerance limits are defined at whole organism level, e.g. by capacity limitations of the circulatory system rather than by a general failure of cellular energy metabolism [24,25].

A number of studies have investigated general mitochondrial functionality and capacities in Antarctic fish [26,27,28,29] as key functional traits in thermal acclimatisation and adaptation as they mediate the integration of molecular adaptations into higher functional levels and reflect the energy demand of cells, tissues and organisms under given environmental conditions [30]. Consisting of some 1500 proteins, mitochondria are complex organelles that besides producing ATP through oxidative phosphorylation are

involved in a broad array of cellular functions [31], such as calcium storage, apoptosis regulation [32], amino acid and haeme biosynthesis [33], and production of nitric oxide and oxygen radicals. In ectotherms, this complex enzymatic system is strongly influenced by temperature [34].

Studies of mitochondrial capacities in Antarctic notothenioids revealed extremely high mitochondrial densities [35,36,37]. Their liver mitochondria were found highly coupled at low capacities and low levels of proton leakage rates, indicating low costs of mitochondrial maintenance [38,39]. Recent studies demonstrated some thermal plasticity with elevated capacities of respiratory chain components upon warm acclimation of Antarctic eelpout that indicate different patterns of warm acclimation and the use of metabolic pathways different than those of temperate fish [23].

Less is known, however, about the biochemical mechanisms that shape adaptation of mitochondrial respiratory activity to low and stable temperatures. The effects of temperature on oxygen consumption rates and on the coupling efficiency between electron transport and ATP synthesis in mitochondria of *Trematomus bernacchii* revealed an unusual sensitivity to temperature in line with strong adaptation to cold and high stenothermy at the whole organism level [26]. How this high level of mitochondrial adaptation evolved, has only rarely been studied. In this context, a translocation of the mitochondrial genes encoding NADH dehydrogenase subunit 6 (*ND6*) and the adjacent $tRNA^{Glu}$ has recently been reported [40,41] for some high-Antarctic notothenioids, with an unclear functional background. *ND6* is seen as an indispensable part of complex I (CI, NADH dehydrogenase; or NADH:quinone oxidoreductase; EC 1.6.5.3) of the mitochondrial electron transport system (ETS). Complex I is the largest (~1 MDa) and least understood component of the ETS [42], which provides about 40% of the proton-motive force required to synthesise ATP in vertebrates [43]. Only 7 of the approximately 45 constituent subunits are encoded by mitochondrial genes, namely *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6* [44]. Complex I deficiencies and mutations can lead to numerous severe diseases in humans [45], for example, in Parkinson's disease. In zebrafish, the most common gene mutation during onset of Parkinson in humans leads to a reduced function of complex I [46]. Especially *ND4* and *ND6* are essential subunits that ensure correct integration of other subunits into complex I [47,48,49]. The translocation of the *ND6* and $tRNA^{Glu}$ genes appears to have occurred only in the more derived five high-Antarctic notothenioid families, among those the Nototheniidae, whereas the basal non-Antarctic families (e.g. Eleginopidae) possess the canonical mitochondrial genome arrangement as commonly found in fish [40,41]. Along with the translocation of the *ND6* gene, the amino acid sequence of *ND6* has been under evolutionary selection and changed considerably [41]. It has therefore been speculated whether complex I in notothenioid fish was functionally modified [40] or impaired after the translocation of the *ND6* and $tRNA^{Glu}$ genes [41]. Zhuang and Cheng [41] detected signals indicating positive selection within control regions containing the translocated *ND6* gene in the Antarctic notothenioid clade, and selection on several residues within *ND6* genes, suggesting diversifying adaptive change of the protein. They suggested *ND6* modifications may (I) improve protein conformation and therefore complex I subunit interactions at subzero temperatures, and/or (II) a role in modulating mitochondrial complex I redox potential and reactive oxygen species (ROS) production. As ROS production has been attributed to complex I [50,51], the down regulation of complex I activity under thermal stress could alter reactive oxygen species production. The functional consequences of positioning and the structural change in nototheniid *ND6* have never been analysed, and therefore form the central focus of this study.

We addressed the function and thermal sensitivities of the ETS with particular emphasis on the relative contributions of complex I and complex II (CII, succinate dehydrogenase; EC 1.3.5.1) and the enzymatic capacities of selected proteins in two Antarctic nototheniids. At different temperatures from 0 to 15°C, we measured the oxygen consumption rates and membrane potential in isolated liver mitochondria respiring on several substrates. In addition, we determined enzymatic capacities (NADH dehydrogenase; cytochrome c oxidase, COX) within membrane fraction enriched protein extracts. We further compared the amino acid content of eight notothenioid and non-notothenioid fish species from sub-Antarctic, temperate, tropical and Arctic waters in order to identify possible differences that may provide notothenioid *ND6* with unique biochemical properties and underline their adaptation to the cold.

As behavioural and morphological differences between species can also relate to mitochondrial plasticity and capacities, we compared two endemic and sympatric Antarctic nototheniid species from King George Island (South Shetland Islands), *Notothenia coriiceps* (yellowbelly rockcod) and *Notothenia rossii* (marbled rockcod). Both species have a wide circum-Antarctic distribution, particularly in shelf areas of the Scotia Arc [52], extending to the Antarctic continental shelf in the case of *N. coriiceps*. Functional capacities may differ according to lifestyle requirements [53], which in turn contribute to defining the width of the thermal tolerance window [54]. The two selected species show different adaptations to life in the water column or in benthic habitats, in line with their respective external morphologies: *N. coriiceps* is demersal and sedentary [55], undergoes winter dormancy associated with metabolic suppression [56], and feeds mainly on benthic organisms. *N. rossii* is semipelagic and migratory, and in addition feeds on water column prey during the summer months [57,58].

Materials and Methods

2.1 Ethics statement

All work on fish was carried out according to the ethics and guidelines of German law. The experiments in this study have been approved according to § 8 Tierschutzgesetz (18.05.2006; 8081. I p. 1207) by the ethics committee of the Senatorin für Arbeit, Frauen, Gesundheit, Jugend und Soziales, Abt. Veterinärwesen, Lebensmittelsicherheit und Pflanzenschutz, Bahnhofsplatz 29, 28195 Bremen, Germany, under the permit number Az.: 522-27-11/02-00 (93) on Jan 15th, 2008 (permit valid until Jan 14th 2012).

2.2 Animal capture and handling

Specimens of *Notothenia coriiceps* (Richardson) and *Notothenia rossii* (Richardson) were caught in Potter Cove, Isla 25 de Mayo/King George Island (62°14′S; 058°41′W) by means of baited traps and trammel nets operated from rubber boats in February and March 2009. The traps were 124 cm long, 64 cm wide and 56 cm high, the mesh size was 25 mm, and the opening 240 by 100 mm wide. Trammel nets were 15 m long, the inner mesh was 25 mm. Traps were deployed in depths ranging from 5 to 25 m, water temperature was 1.72±0.13°C and salinity 34.03±0.07 throughout this period. Fish ranged between 30.0 and 36.5 cm standard length (mean: 33.75±2.8 cm, all errors presented as standard deviation of the mean, SD) and 856±251 g weight for *N. coriiceps* and between 30.0–36.5 cm standard length (33.0±2.1 cm) and

601±100 g weight for *N. rossii*. Species were identified morphologically [59].

Fish were kept in several flow-through aquaria systems of about 600 l at Dallmann Laboratory facilities, Jubany Base, King George Island, at 1.0±0.5°C, >90% O_2 saturation and ambient seawater salinity for at least one week before experimentation.

The animals (n = 10 for each species) were anaesthetised with 0.5 g/l tricaine methane sulphonate (MS 222), blood samples were taken from the caudal vein for further analysis and the liver was excised and stored on ice. The animals were then killed by severing their spinal cord behind the head plates. Blood lactate levels were measured in 20 µl fresh blood with an Accutrend Lactate Analyser (Roche Diagnostics, Germany), haematocrit (Hct) was determined with a Hct microcentrifuge (Compur Microspin M1100, Bayer Diagnostic, Germany).

2.3 Mitochondrial isolation

After dissection, the liver was cleaned from blood and total liver weight was taken. A small subsample of 100–200 mg liver tissue was instantaneously frozen in liquid nitrogen for later enzymatic essays, the remaining tissue was weighed and washed in 5 ml/g tissue ice-cold wash buffer (80 mM sucrose, 85 mM KCl, 5 mM EGTA, 5 mM EDTA, 50 mM HEPES, pH 7.1 at 20°C). Subsequently, the tissue was put into 5 ml/g ice-cold isolation buffer (wash buffer+1% w/v fatty acid free BSA, 1 µg/ml aprotinin) and finely minced with scissors. The mixture was then put into a 30 ml Potter-Elvehjem glass homogenizer (VWR International, Germany) and slowly homogenised with three strokes at 80 revolutions/minute. The homogenate was centrifuged (1,300 g, 12 min, 2°C), the supernatant collected and the pellet resuspended in ice-cold isolation buffer and homogenized and centrifuged a second time. Supernatants were then joined and centrifuged (10,500 g, 10 min, 2°C). The supernatant was discarded and the pellet resuspended in ice-cold assay buffer (80 mM sucrose, 85 mM KCl, 5 mM KH_2PO_4, 50 mM HEPES, 1% w/v fatty acid free BSA, 1 µg/ml aprotinin, pH 7.1 at 20°C) at a dilution of 1 ml/g initial weight. This stock solution was kept on ice and away from light.

2.4 Mitochondrial respiration analysis

A duplicate analysis of each mitochondrial extract was conducted in 2 thermostatted perspex respiration chambers of 3 ml volume (World Precision Instruments, Inc., USA), equipped with an adjustable stopper and ports for triphenylmethylphosphonium (TPMP) and reference electrodes, as well as a titration port for metabolites and inhibitors and one for a TX micro-optode (Presens GmbH, Germany). Micro-optodes were used for fluoroptic measurement of PO_2, membrane potential was measured with a TPMP electrode [60] and a Dri-Ref reference electrode (World Precision Instruments, Inc., USA). The electrodes were connected to a PHM220 voltmeter (Radiometer analytical, France). The voltage output was recorded simultaneously with the oxygen traces by means of a PowerLab recording unit connected to a laptop computer running Chart v5.5.6 software (ADInstruments GmbH, Germany).

Measurements were carried out in assay buffer in a volume of 1.5 ml with mitochondrial concentrations adjusted to about 3 mg mitochondrial protein per ml, at 0, 3, 6, 9, 12 and 15±0.1°C, respectively. Mitochondrial membrane potential was measured as mitochondrial proton-motive force according to Brand [60], with an initial addition of nigericin (80 ng/ml) to clamp ΔpH to zero and six subsequent additions of TPMP (1 mM stock) to a final concentration of 6 µM.

Initial respiration and potential were recorded and malate and glutamate added to a final saturating concentration of 1 mM and 1.3 mM, respectively, as substrates providing NADH for complex I. Then ADP (final concentration 0.1 mM) was added and state III (stIII, maximum slope) and state IV (stIV, ADP depleted) respiration and potential recorded. After that, complex I was inhibited with 0.01 mM rotenone (state IV_{Rot}) and state II respiration (stII) of complex II activated with $FADH_2$ provided by the addition of 2 mM succinate. To record state III and IV again, 0.2 mM ADP were added. State IV^+ was initiated by 1.3 µg/ml oligomycin. Finally, mitochondria were uncoupled with 0.6 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).

2.5 Enzymatic assays

Functional capacities of the NADH:cytochrome c oxidoreductase (complex I&III) and cytochrome c oxidase (complex IV, COX) were determined in membrane preparations of liver extracts. Proteins were extracted from liver tissue by homogenisation of frozen tissue in 10 vol. ice-cold extraction buffer (30 mM Tris/HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, protease inhibitor cocktail for animal tissue (Sigma, Germany)) with a glass homogenizer followed by three treatments with an ultra-turrax (IKA Labortechnik, Germany) for 10 s and intermediate cooling in ice water. Cellular debris was removed by 10 min centrifugation at 1,000 g and 2°C. The supernatant was carefully transferred into a new tube, avoiding co-transferring the upper lipid layer present in the liver preparations. The supernatant was then centrifuged at 218,000 g for 45 minutes. The supernatant was removed and tested for residual activities; the pellet (total membrane fraction) was suspended in 1/5 of starting buffer volume.

All enzyme measurements were conducted in a thermostatted spectrophotometer (Beckman, Fullerton, CA, USA) at 0, 3, 6, 9, 12, 15°C. COX activity was determined according to a protocol modified from Moyes et al. [61] with 2–10 µl of membrane suspension in 1 ml containing 20 mM Tris/HCl, pH 8.0, 0.05% Tween 20 and 0.05 mM reduced cytochrome c. The decrease in absorbance at 550 nm through oxidation of cytochrome c (E_{550} = 19.1 mM^{1} cm^{1}) was followed over time. The oxidation of NADH by complex I was followed by monitoring the transfer of electrons to oxidised cytochrome c thus representing the overall capacities of complex I and III, based on a protocol by Möller and Palmer [62] in 25 mM imidazole/HCl, pH 7.4, 125 mM sucrose, 2 mM $MgCl_2$, 4 mM sodium azide and 80 µM oxidised cytochrome c. The reaction was started by adding 0.2 mM NADH. The increase in absorbance at 550 nm through reduction of cytochrome c was followed over time.

Protein content was measured in all cellular fractions according to Bradford [63] using a bovine serum albumin (BSA) standard. For mitochondrial protein, the protein content of the assay buffer was considered.

2.6 Analysis of amino acid composition

For a comparison of *ND6*, *ND2* and *COI* (cytochrome c oxidase I) structures among eight notothenioid and non-notothenioid fish species from sub-Antarctic, temperate, tropical and Arctic waters, amino acid sequences were retrieved from GenBank (Table S1 for GenBank ID and species names). The amino acid compositions and the instability indexes for each species were computed from the protein sequence using the Expasy ProtParam prediction server (http://us.expasy.org/tools/protparam.html, [64]).

2.7 Statistics

Statistical analyses of differences among treatments by ANOVA, ANCOVA, regression analysis and Student's t-tests

were carried out using Prism v5.0b, InStat v3.0b (GraphPad Software, Inc.) and Sigma Stat v3.5 (Systat Software, Inc.). The specific test used is given in the respective legend. Differences were considered significant if $p<0.05$. All data are presented as means ± standard error of the mean (SEM), unless stated otherwise.

Results

3.1 Animal parameters

At the time of sampling, fish of both species were of the same size and weight range, but displayed different hepatosomatic indices (liver weight/body weight, HSI) of 2.22±0.33% (*N. coriiceps*) and 1.57±0.06% (*N. rossii*), respectively. Condition factors (weight*100/(standard length)^3; CF) also differed significantly between the species with 2.18±0.1 for *N. coriiceps* and 1.67±0.04 for *N. rossii*. Haematocrit (Hct) was significantly higher in *N. coriiceps* (30.6±1.1) than in *N. rossii* (27.5±0.8; $p=0.034$). Blood lactate levels were also higher in *N. coriiceps* (1.7±0.2) than in *N. rossii* (1.0±0.1 mM; $p=0.004$).

3.2 Mitochondrial respiration

In *N. coriiceps* (fig. 1a) and *N. rossii* (fig. 1b), mitochondrial state III respiration rose with temperature and clearly comprised of contributions by complex I (25–50%) and complex II (50–75%). For *N. coriiceps*, thermal effects on respiration became significant at 9 (CI) and 12°C (CII), respectively, when respiration rates were significantly higher than control respiration at habitat temperature (0°C). In *N. rossii*, mitochondrial respiration reached significantly increased levels already at and above 6°C, which in this species marked the maximum mitochondrial respiratory activity (fig. 1b).

Q_{10} values for total state III respiration were greatest between habitat temperature (0°C) and 3°C ($Q_{10}=9.61\pm2.39$ in *N. coriiceps*; 4.38±1.47 in *N. rossii*), levelling off to values around 3 after 6°C in *N. coriiceps* (significantly lower for 6, 12 & 15°C) and around 2 at 12°C and above in *N. rossii* (fig. 1b). For the whole thermal range of 0–15°C, Q_{10} was 2.60±0.47 for *N. coriiceps* and 1.71±0.33 for *N. rossii*.

Ratios of complex I over complex II contributions to state III respiration are depicted in figure 2. For *N. coriiceps* the ratio was about 0.5 between 0 and 9°C, at temperatures above the ratio decreased significantly, displaying a lower fraction of CI in mitochondrial metabolism at 12 and 15°C. In contrast, *N. rossii* mitochondria showed a slight trend to increasing contributions of CI with rising temperatures.

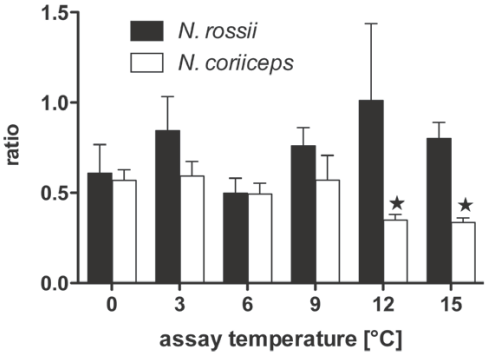

Figure 2. Complex I/complex II ratios of mitochondrial state III respiration for *N. coriiceps* and *N. rossii*. Bars (white for *N. coriiceps*, grey for *N. rossii*) depict means ± SEM, n=7. Star symbols indicate significant differences from data at 0°C.
doi:10.1371/journal.pone.0031860.g002

Figure 3 illustrates an Arrhenius plot for state III respiration in mitochondria from both *N. coriiceps* and *N. rossii*. In *N.* coriiceps, average activation energy for state III respiration over the whole thermal range was 58 kJ * mol^{-1}, starting with 85 kJ*mol^{-1} between 0 and 9°C and then levelling off to about 10 kJ * mol^{-1} between 9 and 15°C in *N. coriiceps*. *N. rossii* showed an overall activation energy of 59 kJ * mol^{-1} (0–9°C), starting with 84 kJ * mol^{-1} between 0 and 6°C and levelling off to about 10 kJ * mol^{-1} between 9 and 15°C.

Respiratory Control Ratios (RCR (stIII/IV)and RCR$^+$ (stIII/IV$^+$ and stIII/IV$_{Rot}$, respectively)) were stable over the experimental thermal range. Mean RCR$^+$ were 5.57±0.33 (CI) and 5.49±1.2 (CII) for *N. coriiceps*; and 4.77±0.59 (CI) and 4.88±0.46 (CII) for *N. rossii* (further RCR data presented in Table S4). Thus, maximum proton leak rates (involving CI and CII) accounted for about 18% of the physiological oxidative capacities in *N. coriiceps*, and for about 21% in *N. rossii*.

Although Acceptor Control Ratios (ACR, the dependence of the O_2 consumption rate on the P_i acceptor ADP, i.e. state III/II)

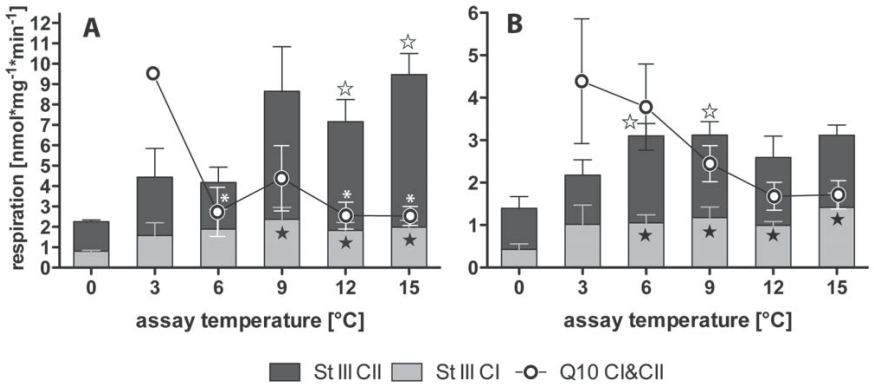

Figure 1. State III respiration and Q_{10} of *N. coriiceps* and *N. rossii* mitochondria. Grey bars represent complex I and black bars complex II respiration for *N. coriiceps* (A) and *N. rossii* (B). Respiration rates significantly different from those at 0°C are indicated by black (complex I) and white (complex II) star symbols. Round symbols indicate Q_{10}, calculated between 0°C and the respective temperature. Q_{10} values significantly different from that between 0 and 3°C are indicated by asterisks.
doi:10.1371/journal.pone.0031860.g001

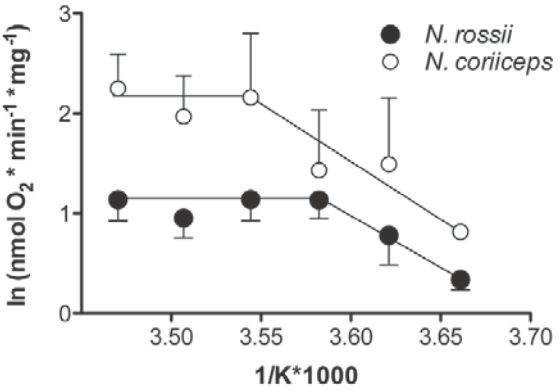

Figure 3. Arrhenius plots of state III respiration of *N. coriiceps* and *N. rossii*. Data are presented as white symbols for *N. coriiceps* and grey symbols for *N. rossii* and represent means ± SEM, n = 7.
doi:10.1371/journal.pone.0031860.g003

appeared to decrease above 3°C, there are no significant differences in ACR between assay temperatures. Mean ACR for *N. coriiceps* were 2.29 (CI, malate), 2.17 (CI, malate+glutamate) and 2.74 (CII, succinate+rotenone), for *N. rossii* ratios of 1.60 (CI, malate), 1.56 (CI, malate+glutamate) and 1.96 (CII, succinate+rotenone) were estimated.

ADP/O ratios were stable over the thermal range tested (and somewhat higher for complex I; c.f. Table S4), they did not differ significantly between complex I and II, but between species: in *N. coriiceps*, mean ADP/O ratios were 2.44±0.11, in *N. rossii* 1.97±0.15.

3.4 Enzymatic capacities

NADH/cytochrome c oxidoreductase activity showed a strong temperature dependency in both species (Table 1; fig. 4a) ranging from 7.9±0.6 to 29.2±2.5 µmol*h g fwt^{-1} between 0° and 15°C in *N. coriiceps* and 6.7±0.5 to 26.3±2.6 µmol*h g fwt^{-1} between 0° and 15°C in *N. rossii*, respectively. Similarly, cytochrome c oxidase (complex IV) activity rose from 77.4±14.4 and 76.0±11.9 to 280.6±44.6 and 273.0±33 µmol*h g fwt^{-1} between 0° and 15°C in *N. coriiceps* and *N. rossii*, respectively (fig. 4b). No significant differences were found between the species at any assay temperature. The Arrhenius plots revealed discontinuous slopes for both enzymes and species (fig. 4), which became visible in the respective activation energies when compared in steps of 6°C over the thermal range investigated (fig. 5).

In all cases, activation energies were highest between 0 and 9°C and significantly reduced in the range of 9 to 15°C. This drop in thermal sensitivity was more pronounced in *N. rossii* with an about 2 to 3-fold reduction for NADH/cytochrome c oxidoreductase (74.8±8.4 kJ*mol^{-1} to 27.9±4.3 kJ*mol^{-1}; fig. 5a) and about 4-fold for cytochrome c oxidase (79.3±9.5 kJ*mol^{-1} to 18.6±11.3 kJ*mol^{-1}; fig. 5b; *N. coriiceps*: NADH/cyt c oxred: 74.8±6.2 to 41.0±4.5 kJ*mol^{-1}; COX: 65.9±6.2 to 33.0±7.2 kJ*mol^{-1}). Due to the higher variability in the measurements at higher temperature, no significant difference between the species could be demonstrated in that range (NADH/Cyt c oxred: $p = 0.065$).

When expressed per µmol cytochrome c, cytochrome c oxidase capacities were found to be about 10- to 12-fold in excess compared to NADH/cytochrome c oxidoreductase in both species at all assay temperatures.

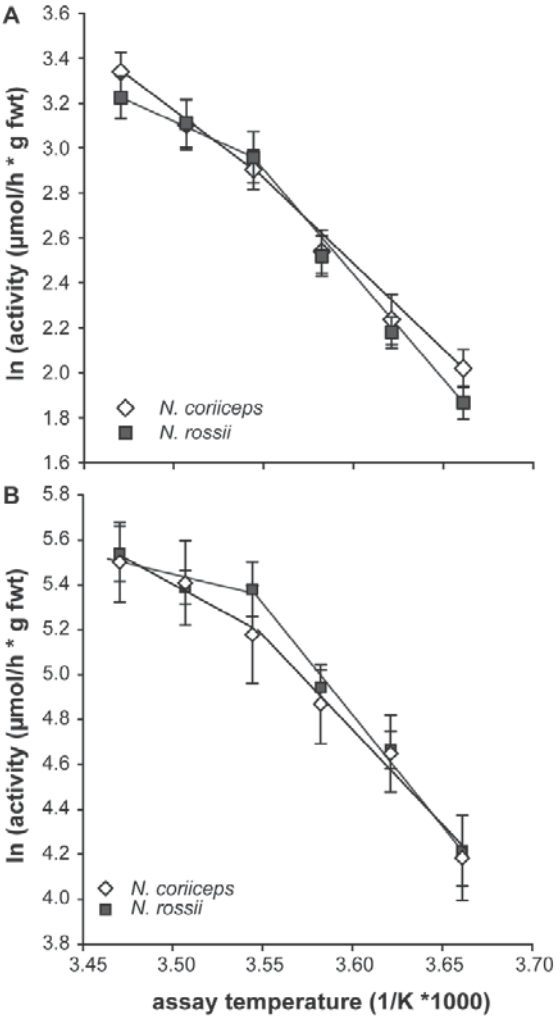

Figure 4. Arrhenius plots for NADH:cytochrome c oxidoreductase and cytochrome c oxidase of *N. coriiceps* and *N. rossii*. Panel A presents data for NADH:cytochrome c oxidoreductase, panel B for cytochrome c oxidase (white symbols: *N. coriiceps*, grey symbols: *N. rossii*). Arrhenius break temperatures are located around 9°C (3.54 K*1000^{-1}) for both enzymes and species. Data are presented as means ± SEM, n = 10.
doi:10.1371/journal.pone.0031860.g004

3.5 Mitochondrial membrane potential

The two panels in figure 6 display both the mitochondrial membrane potentials realised during state II respiration (circles) and the ratio of state II membrane potential over the respective oxygen consumed (bars); CI and CII were analysed separately.

Membrane potentials decreased significantly with temperature in *N. coriiceps* (CI $p = 0.011$, slope: −3.7±0.8; CII $p = 0.001$, slope: −3.6±0.5) but not so in *N. rossii* (CI $p = 0.26$; CII $p = 0.86$; fig. 6, round symbols). In *N. coriiceps* potentials were significantly reduced beyond 3 (CII) and 6°C (CI), respectively (fig. 6a). In *N. rossii*, the membrane potential remained rather stable, it was significantly reduced only at 9°C in CII and at 12°C in CI, and significantly increased at 15°C in CII (fig. 6b).

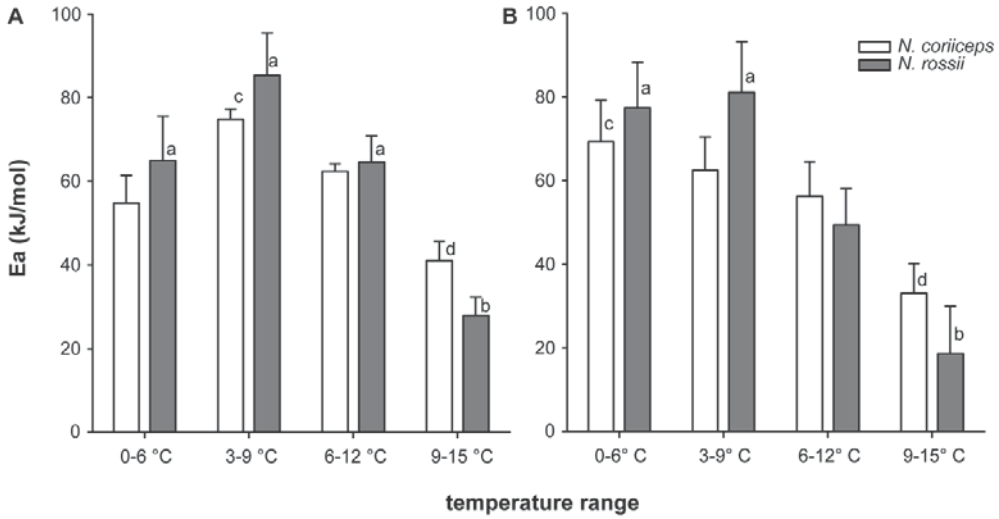

Figure 5. Activation energies for NADH:cytochrome c oxidoreductase and cytochrome c oxidase of *N. coriiceps* and *N. rossii*. Panel A presents data for NADH:cytochrome c oxidoreductase, panel B for cytochrome c oxidase (white bars: *N. coriiceps,* grey bars: *N. rossii*). Differences in E_a over the thermal range were tested using RM-ANOVA and are indicated by different letters (a/b; c/d). Data are presented as means ± SEM, n = 10.
doi:10.1371/journal.pone.0031860.g005

The membrane potential generated per mole of oxygen consumed in state II (solid bars in fig. 6 A, B) decreased with temperature to a similar extent in both species and for both complexes (*N. coriiceps*: CI $p = 0.016$, slope: -20.4 ± 5.1; CII $p = 0.002$, slope: -18.2 ± 2.7 and *N. rossii*: CI $p = 0.011$, slope: -19.7 ± 4.4; CII $p = 0.022$, slope: -12.9 ± 3.5). In *N. coriiceps* (fig. 6a), the ratios of potential/O_2 were significantly reduced at 9 and 15°C in CI and from 6 to 15°C for CII. *N. rossii* showed significant reductions in potential/O_2 at 3°C and between 9 and 15°C for CI and from 6 to 15°C for CII (fig. 6b). As decreasing potential/O_2 ratios can mainly be attributed to increasing electron flux with temperature, Q_{10} values for membrane potential/$O_{2,\ stII}$ and $1/O_{2,\ stII}$ were compared by t-test for both complexes and species (0–15°C) to identify whether decreased efficiencies to generate high membrane potentials during warming further reduced the ratios of potential/O_2. In *N. coriiceps*, decreasing membrane potentials significantly contributed to a further reduction in potential realised per mol O_2 consumed in CII (individual Q_{10} data presented in Table S4). This implies that mitochondrial metabolism could not be increased with rising temperature to an extent to keep state II membrane potential constant.

Table 1. Functional capacities of NADH/cytochrome c oxidoreductase and cytochrome c oxidase.

Species	Temp.	NADH/Cytc ox/red	Cytochrome c oxidase	Ratio
N. coriiceps	0	7.9±0.6	77.4±14.4	9.8
	3	9.9±1.0	118.6±18.8	12.0
	6	13.2±1.3	148.7±24.0	11.2
	9	18.9±1.7	214.4±38.8	11.3
	12	23.5±2.5	259.1±43.6	11.0
	15	29.2±2.5	280.6±44.6	9.6
N. rossii	0	6.7±0.5	76.0±11.9	11.3
	3	9.1±0.6	109.5±9.1	12.2
	6	12.9±1.3	144.5±11.6	11.2
	9	20.5±2.6	232.6±29.6	11.3
	12	23.6±2.4	225.2±16.6	9.5
	15	26.3±2.6	273.0±33.0	10.4

Activities of both enzyme complexes are given in µmol cytochrome c * h g fwt^{-1}. Ratios between NADH/Cyt c ox/red and COX are given in the last column.
doi:10.1371/journal.pone.0031860.t001

3.6 Analysis of amino acid composition

The instability indices presented in table 2 provide protein instability estimates based on a statistical analysis of amino acid composition [65]. A protein with an instability index less than 40 is considered stable, whereas values above 40 predict instability. For *ND6* (Table 2, first column), it is apparent that the three Antarctic species and the sub-Antarctic notothenioid have indices close to or above 40, while the Arctic, temperate and tropical species are significantly below this critical threshold (t-test, $p = 0.002$). *ND2* displays a high instability in all the species analysed (Table 2, second column), owing to a much more similar amino acid composition (data not shown, for further information refer to Table S2). *COI* appears to be much more stable, with lower instability indices throughout (Table 2, third column, and Table S3).

Discussion

4.1 Animal parameters

Animal data indicate that stress levels were generally low and the experimental animals in good condition, comparable to specimens caught regularly in Potter Cove and its direct vicinity [57,66]. In *N. coriiceps* of the same size class and area, Kamler and colleagues [67] found similar HSI of 2.6±1.0 and condition factors of 2.4±0.3.

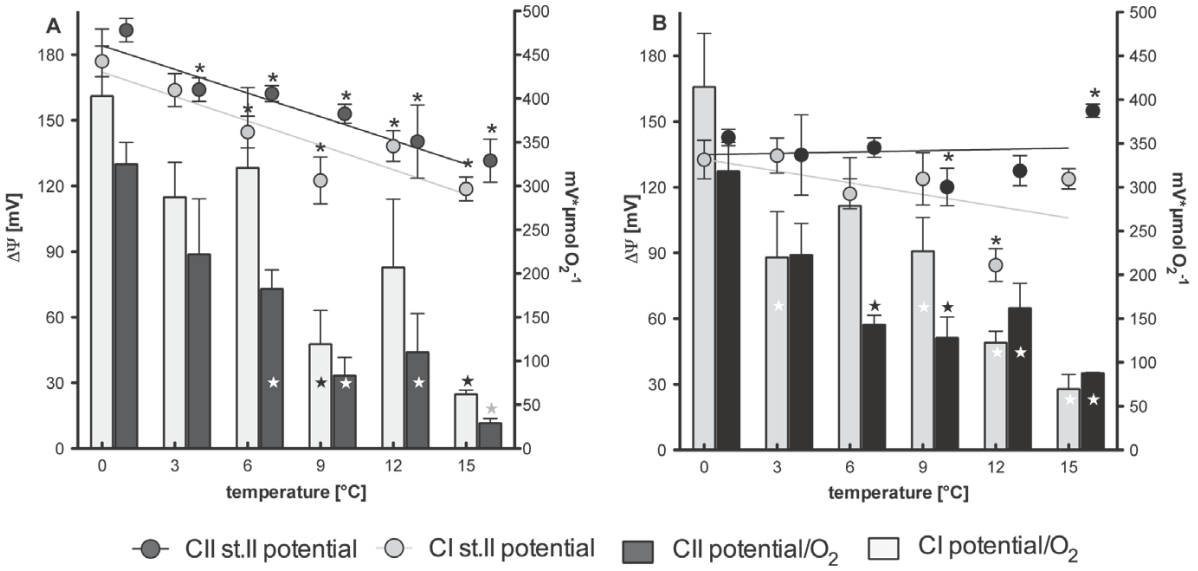

Figure 6. State II membrane potential and potential realised per mol O_2 consumed for *N. coriiceps* and *N. rossii*. Panel A presents data for *N. coriiceps* and panel B for *N. rossii*. Mitochondrial membrane potential (symbols) is given as $\Delta\Psi$ (left ordinate) for respiration involving complex I (grey symbols) and respiration involving complex II (black symbols). Bars represent the potential realised per µmol O_2 consumed (right ordinate), grey bars depict values for complex I, black bars for complex II. Data are presented as means ± SEM, n = 7.
doi:10.1371/journal.pone.0031860.g006

In *N. coriiceps* that had been transported to and kept in the UK, Egginton [68] found somewhat lower Hct (16–19%) and lactate levels (0.05–0.4 mM) than presented here. Heise and Abele [69] reported similar Hct (24%), but much higher lactate levels (around 7.5 mM) for *N. coriiceps* from the same site, while Beers and Sidell [70] even found a Hct of 35% and lactate levels around 1 mM around the Western Peninsula. At Signy Island, *N. coriiceps* and *N. rossii* display haematocrits of 24–25% [71]. This variability, especially in lactate concentrations, may be due to (long-distance) transportation and handling stress, yet it generally shows that the genus Notothenia does produce lactate, despite the reduced glycolytic capacities found by Dunn & Johnston [72].

Table 2. The table reports for each species the instability indices of the *ND6*, *ND2* and *COI* proteins computed using Expasy's ProtParam (http://us.expasy.org/tools/protparam.html) prediction server [64].

Species	Instability index *ND6*	Instability index *ND2*	Instability index *COI*
Notothenia coriiceps	39.19	38.48	28.23
Notothenia rossii	38.92	38.17	22.64
Pachycara brachycephalum	38.29	37.29	24.75
Eleginops maclovinus	40.92*	42.38*	24.87
Boreogadus saida	21.07	39.55	24.61
Arctogadus glacialis	20.58	38.95	24.61
Gadus morhua	20.58	39.30	24.61
Chlorurus sordidus	32.83	35.51	25.60

*protein classified as unstable (threshold: 40).
doi:10.1371/journal.pone.0031860.t002

The differences in hepatosomatic indices and condition factors, both of which were higher in *N. coriiceps* (cf. 3.1), relate to the morphological and physiological differences consistent with the differential adaptations to inhabit the water column of the benthic *N. coriiceps* and the bentho-pelagic *N. rossii*. This becomes evident by a greater density, expressed as mean percentage buoyancy of *N. coriiceps* (4.34%) over *N. rossii* (3.82%) [73]. *N. coriiceps* is a heavy rugged fish, and field observations with underwater cameras showed that it is a rather inactive sit-and-wait predator [55]. In comparison to *N. rossii*, *N. coriiceps* has a greater weight per unit length that is associated with a thicker body; skeletal weight as a percentage of body weight was also significantly greater in *N. coriiceps* (2.46%) than *N. rossii* (1.65%; $t = 5.611$, $P<0.03$) [73]. The more active *N. rossii* is more gracile with a laterally compressed and streamlined body. Its vertebrae morphology consists of bone that is more spongy or porous and of neural arches thinner than those of *N. coriiceps* [73]. *N. rossii* occurs in Potter Cove from 0 to 6–7 years exclusively in the juvenile stage [74,75], after which they migrate offshore to join and spawn with the adult population [76]. Data on age, gonadal stages and depth distribution of *N. coriiceps* at Potter Cove suggest that sexual maturity is first reached at about 6 years and that the species may remain near shore during its whole life cycle [57]. With regard to whole animal performance, it is therefore not surprising that Egginton [68] found a more pronounced drop in arterial PO_2 and higher lactate levels during and after exhaustive exercise in *N. coriiceps* compared to *N. rossii*.

4.2 Mitochondrial function

This study provides evidence for the functional integrity of complex I in the Antarctic notothenioids *N. coriiceps* and *N. rossii*, despite the translocation of *ND6*. To our knowledge, only a few studies presently exist that investigate complex I function individually in fish (let alone the consequences of genetic defects), yet it has been reported by Hilton and colleagues that in triplefin

fishes CI contribution decreases with temperature from about 50 to 30% [77]. In sea bass (*Dicentrarchus labrax*), complex I contribution is around 30–40% (Mark et al., in prep). In this light, complex I/II ratios of 0.3 to 1.0 point at a regular contribution of complex I to total mitochondrial energy metabolism.

Mitochondrial respiration in state III (in figs. 1 expressed as the sum of the maximal capacities of complex I and II) at 0°C were around 2.2±0.1 and 1.4±0.4 nmol O_2*min mg protein^{-1} for *N. coriiceps* and *N. rossii*, respectively. Hardewig and colleagues [27] found 3.7±1.2 nmol O_2*min mg protein^{-1} for *L. nudifrons* liver mitochondria, complex I inhibition with rotenone (8 µM) resulted in 50% respiration reduction, even during respiration on succinate (3.3 mM) alone. Johnston and colleagues reported higher values for red muscle mitochondria at 0°C, in *N. coriiceps* they found 7.7 [78], in *L. nudifrons* 11.8±6 nmol O_2*min mg protein^{-1} (−1°C, [36]) and in the sub-Antarctic *E. maclovinus* (4°C) 24.2±3.5 nmol O_2*min mg protein^{-1}. All these data fall in the same range when taking into account that there are capacity differences between liver and red muscle mitochondria [79], which in goldfish results in an about two-fold discrepancy in favour of muscle tissue [80].

At 0°C, mitochondrial respiration of *N. rossii* was up to 30% lower than in *N. coriiceps*, this may relate to the generally higher respiration rates in *N. coriiceps*: Ralph & Everson [81] estimated whole animal metabolic rates to be 1.75 mmol*kg h^{-1} in *N. coriiceps* and 1.19 mmol*kg h^{-1} in *N. rossii*, reflecting the ratio of mitochondrial capacities.

However, full state III capacity under CI and CII substrates and saturating ADP (denoted OXPHOS) is non additive. CI and CII analysed individually (CI: NADH related substrates; CII: succinate & rotenone) only reach up to about 70 and 80% of total OXPHOS, respectively. This is a frequent finding (in human muscle fibres, there is an approx. 35% discrepancy between OXPHOS capacities and the sum of CI & CII capacities) and may be caused by a downstream limitation in mitochondrial complex III (Cytochrome c Reductase) and/or complex IV (Cytochrome c Oxidase), after the Q cycle where the branches of the ETS converge. Further substrate oxidation in the Krebs cycle (after succinate dehydrogenase) that yields more NADH is also unlikely due to diffusive loss of Krebs cycle products out of the mitochondria [82]. Following analysis of complex I and II capacities individually, simple addition of individual fluxes will overestimate maximal OXPHOS capacities.

4.3 Mitochondrial metabolism

In both species, the decrease of state III Q_{10} values indicates decreased mitochondrial scope beyond 6 (*N. coriiceps*) and 9°C (*N. rossii*), respectively. Similar values reported for *L. nudifrons* [27] and *N. coriiceps* red muscle mitochondria [78] at elevated temperatures corroborate these findings. High Q_{10} values between 0 and 9°C are mirrored by high Arrhenius Activation Energies (E_a) for mitochondrial state III respiration, which fall to much lower E_a values beyond 9°C. This is typical for Antarctic animals, values of around 60 kJ*mol^{-1} have been found in gill mitochondria of the Antarctic bivalve *Laternula elliptica* [39] between 0 and 3°C, which are similar to the values found in liver mitochondria from the notothenioid *L. nudifrons* (47.5 kJ*mol−1, [27]) or red muscle mitochondria from *N. coriiceps* (73 kJ*mol−1, calculated from data presented in [83], between −1.5 and −2.5°C). At temperatures above 5°C, Weinstein & Somero [26] already observed a decrease of E_a in the notothenioid *Trematomus bernacchii* (38.3 kJ*mol^{-1}).

The transition from high to low E_a is sharp in *N. rossii* and *N. coriiceps* and is characterised by an Arrhenius Break Temperature (ABT, fig. 3) at 6 and 9°C, respectively. These are similar to *L. elliptica* [39]. While the E_a differed between *N. rossii* and *N. coriiceps*, the activation energies before and after the ABT similar for the two fish species (cf. 3.2, fig. 3). This cannot be quantified more precisely here, as at present there are no data available for temperatures between 6 and 9°C. ABTs can be assumed to be closer to 9 than to 6°C. In some contrast to the findings presented here, mitochondrial state III ABTs occurred above 15°C [27] and beyond 20°C [26] in the Antarctic nototheniids *L. nudifrons* and *T. bernacchii*, respectively.

Mitochondrial capacities showed a clear thermal limitation, in that increased temperatures led to decreased membrane potentials and no further increments in respiration rate beyond a certain thermal threshold characterised by the ABT. At first sight, this limitation might contribute to setting the whole organism *pejus* temperature, where capacity limitations set in [84]. Yet, due to their lower level of organisational complexity, thermal tolerance windows of organelles generally span a wider temperature range than those of the whole organism [25]. Notably however, Bilyk & De Vries [18] and Beers & Sidell [70] found acute critical thermal maxima (CT_{max}) for *N. coriiceps* and *N. rossii* around 16–17°C (when acutely warmed from −1°C by 0.3°C*min^{-1} and 3.6°C*hour^{-1}, respectively). Chronic heat tolerance limits of Antarctic fish are found at much lower temperatures: the Antarctic notothenid *Pagothenia borchgrevinki* displays first cardiac limitations when acutely warmed to 6°C [85] and has been shown to be able to adapt to a chronic exposure of 4°C [17,20]. *N. rossii* can be acclimated to up to 7°C for several weeks (authors' personal observations), thus the *pejus* range for Antarctic notothenids can be assumed to be generally located between 4 and 9°C, with critical temperatures located beyond 7–8°C for *N. coriiceps* and *N. rossii*.

Nonetheless, mitochondrial efficiency appears to be safeguarded in the two notothenids: RCRs were stable over the experimental temperature range, indicating rather static mitochondrial leak rates independent of temperature. Hardewig and colleagues [27] as well as Johnston and colleagues [78] report RCR^+ values between 7 and 10 for Antarctic notothenioids, which correspond to apparent proton leak rates of 10–15%. The mean leak rates observed for liver mitochondria of the two nototheniids in this study were only slightly higher than these values (18–21%).

As a possible consequence of stable RCR^+, ADP/O ratios also remained unchanged over the thermal range in this study in both nototheniids. They were higher than the values reported by Hardewig and colleagues [27] for *L. nudifrons* (around 1.5), but similar to those observed in short-horn sculpin *M. scorpio* [86] and rainbow trout [87]. In the range of 0–15°C, both complexes display ADP/O ratios similar to or even higher than active temperate fish species, especially so in *N. coriiceps*. In terms of ADP generation, complex I can be thus assumed to be as efficient and thermally stable as complex II in the two nototheniids.

4.4 Enzymatic function

Function of the mitochondrial complexes, as evidenced by the enzymatic assays of complex I/III and complex IV, mirrored (and thus corroborated) the results of the respiratory studies in isolated mitochondria. As observed for mitochondrial state III respiration (cf. 3.2), ABTs were located around 9°C (fig. 4) and activation energies were highest in the range from 0–9°C in both enzymatic complexes and species (fig. 5). E_a of the two enzyme complexes (66–80 kJ*mol^{-1}) were very close to the activation energies observed in mitochondrial state III respiration (84 kJ*mol^{-1}, cf. 4.3), which can be taken as a sign of good mitochondrial coupling. Activation energies for COX were also higher than in temperate fish: in Mediterranean sea bass (*D. labrax*), E_a for COX in the

range of 3–20°C is between 10–13 kJ*mol^{-1} (depending on acclimation, [88]), whereas in cold acclimated temperate eelpout *Z. viviparus* [89] values were around 35 kJ*mol^{-1} (similar to those found in *Notothenia sp.* between 9–15°C).

Higher values (84 kJ*mol^{-1}) thus appear typical for Antarctic fish and corroborate the general concept of higher activation energies of mitochondrial enzymes in cold-adapted ectoterms [27,39,90]: mitochondrial densities are increased in the cold putatively to shorten diffusion distances [91], as a consequence, the activities of the resulting high number of mitochondrial enzymes have to be kept at a physiological level by increasing activation energies accordingly, especially in stenotherms. This becomes evident by the total activities for COX: with values of around 80 µmol * h g fwt^{-1}, they are in the same range or even lower than those in the Antarctic eelpout *P. brachycephalum* (around 210 µmol*h g fwt−1, [89]), in liver of temperate cod *G. morhua* (8°C) (90–200 µmol*h g fwt−1, [92]), or in temperate and Arctic cottids and zoarcids (1°C) (120–180 µmol*h g fwt−1, [93]).

4.5 Energetic coupling of complex I

When compared on the basis of cytochrome c turnover, complex I/III (NADH/cyt c ox/red) displayed a 10-fold lower activity than complex IV (Cyt c oxidase). Under *in vivo* conditions, complex II, flavines and glycerophosphate dehydrogenase (GpDH) also contribute electrons via the ubiquinone pool to complex III. Complex III activity was hence limited to maximal complex I activity in the protocol used here and presumably would have shown higher capacities under further electron contributions from complex II (and perhaps GpDH). Secondly, excess capacities are frequently found downstream in the ETS, which for complex IV can be quite dramatic in invertebrates [94], and have been found to be at least 2.5–3 fold with respect to OXPHOS capacities in triplefin blennies [77]. Complex IV is a potential rate-limiting enzyme in the ETS [95] and acts as an electron sink. High activities of CIV can effectively elevate the mitochondrion's affinity for O_2 [96].

Finally, the different activities may also reflect a decreased CI capacity: expressed as protons pumped into the intermembrane space per pair of electrons translocated (or per mol O consumed), the theoretical stoichiometry between complex I and II is 10:6 (complex I and III pump 4 protons each, complex IV pumps 2; c.f. 43). Therefore, at 4 protons per ATP (3 for synthesis, 1 for translocation by the ANT), this translates into 2.5 vs 1.5 ATP per pair of electrons, or an ADP/O ratio of 2.5 for complex I and 1.5 of complex II. The ADP/O ratios found in this study are not fully concordant with these theoretical values. In both species, ADP/O were only slightly higher for complex I than for complex II and under OXPHOS respiration in state III, complex I respiration only equals, or is even smaller than that of complex II, which comprises 50–75% of mitochondrial respiration (fig. 2). Although one may interpret this as defects in complex I, mean ADP/O ratios approaching 2.5 (*N. coriiceps*) and 2.0 (*N. rossii*) may not indicate ineffective phosphorylation rates but differences in electron flow from complexes I and II and convergence on complex III (cf. 4.2).

The efficiencies of complex I and II can be compared on basis of their ability to generate membrane potential: For every pyruvate that enters the Krebs cycle, 4 NADH are oxidised by complex I and 1 succinate is oxidised by complex II, resulting in 40 protons (10 ATP) and 6 protons being pumped (1.5 ATP) by the respective complexes. In terms of ATP production, complex I is 6 times more efficient than complex II (10 vs. 1.5 ATP) and should contribute 4 times more to the overall mitochondrial phosphorylation capacity than complex II (4 vs. 1 pair of electrons). As these contributions cannot be differentiated by respiration analyses alone, measurement of membrane potential in leak respiration states (i.e. in state II) theoretically should reveal that complex I was 1.66 times more effective at generating membrane potential than complex II (proton stoichiometry of 10:6).

Figure 6 depicts these ratios of membrane potential per state II oxygen consumption (bars) and generally, complex I shows the expected pattern with mean ratios of complex I *vs.* II of 1.62±0.14 (*N. coriiceps*), and 1.26±0.21 (*N. rossii*). Again, *N. coriiceps* presents a fully functional complex I, operating close to the theoretical optimum. However, these ratios are lower and decrease further with rising temperature for *N. rossii*.

A decrease of complex I function with rising temperature has been reported for triplefin blennies [77] and supports the concept of a thermally sensitive, but otherwise properly working complex I. There are few studies comparing complexes I and II in ectotherms at different temperatures and it is therefore not possible to compare our data to further fish species with the typical canonical gene order for *ND6*. It is clear that complex I has a relatively high thermal sensitivity, especially so in *N. rossii*, which may result from structural peculiarities of notothenioid *ND6*.

4.6 *ND6* structure

The protein instability indices (Table 2) underline the general notion that decreased thermal stabilities of cold-adapted enzymes are the side effects of an increased flexibility, which is considered a precondition for proper function at low temperatures [97] and may also have been a pre-adaptation for the Antarctic notothenioid lineages to radiate into the Southern Ocean (even before *ND6* translocation). Modifications to increase flexibility may include a decrease in weak interactions and hydrophobicity, as well as substitution and deletion of specific amino acids [98]. In this respect, nototheniid *ND6* may not only have undergone a translocation, but also some changes in composition. Table 3 lists the percentages of the individual amino acids in *ND6* (for *ND2* and *COI* amino acid composition, refer to Tables S2 and S3, respectively). In fact, there are not only composition differences between the Notothenioid/eelpout and temperate/Arctic/tropical (i.e. non-Antarctic) group but also between the two nototheniid species (*N. coriiceps*, *N. rossii*) and the related sub-Antarctic notothenioid *E. maclovinus* (Eleginopidae). *E. maclovinus* has been described as 'notably divergent from the rest of the notothenioids' in terms of protein composition [99], which appears to carry characteristics of both groups in its *ND6* composition: the 3 Antarctic species and the sub-Antarctic notothenioid bear lower leucine contents than the non-Antarctic groups, but higher percentages of cysteine, which is even more prominent in the nototheniids (*ND6* translocated). All notothenioids lack histidine in their *ND6* structure, the Antarctic nototheniids possess lysine, which is not found in any of the species with canonical gene order. Glutamine also is only present in the Antarctic group (including the zoarcid), with a fourfold difference between nototheniids and the species with canonical gene order. These changes in amino acid composition may be indicative of cold adaptation in the notothenioids and the Antarctic eelpout, rendering *ND6* more flexible at cold temperatures but in turn also bring about a higher thermal sensitivity of the protein. This flexibility becomes evident in the instability indices, also reported in Table 2 for *ND2* and cytochrome c oxidase I (*COI*). *ND2* displays similarly high instability indices as *ND1*, thereby corroborating the commonly observed high thermal sensitivities of complex I [77]. Complex IV (*COI*) on the other hand, is thermally very stable, has high Q_{10}, low instability indices (cf. Table 2) and is generally found to be very

much conserved throughout the animal kingdom (and is therefore often used for phylogenetic analyses). Of the three proteins analysed, only *ND6* showed significant differences in instability indices between the notothenioids and the other species, which may indicate a further cold-adaptation of this protein. It remains unclear, however, whether cold-adaptation only became possible with the translocation of the *ND6* gene.

4.7 Conclusions

In the light of the translocation of the *ND6* gene, we focused our interest on the performance and capacity of the individual mitochondrial complexes I and II. We measured mitochondrial performance during warming, in two stenothermal notothenioid fish species that show slightly different distribution, ecology and life histories.

By specific analysis of CI efficiency, here we demonstrate that despite *ND6* translocation CI remains functional and well coupled. For *N. coriiceps* CI coupling appears to be greater for *N. rossii*, and this is most apparent in complex I ADP/O ratios. These indicate a generally better mitochondrial performance of *N. coriiceps*, which was observed in most parameters that were investigated in this study. Only state II membrane potential (fig. 6) was found to be more stable over the thermal range in *N. rossii*, pointing at a somewhat tighter inner mitochondrial membrane or better coordination and thermal stability of the enzymes involved into the generation of membrane potential.

Overall, mitochondrial thermal responses were similar in both notothenioids: in mitochondrial respiration (and all resulting ratios, figs. 1 & 3) and enzymatic function (figs. 4 & 5), capacities increased until close to 9°C, above which respiration and enzymatic activity levelled off. This break, as characterised by the ABT, was more pronounced on the mitochondrial level (fig. 3) than in the enzymatic complexes analysed individually. This is in line with the general notion that higher degrees of functional integration bring about higher thermal sensitivity [30]. *Notothenia coriiceps* generally displayed a higher amplitude in thermal response (figs. 1 & 3), while *N. rossii* showed higher enzymatic E_a (fig. 5) and in part also higher enzymatic activities (fig. 4, COX). ABTs may be found at slightly lower temperature in *N. rossii* (cf. fig. 3), however greater resolution between 6 and 9°C is required. These differences in mitochondrial metabolism between the two species could also be regarded as a trade-off for different liver sizes (HSI) and compositions (elevated fats) and ontogeny rather than as a sign of differential thermal adaptation between the two species (cf. 4.1). The low thermal tolerance thresholds in terms of ABT values presented here for notothenioid mitochondria most likely reflect the trade-offs in cold adaptation of mitochondrial proteins, in that increased flexibility at very low temperatures go hand in hand with reduced thermal stability, as demonstrated above for *ND6*.

In the light of the present study, one has to ask for the long-term perspective for the two species under the current scenarios of global warming (and ocean acidification), which are particularly dramatic along the Antarctic Peninsula [100,101,102,103]. Some physiological adjustments after warm acclimation to 4°C have been found in the cardiovascular system [16,19,20,104] and the metabolic rate of the cryo-pelagic Antarctic notothenioid *Pagothenia borchgrevinki* [17]. At the mitochondrial level, there is further evidence for limited acclimation capacities in the Antarctic eelpout *P. brachycephalum* [21,23,105]. Whether mitochondrial metabolism in *N. rossii* and *N. coriiceps* will be able to similarly adapt and how molecules, organelles and cells in general will respond to long-term environmental changes remain important and stimulating topics for future studies.

Supporting Information

Table S1 ND6, ND2 and COI amino acid sequences compared in this study. The table reports: species names, general description of habitat, protein (aa) sequence GenBank accession number for each protein and reference.
(DOC)

Table S2 Amino acid composition and instability index of the ND2 protein. The table reports for each species the amino acid composition of ND2 protein and the instability index computed using the Expasy's ProtParam (http://us.expasy.org/tools/protparam.html) prediction server [64].
(DOC)

Table S3 Amino acid composition and instability index of the COI protein. The table reports for each species the amino acid composition of COI protein and the instability index computed using the Expasy's ProtParam (http://us.expasy.org/tools/protparam.html) prediction server [64].
(DOC)

Table S4 ADP/O ratios, ACR, RCR, RCR$^+$ and Q_{10} analysis (0–15°C) of membrane potentials for complex I and II individually (all values are expressed as means ±SEM).
(DOC)

Table 3. Amino acid composition of *ND6*.

	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
N. coriiceps	10.3	1.7	0.6	0.6	**4.0**	**2.3**	4.6	13.8	*0.0*	2.3	**14.9**	**1.1**	5.7	5.2	3.4	8.0	2.9	1.7	4.6	12.1
N. rossii	10.9	1.7	0.6	1.1	**4.0**	**2.3**	4.0	13.8	*0.0*	2.3	**14.4**	**1.1**	6.3	4.6	3.4	8.0	2.9	1.7	4.6	12.1
P. brachycephalum	11.1	3.5	0.6	1.8	2.3	0.6	4.7	14.0	**0.6**	4.7	**14.6**	*0.0*	1.2	5.8	4.1	7.6	2.3	2.9	4.7	12.9
E. maclovinus	7.5	1.7	0.6	1.2	2.3	0.6	4.0	15.0	*0.0*	2.9	**14.5**	*0.0*	6.4	8.1	2.9	8.7	2.9	0.6	5.2	15.0
B. saida	11.0	1.7	0.6	1.2	*1.2*	*0.0*	4.0	14.5	**0.6**	2.9	*20.2*	*0.0*	4.0	3.5	2.9	6.9	1.7	4.0	4.6	14.5
A. glacialis	11.6	1.7	0.6	1.2	*1.2*	*0.0*	4.0	14.5	**0.6**	2.9	*19.7*	*0.0*	4.0	3.5	2.9	6.9	1.7	4.0	4.6	14.5
G. morhua	11.0	1.7	0.6	1.2	*1.2*	*0.0*	4.0	14.5	**0.6**	2.9	*19.7*	*0.0*	4.0	3.5	2.9	6.9	1.7	4.0	4.6	15.0
C. sordidus	13.3	2.3	0.6	2.3	*0.6*	*0.0*	3.5	12.7	**0.6**	3.5	*18.5*	*0.0*	2.9	5.8	2.9	5.8	2.9	2.9	5.8	13.3

The table reports for each species the amino acid composition of the *ND6* protein computed using Expasy's ProtParam (http://us.expasy.org/tools/protparam.html) prediction server [64].
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Author Contributions

Conceived and designed the experiments: FCM ML LZ TP HOP CP. Performed the experiments: FCM ML AS EBO NK CP. Analyzed the data: FCM ML CP. Contributed reagents/materials/analysis tools: FCM ML EBO LZ HOP CP. Wrote the paper: FCM CP ML. Organised the expedition: FCM ML AS NK EBO CP.

References

1. Pörtner HO (2006) Climate-dependent evolution of Antarctic ectotherms: An integrative analysis. Deep-Sea Res Pt II 53: 1071–1104.
2. Karentz D (1991) Ecological Considerations of Antarctic Ozone Depletion. Antarct Sci 3: 3–11.
3. Hagen W, Kattner G, Friedrich C (2000) The lipid compositions of high-Antarctic notothenioid fish species with different life strategies. Polar Biol 23: 785–791.
4. Sidell BD, Wujcik JM, Wang G, Eastman JT (2007) Morphometry of retinal vasculature in Antarctic fishes is dependent upon the level of hemoglobin in circulation. J Exp Biol 210: 815–824.
5. Chen Z, Cheng CH, Zhang J, Cao L, Chen L, et al. (2008) Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. Proc Natl Acad Sci U S A 105: 12944–12949.
6. DeVries AL (1971) Glycoproteins as biological antifreeze agents in antarctic fishes. Science 172: 1152–1155.
7. Stepanov V, Mateo P, Gillet B, Beloeil JC, Lechene P, et al. (1997) Kinetics of creatine kinase in an experimental model of low phosphocreatine and ATP in the normoxic heart. Am J Physiol 273: C1397–C1408.
8. Cocca E, Ratnayakelecamwasam M, Parker SK, Camardella L, Ciaramella M, et al. (1995) Genomic Remnants of Alpha-Globin Genes in the Hemoglobinless Antarctic Icefishes. Proc Natl Acad Sci U S A 92: 1817–1821.
9. Sidell BD, O'Brien KM (2006) When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. J Exp Biol 209: 1791–1802.
10. Wohlschlag DE (1963) An Antarctic fish with unusually low metabolism. Ecology 44: 557–564.
11. Somero GN, Giese AC, Wohlschlag DE (1968) Cold adaptation of the Antarctic fish *Trematomus bernacchii*. Comp Biochem Physiol 26: 223–233.
12. Clarke A (1991) What is cold adaptation and how should we measure it? Amer Zool 31: 81–92.
13. Somero GN, DeVries AL (1967) Temperature tolerance of some Antarctic fishes. Science 156: 257–258.
14. Gonzalez-Cabrera PJ, Dowd F, Pedibhotla VK, Rosario R, Stanley-Samuelson D, et al. (1995) Enhanced hypo-osmoregulation induced by warm-acclimation in antarctic fish is mediated by increased gill and kidney Na+/K (+)-ATPase activities. J Exp Biol 198: 2279.
15. Podrabsky JE, Somero GN (2006) Inducible heat tolerance in Antarctic notothenioid fishes. Polar Biol 30: 39–43.
16. Robinson E, Egginton S, Davison W (2011) Warm-induced bradycardia and cold-induced tachycardia: mechanisms of cardiac and ventilatory control in a warm-acclimated Antarctic fish. Polar Biol 34: 371–379.
17. Robinson E, Davison W (2008) The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption. Polar Biol 31: 317–326.
18. Bilyk KT, DeVries AL (2011) Heat tolerance and its plasticity in Antarctic fishes. Comp Biochem Physiol A: 158: 382–390.
19. Seebacher F, Davison W, Lowe CJ, Franklin CE (2005) A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. Biol letters 1: 151–154.
20. Franklin CE, Davison W, Seebacher F (2007) Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in Pagothenia borchgrevinki. J Exp Biol 210: 3068–3074.
21. Lannig G, Storch D, Pörtner H-O (2005) Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. Polar Biol 28: 575–584.
22. Brodte E, Knust R, Pörtner HO (2006) Temperature-dependent energy allocation to growth in Antarctic and boreal eelpout (Zoarcidae). Polar Biol 30: 95–107.
23. Windisch HS, Kathöver R, Pörtner H-O, Frickenhaus S, Lucassen M (2011) Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways. Am J Physiol 301(5): R1453–R1466.
24. Mark FC, Bock C, Pörtner HO (2002) Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. Am J Physiol 283: R1254–1262.
25. Mark FC, Hirse T, Pörtner HO (2005) Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol 28: 805–814.
26. Weinstein RB, Somero GN (1998) Effects of temperature on mitochondrial function in the Antarctic fish *Trematomus bernacchii*. J Comp Physiol B 168: 190–196.
27. Hardewig I, Pörtner H, Peck L (1999) Thermal sensitivity of mitochondrial function in the Antarctic Notothenioid *Lepidonotothen nudifrons*. J Comp Physiol B 169: 597–604.
28. Urschel MR, O'Brien KM (2008) High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis. J Exp Biol 211: 2638.
29. Urschel MR, O'Brien KM (2009) Mitochondrial function in Antarctic notothenioid fishes that differ in the expression of oxygen-binding proteins. Polar Biol 32: 1323–1330.
30. Pörtner HO (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp Biochem Physiol 132A: 739–761.
31. McDonald TG, Van Eyk JE (2003) Mitochondrial proteomics. Basic Res Cardiol 98: 219–227.
32. Hand SC, Menze MA (2008) Mitochondria in energy-limited states: mechanisms that blunt the signaling of cell death. J Exp Biol 211: 1829–1840.
33. Scheffler IE (1999) Mitochondria. New York: Wiley.
34. Duchen MR (1999) Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. Physiological Soc. pp 1–17.
35. Dunn JF, Archer SD, Johnston IA (1989) Muscle-Fiber Types and Metabolism in Post-Larval and Adult Stages of Notothenioid Fish. Polar Biol 9: 213–223.
36. Johnston IA, Calvo J, Guderley H, Fernandez D, Palmer L (1998) Latitudinal Variation in the Abundance and Oxidative Capacities of Muscle Mitochondria in Perciform Fishes. J Exp Biol 201: 1–12.
37. O'Brien K, Sidell B (2000) The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. J Exp Biol 203: 1287–1297.
38. Hardewig I, Van Dijk PLM, Moyes CD, Pörtner HO (1999) Temperature-Dependent Expression of Cytochrome-C Oxidase in Antarctic and Temperate Fish. Am J Physiol 277: R508–R516.
39. Pörtner HO, Hardewig I, Peck LS (1999) Mitochondrial Function and Critical Temperature in the Antarctic Bivalve, *Laternula elliptica*. Comp Biochem Physiol A 124: 179–189.
40. Papetti C, Lio P, Ruber L, Patarnello T, Zardoya R (2007) Antarctic fish mitochondrial genomes lack ND6 gene. J Mol Evol 65: 519–528.
41. Zhuang X, Cheng CH (2010) ND6 gene "lost" and found: evolution of mitochondrial gene rearrangement in Antarctic notothenioids. Mol Biol Evol 27: 1391–1403.
42. Walker JE, Carroll J, Fearnley IM, Skehel JM, Shannon RJ, et al. (2006) Bovine complex I is a complex of 45 different subunits. J Biol Chem 281: 32724–32727.
43. Efremov RG, Baradaran R, Sazanov LA (2010) The architecture of respiratory complex I. Nature 465: 441–445.
44. Bai Y, Hu P, Park JS, Deng J-H, Song X, et al. (2004) Genetic and Functional Analysis of Mitochondrial DNA-Encoded Complex I Genes. Ann NY Acad Sci 1011: 272–283.
45. Lazarou M, Thorburn DR, Ryan MT, McKenzie M (2009) Assembly of mitochondrial complex I and defects in disease. BBA - Mol Cell Res 1793: 78–88.
46. Flinn L, Mortiboys H, Volkmann K, Koster RW, Ingham PW, et al. (2009) Complex I deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish (*Danio rerio*). Brain 132: 1613–1623.
47. Bai Y, Attardi G (1998) The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. Embo J 17: 4848–4858.
48. Ugalde C, Triepels RH, Coenen MJH, Van Den Heuvel LP, Smeets R, et al. (2003) Impaired complex I assembly in a Leigh syndrome patient with a novel missense mutation in the ND6 gene. Ann Neurol 54: 665–669.
49. Yadava N, Houchens T, Potluri P, Scheffler IE (2004) Development and characterization of a conditional mitochondrial complex I assembly system. J Biol Chem 279: 12406.
50. Antonova E (2010) Short-term thermal compensatory-adaptive reaction mechanisms of the liver in *Carassius auratus gibelio*. Contemp Probl Ecol 3: 57–62.
51. Mortelette H, Moisan C, Sébert P, Belhomme M, Amérand A (2010) Fish as a model in investigations about the relationship between oxygen consumption and hydroxyl radical production in permeabilized muscle fibers. Mitochondrion 10: 555–558.

52. DeWitt HH, Heemstra PC, Gon O (1990) Nototheniidae. In: Gon O, Heemstra PC, eds. Fishes of the Southern Ocean. Grahamstown, South Africa: J. L. B. Smith Institute of Ichthyology. pp 279–331.
53. Clarke A, Johnston NM (1999) Scaling of metabolic rate with body mass and temperature in teleost fish. J Anim Ecol 68: 893–905.
54. Pörtner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881–893.
55. North A (1996) Locomotory activity and behaviour of the Antarctic teleost *Notothenia coriiceps*. Mar Biol 126: 125–132.
56. Campbell HA, Fraser KP, Bishop CM, Peck LS, Egginton S (2008) Hibernation in an antarctic fish: on ice for winter. PLoS ONE 3: e1743.
57. Casaux R, Mazzotta A, Barrera-Oro E (1990) Seasonal aspects of the biology and diet of nearshore nototheniid fish at Potter Cove, South Shetland Islands, Antarctica. Polar Biol 11: 63–72.
58. Barrera-Oro E (2003) Analysis of dietary overlap in Antarctic fish (Notothenioidei) from the South Shetland Islands: no evidence of food competition. Polar Biol 26: 631–637.
59. Gon O, Heemstra P (1990) Fishes of the Southern Ocean. Grahamstown, South Africa: J.L.B. Smith Institute for Ichthyology.
60. Brand MD (1995) Measurement of mitochondrial protonmotive force; BrownG, Cooper C, eds. Oxford, UK: Oxford University Press.
61. Moyes C, Mathieu-Costello O, Tsuchiya N, Filburn C, Hansford R (1997) Mitochondrial biogenesis during cellular differentiation. Am J Physiol 272: C1345.
62. Möller IM, Palmer JM (1982) Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria. Physiol Plantarum 54: 267–274.
63. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
64. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, et al. (2005) Protein identification and analysis tools on the ExPASy server. The proteomics protocols handbook. pp 571–607.
65. Guruprasad K, Reddy B, Pandit MW (1990) Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. Protein Eng 4: 155.
66. Casaux R, Barrera-Oro E (2002) Effect of a shore-based sampling programme on *Notothenia coriiceps* populations. Antarct Sci 14: 221–224.
67. Kamler E, Krasicka B, Rakusa-Suszczewski S (2001) Comparison of lipid content and fatty acid composition in muscle and liver of two notothenioid fishes from Admiralty Bay (Antarctica): an eco-physiological perspective. Polar Biol 24: 735–743.
68. Egginton S (1997) A comparison of the response to induced exercise in red- and white-blooded Antarctic fishes. J Comp Physiol B 167: 129–134.
69. Heise K, Abele D (2008) Response of blood parameters of the Antarctic fish Notothenia coriiceps (Richardson, 1844) to warming and hypoxia, The Potter Cove coastal ecosystem, Antarctica. In: Wiencke C, Ferreyra GA, Abele D, Marenssi S, eds. Reports on Polar and Marine Research: The Antarctic ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo) Synopsis of research performed 1999–2006 at the Dallmann Laboratory and Jubany Station Bremerhaven, Germany. pp 320–330.
70. Beers JM, Sidell BD (2011) Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. Physiol Biochem Zool 84: 353–362.
71. Everson I, Ralph R (1968) Blood analyses of some Antarctic fish. Br Antarct Surv Bull 15: 59–62.
72. Dunn J, Johnston I (1986) Metabolic constraints on burst-swimming in the Antarctic teleost Notothenia neglecta. Mar Biol 91: 433–440.
73. Eastman JT, Barrera-Oro E, Moreira E (2011) Adaptive radiation at a low taxonomic level: divergence in buoyancy of the ecologically similar Antarctic fish *Notothenia coriiceps* and *N. rossii*. Mar Ecol Prog Ser 438: 195–206.
74. Barrera-Oro E, Casaux R (1992) Age estimation for juvenile *Notothenia rossii* from Potter Cove, South Shetland Islands. Antarct Sci 4: 131–136.
75. Barrera-Oro E, Alescio N, Moreira E, Marschoff E (2010) Using scales to clarify the transition from blue-phase to brown-phase fingerling in *Notothenia rossii* from the South Shetland Islands. Polar Biol 33: 877–884.
76. Burchett M, Sayers P, North A, White M (1983) Some biological aspects of the nearshore fish populations at South Georgia. Br Antarct Surv Bull 59: 63–74.
77. Hilton Z, Clements KD, Hickey AJ (2010) Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. J Comp Physiol B 180: 979–990.
78. Johnston I, Guderley H, Franklin C, Crockford T, Kamunde C (1994) Are mitochondria subject to evolutionary temperature adaptation? J Exp Biol 195: 293.
79. Hulbert A, Turner N, Hinde J, Else P, Guderley H (2006) How might you compare mitochondria from different tissues and different species? J Comp Physiol B 176: 93–105.
80. van den Thillart G, Modderkolk J (1978) The effect of acclimation temperature on the activation energies of state III respiration and on the unsaturation of membrane lipids of goldfish mitochondria. BBA - Biomembranes 510: 38–51.
81. Ralph R, Everson I (1968) The respiratory metabolism of some Antarctic fish. Comp Biochem Physiol 27: 299–307.
82. Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle: New perspectives of mitochondrial physiology. Int J Biochem Cell B 41: 1837–1845.
83. Guderley H (1998) Temperature and growth rates as modulators of the metabolic capacities of fish muscle. Cold ocean physiology. HO. Pörtner, RC. Playle, eds. Cambridge: Cambridge University Press. pp 58–87.
84. Pörtner HO, Mark FC, Bock C (2004) Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. Respir Physiol Neurobiol 141: 243–260.
85. Lowe CJ, Seebacher F, Davison W (2005) Thermal sensitivity of heart rate and insensitivity of blood pressure in the Antarctic notothenid fish *Pagothenia borchgrevinki*. J Comp Physiol B 175: 97–105.
86. Guderley H, Johnston I (1996) Plasticity of fish muscle mitochondria with thermal acclimation. J Exp Biol 199: 1311.
87. Suarez RK, Hochachka PW (1981) Preparation and properties of rainbow trout liver mitochondria. J Comp Physiol B 143: 269–273.
88. Trigari G, Pirini M, Ventrella V, Pagliarani A, Trombetti F, et al. (1992) Lipid composition and mitochondrial respiration in warm-and cold-adapted sea bass. Lipids 27: 371–377.
89. Lucassen M, Schmidt A, Eckerle LG, Pörtner HO (2003) Mitochondrial proliferation in the permanent vs. temporary cold: enzyme activities and mRNA levels in Antarctic and temperate zoarcid fish. Am J Physiol 285: R1410–1420.
90. Pörtner HO, van Dijk PLM, Hardewig I, Sommer A (2000) Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. In: Davison W, Williams CW, eds. Antarctic Ecosystems: Models for a wider understanding. ChristchurchNew Zealand: Caxton Press. pp 109–122.
91. Londraville RL, Sidell BD (1990) Maximal diffusion-distance within skeletal muscle can be estimated from mitochondrial distributions. Respir Physiol 81: 291–302.
92. Lannig G, Eckerle LG, Serendero I, Sartoris F-J, Fischer T, et al. (2003) Temperature adaptation in eurythermal cod (*Gadus morhua*): a comparison of mitochondrial enzyme capacities in boreal and Arctic populations. Mar Biol 142: 589–599.
93. Speers-Roesch B, Ballantyne JS (2005) Activities of antioxidant enzymes and cytochrome c oxidase in liver of Arctic and temperate teleosts. Comp Biochem Physiol A 140: 487–494.
94. Iftikar FI, MacDonald J, Hickey AJR (2010) Thermal limits of portunid crab heart mitochondria: Could more thermo-stable mitochondria advantage invasive species? J Exp Mar Biol Ecol 395: 232–239.
95. Arnold S (2011) The power of life - Cytochrome c oxidase takes center stage in metabolic control, cell signalling and survival. Mitochondrion;in press (DOI: 10.1016/j.mito.2011.05.003).
96. Gnaiger E, Lassnig B, Kuznetsov A, Rieger G, Margreiter R (1998) Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. J Exp Biol 201: 1129.
97. D'Amico S, Claverie P, Collins T, Georlette D, Gratia E, et al. (2002) Molecular basis of cold adaptation. Philos T R Soc B 357: 917–925.
98. Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53: 830–841.
99. Fields PA, Somero GN (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci U S A 95: 11476–11481.
100. Gille ST (2002) Warming of the Southern Ocean Since the 1950s. Science 295: 1275–1277.
101. Clarke A, Murphy EJ, Meredith MP, King JC, Peck LS, et al. (2007) Climate change and the marine ecosystem of the western Antarctic Peninsula. Philos T R Soc B 362: 149–166.
102. Mayewski PA, Meredith MP, Summerhayes CP, Turner J, Worby A, et al. (2009) State of the Antarctic and Southern Ocean climate system. Rev Geophys 47: RG1003.
103. Patarnello T, Verde C, di Prisco G, Bargelloni L, Zane L (2011) How will fish that evolved at constant sub zero temperatures cope with global warming? Notothenioids as a case study. Bioessays 33: 260–268.
104. Lowe C, Davison W (2005) Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic notothenid fishes to acute and chronic thermal change. J Fish Biol 67: 752–766.
105. Mark FC, Lucassen M, Pörtner HO (2006) Thermal sensitivity of uncoupling protein expression in polar and temperate fish. Comp Biochem Physiol D 1: 365–374.

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Impact of Climate Change on Fishes in Complex Antarctic Ecosystems

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Abstract

Antarctic marine ecosystems are increasingly threatened by climate change and are considered to be particularly sensitive because of the adaptation of most organisms to cold and stable environmental conditions. Fishes play a central role in the Antarctic marine food web and might be affected by climate change in different ways: (i) directly by increasing water temperatures, decreasing seawater salinity and/or increasing concentrations of CO_2; (ii) indirectly by alterations in the food web, in particular by changes in prey composition, and (iii) by alterations and loss of habitat due to sea ice retreat and increased ice scouring on the sea floor. Based on new data and data collected from the literature, we analyzed the vulnerability of the fish community to these threats.

The potential vulnerability and acting mechanisms differ among species, developmental stages and habitats. The icefishes (family Channichthyidae) are one group that are especially vulnerable to a changing South Polar Sea, as are the pelagic shoal fish species *Pleuragramma antarcticum*. Both will almost certainly be negatively affected by abiotic alterations and changes in food web structure associated with climate change, the latter additionally by habitat loss. The major bottleneck for the persistence of the majority of populations appears to be the survival of early developmental stages, which are apparently highly sensitive to many types of alterations. In the long term, if climate projections are realized, species loss seems inevitable: within the demersal fish community, the loss or decline of one species might be compensated by others, whereas the pelagic fish community in contrast is extremely poor in species and dominated by *P. antarcticum*. The loss of this key species could therefore have especially severe consequences for food web structure and the functioning of the entire ecosystem.

1. INTRODUCTION

Climate change in the Antarctic is not simply a future scenario but already a well-established fact (e.g. Curran et al., 2003; Gille, 2002; Jacob et al., 2011; Murphy et al., 2007, Rignot et al., 2008). Its impacts are most evident in the Antarctic Peninsula region (including the southern Bellingshausen and Amundsen seas), where average temperatures at the sea surface have increased by nearly 3 °C within just the past 50 years (~0.56 °C increase decade^{-1}; e.g. Domack et al., 2003; Turner et al., 2005), and winter temperatures have increased by 5–6 °C (Vaughan et al., 2003). This represents a dramatic increase in air temperature in this region far above the global mean and exceeds any other warming rate observed on Earth at comparable spatial scales; the causes, however, are still under discussion (Gille, 2008; Vaughan et al., 2003).

The corresponding warming of the seawater is less pronounced, though again highly significant: the upper water layers (down to about 50 m water depth) west off the Antarctic Peninsula have warmed by ~1 °C since 1955 (Meredith and King, 2005) and are predicted to rise by another 2 °C over the next century (Murphy and Mitchel, 1995). In Palmer Deep, West Antarctic Peninsula, bottom water temperature has been increasing at a rate of about 0.01 °C $year^{-1}$ since the 1980s (Smith et al., 2012). Moreover, there is some indication of a warming trend emerging in the deep waters of the Ross and Weddell seas (Ozaki et al., 2009; Robertson et al., 2002).

Increasing temperatures significantly affect ice dynamics: the warming has already resulted in a significant reduction in extent and duration of sea ice in the Antarctic Peninsula region, the Amundsen and Bellingshausen seas (Jacobs and Comiso, 1997; Loeb et al., 1997; Stammerjohn et al., 2008a,b) and has also contributed to disintegration and collapses of large ice shelves, such as the northern part of the Larsen ice shelf in the northwestern Weddell Sea (Domack et al., 2005; Marshall et al., 2006). In some shelf regions at the Antarctic Peninsula and in the Ross Sea, melting ice shelves, increased glacial meltwater runoff and reduced sea ice production have led to reduced seawater salinity, particularly in surface water layers (Jacobs et al., 2002; Moline et al., 2004). In Potter Cove, King George Island (South Shetland Islands, west Antarctic Peninsula), a glacier retreat of hundreds of metres and significant freshening of the upper water column have been observed within the past 15 years alone (Schloss et al., 2008).

Ocean acidification is considered a major threat for marine ecosystems that is concomitant with warming and atmospheric change (IPCC, 2007). Anthropogenic CO_2 emissions have increased atmospheric CO_2 concentrations since the industrialization in the 1850s, and about one-third of anthropogenic CO_2 from the atmosphere is absorbed by the world's oceans (Sabine et al., 2004). CO_2 is physically dissolved in seawater and this leads to progressive ocean acidification: several models predict a drop of seawater pH by 0.3–0.5 units by the year 2100 (atmospheric pCO_2 of 1000 μatm) and up to 0.77 units until the year 2300 (atmospheric pCO_2 of 1900–2300 μatm; Caldeira and Wickett, 2003, 2005; Feely et al., 2004; IPCC, 2007). So far, information on CO_2 changes in the Antarctic marine ecosystem is scarce, but local measurements of atmospheric CO_2 concentrations recorded at the permanent Argentinian station 'Carlini[1]', at the shoreline of Potter Cove, revealed a trend of increasing concentrations over a

[1] Formerly known as 'Jubany' (renamed in March 2012).

relatively short time (from 356 µatm in 1994 to 379 µatm in 2006; Ciattaglia et al., 2008).

Though all the mechanisms involved and their interactions are not yet fully understood, there is little doubt that many of these observed changes are beyond that associated with natural variability but caused at least in part by anthropogenic climate change. In the light of ongoing global climate change, it is most likely that those regions of the Antarctic where alterations are not yet evident will also be affected in the near future.

Extant Antarctic marine communities have already been significantly affected by these environmental changes. In Potter Cove, clear shifts in benthic community composition have been observed that appear to be related to increased sediment load in the water column and ice impact due to melting and disintegration of the glacier (Sahade et al., 2008). Changes in salinity alter seawater density and thus can affect stratification of the water column and the depth of the mixed layer: salinity and surface water stratification are two main factors determining phytoplankton composition (Arrigo et al., 1998; Moline et al., 2004). Off the west Antarctic Peninsula, Moline et al. (2004) observed a recurrent change in phytoplankton community structure, with a spatiotemporal shift from large diatoms towards small cryptophytes as salinity declined. Alterations in community structure are also evident in consumers higher in the food chain: since the 1970s, the abundance of krill (*Euphausia superba*) has declined in the southwestern Atlantic and salps have become more abundant (Atkinson et al., 2004). As life cycle and overwintering strategy of Antarctic krill are closely coupled to the sea ice, its accelerating retreat will suppress krill abundance (Atkinson et al., 2004; Loeb et al., 1997). Other factors contributing to krill decline might be water temperature itself, as krill prefers cooler water compared to salps (e.g. Pakhomov et al., 2002), and indirect (trophodynamic) effects, including predation of early krill stages by salps (Huntley et al., 1989) and the inability of krill to efficiently graze on small cryptophytes (see Moline et al., 2004 and references therein). Moreover, salps feed efficiently on a wide range of particles even when phytoplankton concentrations are low (Hopkins, 1985; Kremer and Madin, 1992; Madin, 1974) and are able to attain large population sizes and biomass rapidly (e.g. Mianzan et al., 2001). Under favourable environmental conditions, their efficient grazing and high ingestion rates (e.g. Perissinotto and Pakhomov, 1998a,b) could result in the competitive exclusion of other grazers, such as copepods.

Though there are no immediately obvious visible effects reported so far, Antarctic marine communities could be vulnerable to ocean acidification in the future. Recent experimental studies have shown that survival and development of early stages of calcifying invertebrates and Antarctic krill are significantly negatively affected by levels of predicted future CO_2 concentrations (Kawaguchi et al., 2011; Kurihara, 2008).

Increasing warming of the Southern Ocean will facilitate the invasion and/or introduction and colonization by species from adjacent oceans. Invasion by invertebrates via warming deep waters appears to be already underway. Lithodid crabs have been absent from Antarctic waters for millions of years (see e.g. Thatje et al., 2005), but reports of sightings in Antarctic waters have been accumulating in recent years (e.g. García Raso et al., 2005; Thatje et al., 2008). Recently, a large and reproductive population of king crabs (*Neolithodes yaldwyni*) was discovered in Palmer Deep, west of the Antarctic Peninsula (Smith et al., 2012). So far, the distribution of lithodids seems to be restricted to the deeper slope, but assuming a persistent warming of waters of the Antarctic Peninsula region, Smith et al. (2012) speculated that lithodids might migrate upwards and invade the shelf communities within the next 20 years. Invasion of such hitherto absent durophagous (i.e. shell- or skeleton-crushing) predators strongly affects benthic communities (Aronson et al., 2007). The presence of the king crabs in Palmer Deep has been associated with a decrease in diversity of the megabenthos, including an absence of echinoderms (Smith et al., 2012).

Another more direct impact of human activity is in the form of the increasing ship traffic by tourist cruise ships and research vessels, which further enhance the risk of introduction of exotic species to the South Polar Sea (Lee and Chown, 2007; Lewis et al., 2005, 2006). The North Atlantic spider crab *Hyas araneus*, for example, was found in benthic samples from the Antarctic Peninsula: a species usually only found in the North Atlantic and Arctic Ocean that was most likely introduced into the Southern Ocean via ships' sea chest or ballast water (Tavares and De Melo, 2004). Another passive, man-made pathway for invasion is the increasing amount of litter in the world's oceans. Non-indigenous species may be introduced into the Southern Ocean by transport on drifting plastic debris (Barnes, 2002; Lewis et al., 2005 and citations therein).

Invasion/introduction of alien species will most likely lead to strong alterations in food web structure owing to removal of prey for indigenous species, competition and predation (Woodward et al., 2010a). So far, only

invasion by crustaceans has been detected, but if the warming trend continues, it is inevitable that further species, both benthic and pelagic, will invade the Antarctic marine ecosystem. As long as conditions in the South Polar Sea are appropriate for survival but still limit growth and in particular reproduction capacity of invasive species, the threat for indigenous species will remain low. However, once alien species become able to successfully reproduce and to build up populations (as it seems to be the case in the lithodids found in Palmer Deep; Smith et al., 2012), the threat for native Antarctic species significantly increases.

The direction and strength of ecosystem response to environmental change depend strongly upon responses of individual species and their interactions among each other. Fishes are an integral part of marine ecosystems and have been proposed to serve as useful bio-indicators of climate change (Dulvy et al., 2008; McFarlane et al., 2000). As organisms within an ecosystem are linked to each other directly or indirectly via trophodynamics, any kind of change affecting fishes will indirectly affect other members of the food web, with a huge range of potential indirect effects being triggered. For many decades, scientists retained a view that Antarctic food chains were relatively short and simple: essentially a connection from diatoms to krill to consumers. Krill, *E. superba*, in particular was regarded as an inexhaustible resource that underpinned the whole Antarctic food web, supporting fishes, penguins, seabirds, seals and whales (e.g. Murphy, 1962; Tranter, 1982). However, this paradigm has been challenged as being overly simplistic. Although krill does indeed seem to be a key species over large areas, many food chains are independent of it (e.g. Rodhouse and White, 1995), and high species numbers in the South Polar Sea (e.g. Gutt et al., 2004) suggest that the diatom–krill–consumer chain is only one component of a highly complex food web (Clarke, 1985; Jarre-Teichmann et al., 1995). Fishes take a central position in this ecological network: they occupy a variety of trophic niches, are the main consumers of benthos and plankton, and are an important food source for a multitude of species, including cephalopods, piscivorous fishes, penguins, flying birds, seals and whales (for review, see Barrera-Oro, 2002; Hureau, 1994; Kock, 1992; La Mesa et al., 2004). Fishes thus represent an important trophic link that connects small invertebrates and top predators of the Antarctic marine ecosystem, making their potential vulnerability to systemic shifts of particular interest.

In this chapter, we provide an overview of the potential effects of climate change on Antarctic fish species and communities. Based on our own data

collected during several expeditions and data that were taken from the literature, we summarize the characteristics of the Antarctic marine ecosystem and the fish communities, and we evaluate the threats to fishes, the degree of endangerment of particular species and the potential consequences for overall ecosystem functioning.

2. THE ANTARCTIC MARINE ECOSYSTEM

2.1. Geographical and physical conditions

The ocean surrounding the Antarctic continent represents one of the most unique marine environments on Earth, an appreciation of which requires a brief review of the geological and climatological history and settings of the Antarctic and its adjacent waters and land masses (summarized in Clarke and Johnston, 1996; Eastman, 1991). In the Jurassic, Antarctica was still part of the supercontinent Gondwana, which subsequently broke up, leaving Antarctica connected to South America and Australia throughout the Cretaceous (about 65 Ma[2] ago), when the climate was temperate, with water temperatures above 10 °C. Between late Eocene and early Oligocene (about 38 Ma), separation of Antarctica from Australia was most likely completed, and seawater temperatures began to decrease sharply. The separation from South America and the formation of sea ice and the continental ice sheet began between 37 and 34 Ma (e.g. Ehrmann and Mackensen, 1992; Ivany et al., 2008; Pearson et al., 2009). The final separation from South America and the opening of the Drake Passage allowed for the development of the strongest current system in the world, the Antarctic Circumpolar Current (ACC), driven by strong westerly winds. The ACC encircles the whole continent and acts as a thermal barrier by effectively separating lower latitude warmer and higher latitude colder waters (see e.g. Orsi et al., 1995).

The Antarctic continent and shelf areas are now geographically isolated from other continents and shelves by great distances and the large abyssal basins of more than 4000 m water depths that surround it (Fig. 1): the only connection to other continents with water depths less than 2000 m is via the Scotia Ridge composed of numerous islands linking South America to the Antarctic Peninsula (Arntz et al., 2005; Tomczak and Godfrey, 1994). In addition, the continent and the surrounding ocean are thermally isolated by the ACC, which flows eastwards and connects the Atlantic, Indian

[2] Millions of years before present (Ma).

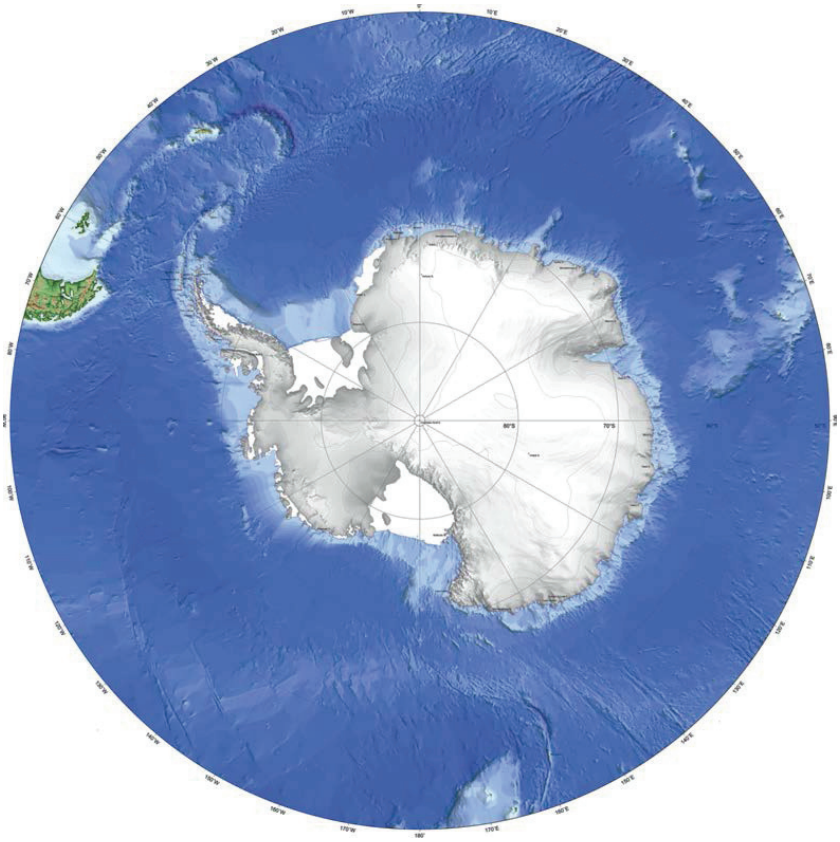

Figure 1 Map of Antarctica and the Southern Ocean *(Source: Centenary edition of the GEBCO Digital Atlas).*

and Pacific basins. This strong current system includes the Antarctic Polar Front, a region of downwelling and sharp temperature change of 3–4 °C (Knox, 1970). As a result, water temperatures in the South Polar Sea are consistently low (ranging from +1 to −1.86 °C close to the continent) with little seasonal variation (Deacon, 1984; Olbers et al., 1992). Close to the continent, the Antarctic Coastal Current (East Wind Drift) flows in the opposite direction and forms clockwise gyres in the Weddell Sea, Ross Sea and Bellingshausen Sea (Gordon and Goldberg, 1970). The region between both current systems is an area of wind- and density-driven upwelling of nutrient-rich circumpolar deep water (Antarctic Divergence), overlaid by Antarctic surface water in the upper layers (e.g. Eastman, 1993).

Beside the unique current system, the most important physical feature structuring the Antarctic marine ecosystem is the ice. The whole Antarctic shelf is narrow and depressed by the large continental ice sheet to depths of

about 200 to >600 m. Shallow benthic habitats are thus extremely rare in the South Polar Sea and mostly restricted to the Islands of the Scotia Arc and along the Antarctic Peninsula. The continental ice sheet extends far beyond the coastline, forming large, floating ice shelves and a major source of calving icebergs (Nicol and Allison, 1997), which significantly affect vast areas of the shelf by grounding and seabed scouring (Gutt, 2001).

Sea ice is present year round but the overall coverage varies strongly with season, ranging from 4×10^6 km^2 in the austral summer to 20×10^6 km^2 in winter (Nicol and Allison, 1997; Zwally et al., 1983). Three major zones are distinguished based on prevailing sea ice conditions (Eicken, 1992): (i) the *high Antarctic zone* is almost permanently covered by ice and includes most areas close to the continent; (ii) the *seasonal sea ice zone* is characterized by open water in summer and ice coverage in winter; (iii) the *marginal ice zone* represents the transition from sea ice to the ice-free open ocean and is a region of enhanced ice drift, fragmentation and deformation. Sea ice dynamics significantly affect stratification of the underlying water column. During autumn, the depth of the mixed layer in the ice-free zone is mainly determined by the wind regime. During ice formation and growth, cold and highly saline (and thereby highly dense) seawater is ejected from the ice into the water below, resulting in thermohaline convection and a deepening of the mixed layer (and the pycnocline) to a depth of 50–200 m. In spring during sea ice melt, the entry of freshwater with low density lowers and stabilizes the pycnocline (Eicken, 1995; Gordon et al., 1984).

Light conditions in the Antarctic and in the upper layer of the South Polar Sea also undergo strong seasonal changes, ranging from 24 h of light in summer to complete darkness during the winter months.

Notwithstanding these strong seasonal fluctuations in ice coverage and light regime, the general physical conditions and cold climate in the South Polar Sea have been stable for more than 15 Ma (Dayton, 1990; Dayton et al., 1994).

2.2. Biological characteristics

The Antarctic marine biota are well adapted to the physical conditions in their environment, particularly in the high Antarctic where primary production, life cycles and strategies are closely coupled to seasonal sea ice dynamics. During winter, autotrophic primary production is low and mostly restricted to the sea ice (Arrigo et al., 1997; Lizotte, 2001). During spring

and summer, when the sea ice is melting, the released ice algae fuel subsequent phytoplankton blooms in the shallow and stable mixed layer of the marginal ice edge (Lizotte, 2001; Smith and Nelson, 1986); these blooms are mainly formed by diatoms and *Phaeocystis* (Estrada and Delgado, 1990; Nöthig et al., 1991). In autumn, sea ice extends again and remaining algae are incorporated into newly formed ice (e.g. Melnikov, 1998). The large microphytoplankton (>20 μm) blooms account for most of the annual primary production (e.g. Scharek and Nöthig, 1995; Smith and Sakshaug, 1990) but their occurrence is limited in time and space. Pico- (0.2 to <2.0 μm) and nanoplankton (2.0 to <20 μm) are present in the water column throughout the whole year but these small size classes achieve much lower biomass and productivity compared with the bloom system (Detmer and Bathmann, 1997; Scharek and Nöthig, 1995).

Primary and secondary consumers in the water column are mainly represented by copepods, hyperiid amphipods, salps, fish larvae, chaetognaths and euphausiids; larger pelagic predators include squids and fishes (Hempel, 1985; Siegel et al., 1992). Antarctic krill, *E. superba*, is a dominate member of the community in the seasonal sea ice zone and the life history pattern of this species is closely linked to the seasonal sea ice cycle (Smetacek et al., 1990). In the high Antarctic zone, *E. superba* is replaced by a smaller congener *Euphausia crystallorophias*, the so-called ice krill (e.g. Hempel, 1985). Most zooplankton species are present and feeding in the upper water column or at the ice underside the whole year round (Bathmann et al., 1991; Marshall, 1988; Øresland, 1995; Smetacek et al., 1990).

Benthic shelf communities in the high Antarctic are characterized by extraordinarily high biomass and diversity (Brey and Gerdes, 1997; Dayton et al., 1994; Gutt et al., 2004) and are characterized by the dominance of suspension and deposit-feeding species such as sponges, ascidians and echinoderms (Dayton et al., 1974; Gutt and Starmans, 1998; Voss, 1988). In many regions, from shallow water coastal zones to deeper high Antarctic shelf areas, benthic community structure is shaped by physical disturbance, in particular by ice (Gutt, 2000, 2001; Sahade et al., 1998; Smale et al., 2008). In the eastern Weddell Sea, for example, the disturbance of the seafloor by grounding icebergs results in a patchy distribution of various successional stages, which increases between-habitat diversity (Gutt, 2000, 2001; Gutt and Piepenburg, 2003; Knust et al., 2003) which adds a spatiotemporal component to changes in the structure of the benthic food webs (Hagen et al., 2012).

In vast areas of the high Antarctic shelf, large sponges form a typical 3-dimensional habitat for a diverse invertebrate and fish community (Arntz et al., 1994; Gutt and Starmans, 1998). Sponges are often used by invertebrates and fishes as a refuge and/or nursery as well as an upper level substrate to benefit from enhanced access to the water column (Fig. 2A,B). In shallow, inshore areas (e.g. Potter Cove in King George Island), benthic macroalgae such as *Desmarestia* spp., *Himantothallus grandifolius* and *Palmaria decipiens* contribute significantly to primary production (Quartino and Boraso de Zaixso, 2008). As with the sponges on the deeper shelf, benthic macroalgae in shallow waters provide an analogously complex habitat and shelter for a multitude of species including fishes in coastal communities (e.g. Barrera-Oro and Casaux, 1990; Gambi et al., 1994; Moreno et al., 1982; Tada et al., 1996; Takeuchi and Watanabe, 2002) and are a major food source for secondary producers (Iken, 1996; Tatián et al., 2004). Below the depth zone of macroalgal presence, benthic consumers depend on pelagic production (e.g. Mincks et al., 2008). On the high Antarctic continental shelf, where benthic macroalgae are absent over vast areas, tight benthopelagic coupling plays an important role in the food web. The high benthic biomass found on the shelf indicates a highly efficient transfer of organic matter from surface waters towards the seafloor (Smith et al., 2006). The vertical export of energy is driven either passively, via sinking particulate organic matter (POM), or actively by migrating organisms.

POM flux on the shelves is dominated by faecal pellets and strings, and large diatoms (Bathmann et al., 1991; Bodungen et al., 1988; Fischer, 1989; Nöthig and Bodungen, 1989). Mass sedimentations of ice algae, *Phaeocystis*

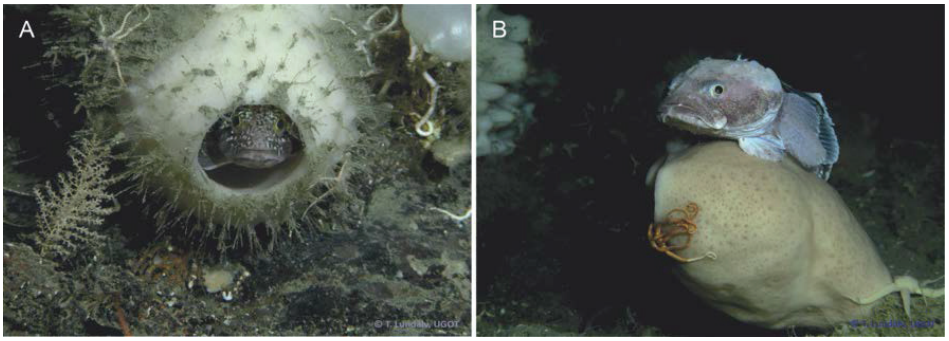

Figure 2 (A) *Trematomus* cf. *nicolai* hiding inside a sponge; (B) *Pogonophryne* sp. on top of a sponge (ANT XXVII-3 in 2011, western Weddell Sea). Photos: ©Tomas Lundälv, University of Gothenburg.

or diatoms after ice melt and termination of blooms are seasonally important export mechanisms (DiTullio et al., 2000; Riebesell et al., 1991; Scharek et al., 1999). Several zooplankton species including Antarctic krill, copepods and salps were observed to undertake extensive vertical migrations within the water column (Atkinson et al., 1992; Gili et al., 2006; Zhou and Dorland, 2004), thereby significantly contributing to the energy export from the euphotic zone towards the seafloor.

The marine living communities of the South Polar Sea are exploited by a multitude of endothermic animals. Whales and seabirds are seasonal visitors that forage in the seasonal sea ice zone and under the pack ice during summer (Boyd, 2002; Murase et al., 2002; Van Franeker et al., 1997). Penguins (mainly Emperor penguin, *Aptenodytes forsteri*, and Adélie penguin, *Pygoscelis adeliae*) and seals (Weddell seal, *Leptonychotes weddellii*; Ross seal, *Ommatophoca rossii*; Crabeater seal, *Lobodon carcinophagus*; Fur seal, *Arctocephalus gazella*; Elephant seal, *Mirounga leonina*) are permanent inhabitants of Antarctic coastal areas. Extensive cracks in the ice shelf covered by sea ice, such as the Drescher Inlet in the Riiser-Larsen Shelf ice (eastern Weddell Sea), represent particularly important breeding and foraging grounds for Weddell Seals and large Emperor Penguin colonies (Plötz et al., 1987).

3. ANTARCTIC FISH COMMUNITIES

3.1. Composition of the modern fauna

Despite the large area covered by the South Polar Sea (>20 million km^2), the modern fish fauna is composed of only about 320 species, belonging to 50 families (Eastman, 2005). This Antarctic ichthyofauna is unique for two reasons: (i) the modern fish fauna is highly endemic, with 88% of all species being confined to the South Polar Sea (Andriashev, 1987), and (ii) the communities are dominated by a single taxonomic group, the perciform suborder Notothenioidei, which accounts for about 35% of all Antarctic fish species (Eastman, 1993). In high Antarctic shelf areas, such as those of the eastern Weddell Sea, notothenioids form up to 98% of the total fish abundance and biomass (R. Knust and K. Mintenbeck, unpublished data). Groups typical of fish communities in temperate or boreal regions, such as clupeids, are absent. Non-notothenioid fish species inhabiting the South Polar Sea mostly belong to typical deep-sea groups such as zoarcids, liparids, macrourids and myctophids. The occurrence of these groups is largely restricted to the

lower slope and the deep sea where notothenioid fishes, except for the two *Dissostichus* spp. (*Dissostichus eleginoides* and *Dissostichus mawsoni*), are absent (Boysen-Ennen and Piatkowski, 1988; Donnelly et al., 2004; Gon and Heemstra, 1990; Kock, 1992).

Close to 100 notothenioid fish species have been described from the South Polar Sea (Eastman and Eakin, 2000) but new species are still being discovered (see e.g. Eakin and Balushkin, 1998, 2000; Eakin and Eastman, 1998; Eakin et al., 2008). Most species belong to just five families: Nototheniidae (notothens), Channichthyidae (icefish), Artedidraconidae (plunderfish), Bathydraconidae (dragonfish) and Harpagiferidae (spiny plunderfish). Endemism within the suborder is extremely high, with 97% of notothenioid species being found only in the Antarctic (Andriashev, 1987).

The diversity of the demersal fish community differs regionally, with a latitudinal shift in species composition (Hureau, 1994; Kock, 1992; Mintenbeck et al., 2003, 2012; Permitin, 1977). In the ice-free zone, on the Sub-Antarctic island shelves, typical members of the demersal fish communities are the channichthyids *Chaenocephalus aceratus* and *Champsocephalus gunnari*, the nototheniids *Patagonotothen guntheri*, *Gobionotothen gibberifrons*, *Lepidonotothen* spp., *Notothenia* spp. and *D. eleginoides* (Patagonian toothfish).

At higher latitudes, in the seasonal sea ice zone, communities are dominated by *Lepidonotothen* spp., *Notothenia* spp., *Chionodraco rastrospinosus* (Channichthyidae) and some species of the genus *Trematomus* (see also Barrera-Oro, 2002). In inshore shallow waters, the harpagiferid *Harpagifer antarcticus* is also abundant (Barrera-Oro, 2002; Barrera-Oro and Casaux, 1998).

The demersal fish fauna in the high Antarctic zone is characterized by several *Trematomus* (Nototheniidae), artedidraconid and bathydraconid species, and the channichthyids *Chionodraco* spp. and *Cryodraco antarcticus* (Donnelly et al., 2004; Eastman and Hubold, 1999; Hubold, 1992; Schwarzbach, 1988). In high Antarctic shelf regions, such as the eastern Weddell Sea shelf, species diversity is much higher than on the Sub-Antarctic island shelves or west of the Antarctic Peninsula (Mintenbeck et al., 2012; Schröder et al., 2001). The major reasons for this high species diversity are the 3-dimensionality of the benthic habitat and the high between-habitat diversity shaped by grounding icebergs, both allowing for small-scale niche separation (horizontally and vertically) and thus for the coexistence of trophically similar species (Brenner et al., 2001; Gerdes et al., 2008; Knust et al., 2003).

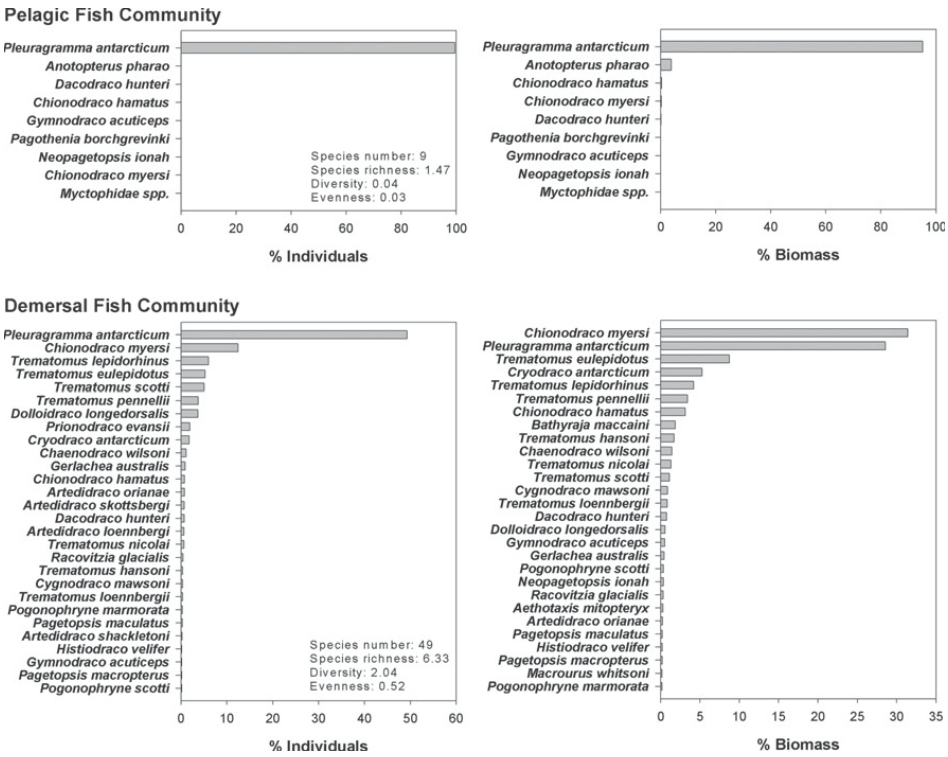

Figure 3 Composition of the pelagic and demersal fish communities on the eastern Weddell Sea shelf between 200 and 600 m water depth (samples from 26 otter trawl hauls and 10 hauls with a bentho-pelagic net taken between 1996 and 2004). Only the 28 out of 49 species contributing >0.15% to overall individuals and biomass are shown for the demersal community. Species number, species richness, diversity and evenness are given for the two communities using different scales.

The pelagic ichthyofauna of the South Polar Sea includes an oceanic and a neritic fish community. The oceanic pelagic communities off the shelves are mainly composed of several myctophid fish species (Barrera-Oro, 2002; Pusch et al., 2004). The neritic pelagic community differs significantly from the oceanic community and is extremely species poor compared with the demersal community on the shelf. In Fig. 3, this difference is exemplified by comparing the pelagic and the demersal fish communities on the eastern Weddell Sea shelf between 200 and 600 m water depth. The neritic pelagic fish community is composed of very few species, and most of them (e.g. the channichthyids *Chionodraco* spp., *Dacodraco hunteri*, *Neopagetopsis ionah* and the bathydraconid *Gymnodraco acuticeps*) are in fact demersal fishes that only occasionally move into the water column. The cryopelagic nototheniid *Pagothenia borchgrevinki* is closely associated with the underside of ice (e.g. Janssen et al., 1991) and is rarely found in open

waters. Both the demersal and pelagic fish communities are distinctly dominated by a single notothenioid species, the Antarctic silverfish *P. antarcticum*. The only other species that attains higher biomass in the demersal community is the large icefish *Chionodraco myersi* (Fig. 3). *P. antarcticum* is an endemic species with circum-Antarctic distribution and is one of the few truly pelagic representatives of the entire suborder Notothenioidei. It is a shoaling species (Eastman, 1985a) and adults undertake diel vertical migrations from the sea floor towards the surface waters (Fuiman et al., 2002; K. Mintenbeck and R. Knust unpublished data; Plötz et al., 2001). This species dominates the pelagic fish biomass in coastal waters of the South Polar Sea by >90% (see also DeWitt, 1970; Donnelly et al., 2004; Hubold and Ekau, 1987). In the southern Weddell Sea, *P. antarcticum* accounts for most of the overall fish production, and stock density was estimated to amount at least 1 ton km^{-2} (Hubold, 1992). Though usually found in the free water column, its life cycle strategy, including its feeding dynamics, seems to be closely associated with the sea ice (Daniels, 1982; La Mesa and Eastman, 2012; Vacchi et al., 2004). Besides adult *P. antarcticum*, larvae and early juveniles of several notothenioid species dominate the neritic pelagic fish community numerically. The notothenioid ichthyoplankton community is also dominated by early life stages of *P. antarcticum*. Notothenioid larvae are mainly concentrated in the upper 50 m in well-stratified surface waters, while juveniles occur in slightly deeper waters (Granata et al., 2002; Hubold, 1984, 1985; Hubold and Ekau, 1987; Kellermann, 1986a, b; Morales-Nin et al., 1998). Due to their dominate role in Antarctic fish communities, this chapter largely focuses on notothenioids.

3.2. Evolution and adaptive radiation

The uniqueness of the Antarctic fish fauna with its high degree of endemism and a single dominant group is the result of a long evolutionary history of adaptive radiation in isolation at sub-zero temperatures. Though fossil records are scarce, there is some evidence that the fish fauna in the Antarctic during the Eocene differed substantially from the modern fauna and that the community was composed of species from many, and more cosmopolitan, families (Eastman, 1993, 2005; Eastman and Grande, 1989). Following the complete separation of Antarctica and the progressive cooling of the region's waters, most components of the Eocene fish fauna vanished from shelf areas. Local extinctions likely occurred due to habitat loss associated with the

massive expansion of the ice sheet and changes in trophic structure (Eastman, 2005). After this period of extinctions, a multitude of niches were available for other species. These niches were filled by species of the suborder Notothenioidei, which have undergone a remarkable diversification by adaptive radiation on the isolated shelf of the Antarctic continent. The lack of competition from other fish groups allowed increased morphological and ecological diversification of notothenioid fish and expansion into various niches (e.g. Eastman and McCune, 2000; Ekau, 1988; Ptacnik et al., 2010). Accordingly, notothenioid fish species now occupy benthic, bentho-pelagic, pelagic as well as cryopelagic habitats. However, due to the lack of a swimbladder in their common ancestor, the majority of recent notothenioid species are demersal (Clarke and Johnston, 1996). Adaptive radiation of notothenioids also included trophic diversification (Ekau, 1988; Schwarzbach, 1988), and notothenioid fishes now occupy a multitude of trophic niches. Kock (1992) distinguished five main feeding types according to their principal prey: benthos feeders, fish and benthos feeders, plankton and fish feeders, plankton and benthos feeders, and plankton feeders. As some species, such as the channichthyid *D. hunteri*, rely almost exclusively on piscivory (Eastman, 1999; Schwarzbach, 1988), a sixth group of pure 'fish feeders' also exists.

The point at which the characteristic modern fauna became established exactly is unknown (Clarke and Johnston, 1996; Eastman, 2005). The few existing fossil records indicate first appearance of this group in the early Tertiary (38 Ma; Balushkin, 1994), and according to a recent phylogenetic study, radiation of notothenioids began near the Oligocene–Miocene transition (~24 Ma), coinciding with the enhanced formation of sea ice (Matschiner et al., 2011).

3.3. Adaptations and characteristics of notothenioid fishes

3.3.1 Physiological and morphological adaptations

Notothenioid fishes are characterized by a multitude of physiological adaptations to life within cold waters, but the key innovation enabling species to survive and diversify was most likely the evolution of antifreeze glycoproteins (AFGPs; Matschiner et al., 2011). AFGPs provide a highly efficient protection from freezing of hypoosmotic (compared to seawater) body fluids by adhering to and blocking the growth of ice crystals (DeVries, 1971; Fletcher et al., 2001). Concentrations of AFGP differ between species and depend on ambient water temperature, depth

distribution, life cycle, activity and phylogeny (Wöhrmann, 1996, 1997). AFGP synthesis is most likely regulated by ambient water temperature (Wöhrmann, 1997).

Beside the risk of freezing, another problem of life at sub-zero temperatures is the temperature dependence of viscosity of body fluids in ectotherms. Viscosity and temperature are strongly, negatively correlated, that is, body liquids become more viscous when cold, which affects membrane fluidity, blood circulation, enzyme kinetics and gas diffusion (e.g. Hochachka and Somero, 2002). Cell membrane fluidity is maintained in the cold by homeoviscous adaptation (see Sinensky, 1974), which involves an increased content of unsaturated fatty acids and specific membrane phospholipids (e.g. Eastman, 1993; Hazel, 1995). An increased blood viscosity is offset by reduced haematocrit and haemoglobin concentrations in notothenioid fishes (Egginton, 1996, 1997a,b; Kunzmann, 1991). The only known exceptions of fish with a relatively high haematocrit are *Notothenia coriiceps* and *N. rossii* from the Antarctic Peninsula (Beers and Sidell, 2011; Mark et al., 2012; Ralph and Everson, 1968). Owing to the low metabolic demands of notothenioids (e.g. Clarke, 1983; Clarke and Johnston, 1996) and the increased physical oxygen solubility in seawater, blood and cytosol at cold temperatures, the reduction in haematocrit and respiratory pigment is not detrimental to aerobic performance. In species of the family Channichthyidae, the so-called icefishes or white-blood fishes, functional red blood cells are completely absent: these fishes do not possess any oxygen-binding pigment (haemoglobin) in their blood and some species also lack intracellular myoglobin (Montgomery and Clements, 2000; Sidell and O'Brien, 2006).

The limited oxygen-carrying capacity of the blood is compensated by a multitude of secondary adaptive body modifications in icefishes, for example, a larger ventricle, increased blood volume and cardiac output, and increased skin vascularity (Kock, 2005a; O'Brien and Sidell, 2000; O'Brien et al., 2003; Sidell, 1991). Molecular adaptations also include the absence of heat-shock protein expression in some notothenioids (Carpenter and Hofmann, 2000) and a rearrangement of the mitochondrial genome that may have supported cold adaptation of mitochondrial properties (Mark et al., 2012; Mueller et al., 2011; Papetti et al., 2007; Zhuang and Cheng, 2010).

The increased viscosity of body fluids, together with cold temperatures, affect enzyme kinetics and cytosolic diffusion processes (Sidell, 1991). Both gas diffusion and enzyme kinetics are temperature dependent and decelerate rapidly at cold temperatures. Mitochondrial oxidative capacity of notothenioid fishes is low compared with warm water species

(Johnston et al., 1994), and reduced diffusion of gas and metabolites to and from mitochondria entails an additional reduction of available energy and oxygen. The negative effects of temperature and viscosity on enzymes and diffusion are counterbalanced in Antarctic fishes by two metabolic adaptations. First, these fishes have increased quantities and capacities of intracellular enzymes (Crockett and Sidell, 1990), which reduce diffusion distance and increase efficiency (Pörtner et al., 2000). Second, they display mitochondrial proliferation, an increase in mitochondrial abundance and ultra-structural density (Guderley and Johnston, 1996). Up to 60% of muscle fibre volume of the slow-swimming, pelagic notothenioid *P. antarcticum* is occupied by mitochondria (Clarke and Johnston, 1996). Additionally, many species have relatively high intracellular concentrations of lipids which may be used as energy stores (Crockett and Sidell, 1990; Eastman and DeVries, 1981) and aid gas diffusion (Kamler et al., 2001). These intracellular lipids also play a role in buoyancy (see below).

Notothenioid fishes are thus well adapted to cold waters. Nevertheless, these adaptations apparently involve an extreme stenothermy of physiological functions and seem to result in narrow thermal tolerance windows of this group (Johnston, 2003; Mueller et al., 2011; Pörtner and Peck, 2010; Somero and DeVries, 1967).

However, the success of notothenioid species in the South Polar Sea ecosystem is not only based on physiological adaptations, but also on morphological modifications related to buoyancy (Eastman, 2005; Eastman et al., 2011). Notothenioids lack a swim bladder and, without this organ, the exploitation of bentho-pelagic or pelagic food sources is extremely energy consuming. To compensate for the lack of a swim bladder, some notothenioid species developed modifications in body structure, which allow them to inhabit and to exploit the pelagic realm without an energetic disadvantage. In these species, mineralization of skeleton and scales is reduced, and the skeleton contains a high proportion of cartilage, which is less dense than bone (DeVries and Eastman, 1978; Eastman and DeVries, 1981, 1982; Eastman et al., 2011). *P. antarcticum* has a persistent notochord and large amounts of lipids (accounting for ~39% of muscle dry mass) are stored in subcutaneous and intramuscular lipid sacs which provide static lift (DeVries and Eastman, 1978). *D. mawsoni* possess extensive lipid deposits in adipose cells, which account for ~23% white muscle dry weight (Eastman and DeVries, 1981). The lipid deposits in these species mainly consist of triglycerols (Eastman and DeVries, 1981, 1982; Hubold and Hagen, 1997). However, neutral buoyancy is rare in

notothenioids and limited to very few species, for example, *P. antarcticum*, *D. mawsoni* and *Aethotaxis mitopteryx* (Eastman, 2005).

3.3.2 Growth, reproduction and development

Most notothenioids are characterized by a rather sluggish mode of life and high longevity. High Antarctic fish species typically reach ages of 15–21 years (see Kock, 1992; La Mesa and Vacchi, 2001 for review). The pelagic *P. antarcticum* was estimated to live more than 30 years (Radtke et al., 1993). Growth performance of most species is similar to species from boreal or temperate regions (Kock and Everson, 1998; La Mesa and Vacchi, 2001) but there seems to be a trend towards lower growth performance in the high Antarctic notothenioids, relative to their congeners from the seasonal sea ice zone (Kock, 1992). However, in adult fish, growth performance is apparently related to lifestyle and tends to increase from pelagic towards benthic lifestyles (La Mesa and Vacchi, 2001). Pelagic fishes such as *P. antarcticum* are thus characterized by slow growth (e.g. Hubold and Tomo, 1989). Many notothenioids show a distinct seasonal growth pattern with high growth rates in summer and low growth rates in winter (Hureau, 1970; North et al., 1980; White, 1991). The interspecific latitudinal and intraspecific seasonal differences in growth rates of notothenioids most likely (primarily) stem from variations in food supply and/or prey composition, and feeding intensity (Kock, 1992). For example, *N. coriiceps* undergoes winter metabolic suppression and enters a dormant stage with periodic arousals lasting only a few hours, resulting in a net loss of growth rate during the winter months (Campbell et al., 2008). However, most notothenioids seem to feed year round (e.g. Casaux et al., 1990; Hubold, 1992).

Sexual maturity is delayed in most Antarctic fishes. With a few exceptions, species reach maturity at 50–80% of their maximum age and size (Kock and Everson, 1998; La Mesa and Vacchi, 2001). The spawning season is species- and location specific: in the seasonal sea ice zone, most species spawn in autumn/winter and in the high Antarctic zone, most species are summer and autumn spawners (Kock and Kellermann, 1991). Some species have demersal eggs, which are often laid on rocks or in the cavity of sponges. Nest-guarding and other parental care behaviours have been reported increasingly in notothenioids (e.g. Barrera-Oro and Lagger, 2010; Detrich et al., 2005; Kock et al., 2006; Moreno, 1980). Others species, for example, *P. antarcticum*, have pelagic eggs (Faleyeva and Gerasimchuk, 1990; Vacchi et al., 2004). The eggs are usually large

and yolky, so relative fecundity is low in most species, particularly in high Antarctic notothenioids (Hubold, 1992; Kock, 1992; Kock and Kellermann, 1991). The incubation period of eggs is long and usually takes several months (Hubold, 1992; Kock and Kellermann, 1991; North and White, 1987). Larvae of many species apparently hatch in spring and summer (Efremenko, 1983) when food conditions are best; however, some species also hatch in winter (Ekau, 1989; North and White, 1987). Larvae are large at hatching (Kellermann, 1990; North and White, 1987) and the mouth is well-developed, so that even early yolk-sac larvae are able to feed (Kellermann, 1986b). Most, if not all, notothenioid larvae are pelagic.

3.4. Threats to the fish community

Antarctic fish communities are threatened by climate change in multiple ways. On the one hand, fishes might be affected at the physiological level directly by increasing water temperatures and pCO_2, and reduced water salinity. Due to the numerous adaptations to life in the South Polar Sea, fishes are likely to be affected on different organizational levels, from the cellular level up to the population level and beyond. In particular, an increase of water temperatures might pose a major threat to stenothermal species (see e.g. Somero, 2010), whereas increasing concentrations of CO_2 might have more general detrimental effects across many fish species (e.g. Ishimatsu et al., 2005). Whether and to what extent fitness and survival are affected by such changes depends on individual or species-specific physiological plasticity.

Climate change can additionally affect fishes indirectly by secondary effects, such as those due to changes in the abiotic environment that will entail alterations in the food web, as also reported for instance in many freshwaters (Meerhoff et al., 2012). Unfavourable abiotic conditions as well as invasion of Antarctic waters by non-indigenous species may result in changes in trophic structure and dynamics by alterations in composition and population density of prey and predator communities (Woodward et al., 2010a).

Changes in prey species composition will involve alterations in the type of prey available to fishes, particularly in size structure and energy content: a shift from diatoms to cryptophytes is accompanied by a strong shift in size structure of primary producers, and ultimately with a potential size shift in secondary producers; a shift from a krill dominated zooplankton community towards a community dominated by salps involves a drastic decrease in nutritional value of potential prey for higher trophic level consumers (but

see also Gili et al., 2006). In the marine Antarctic, where life cycles are closely coupled to seasonal sea ice dynamics, changes in water temperature itself but also reduced sea ice extent and duration might entail phenological shifts and a trophic mismatch between prey and consumer species (Hagen et al., 2012). Secondary effects of climate change may also involve a reduction of habitat, which could lead to fragmentation of the food web (Hagen et al., 2012): sea ice reduction means a loss of habitat for ice-associated pelagic species, but demersal fish are threatened by habitat reduction as well, as warmer temperatures will most likely result in enhanced disintegration of glaciers and ice shelves. An increased iceberg calving and breakup will lead to a higher frequency of iceberg scouring events and thus to increased seafloor and habitat destruction.

From other ecosystems, it is well-known that many fish species are sensitive to these types of threats, via mechanisms operating directly at the ecophysiological level (e.g. McFarlane et al., 2000; Pörtner and Peck, 2010; Pörtner et al., 2008) but also indirectly at the trophic level (Beaugrand et al., 2003; Benson and Trites, 2002; Drinkwater et al., 2010), as well as by alterations in habitat structure and heterogeneity (Hughes et al., 2002; Yeager et al., 2011).

4. PHYSIOLOGICAL VULNERABILITY OF ANTARCTIC FISHES

4.1. Sensitivity to changes in temperature and salinity

Antarctic fishes have very narrow thermal windows due to cold adaptation (Clarke, 1991; Somero et al., 1968; Wohlschlag, 1963), resulting in high stenothermy in this group (Gonzalez-Cabrera et al., 1995; Podrabsky and Somero, 2006; Robinson et al., 2011; Somero and DeVries, 1967). Most species, for example, the bottom-dwelling *Trematomus bernacchii*, *Trematomus hansoni* and *Trematomus pennellii*, have an upper lethal temperature between just 4 and 6 °C (Robinson, 2008; Somero and DeVries, 1967). Fish performance is already affected well below the lethal limit. However, the paradigm that all notothenioid species are extremely stenothermal without exceptions has recently been revised.

Some species such as *H. antarcticus* and young *N. coriiceps* are frequently found in tide pools in King George Island (South Shetland Islands), where during sunny days individuals are exposed to warm temperatures for many hours (E.R. Barrera-Oro and E. Moreira, personal observation). Thus, these species can at least cope with acute, relatively short-term temperature

increases. A few species are apparently also able to compensate for chronic exposure to higher temperatures, for example, the cryopelagic *P. borchgrevinki* shows some metabolic plasticity: long-term warm acclimation of *P. borchgrevinki* to 4 °C results in a shift of the thermal tolerance window towards warmer temperatures (Bilyk and DeVries, 2011; Franklin et al., 2007; Robinson and Davison, 2008) owing to metabolic compensation (Seebacher et al., 2005) which leads to a reduced performance at low temperatures (Franklin et al., 2007). Recent measurements of routine metabolic rate of *Notothenia rossii* and *Lepidonotothen squamifrons* from the Scotia Arc shelf revealed a partial compensation after long-term acclimation to elevated temperatures (A. Strobel and F.C. Mark, unpublished data). Similarly, long-term warm acclimation of the Antarctic eelpout *Pachycara brachycephalum* involves metabolic rearrangements (Lannig et al., 2005) and indicates an improvement of hepatic metabolism accompanied by a shift of energy sources from lipids to carbohydrates (Brodte et al., 2006, Windisch et al., 2011).

However, these metabolic acclimations apparently do not result in a full compensation and cannot be generalized across all species (c.f. the deepwater zoarcid *Lycodichthys dearborni*; Podrabsky and Somero, 2006), but seem rather dependent on the physical capacities of the circulatory system: studies of energy allocation in isolated cells of Antarctic notothenioids and *P. brachycephalum* suggest that within a thermal range of about −1 to 12 °C, thermal tolerance limits are defined at the whole organism level (Fig. 4), for

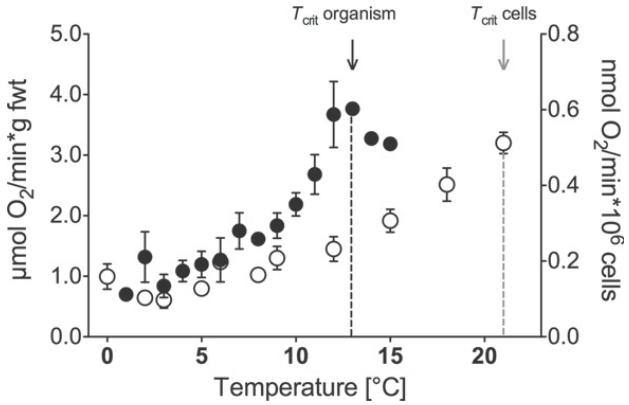

Figure 4 Temperature dependence of whole animal metabolic rate (filled symbols, left axis) and respiration rate of hepatocytes (open symbols, right axis) of the Antarctic eelpout *Pachycara brachycephalum*. Due to organismal complexity, acute whole animal critical temperatures (T_{crit}) that mark the onset of anaerobic metabolism are reached at lower temperatures (~13 °C, black arrow) than in isolated cells (>21 °C, grey arrow; *redrawn from Mark et al., 2002, 2005; with permission from Springer).*

example, by capacity limitations of the circulatory system rather than by a general failure of cellular energy metabolism (Mark et al., 2002, 2005). Due to their lower level of organizational complexity, thermal tolerance windows of organelles generally span a wider temperature range than those of the whole organism (Mark et al., 2005). Thus, acclimatory capacities are mainly defined by the degree of changes in cellular energy metabolism and depend strongly on the mitochondrial oxidative capacities. The acute thermal tolerance of oxidative capacity varies between species and tissues (Mark et al., 2012; Urschel and O'Brien, 2009). However, these acclimatory capacities are always constrained within the frame of the thermal window set by the whole organism's physiological plasticity.

Fishes possess the flexibility to respond to chronically elevated temperatures via mitochondrial proliferation—modifications of the amount and volume of mitochondria to adjust aerobic capacity (Tyler and Sidell, 1984; Urschel and O'Brien, 2008). Mitochondrial oxygen demand rises with increasing temperature and aerobic capacities need to be adjusted accordingly. Only a few studies have demonstrated a full compensation of the increased oxygen demand at the mitochondrial level after warm acclimation, and these studies were exclusively performed on non-Antarctic organisms (e.g. Dahlhoff and Somero, 1993; Sloman et al., 2008). This indicates that there are limitations to mitochondrial acclimation, ultimately co-defining the rather narrow bandwidth of thermal acclimation, especially in Antarctic fishes (Mark et al., 2006). Increased mitochondrial oxygen demand in warmer conditions may be met by altering haemoglobin affinities (Tetens et al., 1984) and raising the haematocrit to optimize the oxygen-carrying capacity of the blood. Yet, haematocrit levels are correlated with haemoglobin expression (Beers and Sidell, 2011). Thus, species with low or no haemoglobin levels have less capacity to adjust haematocrit. This is further exacerbated by the fact that rising temperatures result in lower levels of physically dissolved oxygen in the blood. Therefore, icefishes that lack haemoglobin will be more vulnerable to warming than red-blooded species, because they cannot increase the oxygen-carrying capacity of the blood. According to Beers and Sidell (2011), Antarctic fishes with higher haematocrit levels thus possess higher temperature acclimatory capacities than species with lower haematocrit.

Studies on the impact of salinity changes on notothenioid fishes are extremely scarce. O'Grady and DeVries (1982) investigated the capacity for osmoregulation of adult *P. borchgrevinki* and *Trematomus* spp. at a wide range

of salinities (25–200% of the salinity in their natural habitat, 35 psu) and found these species to be rather tolerant towards even large fluctuations from 50% to 175% normal salinity. Blood serum osmolarities in Antarctic fishes are among the highest in marine teleosts, which has been interpreted as an additional antifreezing protection (O'Grady and DeVries, 1982). After warm acclimation, serum osmolarities reduced to the levels found in temperate teleosts were measured in *T. bernacchii*, *T. newnesi* and *P. borchgrevinki* (Gonzalez-Cabrera et al., 1995; Hudson et al., 2008; Lowe and Davison, 2005), and also in *N. rossii* and *L. squamifrons* (A. Strobel and F.C. Mark, unpublished data). Thus, adult notothenioids are apparently capable of efficient osmoregulation and seem able to adapt blood osmolarity to ambient environmental conditions.

Almost all studies on physiological sensitivity of notothenioid fishes to changing abiotic parameters have been conducted on adults. Data from non-Antarctic fish species suggest an ontogenetic shift in temperature tolerance, with narrow thermal tolerance windows in eggs and larvae (e.g. Pörtner and Farrell, 2008; Pörtner and Peck, 2010). Most teleost fishes are able to osmoregulate at hatch, but the efficiency seems to be higher in more advanced developmental stages (Varsamos et al., 2005). To our knowledge, there are no experimental data for early developmental stages of notothenioid fishes, but indirect evidence from abundance and distribution of *P. antarcticum* larvae and juveniles indicate that these early stages likely have limited ability to tolerate changes in temperature and salinity. Larvae and juveniles of this species are mostly found within water masses of particular temperature and salinity (e.g. Granata et al., 2002; Guglielmo et al., 1998; Hubold, 1984; Kellermann, 1986a). West of the Antarctic Peninsula, *P. antarcticum* larvae and juveniles were clearly confined to cold and high salinity water masses originating from the Weddell Sea (Slósarczyk, 1986). Based on combined datasets of fish abundances in waters of Weddell Sea and Bellinghausen Sea origin, Slósarczyk (1986) calculated the range of approximate optimum conditions for larvae and juveniles in the Bransfield Strait: Abundances suggested optimal ranges in temperature and salinity of −0.50 to +0.45 °C and ~34.10–34.62 psu, respectively.

Though changes in salinity induced by climate change might be a locally restricted phenomenon, dense aggregations of larvae and juveniles that are concentrated close to the coast/shelf ice in the upper water layers might be significantly affected. Moline et al. (2004) found vast areas west of the Antarctic Peninsula covered by low salinity water (33.4–33.6 psu), and the meltwater plume extended to depths as great as 50 m (Dierssen

et al., 2002). It still needs to be verified whether and to what degree temperatures and salinities outside the narrow ranges given by Slósarczyk (1986) limit physiological performance and survival of larvae and juveniles, but tolerance indeed seems to be low: *P. antarcticum* larvae acclimated to cold Weddell Sea water were observed to shrink and to die immediately at water temperatures $>0\,^{\circ}C$ (Hubold, 1990). Whether or not this limited tolerance holds true for early stages of other species needs further investigation, but at least *P. antarcticum* larvae seem to be highly vulnerable to changes in the abiotic environment.

4.2. Sensitivity to increasing pCO_2

Ocean acidification, as an additional stressor in parallel to ongoing climate warming (Woodward et al., 2010a), may prove to be particularly threatening to polar ecosystems owing to enhanced CO_2 solubility in cold waters and body fluids. Thermal tolerance windows are narrow in most species and, thus, sensitivities to combined stressor effects are likely to be higher in cold-adapted polar compared to temperate species. Many notothenioids will eventually find themselves at the upper end of their thermal tolerance range, implying that they are energetically limited and their physiological performance is highly susceptible to further stressors, such as the increasing concentration of carbon dioxide (Pörtner and Peck, 2010).

Previous research on the effects of elevated CO_2 levels on marine fishes led to the general notion that fishes are not particularly vulnerable to the direct effects of ocean acidification alone, due to their powerful mechanisms of ion regulation (Fivelstad et al., 2003). Most adult fishes are able to compensate for acid–base disturbances (Larsen et al., 1997) and show only minimal effects of hypercapnia on physiological performance (Melzner et al., 2009) including an incomplete compensation of extracellular pH (Michaelidis et al., 2007).

However, several studies on different non-Antarctic fish species demonstrated chronic effects of environmental hypercapnia, with early developmental stages being particularly affected: exposure to elevated CO_2 concentrations impairs embryonic metabolism (Franke and Clemmesen, 2011), survival and growth of eggs and larvae (Baumann et al., 2012), and growth of juveniles (Moran and Støttrup, 2010), and causes severe to lethal tissue damage in many internal organs of larvae (Frommel et al., 2012). The sensitivity to ocean acidification may generally be enhanced

by ocean warming, which has been confirmed in tropical fishes (Nilsson et al., 2009; Pörtner and Farrell, 2008). Still very little is known about how the physiology and distribution of Antarctic fishes and their various life stages may be altered by the additional effects of hypercapnia, but several current projects are dealing with this topic, and initial results indicate that chronic hypercapnia leads to significant reductions of mitochondrial capacities in *N. rossii* (A. Strobel and F.C. Mark, unpublished data), on top of the thermal sensitivity of its mitochondrial metabolism (see Section 4.1 above; Mark et al., 2012). Hypercapnia-induced regulatory shifts in intracellular metabolic pathways and capacities therefore may exacerbate the effects of increased temperature on cellular and whole animal metabolism.

We currently lack sufficient data on Antarctic fishes to be able to generalize as to whether all life stages respond similarly or whether early developmental stages represent potential bottlenecks for population survival. Another topic that needs to be addressed is how hypercapnia will modify interactions between species already affected by the warming trend. Recent findings in tropical coral reef fish demonstrated behavioural disturbances by moderate levels of ocean acidification (1050 ppm CO_2, pH 7.8, year 2100 scenario), presumably elicited by hypercapnia effects on the central nervous system (Munday et al., 2009). Hypercapnia therefore may also alter trophodynamic interactions in a particular ecosystem (cf. Ferrari et al., 2011), beyond those of the direct lethal effects on interacting organisms. These aspects have never been studied in Antarctic fishes and clearly need further research.

5. TROPHIC VULNERABILITY OF ANTARCTIC FISHES

5.1. Vulnerability to general changes in trophic structure and dynamics

The vulnerability of a particular species to changes in food web structure and dynamics depends on its ability to cope with both 'bottom-up' and 'top-down' effects (Jacob et al., 2011; Melian et al., 2011; O'Gorman and Emmerson, 2010). Trophic plasticity, that is, the capability to cope with fluctuations in resource availability, is positively related to prey diversity (specialist vs. generalist consumers; Johnson, 2000; Mihuc and Minshall, 1995). Predator-induced mortality is the principal 'top-down' effect, and suppression of a particular species strongly increases with increasing predator diversity (Snyder et al., 2006). Vulnerability to 'top-down'

effects and resilience capability are thus related to the number of predator species. Accordingly, species vulnerability to food web-mediated alterations is expected to decrease with prey diversity and to increase with predator diversity. Whether and how the complete loss of one species will affect overall food web structure and ecosystem functioning depends on the community's capacity for functional compensation, that is, species trophic redundancy (Johnson, 2000; Naeem, 1998).

Here, the relative trophic vulnerability of the adult notothenioid fish community inhabiting the eastern Weddell Sea shelf (between 200 and 600 m water depth) to alterations in the food web was estimated. A simple, quantitative measure based on the number of feeding links to prey and predator species was used to assess vulnerability. Information on trophic linkages was extracted from the extensive trophic database published in Jacob et al. (2011) that includes information on feeding relations of 489 consumer and resource species from the Antarctic Weddell Sea (for detailed information and sources, see Jacob, 2005; Jacob et al., 2011). For seven more fish species, additional information on prey composition was collected from Foster et al. (1987), Gon and Heemstra (1990), La Mesa et al. (2004) and Schwarzbach (1988). All in all, information on prey composition and links to predators was available for 37 of the 42 notothenioid species inhabiting the shelf.

For each fish species i, the total number of prey species $\sum P_i$, the number of prey species belonging to the functional groups 'Benthos', 'Plankton' and 'Fish', $\sum P_{\mathrm{B},i}$, $\sum P_{\mathrm{P},i}$, $\sum P_{\mathrm{F},i}$, and the number of predators $\sum C_i$ were extracted from the database. Both $\sum P$ and $\sum C$ are common descriptors in theoretical food web ecology and usually referred to as 'generality' ($\sum P$) and 'vulnerability' ($\sum C$; see e.g. Memmot et al., 2000; Schoener, 1989). Here, both variables were combined to calculate consumers' relative trophic vulnerability, a comparative index with values located between 0 and 1. $\sum C$ was taken as a measure of vulnerability to top-down effects, and $\sum P$ as an (inverse) measure of vulnerability to bottom-up effects.

The relative trophic vulnerability VI_i of fish species i can thus be computed by

$$\mathrm{VI}_i = \frac{\sum_{i=1}^{m} C_i}{\sum_{i-1}^{n} P_i + \sum_{i=1}^{m} C_i} \qquad [1]$$

where m is the total number of consumer species and n is the total number of prey species of fish species i. $\sum P_i + \sum C_i \geq 1$ and $0 \leq \mathrm{VI}_i \leq 1$; $\sum P_i = \sum P_{\mathrm{B},i} + \sum P_{\mathrm{P},i} + \sum P_{\mathrm{F},i}$. In this basic equation (Eq. 1), each C_i and P_i count 1.

However, there is a difference in relative top-down and bottom-up effects depending on (i) whether a particular consumer is a generalist feeder or specialized on fish species i and (ii) whether a particular prey species is exclusively consumed by fish species i or exploited by a multitude of predators. To account for these differences, each consumer of fish species i, C_i, was weighted by the number of its own prey species (P_j) and each prey species of fish species i, P_i, was weighted by the number of its own consumer species (C_k). These weighted consumer and prey values of fish species i are referred to as WC_i and WP_i, respectively. Accordingly, the calculation of the relative trophic vulnerability index VI_i of fish species i was adapted by

$$\mathrm{VI}_i = \frac{\sum_{i=1}^{m} \mathrm{WC}_i}{\sum_{i=1}^{n} \mathrm{WP}_i + \sum_{i=1}^{m} \mathrm{WC}_i} \qquad [2]$$

with

$$\mathrm{WP}_{i,k} = \left(\frac{1}{\sum_{k=1}^{m} C_k} \right) \text{ and } \mathrm{WC}_{i,j} = \left(\frac{1}{\sum_{j=1}^{n} P_j} \right); \sum \mathrm{WP}_i + \sum \mathrm{WC}_i > 0$$

and $0 \leq \mathrm{VI}_i \leq 1$; $\sum \mathrm{WP}_i = \sum \mathrm{WP}_{\mathrm{B},i} + \sum \mathrm{WP}_{\mathrm{P},i} + \sum \mathrm{WP}_{\mathrm{F},i}$. Here, m is the total number of weighted consumer species and n is the total number of weighted prey species of fish species i. This index was used as an indicator of species' risk to be negatively affected by changes in the food web. Spearman's rank correlation was used to analyze relationships between all parameters with the aim to rank $\sum \mathrm{WC}$, $\sum \mathrm{WP}$, $\sum \mathrm{WP_B}$, $\sum \mathrm{WP_P}$ and $\sum \mathrm{WP_F}$ according to their effect on VI.

The number of prey items $\sum P$ ranged from 5 in some planktivorous fishes to >100 in benthos feeders. The number of predators $\sum C$ ranged from 12 to 47 (Table A1). The majority of notothenioid fish species are benthos feeders and mixed feeders, consuming varying proportions of benthos and plankton (Fig. 5). The number of pure plankton feeders and mixed feeders of plankton and fish is comparatively low and pure piscivorous species are extremely scarce. Relative vulnerability VI is related to

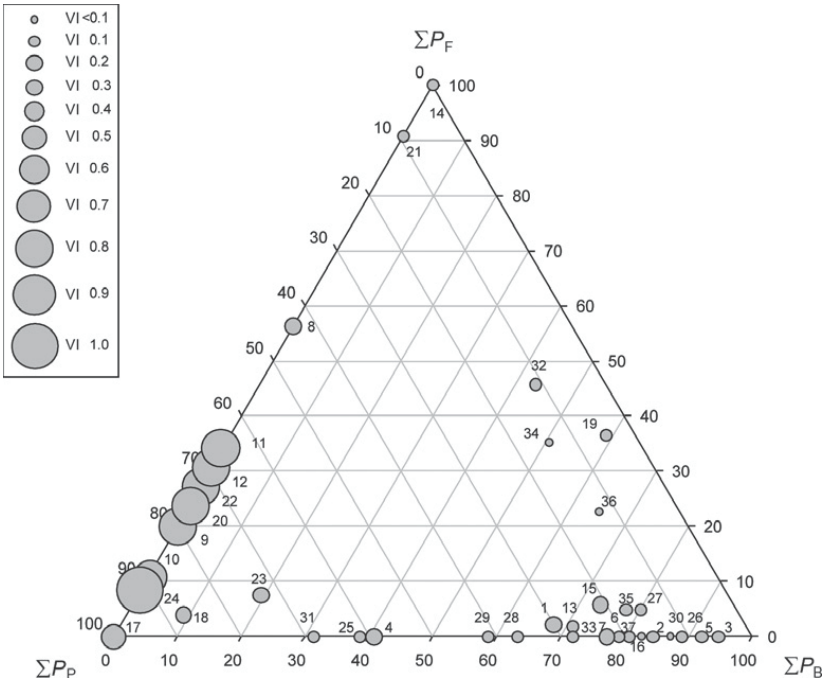

Figure 5 Relative proportions [%] of benthos ($\sum P_B$), plankton ($\sum P_P$) and fish ($\sum P_F$) in the diet of notothenioid fish species. Each circle represents one species; circle diameter indicates relative trophic vulnerability (VI). For species code numbers, see Table A1.

the distribution of prey species among the functional groups 'Benthos', 'Plankton' and 'Fish'. VI is lowest in benthos feeders (VI 0.02–0.11, mean ± SD 0.07 ± 0.03), fish feeders (VI 0.1, mean ± SD 0.1 ± 0) and benthos and fish feeders (VI 0.03–0.07, mean ± SD 0.05 ± 0.02), intermediate in mixed feeders of benthos and plankton (VI 0.02–0.28, mean ± SD 0.16 ± 0.07) and highest in species feeding almost exclusively on planktonic prey (VI 0.15–0.96, mean ± SD 0.56 ± 0.34) or on a mixture of plankton and fish (VI 0.28–0.77, mean ± SD 0.69 ± 0.20). The highest VI of 0.96 is found in the plankton-feeding *P. antarcticum,* followed by some channichthyid species such as *C. myersi* and *C. antarcticus* with VIs of 0.77 (see Table A1; Fig. 5). It appears that there is a certain accumulation of risk in the trophic group of plankton feeders. VI is correlated more strongly with $\sum$WP (Spearman's $r = -0.980$, $p < 0.0001$; Table 1) than to $\sum$WC ($r = 0.614$, $p < 0.0001$). In notothenioid fishes, differences in relative vulnerability VI between species are thus mainly determined by the number of prey items, that is, by the degree of generalism (see Table 1). The effect of predator diversity is of less significance, as most fish species share a similar number of potential predators that feed non-selectively.

Table 1 Spearman's rank correlations between relative trophic vulnerability index VI, weighted number of consumer species $\sum$WC and weighted number of prey species $\sum$WP, with the functional prey groups benthos $\sum WP_B$, plankton $\sum WP_P$ and fish $\sum WP_F$

	VI	$\sum$WC	$\sum$WP	$\sum WP_B$	$\sum WP_P$
$\sum$WC	$r=0.614$ $p<0.0001$	–	–	–	–
$\sum$WP	$r=-0.980$ $p<0.0001$	$r=-0.505$ $p=0.0014$	–	–	–
$\sum WP_B$	$r=-0.861$ $p<0.0001$	$r=-0.648$ $p<0.0001$	$r=0.817$ $p<0.0001$	–	–
$\sum WP_P$	$r=-0.389$ $p=0.0174$	$r=-0.469$ $p=0.0034$	$r=0.347$ $p=0.0357$	$r=0.490$ $p=0.0021$	–
$\sum WP_F$	$r=0.186$ ns	$r=0.500$ $p=0.0016$	$r=-0.101$ ns	$r=-0.436$ $p=0.0070$	$r=-0.449$ $p=0.0053$

For each parameter combination, correlation coefficient r and level of significance (p value) are given (ns, not significant, that is, $p>0.05$). With respect to VI, r and p are interpreted as indicators of effect strength with signs indicating the direction of the effect.

Among functional prey groups, the number of benthic prey items $\sum WP_B$ exerts the strongest effect on VI ($r=-0.861$, $p<0.0001$), followed by planktonic prey $\sum WP_P$ ($r=-0.389$, $p=0.0174$). The number of fish species in the diet $\sum WP_F$ is not significantly related to VI ($p>0.05$). The pattern of high benthic biomass and diversity on the high Antarctic shelf (see Section 2.2) is obviously reflected in notothenioid prey diversity and thus in trophic vulnerability: the number of benthic prey species $\sum WP_B$ exerts by far the strongest effect on VI; the higher the share of benthic species in the diet, the lower is VI (Table 1; Fig. 5).

The resilience of the entire system, that is, to what extent the extinction of particular consumer species from the system impacts overall food web stability and ecosystem functioning, strongly depends on the systems' ability to compensate for the loss by co-occurring species (Johnson, 2000; Naeem, 1998). As the majority of species include a certain proportion of benthic prey in their diet, functional redundancy seems to be high among benthos feeders (see Fig. 5). Feeding on the benthos is associated with a high degree of trophic generalism and functional redundancy, and hence with a certain capability to adapt food choice to prey availability and to dampen bottom-up effects. Plankton consumers tend to have higher vulnerability: specializing on a comparatively narrow prey spectrum makes them more sensitive to changes in prey availability. As there are

fewer plankton-feeding species in the system, the potential for functional compensability is lower, too, making this part of the food web particularly sensitive to change.

Larvae and juveniles were not considered in this analysis because information on diet composition of early stages is not as complete as that of the adults for most species (except for *P. antarcticum*; see e.g. Granata et al., 2009; Kellermann, 1987; Vallet et al., 2011). However, as early stages of most notothenioids are pelagic, it is most likely that the relative trophic vulnerability is high compared with adult benthic stages. The number of potential predators ($\sum C$) is presumably size- (see Section 5.2) and/or density-dependent (e.g. Woodward et al., 2010b). Early stages of *P. antarcticum*, for example, occur in dense aggregations and are heavily preyed upon by other notothenioids (Eastman, 1985b; Hubold and Ekau, 1990; La Mesa et al., 2011). The degree of trophic generalism ($\sum P$) seems to differ strongly among families. Nototheniid larvae (including *P. antarcticum*) feed mainly on early copepod stages and eggs, whereas early juveniles feed on small copepod species. Compared with nototheniids, larvae and juveniles of the family Channichthyidae (e.g. *C. myersi*, *C. antarcticus*) are trophic specialists, with a narrow food spectrum that is exclusively composed of early developmental stages of krill and fish fry (Hubold and Ekau, 1990; Kellermann, 1986b, 1987, 1989). Accordingly, relative trophic vulnerability of larval and juvenile channichthyids is expected to be very high.

5.2. Vulnerability to changes in size structure and prey quality

5.2.1 Prey size

Body size is one of the major factors determining who eats whom in aquatic food webs (e.g. Brose et al., 2006; Castle et al., 2011; Woodward et al., 2005, 2010a,b). In particular for early developmental stages of fishes, the size of their prey seems to play an important role as the upper limit of consumable prey size is strongly limited by mouth width (Kellermann, 1986b, 1987). Accordingly, early stages always feed on a relatively narrow prey size range (Hubold and Ekau, 1990; Kellermann, 1986b). However, size of ingested prey is not only determined by morphological constraints but may also be the result of selective feeding behaviour. In postlarval and juvenile *P. antarcticum*, prey selection was found to be a function of prey density: at low food density conditions, larger prey species were selectively chosen, but when food density was high, size-selective feeding behaviour was distinctly less pronounced (Kellermann, 1986b, 1987).

There is also some evidence of size-selective feeding in adult *P. antarcticum*, with a negative selection of highly abundant small prey (K. Mintenbeck, unpublished data). However, whether this is the result of density-dependent selection (as observed in early stages) or due to other restrictions still needs to be verified.

In adult fish, mouth gape is less restrictive for prey handling than in larvae and juveniles, but sensory capabilities might be a limiting factor for efficient detection of small-sized prey. Depending on species' sensory capabilities, detection, capture success and feeding efficiency are likely to vary with prey size. To test for the impact of prey size on fish detection capability and feeding efficiency, feeding experiments with two nototheniid species were carried out during the expedition ANT/XXVII-3 with RV *Polarstern* in 2011.

N. coriiceps and *N. rossii* were caught by means of baited traps in February 2011 in Potter Cove. Both species belong to the family Nototheniidae and are common components of the inshore demersal fish fauna in waters of the northwestern Antarctic Península region (Barrera-Oro, 2003). Fishes were held unfed in large tanks at a water temperature of 0 °C for about 2 months prior to the experiments. Ten individuals were selected of each species (size ranges: *N. coriiceps* 24.2–33.6 cm standard length (SL), *N. rossii* 24.0–35.1 cm SL) and transferred into individual 85-l aquaria (0 °C water temperature, dim light conditions) 24 h before the feeding experiment started. Five different prey size classes were offered to each fish (prey size categories 1 (small) to 5 (large); see Table 2), starting with the smallest size category. Prey density was constant with 30 prey individuals per fish in all feeding trials. Times of first reaction, and each

Table 2 Food used in the feeding experiments to test for the impact of prey size on fish detection capability and feeding efficiency included five different prey size categories

Size category	Type	Size range [cm] (min–max)
1	Cyclopoid copepods	0.8–1.3
2	Daphnia	2.2–3.0
3	Mysids	9.2–14.5
4	Juvenile euphausiids	12.2–18.9
5	Adult euphausiids	27.9–33.5

Categories 1–4 were commercial frozen fish food (Erdmann Frostfutter, Germany) and category 5 was adult ice krill (*Euphausia crystallorophias*) caught during the RV *Polarstern* expedition. The food was defrosted prior to experiments.

Table 3 Number (*N*) of feeding individuals (*N. coriiceps* and *N. rossii*), and mean times (min:s; ± standard deviation) until first reaction to prey addition (movement of head towards prey), first detection of a prey item that was followed by an attack and first consumption are given for each prey size class (categories 1–5)

Prey category	*N* of feeding fish	First reaction	First prey detection	First consumption
Notothenia coriiceps				
1	0	–	–	–
2	0	–	–	–
3	6	2:12 ± 2:53	3:34 ± 3:50	3:36 ± 3:50
4	10	1:27 ± 3:21	1:47 ± 3:18	1:48 ± 3:17
5	10	0:10 ± 0:16	0:10 ± 0:16	0:12 ± 0:15
Notothenia rossii				
1	0	–	–	–
2	1	0:37	0:37	0:40
3	5	4:12 ± 6:04	4:12 ± 6:04	4:13 ± 6:04
4	9	1:18 ± 3:19	1:31 ± 3:16	1:34 ± 3:15
5	10	0:10 ± 0:08	0:10 ± 0:08	0:12 ± 0:07

particular prey detection and consumption were registered; overall experimental duration was 15 min. Depending on the amount of food consumed, the time lag between particular experiments was up to 3 days to avoid an effect of satiation on feeding behaviour. The offered food was not alive, but prey items were in motion in the tanks all the time owing to aeration and steady inflow of fresh seawater.

None of the fish fed on the smallest prey (category 1) and only one small *N. rossii* consumed a single prey item of category 2 (Table 3). Except for this single individual, no reaction to prey of categories 1 or 2 was observed. Both *N. coriiceps* and *N. rossii* started to react when prey of size category 3 was offered: a total of six *N. coriiceps* and five *N. rossi* detected and consumed prey of this category. All but one fish fed on size categories 4 and 5. Time until first reaction to prey addition (movement of the head towards prey), time of first prey detection that was followed by an attack and time of first consumption were all inversely related to prey size in both species (Table 3).

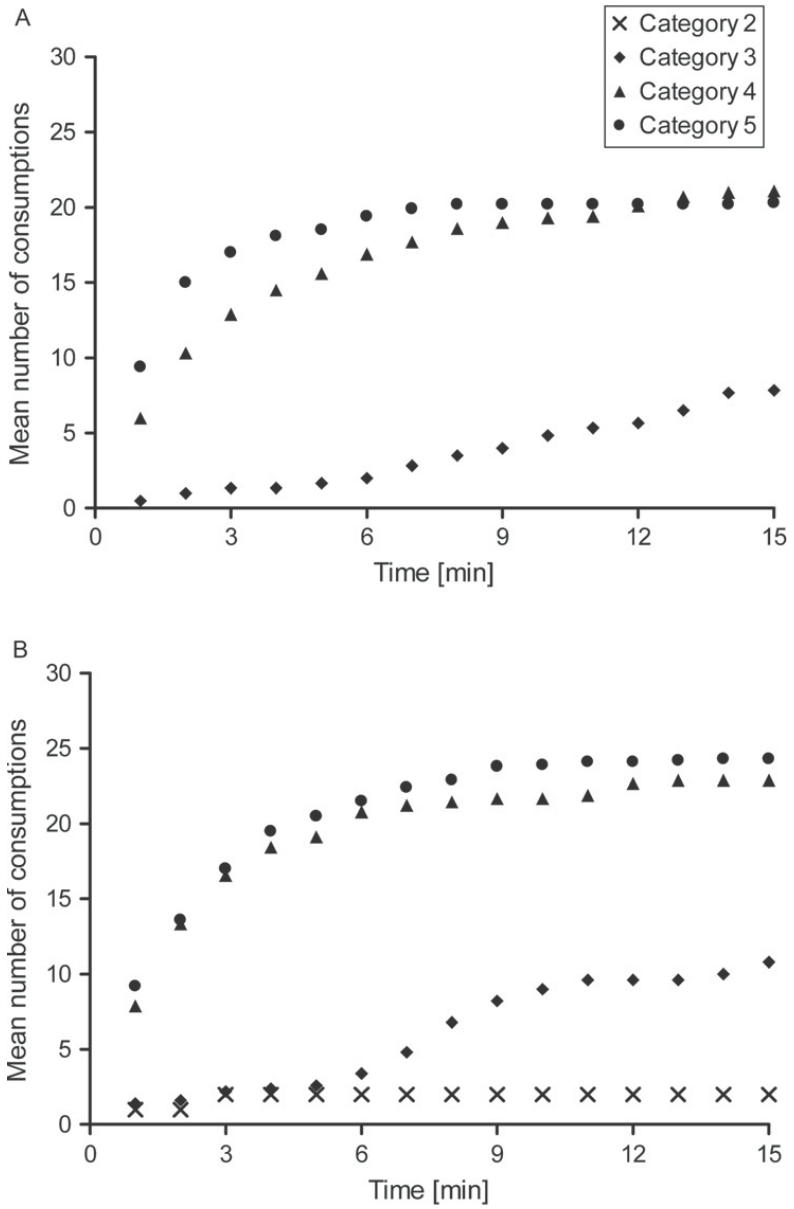

Figure 6 Mean consumption rate (number of consumptions × time^{-1}) in *N. coriiceps* (A) and *N. rossii* (B) depending on prey size category (2–5; 2: daphnia, 3: mysids, 4: juvenile euphausiids, 5: adult euphausiids). For the number (*N*) of feeding individuals, see Table 3.

The mean consumption rate (number of consumptions × time^{-1}) depended upon prey size category in both species (Fig. 6; Kruskal–Wallis ANOVA, *N. coriiceps*: $H = 29.45$, $p < 0.0001$; *N. rossii*: $H = 46.99$, $p < 0.0001$). The feeding rates were low when fish were offered size categories 2 and 3, and high for the two largest prey size categories. The feeding

rates of fish offered the two small size categories (2 and 3) were significantly lower than the feeding rates of fish offered large prey (4 and 5; Dunn's post hoc test, $p < 0.01$), while no differences were found among the two small nor the two large categories (Dunn's post hoc test, $p > 0.05$). The mean time between detection and consumption was independent of prey size in both species (Kruskal–Wallis ANOVA, $p > 0.05$). Neither the total sum of consumed prey nor time until first reaction and mean time between detection and consumption were significantly correlated with fish size for any prey size category (Spearman's rank correlation, $p > 0.05$). However, the fish used in these experiments did not differ much in size, hence, further experiments using a broader range of fish sizes will be needed to verify the relationship between fish size and these parameters.

Nevertheless, these data clearly show that feeding rates depend strongly on prey size. Both species are obviously not capable (and/or willing) to attack small prey items and feeding efficiency is low below a certain prey size limit. This prey size-dependent detection and consumption rate are most likely not only found in these two species, but might be a limitation in many other Antarctic species (if not all).

5.2.2 Prey quality

The importance of prey quality for consumers is widely accepted, but only a few studies have dealt with this issue in fishes. Malzahn et al. (2007) found nutrient limitation of primary producers to propagate along the food chain, finally affecting condition of fish (larval herring *Clupea harengus*; condition assessed based on RNA/DNA ratios) feeding on herbivorous zooplankton. Based on the histology of the digestive organs, Koubbi et al. (2007) investigated the condition of larval *P. antarcticum* off Terre Adélie in relation to prey composition and found that larvae feeding on copepods were in better condition than those feeding on diatoms.

The energy contents of Antarctic and Sub-Antarctic species from various taxonomic groups are well studied (Ainley et al., 2003; Barrera-Oro, 2002; Clarke et al., 1992; Croxall and Prince, 1982; Donnelly et al., 1990, 1994; Eder and Lewis, 2005; Lea et al., 2002; Tierney et al., 2002; Torres et al., 1994) and are summarized in Fig. 7. Fishes and decapods have the highest energy contents; squid and crustaceans such as mysids, euphausiids and copepods have moderate energy contents. By far, the lowest energy content is found in gelatinous zooplankters such as salps, chaetognaths and cnidarians. Shifts in the zooplankton community with crustaceans being replaced by salps (see Section 1) thus involve a drastic decrease in energy density and nutritive value of prey for consumers such as fish.

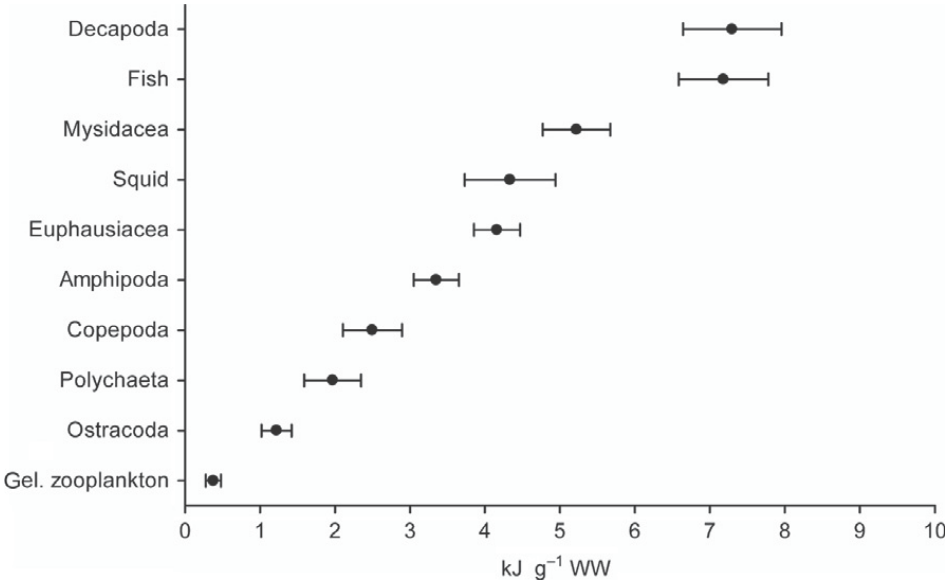

Figure 7 Energetic value (kJ g^{-1} wet weight; means ± SE) of Antarctic and Sub-Antarctic species belonging to several taxonomic groups (for details and data sources for each group, see Mintenbeck, 2008 and references therein).

Some species, such as *N. coriiceps*, feed on crustaceans (mainly krill and amphipods) and even on macroalgae (Iken, 1996; Iken et al., 1997). However, in feeding experiments, algae are only ingested in the absence of alternative animal prey (e.g. crustaceans; Fanta, 1999; K. Mintenbeck, unpublished data), providing evidence that macroalgae are not a favoured food source (see also Fanta et al., 2003). If krill is not available due to a shift in zooplankton composition, omnivorous fish species such as *N. coriiceps* might, however, be increasingly forced to feed on such low energy macroalgae.

Low energy food might affect survival, growth, body condition and reproductive output of consumers and ultimately might make fish species itself a low quality prey for its endothermic predators (see Österblom et al., 2006, 2008).

6. VULNERABILITY OF ANTARCTIC FISHES TO HABITAT DESTRUCTION

6.1. The impact of sea ice reduction

There is no doubt that a reduction in sea ice extent and duration of coverage due to climate-driven warming will affect the sympagic community living within the ice and invertebrates such as Antarctic krill, *E. superba*, whose life

cycle is coupled to seasonal sea ice dynamics (see Moline et al., 2008 for review). However, there are also fish species whose life cycles and life styles are closely associated to the sea ice, namely *P. borchgrevinki* and *P. antarcticum*.

P. borchgrevinki is morphologically well adapted for a cryopelagic life (Eastman and DeVries, 1985) and is usually found closely associated with the underside of ice, where it frequently hides in crevices (e.g. Davis et al., 1999). Main prey items of this species include sympagic copepods (Hoshiai et al., 1989): sea ice thus provides the consumer with a habitat, refuge and feeding ground. Though *P. antarcticum* is usually found in the free water column, sea ice seems to be an important feeding ground for this species as well. Huge shoals of several thousand individuals have been observed feeding under the fast ice west off the Antarctic Peninsula (Daniels, 1982). The sea ice region is apparently also the spawning ground (La Mesa and Eastman, 2012) as the pelagic eggs were found floating under the sea ice (Vacchi et al., 2004). The reproductive cycle of *P. antarcticum* seems to be closely coupled to seasonal sea ice dynamics, and early stages depend on the temporal and spatial match with the seasonal zooplankton production (La Mesa and Eastman, 2012; La Mesa et al., 2010). The hatching period of *P. borchgrevinki* seems to be less strongly coupled to production peaks (Pankhurst, 1990), but both species are expected to be significantly affected by alterations in seasonal sea ice dynamics by loss of habitat/refuge and spawning ground and alterations at the base of the food web.

6.2. The impact of increased iceberg scouring

6.2.1 *The role of habitat structure and disturbance events for species richness*

For freshwater habitats (lakes and rivers) as well as for marine habitats, such as coral reefs and hard-substrate environments in the Mediterranean, a high diversity in habitat structures often promotes high species richness in the associated fish communities (Feld et al., 2011; Garcia and Ruzafa, 1998; Gratwicke and Speight, 2005; Guégan et al., 1998; Öhman and Rajasuriya, 1998). The benthic communities of the eastern Weddell Sea shelf in water depths between 200 and 450 m are characterized by a patchwork of structurally different successional stages; the two extremes are areas with a diverse epifauna forming the rich 3-dimensional habitat (Fig. 8A) on the one hand and desert-like areas with nearly no epibenthic fauna (Fig. 8B) on the other. This patchwork is the result of mechanical disturbance events by grounding icebergs, calving from the shelf ice and grounding at water depths between 200 and 500 m. This kind of

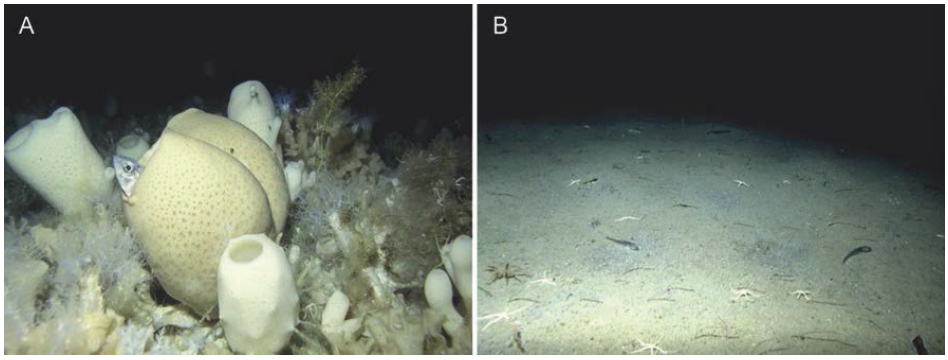

Figure 8 (A) Typical undisturbed site with a rich 3-dimensional habitat and *Trematomus* cf. *eulepidotus* hiding inside a large sponge; (B) Fresh iceberg scour habitat without any 3-dimensional megafauna species, only with a pycnogonid, some ophiuroids and *Prionodraco evansii*. Photos: ©Julian Gutt, AWI Bremerhaven.

disturbance by ice is a common phenomenon in polar regions and affects the sea floor up to a maximum water depth of 550 m (Conlan and Kvitek, 2005; Conlan et al., 1998; Gutt, 2001). Beside forest fires and hurricanes, iceberg scouring is one of the most significant natural physical perturbations imposed on ecosystems (Garwood et al., 1979; Gutt and Starmans, 2001; Peck et al., 1999) and is considered to be one of the strongest physical forces structuring the benthic environment in polar regions (Gutt and Piepenburg, 2003).

In the area of Austasen and Kapp Norvegia (eastern Weddell Sea), about 7% of the coastal zone (< 400 m) is covered by iceberg aggregations (Fig. 9; Knust et al., 2003). Gutt and Starmans (2001) analyzed video and photo material from this area and calculated that 42–70% of the sea bed is affected by iceberg scouring (Fig. 10). Depending on sea floor morphology, nearly 10–40% of the area showed young scour marks or early recolonization stages. On a smaller (local) scale, these iceberg disturbances completely destroy most of the in- and epifauna. However, on a regional scale, the sea floor destruction by icebergs creates new space for pioneer species that are specialized in recolonization (Gerdes et al., 2008; Teixidó et al., 2004), and the patchwork of structurally different successional stages increases the overall gamma diversity (Gray, 2000). Following the Intermediate Disturbance Hypothesis, which predicts a high diversity caused by intermediate levels of disturbance (Huston, 1979), Gutt and Piepenburg (2003) calculated an increased species diversity for the epibenthic megafauna on a regional scale (1–100 km) due to iceberg disturbances.

However, iceberg scouring not only affects benthic organisms, but also the demersal fish fauna, the composition of which differs significantly

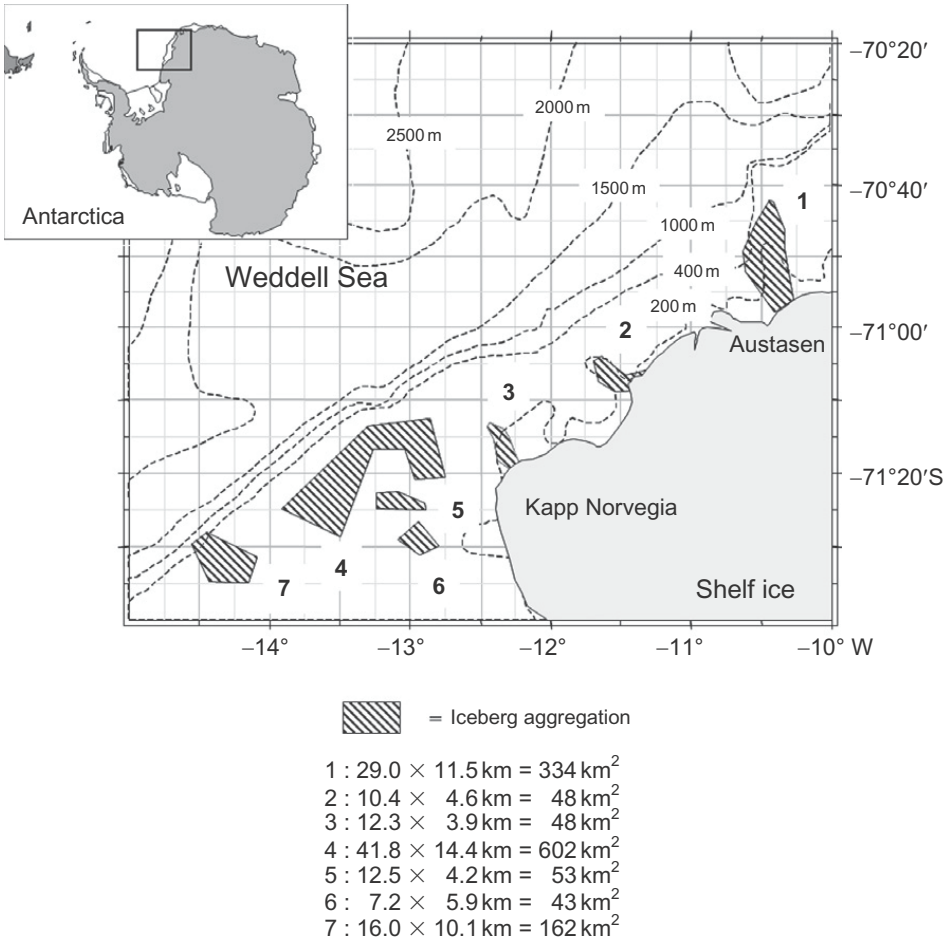

Figure 9 Iceberg aggregations on the high Antarctic shelf off Austasen and Kapp Norvegia (eastern Weddell Sea) in March 2000 *(redrawn from Knust et al., 2003; with permission from Backhuys Publishers).*

between undisturbed and recently disturbed sites, with higher species richness and a higher diversity (Shannon's diversity) in undisturbed areas, and in species identity (Fig. 11; Knust et al., 2003). Some species, such as *Trematomus scotti*, *C. antarcticus*, *Pagetopsis maculatus* and *Artedidraco loennbergi*, are typical members of the fish community in undisturbed areas, while *T. pennellii*, *Trematomus nicolai* and *Prionodraco evansii* are specialized to live at disturbed sites. Iceberg disturbance events play a key role in small-scale niche separation of fishes, as the structurally different habitats allow for the coexistence of trophically similar species (Brenner et al., 2001; Hagen et al., 2012; Ptacnik et al., 2010).

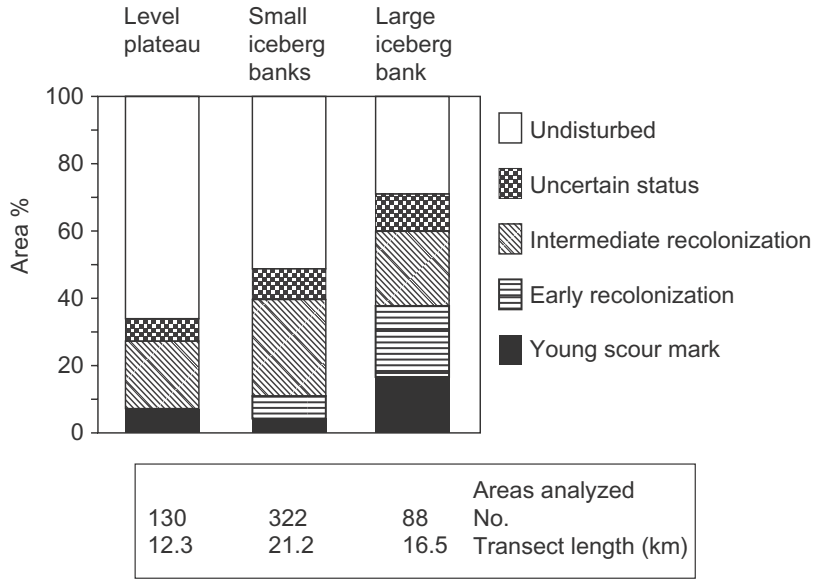

Figure 10 Proportions of different recolonization stages at different morphological condition in the shelf area off Austasen and Kapp Norvegia (eastern Weddell Sea) *(redrawn from Knust et al., 2003, based on data from Gutt and Starmans, 2001; with permission from Backhuys).*

In view of climate change, the major questions are (i) whether the pattern of increased diversity at intermediate disturbance levels found for benthic megafauna (e.g. Gutt and Piepenburg, 2003; see above) does also hold true for the demersal fish community and (ii) how diversity and community structure will respond to increased disturbance events. To answer these questions, we analyzed the relationship between the level of disturbance events and the gamma diversity of the demersal fish fauna on the eastern Weddell Sea shelf using a simple simulation model.

6.2.2 The disturbance simulation model

The calculations of the model are based on abundance data from several bottom trawls (standard otter trawl, 20-mm mesh in codend) taken in undisturbed and disturbed areas off Austasen and Kapp Norvegia (eastern Weddell Sea) during four expeditions between 1996 and 2004. The position of iceberg scours was identified by side scan sonar, ROV underwater video and underwater photography. The composition of invertebrate bycatches of the otter trawls was used as an indicator for the degree of disturbance. In particular, the presence/absence of large, old hexactinellid sponges indicated the disturbance level of the sea floor (Gerdes et al.,

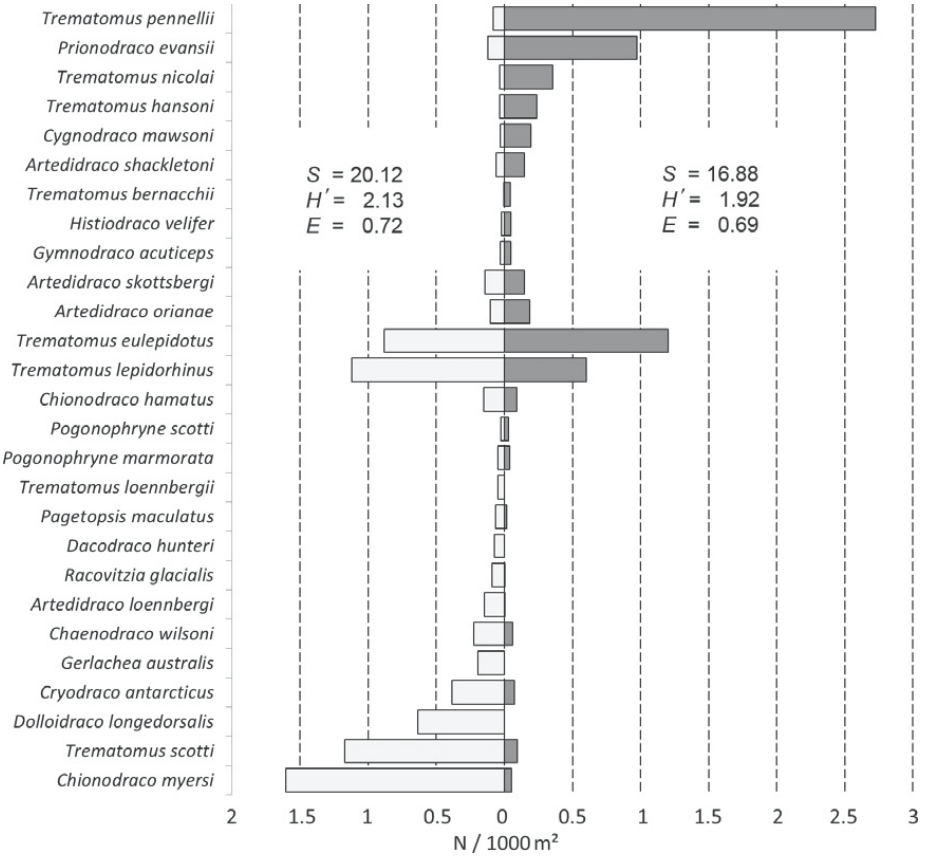

Figure 11 Mean abundance (N/1000 m^2) of the dominating fish species in undisturbed areas and on young iceberg scours (recalculated with data from Knust et al., 2003; R. Knust and K. Mintenbeck, unpublished data). For both communities, average number of species (*S*), average Shannon diversity (H') and average evenness (*E*) are given.

2008; Knust et al., 2003). Overall 30 stations were sampled, 22 in undisturbed areas and 8 in disturbed areas. Fish abundance data were calculated based on 1000 m^2 swept area.

To simulate different disturbance levels, the abundance data of each catch from disturbed and undisturbed areas were randomly combined in a 200 station matrix by a Monte Carlo simulation. To simulate a completely undisturbed situation (disturbance level 0.0), data from exclusively undisturbed stations were randomly combined. The disturbance level 1.0 (the entire shelf is disturbed) was simulated by a random combination of catches from exclusively disturbed areas. For a disturbance level of 0.05 (5% of the shelf area is disturbed by iceberg scouring), 5% of the 200 stations in the matrix were filled with abundance data from disturbed stations and 95%

were filled with abundance data from undisturbed station. The stations were randomly selected. The disturbance level was increased in 0.05 steps. For each disturbance level, 100 iterations of random combination were computed.

For each disturbance level, the averages of total number of species (S), gamma diversity (Shannon's diversity, H') and evenness (Pielou, E) were calculated:

$$H' = \sum_{i=1}^{S} p_i \cdot \ln(p_i) \quad [3]$$

where $p_i = n_i/N$, with n_i is the abundance of species i and N the sum of all individuals;

$$E = \frac{H'}{H'_{\max}} \quad [4]$$

where $H'_{\max} = \log_2(S)$. To avoid an oversized effect of very rare species on the total number of species, only species with an abundance of >0.001 individuals per 1000 m^2 were taken into account for each combination.

The results of the Monte Carlo simulation are shown in Fig. 12. The results of the different catch combinations were fitted to a univariate, second-order polynomial (solid line), the dots represent the particular results of each combination. All three parameters increased with increasing disturbance level up to a maximum between disturbance values of 0.25–0.40, representing 25–40% of the shelf area disturbed by icebergs. Average species richness was highest (57.9 species) at a disturbance level of 0.25, average gamma diversity was highest ($H' = 2.80$) at a level of 0.34 and average evenness (0.69) was highest at a disturbance level of 0.40.

The comparison of these results with the estimated disturbance level on the shelf of the eastern Weddell Sea (Gutt and Starmans, 2001; see above) shows that the fish fauna on the eastern Weddell Sea shelf is obviously well adapted to this kind of mechanical disturbances and to the average level of disturbance occurrence there. The gamma diversity (and evenness) of the fish fauna is highest at the disturbance level we find nowadays, but rapidly decreases at higher levels according to the model predictions. Future climate scenarios suggest an increasing rate of iceberg calving in the shelf ice areas of Antarctica, with an enhanced risk of iceberg groundings and an increasing disturbance level in the benthic communities.

Such a reduction in habitat structure and heterogeneity means on the one hand a reduction of habitat and refuge for demersal fishes (e.g. Moreno et al.,

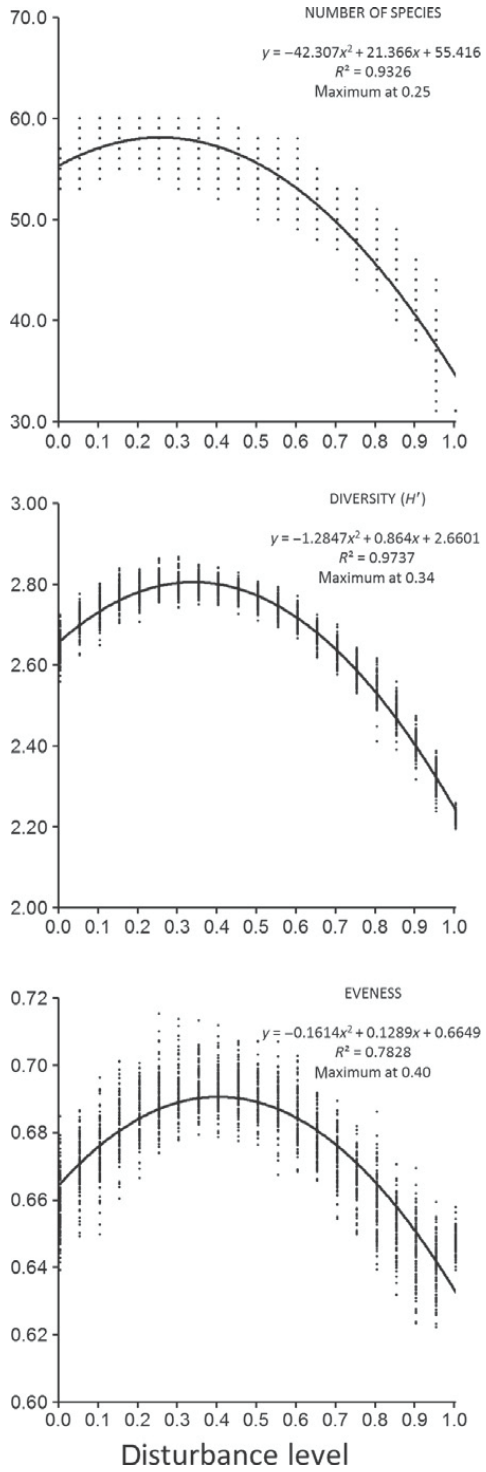

Figure 12 Results of the Monte Carlo simulation: total number of species, gamma diversity (H') and evenness (E) of the demersal fish community depending on disturbance rates. The calculations are based on fish abundance data from disturbed and undisturbed areas on the eastern Weddell Sea shelf (R. Knust and K. Mintenbeck unpublished data).

1982), with an increased predation risk for all developmental stages. On the other hand, a reduction in habitat diversity will result in an increased competition among species with overlapping trophic niches, which at the present disturbance level perfectly avoid competition by small-scale niche separation (Brenner et al., 2001). If the disturbance level increases in the future, a loss in species diversity seems to be inevitable.

7. DISCUSSION

7.1. The impact of climate change on Antarctic fish species

Notothenioid fishes are well adapted to their habitat, and alterations in the abiotic environment directly affect physiological functions. Increasing water temperatures, particularly in combination with ocean acidification, pose a major thread to the persistence of notothenioid fishes. Some species such as *P. borchgrevinki* and *Notothenia* spp. show some physiological plasticity and are able to compensate for increasing oxygen demand, for example, by mitochondrial proliferation and/or increased haematocrit. However, these compensatory mechanisms are limited and most notothenioid species are in fact stenothermal and are not capable to adjust metabolic functioning. Channichthyids are highly vulnerable to changes in the abiotic environment as they lack any capacity to adjust blood parameters to an increasing oxygen demand. Early developmental stages as well seem to be highly vulnerable to all kinds of abiotic alterations, including salinity.

General vulnerability to changes in food web structure and dynamics was analyzed using a conceptual approach, with a quantitative measure (VI) that served as an indicator of the risk of consumer species to be negatively affected by such changes. Relative trophic vulnerability was found to be low in all fish species that include a certain proportion of benthic organisms in their diet. Obviously, feeding on benthos goes along with a high degree of trophic generalism and, hence, with a certain capability to adapt food choice to prey availability and to dampen bottom-up effects. Plankton consumers displayed a distinctly higher vulnerability, as these species tend to specialize on a comparatively narrow prey spectrum, which makes them more sensitive to changes in prey availability. Thus, there exists an accumulation of risk in the trophic group of plankton feeders, making this part of the food web particularly sensitive to change. Highest trophic vulnerability was found in channichthyids, such as *C. myersi*, *C. antarcticus* and *P. maculatus*, which are all specialized on very few prey items, and in the nototheniid *P. antarcticum*, which not only has a

narrow food spectrum but is additionally exploited by a multitude of predators. Relative trophic vulnerability of plankton-feeding larvae and early juveniles is expected to be very high, as well.

Trophic vulnerability to shifts in prey size was investigated in two fish species, *N. coriiceps* and *N. rossii*. In the two species studied, feeding efficiency and prey detection capability were found to strongly depend upon prey size, with a complete detection failure (or ignorance) of smallest prey. However, the general susceptibility to prey size shifts might differ among species and age/size classes. As large fish species/individuals usually have a larger size range of available prey than small species/specimens, large fish are expected to be less susceptible to shifts in prey size compared with small fish. Trophic vulnerability to shifts in energy content is likely similar for most fish species. Nutritive value varies strongly among taxonomic groups, with lowest energy contents found in gelatinous zooplankton. Salps with their guts filled with fresh phytoplankton are a valuable food source for some Antarctic benthic suspension feeders, which usually depend on more or less degraded POM (Gili et al., 2006). Nevertheless, in comparison with other zooplankton species such as decapods, euphausiaceans and copepods, their nutritive value for fishes is extremely low. Prey of inappropriate size and/or quality affects the nutritional status and condition of fishes (see also Beaugrand et al., 2003; Koubbi et al., 2007; Malzahn et al., 2007), and in the worst case even survival.

The loss of habitat poses a threat to the majority of Antarctic fish species. There is no doubt that a reduction in sea ice extent and duration due to climate-driven warming will particular affect the life stages of those fish species that are strongly associated with sea ice, namely *P. borchgrevinki* and *P. antarcticum*. Habitat structure and heterogeneity are of particular importance for the demersal fish community because their loss would imply a loss of refuge and shelter for juveniles, adults and eggs (see Barrera-Oro and Casaux, 1990; Moreno, 1980; Moreno et al., 1982), with the consequent increase in competition among trophically similar species (e.g. Brenner et al., 2001). Model simulations based on abundance data indicated that an increase in ice scouring will lead to a steep decrease in diversity and evenness, and to the loss of species.

7.2. Effects of climate change in other marine systems

In general, the changes detected so far in the South Polar Sea resemble many of those observed on a worldwide scale. The world's oceans are warming, atmospherical pCO_2 is rising, leading to potential ocean acidification

(IPCC, 2007), and seawater salinity is decreasing in the vicinity of melting ice and glaciers (e.g. Curry and Mauritzen, 2005). Fishes, for example, in temperate regions, have been shown to be significantly affected at the physiological level, in particular by increasing water temperatures, resulting in reduced growth performance, recruitment and abundance (e.g. Pörtner and Knust, 2007; Pörtner et al., 2008; Sirabella et al., 2001). In response to such environmental alterations, several fish species have already shifted their distributional ranges and have migrated into waters with more favourable conditions (Dulvy et al., 2008; Perry et al., 2005).

The risk of habitat loss and alteration of habitat structure and heterogeneity due to sea ice retreat (Stroeve et al., 2007) and increasing occurrence of iceberg scouring events (Conlan et al., 1998; Gutt et al., 1996) is comparable in the Arctic Ocean. Invasion by lithodids as observed west of the Antarctic Peninsula is also found in the northern hemisphere. The most popular example is the red king crab (*Paralithodes camtschaticus*), which was introduced into the Barents Sea and subsequently invaded Norwegian waters. In the presence of these invaders, benthic biomass and diversity is drastically reduced; these crabs thus remove potential prey for benthos feeding fish species and even prey upon fish eggs (reviewed in Falk-Petersen et al., 2011).

Alterations in plankton community composition due to climate forcing are common in many ecosystems and often include a shift from larger to smaller phytoplankton (Yvon-Durocher et al., 2011) and zooplankton species, in particular in copepods (e.g. in the North Sea, Beaugrand et al., 2003; Helaouët and Beaugrand, 2007; and in the Humboldt Current ecosystem reviewed in Alheit and Niquen, 2004). Substantial increases in gelatinous zooplankton have been observed in different marine systems in recent years (e.g. Attril et al., 2007; Brodeur et al., 1999; Purcell, 2005). High abundances of gelatinous zooplankton are often related to water temperature and salinity, suggesting that population density of gelatinous zooplankton will further increase under future climate change scenarios (reviewed in Purcell, 2005). In the central North Sea, the occurrence of jellyfish was also negatively correlated with seawater pH, thus, future levels of CO_2 may synergistically promote the presence of gelatinous zooplankton (Attril et al., 2007). Gelatinous zooplankton prey upon fish eggs and larvae (Doyle et al., 2008; Purcell, 1985) and also represent strong competitors for plankton-feeding fish by efficiently removing potential prey, such as copepods (Purcell and Decker, 2005) or euphausiids (Suchman et al., 2008). Some gelatinous zooplankton such as

salps or ctenophores are occasionally consumed by fishes (including notothenioids), but appear to be a form of 'survival food' when preferred zooplankton prey are not abundant (Kashkina, 1986; Mianzan et al., 2001).

Taking a closer look at the effects of climatic shifts in the world's oceans, an intriguing pattern seems to emerge: the most severe (or most rapid) effects of climate forcing on marine biota appear to be found within pelagic communities (Alheit, 2009; Alheit and Niquen, 2004; Alheit et al., 2005; Arntz, 1986; Beaugrand et al., 2003; Benson and Trites, 2002). These observed changes in the pelagic realm are not restricted to one or two trophic levels, or to specific species, but usually involve strong alterations in food web structure and ecosystem functioning and dynamics. In the central Baltic and the North Sea (Alheit et al., 2005; Beaugrand et al., 2003), in the Bering Sea and the North Pacific (Benson and Trites, 2002) as well as in the Humboldt Current ecosystem (Alheit and Niquen, 2004; Arntz, 1986; Arntz and Fahrbach, 1991), all trophic levels in the pelagial, from primary producers to apex predators, were affected by direct and/or indirect climate forcing. In particular, pelagic fishes with short plankton-based food chains, such as clupeids, may undergo strong fluctuations in stock density (Alheit and Niquen, 2004), with severe consequences for their endothermic consumers (e.g. Alheit, 2009; Arntz, 1986; Cury et al., 2000).

To our knowledge, there are no reports on such extensive severe effects of climatic shifts on benthic biota, suggesting that they are less affected, but whether this is really a common pattern or just due to a greater focus on the pelagic realm still needs to be verified.

Overall, the general effects of climate forcing and the potential direct and indirect impact on marine living communities thus appear to be similar in the South Polar Sea compared with marine systems worldwide. Nevertheless, there are some significant differences: (i) fishes inhabiting temperate and tropical regions often have the opportunity to emigrate into waters with more favourable abiotic and biotic (prey) conditions (see Arntz, 1986; Dulvy et al., 2008; Perry et al., 2005). For notothenioid fishes, particularly for high Antarctic species, emigration is strongly limited by stenothermy and the lack of alternative habitats, as they are already living at the highest latitudes. (ii) Fish species such as clupeids in upwelling systems are evolutionarily adapted to strong environmental fluctuations by possessing traits associated with fast growth (Cubillos et al., 2002) and high fecundity (Alheit and Alegre, 1986), both facilitating population recovery after stock decline. In tropical reef fish, a rapid transgenerational

acclimation to increasing water temperatures has also been observed (Donelsen et al., 2012). In contrast, notothenioid fish species are characterized by slow development rates and low fecundity, and their recovery potential is thus strongly limited. Due to the low recovery capacity, even a modest increase in the amplitude of interannual climate fluctuations could affect long-term population dynamics of notothenioid fish, with ramifications that would ripple through the wider food web. Given the rate of alterations due to climate change observed off the Antarctic Peninsula and fish species' life history characteristics, an evolutionary adaptation of notothenioid fishes that keeps pace with the rate of change in conditions is unlikely, if not impossible (see Somero, 2010).

7.3. Antarctic fish community persistence—Winners and losers

Given the current state of knowledge, it is unlikely that there will be any true 'winners' of climate change among notothenioid fish species. There will be only 'survivors' and many 'losers'. *P. borchgrevinki*, for example, might be among the survivors in a changing South Polar Sea as this species is characterized by relatively high metabolic plasticity and a wide thermal tolerance window (Robinson and Davison, 2008), and low trophic vulnerability. But still, *P. borchgrevinki* is threatened by habitat reduction owing to retreat of sea ice and so far it is unknown whether this species can cope with loss of its ice habitat. Demersal fish species (except for plankton-feeding channichthyids) show low relative trophic vulnerability, but will be significantly affected by the loss of habitat structure and diversity. Which species will ultimately survive and which will not in the long run depends on a combination of species-specific physiological and trophical plasticity, and population dynamics, in addition to higher-level food web effects. Trophic plasticity is apparently high in benthos feeders, but acclimation capacity seems to differ strongly among species: some, such as *N. rossii*, possess partially compensatory mechanisms (A. Strobel and F.C. Mark, unpublished data), whereas the potential for acclimation is apparently low for many others (e.g. high Antarctic Trematomus spp.; Robinson, 2008). However, which demersal species exactly will be lost due to habitat loss on the high Antarctic shelf remains unknown for now. Population dynamic parameters such as relative fecundity and growth rate are rather similar among most demersal high Antarctic notothenioids (e.g. Kock and Kellermann, 1991; La Mesa and Vacchi, 2001), but much more work is needed on the effects that changes in the abiotic environment exert on population dynamics.

Icefishes, however, are one group that will almost certainly be on the losing side in a warming South Polar Sea with increasing levels of CO_2, as the oxygen-carrying capacity of their blood is limited, and there is no potential for physiological acclimation to satisfy the increasing tissue oxygen demand. Moreover, many channichtyids, such as *C. myersi*, are specialist consumers with a high relative trophic vulnerability, making this group additionally susceptible to changes in food web structure and dynamics. Another potential 'loser' which is affected by direct and indirect effects of climate change is the currently dominant pelagic species, *P. antarcticum*, which will be most likely affected by sea ice reduction. It also seems to be highly vulnerable to alterations in the food web, and indirect evidence suggests that at least larvae and juveniles are highly vulnerable to abiotic changes. Theoretically, all fish species are threatened by a shift in prey size structure and a decrease in prey nutritive value. However, the pelagic realm in the South Polar Sea (and elsewhere) will likely be among the first to react to climate fluctuations, and the shifts in question have thus so far only been observed in the plankton community. Plankton consumers are, therefore, especially vulnerable. Shifts in size distribution from large to small phytoplankton organisms in the marine Antarctic (as observed west off the Antarctic Peninsula; Moline et al., 2004) will thus most likely favour the prevalence of small zooplankton species, such as cyclopoid copepods (as observed in other marine systems). Given the size dependency of prey detection and feeding efficiency in notothenioid fish, it is questionable whether plankton consumers such as *P. antarcticum* but also icefish can cope with such a prey size shift. Feeding on low quality 'survival food' such as salps is not a suitable alternative to energy-rich crustaceous zooplankton in the long run. In particular, at the edge of their thermal tolerance window, fish species will be highly sensitive to such additional stressors.

However, the major bottleneck for the persistence of most (if not all) species' populations is most likely the survival of early developmental stages. Eggs and larvae appear particularly sensitive to alterations in the abiotic environment, some might be affected by sea ice reduction (e.g. early stages of *P. antarcticum*), and larvae and juveniles are apparently vulnerable to indirect food-web-mediated effects of climate change. For non-Antarctic pelagic larval fish, three key parameters were identified: prey abundance, prey type and seasonal timing (Beaugrand et al., 2003). Shifts in any of these three parameters might significantly compromise larval condition and survival. Most notothenioid larvae depend on seasonal timing as well (Efremenko, 1983; La Mesa and Eastman, 2012; La Mesa et al., 2010), and the capacity to avoid a

mismatch depends on species' plasticity in their reproductive cycle. In some species, a certain plasticity to adapt the reproductive cycle might exist, as indicated by differences in spawning time among populations in different locations (see Kock and Kellermann, 1991), but it is unknown whether all species are able to adapt their reproductive cycle and by which factors the timing is triggered. Without offspring sustaining the stocks, populations will progressively age and density will inexorably decline.

So far, our knowledge on species-specific vulnerability, potential plasticity, and acclimation and/or adaptation potential is still limited. However, based on what we already know from the Antarctic and from what we can observe in other systems, we can at least identify some potential bottlenecks. Nevertheless, there is an urgent need for more experimental studies on a broad range of species to gain a cause-and-effect understanding of the consequences of the potentially complex interactions between abiotic and biotic mechanisms (Woodward et al., 2010a).

7.4. Consequences of fish species loss for the marine Antarctic ecosystem

Fishes of the South Polar Sea will be affected by climate change in multiple ways, with the potential vulnerability and mechanisms differing among species, developmental stages and habitats. What we know about the effects of climate change and the vulnerability of Antarctic fish species leaves little doubt that the population density of many species will decline and some species will go extinct in the long run. The resilience of the entire system, that is, to what extent the decline or extinction of particular notothenioid fish species from the system impacts overall food web stability and ecosystem functioning, depends strongly on the species' functional role and the systems' ability to absorb for the loss by compensatory mechanisms among co-occurring species (Johnson, 2000; Naeem, 1998).

In the South Polar Sea, demersal fish communities are characterized by relatively high species richness in comparison with the very limited diversity of the pelagic ichthyofauna. The majority of demersal fish species are opportunistic generalist consumers with high trophic niche overlap, indicating a high functional redundancy. It is therefore likely that within the demersal community, the decline or loss of some species can be compensated for by others. However, the model on the impact of disturbance events on demersal fish communities indicated not only a future decrease in species number but also in evenness. Thus, one or a few species might become extremely dominant, and the impact of such an alteration in community composition

on overall ecosystem functioning ultimately depends on a species' identity, its specific traits and its potential to serve as valuable prey for top predators. As the role of demersal fish as a food source for endothermic predators is more important in inshore compared to offshore areas (Barrera-Oro, 2002), the effects of alterations in the demersal community on fish consumers might be stronger in shallow coastal zones. In inshore waters of the South Shetland Islands, for example, *N. coriiceps* proliferated and became the dominant demersal fish species after the stocks of the demersal *N. rossii* and *G. gibberifrons* were drastically diminished in the early 1980s due to anthropogenic actions (Barrera-Oro et al., 2000; see Section 7.5 below). Simultaneously, the breeding populations of one of their most important consumers in this area, the Antarctic shag *Phalacrocorax bransfieldensis*, now mainly preying upon *N. coriiceps*, steadily declined. Thus, though *N. coriiceps* is similar to *N. rossii* and *G. gibberifrons* in terms of ecology and body size, it could apparently not fully compensate for the reduction of the two fish species within the food web (Casaux and Barrera-Oro, 2006).

The extent to which the loss of channichthyids such as *C. myersi* or *C. antarcticus* can be compensated by other fish species is not clear. Channichthyids feed almost exclusively on krill and fish (reviewed in Hureau, 1994; Kock, 2005a; La Mesa et al., 2004). While a multitude of other fish species also feed on krill, true piscivores are rare among other notothenioids (e.g. Hureau, 1994). Some species occasionally feed on fish (see Fig. 5; La Mesa et al., 2004) but to a much lesser extent compared to channichthyids. Hence, the loss of channichthyids from the system might release some fish populations from top-down control. Though icefish are an abundant component in Antarctic fish communities, they seem to be of minor importance in the diet of endothermic predators, but their importance increases regionally when other prey, such as krill, is scarce (Kock, 2005a,b and references therein). Compared with most other notothenioids, adult channichthyid fishes are large and show only weak escape responses (authors' personal observation from ROV videos), making them an easy-to-catch prey for large consumers. At our current state of knowledge, we can only speculate, but it is likely that the loss of channichthyids will have detrimental effects on many components of the Antarctic marine food web.

The pelagic fish community is composed of very few species only, and the whole community is dominated by a single fish species, *P. antarcticum* (see above), which on the high Antarctic shelf seems to occupy a similar ecological key role in the food web as Antarctic krill in the seasonal sea ice zone

(Hubold, 1992; Hureau, 1994; La Mesa et al., 2004; Takahashi and Nemoto, 1984). It is one of the principal consumers of zooplankton, and all developmental stages are among the most important food sources for a multitude of predators, in particular for endotherms inhabiting Antarctic shelf areas (e.g. Daneri and Carlini, 2002; Hureau, 1994; La Mesa et al., 2004; Plötz, 1986). This pelagic fish species occurs in loose shoals (Eastman, 1985a; Fuiman et al., 2002) and undertakes nocturnal migrations into upper water layers (K. Mintenbeck and R. Knust, unpublished data; Plötz et al., 2001), where it provides a rich and easily accessible food source. It is thus of critical importance in the Antarctic marine food web. No other species, neither other pelagic notothenioids nor invertebrates (e.g. squid or krill), may be able to provide full functional compensation in the case of its extinction or reduction of the stock, in particular because none combines a pelagic shoaling life style and vertical migration with a comparable size spectrum and energy content (see e.g. Ainley et al., 2003). In its appearance (Fig. 13) and life style, as well as in its central role in a relatively simply structured and highly productive pelagic system, *P. antarcticum* strongly resembles shoaling clupeid fishes in upwelling systems (see Section 7.2 above).

Figure 13 Catch of *Pleuragramma antarcticum* in the eastern Weddell Sea (Photo by J. Plötz, AWI Bremerhaven).

In the eastern South Pacific, for example, El Niño events involve strong reductions in stocks of anchovy and sardine (owing to direct and indirect climate forcing in combination with fisheries effects), causing starvation and mortality in the very top predators, birds and seals (e.g. Arntz, 1986). But life history traits of clupeids and the nototheniid *P. antarcticum* (see Table 4) and recovery potential differ significantly: population doubling time was estimated to be often less than 15 months in clupeid fish species and 5–14 years in *P. antarcticum* (Froese et al., 2002), making populations of the latter extremely vulnerable to any kind of disturbance or systemic shifts.

Nevertheless, seals and penguins do not depend exclusively on pelagic prey but also prey upon demersal fishes (e.g. Casaux et al., 2006; Coria et al., 2000; Plötz et al., 1991). In many high Antarctic shelf areas, exploitation of this resource requires deep diving. Though Weddell seal and Emperor penguin are both excellent divers (Burns and Kooyman, 2001; Wienecke et al., 2007), exploitation of fishes at great depth is energetically disadvantageous for these air-breathing endothermic predators as it involves an increased swimming effort, shorter times at feeding depth, and/or longer dives followed by longer recovery phases (Kooyman, 1989; Kooyman and Kooyman, 1995; Wilson and Quintana, 2004). Moreover, foraging efficiency is higher in shallow dives (Croxall et al., 1985), while encounter rates are probably lower in light-depleted deep waters, as indicated by a lower number of feeding events at depth (see Liebsch et al., 2007; Plötz et al., 2005). Hence, declining stocks or complete loss of *P. antarcticum* will in either case severely affect the top predators in the Antarctic marine ecosystem.

Table 4 Life history traits of clupeid fishes (sardines and anchovies) and the nototheniid *P. antarcticum*

	Clupeids	*Pleuragramma antarcticum*
Von Bertalanffy growth constant K	0.5–0.8[3]	0.05–0.07[5]
Age at first spawning (years)	1–1.5[2,8]	7–9[7]
Relative fecundity (eggs g^{-1} wet weight)	550–600[1]	70–160[4]
Duration of larval phase (days)	~37–74[6]	180–365[5]

Data sources are indicated by superscripts: [1]Alheit and Alegre (1986), [2]Cubillos and Claramunt (2009), [3]Cubillos et al. (2002), [4]Gerasimchuk (1988), [5]Hubold and Tomo (1989), [6]Houde and Zastrow (1993), [7]Kock and Kellermann (1991) and [8]Whitehead (1985).

What are the future perspectives for Antarctic fish communities? There is no doubt that fishes still will be an important and abundant component of the Antarctic marine ecosystem in the future, but the composition of communities will change significantly in the long run. It is likely that, with an ongoing warming trend, Sub-Antarctic demersal fish species such as *Notothenia* spp. (but also non-notothenioids) will move southwards into high Antarctic shelf areas, taking over the role of extinct or declining species in the present-day food web. Possible future scenarios for the pelagic community are the occupation of the 'small pelagic zooplankton feeder' niche by myctophid fishes or by clupeids such as the Falkland sprat, *Sprattus fuegensis*. Whether myctophids or clupeids can effectively replace *P. antarcticum* in its functional role in the food web, however, remains to be seen.

7.5. Final thoughts—Is climate change exclusively to blame?

Though our knowledge is steadily improving, we are in fact just starting to comprehend the structure, dynamics and functioning of the Antarctic marine ecosystem, while the system apparently has already started to respond to climate change. This, however, is not the only threat to marine living communities in the South Polar Sea, and human activities have already caused significant alterations in the past and still affect Antarctic communities today. Commercial fisheries in the Antarctic started in the late 1960s/early 1970s (see Kock et al., 2007 for review), and the destructive impact of bottom trawling on benthic communities is comparable to the impact of iceberg scouring (discussed in Barnes and Conlan, 2007). Commercial sealing and whaling activities in the South Polar Sea in the nineteenth and twentieth centuries (see Kock, 2007; Laws, 1977) resulted in large-scale and long-term alterations of food web structure and population dynamics of prey and competitors (Ainley et al., 2007, 2010; Laws, 1985). Industrial exploitation of Antarctic fish species (and krill) between the 1970s and 1990s resulted in dramatic stock decreases and rapid overexploitation of some species (reviewed in Ainley and Blight, 2008; Kock, 1992, 2007). Since 1982, the fisheries are regulated by CCAMLR (Commission for the Conservation of Antarctic Marine Living Resources), and many were closed between 1985 and 1990 due to overexploitation (Ainley and Blight, 2008; Kock et al., 2007). Nevertheless (to provide some examples

only), regular monitoring of the stocks of many commercially exploited fish species, such as *N. rossii* and *G. gibberifrons*, around the South Shetland Islands, indicates a lack of recovery more than three decades after the end of fisheries (Barrera-Oro and Marschoff, 2007; Barrera-Oro et al., 2000). Similarly, stocks of the channichthyid *C. gunnari* in the Indian Ocean did not recover to pre-exploitation levels after the fishery had ceased for many years (Kock, 2005b). One reason for the slow stock recovery may be the low fecundity and slow development (see Section 3.3.2 above) of many species. However, two additional factors that may adversely affect stock recovery of *C. gunnari* were proposed (Kock, 2005b and references therein): (i) an increase in top-down pressure, that is, increased predation by seals owing to fluctuations in alternative prey (krill) and (ii) possible direct effects of climate change, in particular increasing water temperature. Owing to strict regulation, the numbers of some whale species seem to be increasing again in the South Polar Sea (Branch, 2006, 2007), which is on the one hand desirable, but on the other hand might entail an additional increased top-down pressure on zooplankton and fish communities.

These examples emphasize the complexity of relationships among human activities (historic and current), abiotic climate forcing and altered trophic structure, and how these factors can interact to control fish populations in the South Polar Sea. Thus, multiple drivers act synergistically to affect a particularly sensitive ecosystem, and projecting the future trajectories of fish stocks is particularly challenging, but we are better placed than ever before to start to anticipate and respond to likely scenarios of future change.

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APPENDIX

Table A1 Species names, family and code no. (see Fig. 5) of the species used for the analysis and comparison of the trophic vulnerability to general changes in food web structure and dynamics

No.	Species name	Family	Σ*P*	Σ*C*	VI	TG
1	*Aethotaxis mitopteryx*	Nototheniidae	53	14	0.20	BP
2	*Akarotaxis nudiceps*	Bathydraconidae	79	13	0.09	B
3	*Artedidraco loennbergi*	Artedidraconidae	108	14	0.06	B
4	*Artedidraco orianae*	Artedidraconidae	27	14	0.21	BP
5	*Artedidraco shackletoni*	Artedidraconidae	110	14	0.07	B
6	*Artedidraco skottsbergi*	Artedidraconidae	86	13	0.09	BP
7	*Bathydraco marri*	Bathydraconidae	47	13	0.17	BP
8	*Chaenodraco wilsoni*	Channichthyidae	16	15	0.28	PF
9	*Chionobathyscus dewitti*	Channichthyidae	5	14	0.77	PF
10	*Chionodraco hamatus*	Channichthyidae	10	15	0.67	P
11	*Chionodraco myersi*	Channichthyidae	5	15	0.77	PF
12	*Cryodraco antarcticus*	Channichthyidae	5	15	0.77	PF
13	*Cygnodraco mawsoni*	Bathydraconidae	55	14	0.14	BP
14	*Dacodraco hunteri*	Channichthyidae	37	15	0.10	F
15	*Dissostichus mawsoni*	Nototheniidae	52	21	0.28	BP
16	*Dolloidraco longedorsalis*	Artedidraconidae	142	14	0.04	B
17	*Gerlachea australis*	Bathydraconidae	14	14	0.46	P
18	*Gymnodraco acuticeps*	Bathydraconidae	33	14	0.15	P
19	*Histiodraco velifer*	Artedidraconidae	85	13	0.07	BF
20	*Neopagetopsis ionah*	Channichthyidae	5	14	0.77	PF
21	*Pagetopsis macropterus*	Channichthyidae	43	15	0.10	F
22	*Pagetopsis maculatus*	Channichthyidae	5	15	0.77	PF
23	*Pagothenia borchgrevinki*	Nototheniidae	17	12	0.27	BP
24	*Pleuragramma antarcticum*	Nototheniidae	12	47	0.96	P
25	*Pogonophryne marmorata*	Artedidraconidae	45	14	0.13	BP

Table A1 Species names, family and code no. (see Fig. 5) of the species used for the analysis and comparison of the trophic vulnerability to general changes in food web structure and dynamics—cont'd

No.	Species name	Family	ΣP	ΣC	VI	TG
26	*Pogonophryne permitini*	Artedidraconidae	79	14	0.10	B
27	*Pogonophryne scotti*	Artedidraconidae	78	14	0.11	B
28	*Prionodraco evansii*	Bathydraconidae	88	14	0.08	BP
29	*Racovitzia glacialis*	Bathydraconidae	89	14	0.08	BP
30	*Trematomus bernacchii*	Nototheniidae	93	14	0.02	B
31	*Trematomus eulepidotus*	Nototheniidae	45	14	0.12	BP
32	*Trematomus hansoni*	Nototheniidae	81	14	0.06	BF
33	*Trematomus lepidorhinus*	Nototheniidae	71	14	0.10	BP
34	*Trematomus loennbergii*	Nototheniidae	105	14	0.05	BF
35	*Trematomus nicolai*	Nototheniidae	88	14	0.09	B
36	*Trematomus pennellii*	Nototheniidae	164	14	0.03	BF
37	*Trematomus scotti*	Nototheniidae	121	14	0.06	B

All species are members of the fish community on the eastern Weddell Sea shelf. Species are listed in alphabetical order; for authorities, please consult Gon and Heemstra (1990). For each notothenioid species, the number of prey ($\sum P$) and consumer species ($\sum C$), the relative trophic vulnerability (VI) and trophic group (TG) are given. The index of relative vulnerability VI was calculated from the weighted number of prey species ($\sum$WP) and weighted number of consumer species ($\sum$WC) (see Eq. 2). Data on trophic links are part of the database published in Jacob et al. (2011). Trophic groups were assigned according to main food components as shown in Fig. 5, with B, benthos; P, plankton; F, fish.

REFERENCES

Ainley, D.G., Blight, L.K., 2008. Ecological repercussions of historical fish extraction from the Southern Ocean. Fish Fish. 9, 1–26.

Ainley, D.G., Ballard, G., Barton, K.J., Karl, B.J., Rau, G., Ribic, C.A., Wilson, P.R., 2003. Spatial and temporal variation of diet within a presumed metapopulation of Adélie penguins. Condor 105, 95–106.

Ainley, D., Ballard, G., Ackley, S., Blight, L.K., Eastman, J.T., Emslie, S.D., Lescroël, A., Olmastroni, S., Townsend, S.E., Tynan, C.T., Wilson, P., Woehler, E., 2007. Paradigm lost, or is top-down forcing no longer significant in the Antarctic marine ecosystem? Antarct. Sci. 19, 283–290.

Ainley, D., Ballard, G., Blight, L.K., Ackley, S., Emslie, S.D., Lescroël, A., Olmastroni, S., Townsend, S.E., Tynan, C.T., Wilson, P., Woehler, E., 2010. Impacts of cetaceans on the structure of Southern Ocean food webs. Mar. Mamm. Sci. 26, 482–498.

Alheit, J., 2009. Consequences of regime shifts for marine food webs. Int. J. Earth Sci. 98, 261–268.

Alheit, J., Alegre, B., 1986. Fecundity of Peruvian anchovy, *Engraulis ringens*. ICES CM 1986/H, 60.

Alheit, J., Niquen, M., 2004. Regime shifts in the Humbold Current ecosystem. Prog. Oceanogr. 60, 201–222.

Alheit, J., Möllmann, C., Dutz, J., Kornilovs, G., Loewe, P., Mohrholz, V., Wasmund, N., 2005. Synchronous ecological regime shifts in the central Baltic and the North Sea in the late 1980s. ICES J. Mar. Sci. 62, 1205–1215.

Andriashev, A.P., 1987. A general review of the Antarctic bottom fish fauna. In: Kullander, S.O., Fernholm, B. (Eds.), Proc. V Congr. Europ. Ichthyol. Stockholm 1985, 357–372.

Arntz, W.E., 1986. The two faces of El Niño 1982-83. Meeresforschung 31, 1–46.

Arntz, W.E., Fahrbach, E., 1991. El Niño—Klimaexperiment der Natur. Physikalische Ursachen und biologische Folgen, Birkhäuser Verlag, Basel.

Arntz, W.E., Brey, T., Gallardo, V., 1994. Antarctic zoobenthos. Oceanogr. Mar. Biol. 32, 241–304.

Arntz, W.E., Lovrich, G.A., Thatje, S. (Eds.), 2005. The Magellan-Antarctic connection: links and frontiers at high southern latitudes. Sci. Mar. 69, 1–373.

Aronson, R.B., Thatje, S., Clarke, A., Peck, L.S., Blake, D.B., Wilga, C.D., Seibel, B.A., 2007. Climate change and invasibility of the Antarctic benthos. Annu. Rev. Ecol. Evol. Syst. 38, 129–154.

Arrigo, K.R., Worthen, D.L., Lizotte, M.P., Dixon, P., Dieckmann, G., 1997. Primary production in Antarctic sea ice. Science 276, 394–397.

Arrigo, K.R., Weiss, A.M., Smith Jr., W.O., 1998. Physical forcing of phytoplankton dynamics in the southwestern Ross Sea. J. Geophys. Res. 103, 1007–1021.

Atkinson, A., Ward, P., Williams, R., Poulet, S.A., 1992. Diel vertical migration and feeding of copepods at an oceanic site near South Georgia. Mar. Biol. 113, 583–593.

Atkinson, A., Siegel, V., Pakhomov, E., Rothery, P., 2004. Long-term decline in krill stock and increase in salps within the Southern Ocean. Nature 432, 100–103.

Attril, M.J., Wright, J., Edwards, M., 2007. Climate-related increases in jellyfish frequency suggest a more gelatinous future for the North Sea. Limnol. Oceanogr. 52, 480–485.

Balushkin, A.V., 1994. Fossil notothenioid, and not gadiform, fish *Proeleginops grandeastmanorum* gen. nov. sp. nov. (Perciformes, Notothenioidei, Eleginopidae) from the late Eocene found in Seymour Island (Antarctica). J. Ichthyol. 34, 298–307.

Barnes, D.K.A., 2002. Invasions by marine life on plastic debris. Nature 416, 808–809.

Barnes, D.K.A., Conlan, K.E., 2007. Disturbance, colonization and development of Antarctic benthic communities. Philos. Trans. R. Soc. B 362, 11–38.

Barrera-Oro, E.R., 2002. The role of fish in the Antarctic marine food web: differences between inshore and offshore waters in the southern Scotia Arc and west Antarctic Peninsula. Antarct. Sci. 14, 293–309.

Barrera-Oro, E.R., 2003. Analysis of dietary overlap in Antarctic fish (Notothenioidei) from the South Shetland Islands: no evidence of food competition. Polar Biol. 26, 631–637.

Barrera-Oro, E.R., Casaux, R.J., 1990. Feeding selectivity in *Notothenia neglecta*, Nybelin, from Potter Cove, South Shetland Islands, Antarctica. Antarct. Sci. 2, 207–213.

Barrera-Oro, E.R., Casaux, R.J., 1998. Ecology of demersal fish species from Potter Cove. In: Wiencke, C., Ferreyra, G., Arntz, W.E., Rinaldi, C. (Eds.), The Potter Cove Coastal Ecosystem, Antarctica, Ber. Polarforsch.299, 156–167.

Barrera-Oro, E.R., Lagger, C., 2010. Egg-guarding behaviour in the Antarctic bathydraconid dragonfish *Parachaenichthys charcoti*. Polar Biol. 33, 1585–1587.

Barrera-Oro, E.R., Marschoff, E.R., 2007. Information on the status of fjord *Notothenia rossii*, *Gobionotothen gibberifrons* and *Notothenia coriiceps* in the lower South Shetland Islands, derived from the 2000-2006 monitoring program at Potter Cove. CCAMLR Sci. 14, 83–87.

Barrera-Oro, E.R., Marschoff, E.R., Casaux, R.J., 2000. Trends in relative abundance of fjord *Notothenia rossii*, *Gobionotothen gibberifrons* and *Notothenia coriiceps* at Potter Cove, South Shetland Islands, after commercial fishing in the area. CCAMLR Sci. 7, 43–52.

Bathmann, U.V., Fischer, G., Müller, P.J., Gerdes, D., 1991. Short-term variations in particulate matter sedimentation off Kapp Norvegia, Weddell Sea, Antarctica: relation to water mass advection, ice cover, plankton biomass and feeding activity. Polar Biol. 11, 185–195.

Baumann, H., Talmage, S.C., Gobler, C.J., 2012. Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat. Clim. Chang. 2, 38–41.

Beaugrand, G., Brander, K.M., Lindley, J.A., Souissi, S., Reid, P.C., 2003. Plankton effect on cod recruitment in the North Sea. Nature 426, 661–664.

Beers, J.M., Sidell, B.D., 2011. Thermal tolerance of Antarctic notothenioid fishes correlates with level of circulating hemoglobin. Physiol. Biochem. Zool. 84, 353–362.

Benson, A.J., Trites, A.W., 2002. Ecological effects of regime shifts in the Bering Sea and eastern North Pacific Ocean. Fish Fish. 3, 95–113.

Bilyk, K.T., DeVries, A.L., 2011. Heat tolerance and its plasticity in Antarctic fishes. Comp. Biochem. Phys. A 158, 382–390.

Bodungen, B.v, Nöthig, E.M., Sui, Q., 1988. New production of phytoplankton and sedimentation during summer 1985 in the southeastern Weddell Sea. Comp. Biochem. Physiol. B 90, 475–487.

Boyd, I.L., 2002. Antarctic marine mammals. In: Perrin, W., Würstig, B., Thewissen, J.G.M. (Eds.), Encyclopedia of Marine Mammals. Academic Press, San Diego, pp. 30–36.

Boysen-Ennen, E., Piatkowski, U., 1988. Meso- and macrozooplankton communities in the Weddell Sea, Antarctica. Polar Biol. 9, 17–35.

Branch, T.A., 2006. Humpback abundance south of 60°S from three completed sets of IDCR/SOWER circumpolar surveys. IWC Paper SC/A06/HW6, 1–14.

Branch, T.A., 2007. Abundance of Antarctic blue whales south of 60°S from three complete circumpolar sets of surveys. J. Cetacean Res. Manag. 9, 253–262.

Brenner, M., Buck, B.H., Cordes, S., Dietrich, L., Jacob, U., Mintenbeck, K., Schröder, A., Brey, T., Knust, R., Arntz, W., 2001. The role of iceberg scours in niche separation within the Antarctic fish genus *Trematomus*. Polar Biol. 24, 502–507.

Brey, T., Gerdes, D., 1997. Is Antarctic benthic biomass really higher than elsewhere? Antarct. Sci. 9, 266–267.

Brodeur, R.D., Mills, C.E., Overland, J.E., Walters, G.E., Schumacher, J.D., 1999. Evidence for a substantial increase in gelatinous zooplankton in the Bering Sea, with possible links to climate change. Fish. Oceanogr. 8, 296–306.

Brodte, E., Knust, R., Pörtner, H.O., 2006. Temperature-dependent energy allocation to growth in Antarctic and boreal eelpout (Zoarcidae). Polar Biol. 30, 95–107.

Brose, U., Jonsson, T., Berlow, E.L., Warren, P., Banasek-Richter, C., Bersier, L.F., Blanchard, J.L., Brey, T., Carpenter, S.R., Cattin Blandenier, M.F., Cushing, L., Dawah, H.A., Dell, T., Edwards, F., Harper-Smith, S., Jacob, U., Ledger, M.E., Martinez, N.D., Memmot, J., Mintenbeck, K., Pinnegar, J.K., Rall, B.C., Rayner, T.S., Reuman, D.C., Ruess, L., Ulrich, W., Williams, R.J., Woodward, G., Cohen, J.E., 2006. Consumer-resource body-size relationships in natural food webs. Ecology 87, 2411–2417.

Burns, J.M., Kooyman, G.L., 2001. Habitat use by Weddell seals and Emperor penguins foraging in the Ross Sea, Antarctica. Am. Zool. 41, 99–112.

Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. Nature 425, 365.

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J. Geophys. Res. Oceans 110, 1–12.

Campbell, H.A., Fraser, K.P.P., Bishop, C.M., Peck, L.S., Egginton, S., 2008. Hybernation in an Antarctic fish: on ice for winter. PLoS One 3, e1743.

Carpenter, C.M., Hofmann, G.E., 2000. Expression of 70 kda heat shock proteins in Antarctic and New Zealand notothenioid fish. Comp. Biochem. Phys. A 125, 229–238.

Casaux, R.J., Barrera-Oro, E.R., 2006. Shags in Antarctica: their feeding behavior and ecological role in the marine food web. Antarct. Sci. 18, 3–14.
Casaux, R.J., Baroni, A., Ramón, A., 2006. The diet of the Weddell Seal *Leptonychotes weddellii* at the Danco Coast, Antarctic Peninsula. Polar. Biol. 29, 257–262.
Casaux, R.J., Mazzotta, A.S., Barrera-Oro, E.R., 1990. Seasonal aspects of the biology and diet of nearshore notothenid fish at Potter Cove, South Shetland Islands, Antarctica. Polar Biol. 11, 63–72.
Castle, M.D., Blanchard, J.L., Jennings, S., 2011. Predicted effects of behavioural movement and passive transport on individual growth and community size structure in marine ecosystems. Adv. Ecol. Res. 45, 41–66.
Ciattaglia, L., Rafanelli, C., Araujo, J., Rodriguez, H., 2008. Long-term measurements of the atmospheric carbon dioxide concentration measured at Jubany Station indicate a relationship with "El Niño" In: Wiencke, C., Ferreyra, G., Abele, D., Marenssi, S. (Eds.), The Antarctic Ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo), Ber. Polarforsch. Meeresforsch.571, 390–396.
Clarke, A., 1983. Life in cold water: the physiological ecology of polar marine ectotherms. Oceanogr. Mar. Biol. Ann. Rev. 21, 341–453.
Clarke, A., 1985. Energy flow in the Southern Ocean food web. In: Siegfried, W.R., Condy, P.R., Laws, R.M. (Eds.), Antarctic Nutrient Cycles and Food Webs. Springer Verlag, Berlin, pp. 573–580.
Clarke, A., 1991. What is cold adaptation and how should we measure it? Am. Zool. 31, 81–92.
Clarke, A., Johnston, I.A., 1996. Evolution and adaptive radiation of Antarctic fishes. Trends Ecol. Evol. 11, 212–218.
Clarke, A., Holmes, L.J., Gore, D.J., 1992. Proximate and elemental composition of gelatinous zooplankton from the Southern Ocean. J. Exp. Mar. Biol. Ecol. 155, 55–68.
Conlan, K.E., Kvitek, R.G., 2005. Recolonization of soft-sediment ice scours on an exposed Arctic coast. Mar. Ecol. Prog. Ser. 286, 21–42.
Conlan, K.E., Lenihan, H.S., Kvitek, R.G., Oliver, J.S., 1998. Ice scour disturbance to benthic communities in the Canadian high Arctic. Mar. Ecol. Prog. Ser. 166, 1–16.
Coria, N., Libertelli, M., Casaux, R., Darrieu, C., 2000. Inter-annual variation in the autumn diet of the gentoo penguin at Laurie Island, Antartica. Waterbirds 23, 511–517.
Crockett, E.L., Sidell, B.D., 1990. Some pathways of energy-metabolism are cold adapted in Antarctic fishes. Physiol. Zool. 63, 472–488.
Croxall, J.P., Prince, P.A., 1982. Caloric content of squid (Mollusca: Cephalopoda). Br. Antarct. Surv. Bull. 55, 27–31.
Croxall, J.P., Everson, I., Kooyman, G.L., Ricketts, C., Davis, R.W., 1985. Fur seal diving behaviour in relation to vertical distribution of krill. J. Anim. Ecol. 54, 1–8.
Cubillos, L.A., Claramunt, G., 2009. Length-structured analysis of the reproductive season of anchovy and common sardine off central southern Chile. Mar. Biol. 156, 1673–1680.
Cubillos, L.A., Bucarey, D.A., Canales, M., 2002. Monthly abundance estimation for common sardine *Strangomera bentincki* and anchovy *Engraulis ringens* in the central-southern area off Chile (34-40°S). Fish. Res. 57, 117–130.
Curran, M.A.J., van Ommen, T.D., Morgan, V.I., Phillips, K.L., Palmer, A.S., 2003. Ice core evidence for Antarctic sea ice decline since the 1950s. Science 302, 1203–1206.
Curry, R., Mauritzen, C., 2005. Dilution of the northern North Atlantic Ocean in recent decades. Science 308, 1772–1774.
Cury, P., Bakun, A., Crawford, R.J.M., Jarre, A., Quiñones, R.A., Shannon, L.J., Verheye, H.M., 2000. Small pelagics in upwelling systems: patterns of interaction and structural changes in "wasp-waist" ecosystems. ICES J. Mar. Sci. 57, 603–618.

Dahlhoff, E., Somero, G.N., 1993. Effects of temperature on mitochondria from abalone (genus *Haliotis*): adaptive plasticity and its limits. J. Exp. Biol. 185, 151–168.
Daneri, G.A., Carlini, A.R., 2002. Fish prey of southern elephant seals, *Mirounga leonina*, at King George Island. Polar Biol. 25, 739–743.
Daniels, R.A., 1982. Feeding ecology of some fishes of the Antarctic Peninsula. Fish. B.-NOAA 80, 575–589.
Davis, R.W., Fuiman, L.A., Williams, T.M., Collier, S.O., Hagey, W.P., Kanatous, S.B., Kohin, S., Horning, M., 1999. Hunting behaviour of a marine mammal beneath the Antarctic fast ice. Science 283, 993–996.
Dayton, P.K., 1990. Polar benthos. In: Smith, W.O. (Ed.), Polar Oceanography, Part B: Chemistry, Biology, and Geology. Academic Press, Boston, pp. 631–685.
Dayton, P.K., Robilliard, G.A., Paine, R.T., Dayton, L.B., 1974. Biological accommodation in the benthic community at McMurdo Sound, Antarctica. Ecol. Monogr. 44, 105–128.
Dayton, P.K., Mordida, B.J., Bacon, F., 1994. Polar marine communities. Am. Zool. 34, 90–99.
Deacon, G., 1984. The Antarctic Circumpolar Ocean. Cambridge University Press, Cambridge.
Detmer, A.E., Bathmann, U.V., 1997. Distribution patterns of autotrophic pico- and nanoplankton and their relative contribution to algal biomass during spring in the Atlantic sector of the Southern Ocean. Deep Sea Res. II 44, 299–320.
Detrich III, H.W., Jones, C.D., Kim, S., North, A.W., Thurber, A., Vacchi, M., 2005. Nesting behaviour of the icefish *Chaenocephalus aceratus* at Bouvetøya, Southern Ocean (CCAMLR Subarea 48.6). Polar Biol. 28, 828–832.
DeVries, A.L., 1971. Glycoproteins as biological antifreeze agents in Antarctic fishes. Science 172, 1152–1155.
DeVries, A.L., Eastman, J.T., 1978. Lipid sacs as a buoyancy adaptation in an Antarctic fish. Nature 271, 352–353.
DeWitt, H.H., 1970. The character of the midwater fish fauna of the Ross Sea, Antarctica. In: Holdgate, M.W. (Ed.), Antarctic Ecology, vol. 1. Academic Press, London, pp. 305–314.
Dierssen, H.M., Smith, R.C., Vernet, M., 2002. Glacial meltwater dynamics in coastal waters west of the Antarctic Peninsula. Proc. Natl. Acad. Sci. U.S.A. 99, 1790–1795.
DiTullio, G.R., Grebmeier, J.M., Arrigo, K.R., Lizotte, M.P., Robinson, D.H., Leventer, A., Barry, J.P., VanWoert, M.L., Dunbar, R.B., 2000. Rapid and early export of *Phaeocystis antarctica* blooms in the Ross Sea, Antarctica. Nature 404, 595–598.
Domack, E., Leventer, A., Burnett, A., Bindschadler, R., Convey, P., Kirby, M. (Eds.), 2003. Antarctic Peninsula climate variability: historical and paleoenvironmental perspectives. Antarct. Res. Ser. 79, 260.
Domack, E., Duran, D., Leventer, A., Ishman, S., Doane, S., McCallum, S., Amblas, D., Ring, J., Gilbert, R., Prentice, M., 2005. Stability of the Larsen B ice shelf on the Antarctic Peninsula during the Holocene epoch. Nature 436, 681–685.
Donelsen, J.M., Munday, P.L., McCormick, M.I., Pitcher, C.R., 2012. Rapid transgenerational acclimation of a tropical reef fish to climate change. Nat. Clim. Chang. 2, 30–32.
Donnelly, J., Torres, J.J., Hopkins, T.L., Lancraft, T.M., 1990. Proximate composition of Antarctic mesopelagic fishes. Mar. Biol. 106, 13–23.
Donnelly, J., Torres, J.J., Hopkins, T.L., Lancraft, T.M., 1994. Chemical composition of Antarctic zooplankton during austral fall and winter. Polar Biol. 14, 171–183.
Donnelly, J., Torres, J.J., Sutton, T.T., Simoniello, C., 2004. Fishes of the eastern Ross Sea, Antarctica. Polar Biol. 27, 637–650.
Doyle, T.K., De Haas, H., Cotton, D., Dorschel, B., Cummins, V., Houghton, J.D.R., Davenport, J., Hays, G.C., 2008. Widespread occurrence of the jellyfish *Pelagia noctiluca* in Irish coastal and shelf waters. J. Plankton Res. 30, 963–968.

Drinkwater, K.F., Beaugrand, G., Kaeriyma, M., Kim, S., Ottersen, G., Perry, R.I., Pörtner, H.O., Polovina, J.J., Takasuka, A., 2010. On the processes linking climate to ecosystem changes. J. Mar. Syst. 79, 374–388.

Dulvy, N.K., Rogers, S.I., Jennings, S., Stelzenmüller, V., Dye, S.R., Skjoldal, H.R., 2008. Climate change and deepening of the North Sea fish assemblage: a biotic indicator of warming seas. J. Appl. Ecol. 45, 1029–1039.

Eakin, R.R., Balushkin, A.V., 1998. A new species of toadlike Plunderfish *Pogonophryne orangiensis* sp. nova (Artedidraconidae, Notothenioidei) from the Weddell Sea, Antarctica. J. Ichthyol. 38, 800–803.

Eakin, R.R., Balushkin, A.V., 2000. A new species of *Pogonophryne* (Pisces: Perciformes: Artedidraconidae) from East Antarctica. Proc. Biol. Soc. Wash. 113, 264–268.

Eakin, R.R., Eastman, J.T., 1998. New species of *Pogonophryne* (Pisces, Artedidraconidae) from the Ross Sea, Antarctica. Copeia 4, 1005–1009.

Eakin, R.R., Eastman, J.T., Matallanas, J., 2008. New species of Pogonophryne (Pisces, Artedidraconidae) from the Bellinghausen Sea, Antarctica. Polar Biol. 31, 1175–1179.

Eastman, J.T., 1985a. The evolution of neutrally buoyant notothenioid fishes: their specialization and potential interactions in the Antarctic marine food web. In: Siegfried, W.R., Condy, P.R., Laws, R.M. (Eds.), Antarctic Nutrient Cycles and Food Webs. Springer Verlag, Berlin, pp. 430–436.

Eastman, J.T., 1985b. *Pleuragramma antarcticum* (Pisces, Nototheniidae) as food for other fishes in McMurdo Sound, Antarctica. Polar Biol. 4, 155–160.

Eastman, J.T., 1991. The fossil and modern fish faunas of Antarctica: evolution and diversity. In: Di Prisco, G., Maresca, B., Tota, B. (Eds.), Biology of Antarctic Fish. Springer Verlag, Berlin, pp. 116–130.

Eastman, J.T., 1993. Antarctic Fish Biology—Evolution in a Unique Environment. Academic Press, San Diego.

Eastman, J.T., 1999. Aspects of the biology of the icefish *Dacodraco hunteri* (Notothenioidei, Channichthyidae) in the Ross Sea, Antarctica. Polar Biol. 21, 194–196.

Eastman, J.T., 2005. The nature of the diversity of Antarctic fishes. Polar Biol. 28, 93–107.

Eastman, J.T., DeVries, A.L., 1981. Buoyancy adaptations in a swim-bladderless Antarctic fish. J. Morphol. 167, 91–102.

Eastman, J.T., DeVries, A.L., 1982. Buoyancy studies of notothenioid fishes in McMurdo Sound, Antarctica. Copeia 2, 385–393.

Eastman, J.T., DeVries, A.L., 1985. Adaptations for cryopelagic life in the Antarctic notothenioid fish *Pagothenia borchgrevinki*. Polar Biol. 4, 45–52.

Eastman, J.T., Eakin, R.R., 2000. An updated species list for notothenioid fish (Perciformes; Notothenioidei), with comments on Antarctic species. Arch. Fish. Mar. Res. 48, 11–20.

Eastman, J.T., Grande, L., 1989. Evolution of the Antarctic fish fauna with emphasis on the recent notothenioids. In: Crame, J.A. (Ed.), Origins and Evolution of the Antarctic Biota. Geological Society, London, pp. 241–252 Special Publication No. 47.

Eastman, J.T., Hubold, G., 1999. The fish fauna of the Ross Sea, Antarctica. Antarct. Sci. 11, 293–304.

Eastman, J.T., McCune, A.R., 2000. Fishes on the Antarctic continental shelf: evolution of a marine species flock? J. Fish Biol. 57, 84–102.

Eastman, J.T., Barrera-Oro, E.R., Moreira, E., 2011. Adaptive radiation at a low taxonomic level: divergence in buoyancy of the ecologically similar Antarctic fish *Notothenia coriiceps* and *N. rossii*. Mar. Ecol. Prog. Ser. 438, 195–206.

Eder, E.B., Lewis, M.N., 2005. Proximate composition and energetic value of demersal and pelagic prey species from the SW Atlantic Ocean. Mar. Ecol. Prog. Ser. 291, 43–52.

Efremenko, V.N., 1983. Atlas of fish larvae of the Southern Ocean. Cybium 7, 1–74.

Egginton, S., 1996. Blood rheology of Antarctic fishes: viscosity adaptations at very low temperatures. J. Fish Biol. 48, 513–521.
Egginton, S., 1997a. A comparison of the response to induced exercise in red- and white-blooded Antarctic fishes. J. Comp. Physiol. B 167, 129–134.
Egginton, S., 1997b. Control of tissue blood flow at very low temperatures. J. Therm. Biol. 22, 403–407.
Ehrmann, W.U., Mackensen, A., 1992. Sedimentological evidence for the formation of an East Antarctic ice sheet in the Eocene/Oligocene time. Palaeogeogr. Palaeoclimatol. Palaeoecol. 93, 85–112.
Eicken, H., 1992. The role of sea ice in structuring Antarctic ecosystems. Polar Biol. 12, 3–13.
Eicken, H., 1995. Wie polar wird das Polarmeer durch das Meereis? In: Hempel, I., Hempel, G. (Eds.), Biologie der Polarmeere. Gustav Fischer Verlag, Jena, pp. 58–76.
Ekau, W., 1988. Ökomorphologie nototheniider Fische aus dem Weddellmeer, Antarktis. Ber. Polarforsch. 51, 1–140.
Ekau, W., 1989. Egg development of *Trematomus eulepidotus* Regan, 1914 (Nototheniidae, Pisces) from the Weddell Sea, Antarctica. Cybium 13, 213–219.
Estrada, M., Delgado, M., 1990. Summer phytoplankton distributions in the Weddell Sea. Polar Biol. 10, 441–449.
Faleyeva, T.I., Gerasimchuk, V.V., 1990. Features of reproduction in the Antarctic sidestripe, *Pleuragramma antarcticum* (Nototheniidae). J. Ichthyol. 30, 67–79.
Falk-Petersen, J., Renaud, P., Anisimova, N., 2011. Establishment and ecosystem effects of the alien invasive red king crab (*Paralithodes camtschaticus*) in the Barents Sea—a review. ICES J. Mar. Sci. 68, 479–488.
Fanta, E., 1999. Laboratory test on feeding interactions of some Antarctic fish from Admiralty Bay (King George Island, South Shetlands). Pol. Polar Res. 20, 17–28.
Fanta, E., Rios, F.S., Donatti, L., Cardoso, W.E., 2003. Spatial and temporal variation in krill consumption by the Antarctic fish *Notothenia coriiceps*, in Admirality Bay, King George Island. Antarct. Sci. 15, 458–462.
Feely, R.A., Sabine, C.L., Lee, K., Berelson, W., Kleypas, J., Fabry, V.J., Millero, F.J., 2004. Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. Science 305, 362–366.
Feld, C.K., Birk, S., Bradley, D.C., Hering, D., Kail, J., Marzin, A., Melcher, A., Nemitz, D., Pedersen, M.L., Pletterbauer, F., Pont, D., Verdonschot, P.F.M., Friberg, N., 2011. From natural to degraded rivers and back again: a test of restoration ecology theory and practice. Adv. Ecol. Res. 44, 119–210.
Ferrari, M.C.O., McCormick, M.I., Munday, P.L., Meekan, M.G., Dixson, D.L., Lonnstedt, Ö., Chivers, D.P., 2011. Putting prey and predator into the CO_2 equation—qualitative and quantitative effects of ocean acidification on predator-prey interactions. Ecol. Lett. 14, 1143–1148.
Fischer, G., 1989. Stabile Kohlenstoff-Isotope in partikulärer organischer Substanz aus dem Südpolarmeer (Atlantischer Sektor). Berichte aus dem Fachbereich Geowissenschaften der Universität Bremen No. 5, 161pp. Dissertation, Universität Bremen.
Fivelstad, S., Olsen, A.B., Asgard, T., Baeverfjord, G., Rasmussen, T., Vindheim, T., Stefansson, S., 2003. Long-term sublethal effects of carbon dioxide on Atlantic salmon smolts (*Salmo salar* L.): ion regulation, haematology, element composition, nephrocalcinosis and growth parameters. Aquaculture 215, 301–319.
Fletcher, G.L., Hew, C.L., Davies, P.L., 2001. Antifreeze proteins of teleost fishes. Annu. Rev. Physiol. 63, 359–390.
Foster, B.A., Cargill, J.M., Montgomery, J.C., 1987. Planktivory in *Pagothenia borchgrevinki* (Pisces: Nototheniidae) in McMurdo Sound, Antarctica. Polar Biol. 8, 49–54.

Franke, A., Clemmesen, C., 2011. Effect of ocean acidification on early life stages of Atlantic herring (*Clupea harengus* l.). Biogeosci. Discuss. 8, 7097–7126.
Franklin, C.E., Davison, W., Seebacher, F., 2007. Antarctic fish can compensate for rising temperatures: thermal acclimation of cardiac performance in *Pagothenia borchgrevinki*. J. Exp. Biol. 210, 3068–3074.
Froese, R., Palomares, M.L.D., Pauly, D., 2002. Estimation of Life-History Key Facts. Version of 17 July 2002, http://www.fishbase.org.
Frommel, A., Maneja, R., Lowe, D., Malzahn, A., Geffen, A., Folkvord, A., Piatkowski, U., Reusch, T., Clemmesen, C., 2012. Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nat. Clim. Chang. 2, 43–46.
Fuiman, L., Davis, R., Williams, T., 2002. Behaviour of midwater fishes under the Antarctic ice: observations by a predator. Mar. Biol. 140, 815–822.
Gambi, M.C., Lorenti, M., Russo, G.F., Scipione, M.B., 1994. Benthic associations of the shallow hard bottoms off Terra Nova Bay, Ross Sea: zonation, biomass and population structure. Antarct. Sci. 6, 449–462.
García Raso, J.E., Manjón-Cabeza, M.E., Ramos, A., Olaso, I., 2005. New record of Lithodidae (Crustacea: Decapoda: Anomura) from the Antarctic (Bellingshausen Sea). Polar Biol. 28, 642–646.
Garcia, J.A., Ruzafa, P., 1998. Correlation between habitat structure and a rocky reef assemblage in the Southwest Mediterranean. Mar. Ecol. 19, 111–128.
Garwood, N.C., Janos, D.P., Brokaw, N., 1979. Earthquake-caused landslides: a major disturbance to tropical forests. Science 205, 997–999.
Gerasimchuk, V.V., 1988. On the fecundity of Antarctic sidestripe, *Pleuragramma antarcticum*. J. Ichthyol. 28, 98–100.
Gerdes, D., Isla, E., Knust, R., Mintenbeck, K., Rossi, S., 2008. Response of Antarctic benthic communities to disturbance: first results from the artificial Benthic Disturbance Experiment on the eastern Weddell Sea shelf, Antarctica. Polar Biol. 31, 1469–1480.
Gili, J.-M., Rossi, S., Pages, F., Orejas, C., Teixidó, N., López-González, P.J., Arntz, W.E., 2006. A new trophic link between the pelagic and benthic system on the Antarctic shelf. Mar. Ecol. Prog. Ser. 322, 43–49.
Gille, S.T., 2002. Warming of the Southern Ocean since the 1950s. Science 295, 1275–1277.
Gille, S.T., 2008. Decadal-scale temperature trends in the southern hemisphere ocean. J. Clim. 21, 4749–4765.
Gon, O., Heemstra, P.C., 1990. Fishes of the Southern Ocean. J.L.B Smith Institute of Ichthyology, Grahamstown 462pp.
Gonzalez-Cabrera, P.J., Dowd, F., Pedibhotla, V.K., Rosario, R., Stanley-Samuelson, D., Petzel, D., 1995. Enhanced hypo-osmoregulation induced by warm-acclimation in Antarctic fish is mediated by increased gill and kidney Na+/K (+)-ATPase activities. J. Exp. Biol. 198, 2279–2291.
Gordon, A.L., Goldberg, R.D., 1970. Circumpolar characteristics of Antarctic waters. In: Bushnell, V.C. (Ed.), Antarctic Map Folio Series, Folio 13. American Geographical Society, New York, pp. 1–5.
Gordon, A.L., Chen, C.T.A., Metcalf, W.G., 1984. Winter mixed layer entrainment of Weddell deep water. J. Geophys. Res. 89, 637–640.
Granata, A., Cubeta, A., Guglielmo, L., Sidoti, O., Greco, S., Vacchi, M., La Mesa, M., 2002. Ichthyoplankton abundance and distribution in the Ross Sea during 1987-1996. Polar Biol. 25, 187–202.
Granata, A., Zagami, G., Vacchi, M., Guglielmo, L., 2009. Summer and spring trophic niche of larval and juvenile *Pleuragramma antarcticum* in the Western Ross Sea, Antarctica. Polar Biol. 32, 369–382.

Gratwicke, B., Speight, M.R., 2005. The relationship between fish species richness, abundance and habitat complexity in a range of shallow tropical marine habitats. J. Fish Biol. 66, 650–667.

Gray, J.S., 2000. The measurement of marine species diversity, with an application to the benthic fauna of the Norwegian continental shelf. J. Exp. Mar. Biol. Ecol. 250, 23–49.

Guderley, H., Johnston, I., 1996. Plasticity of fish muscle mitochondria with thermal acclimation. J. Exp. Biol. 199, 1311–1317.

Guégan, J.F., Lek, S., Oberdorff, T., 1998. Energy availability and habitat heterogeneity predict global riverine fish diversity. Nature 391, 382–384.

Guglielmo, L., Granata, A., Greco, S., 1998. Distribution and abundance of postlarval and juvenile *Pleuragramma antarcticum* (Pisces, Nototheniidae) off Terra Nova Bay (Ross Sea, Antarctica). Polar Biol. 19, 37–51.

Gutt, J., 2000. Some "driving forces" structuring communities of the sublittoral Antarctic macrobenthos. Antarct. Sci. 12, 297–313.

Gutt, J., 2001. On the direct impact of ice on marine benthic communities, a review. Polar Biol. 24, 553–564.

Gutt, J., Piepenburg, D., 2003. Scale-dependent impact on diversity of Antarctic benthos caused by grounding of icebergs. Mar. Ecol. Prog. Ser. 253, 77–83.

Gutt, J., Starmans, A., 1998. Structure and biodiversity of megabenthos in the Weddell and Lazarev Seas (Antarctica): ecological role of physical parameters and biological interactions. Polar Biol. 20, 229–247.

Gutt, J., Starmans, A., 2001. Quantification of iceberg impact and benthic recolonisation patterns in the Weddell Sea (Antarctica). Polar Biol. 24, 615–619.

Gutt, J., Starmans, A., Diekmann, G., 1996. Impact of iceberg scouring on polar benthic habitats. Mar. Ecol. Prog. Ser. 137, 311–316.

Gutt, J., Sirenko, B.I., Smirnov, I.S., Arntz, W.E., 2004. How many macrozoobenthic species might inhabit the Antarctic shelf? Antarct. Sci. 16, 11–16.

Hagen, M., Kissling, W.D., Rasmussen, C., De Aguiar, M.A.M., Brown, L., Carstensen, D.W., Alves-Dos-Santos, I., Dupont, Y.L., Edwards, F.K., Genini, J., Guimarães Jr., P.R., Jenkins, G.B., Jordano, P., Kaiser-Bunbury, C.N., Ledger, M., Maia, K.P., Marquitti, F.M.D., Mclaughlin, Ó., Morellato, L.P.C., O'Gorman, E.J., Trøjelsgaard, K., Tylianakis, J.M., Vidal, M.M., Woodward, G., Olesen, J.M., 2012. Biodiversity, species interactions and ecological networks in a fragmented world. Adv. Ecol. Res. 46, 89–210.

Hazel, J.R., 1995. Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? Annu. Rev. Physiol. 57, 19–42.

Helaouët, P., Beaugrand, G., 2007. Statistical study of the ecological niche of *Calanus finmarchicus* and *C. helgolandicus* in the North Atlantic Ocean and adjacent seas. Mar. Ecol. Prog. Ser. 345, 147–165.

Hempel, G., 1985. Antarctic marine food webs. In: Siegfried, W.R., Condy, P.R., Laws, R.M. (Eds.), Antarctic Nutrient Cycles and Food Webs. Springer Verlag, Berlin, pp. 266–270.

Hochachka, P.W., Somero, G.N., 2002. Biochemical Adaptation. Oxford University Press, New York.

Hopkins, T.L., 1985. Food web of an Antarctic midwater ecosystem. Mar. Biol. 89, 197–212.

Hoshiai, T., Tanimura, A., Fukuchi, M., Watanabe, K., 1989. Feeding by the notothenid fish, *Pagothenia borchgrevinki* on the ice-associated copepod, *Paralabidocera antarctica*. Polar Biol. 2, 61–64.

Houde, E.D., Zastrow, C.E., 1993. Ecosystem- and taxon-specific dynamic and energetics properties of fish larvae assemblages. Bull. Mar. Sci. 53, 290–335.

Hubold, G., 1984. Spatial distribution of *Pleuragramma antarcticum* (Pisces: Nototheniidae) near the Filchner- and Larsen ice shelves (Weddell Sea/Antarctica). Polar Biol. 3, 231–236.

Hubold, G., 1985. The early life-history of the high-Antarctic Silverfish, *Pleuragramma antarcticum*. In: Siegfried, W.R., Condy, P.R., Laws, R.M. (Eds.), Antarctic Nutrient Cycles and Food Webs. Springer Verlag, Berlin, pp. 446–451.

Hubold, G., 1990. Seasonal patterns of ichthyoplankton distribution and abundance in the southern Weddell Sea. In: Kerry, K.R., Hempel, G. (Eds.), Antarctic Ecosystems. Ecological Change and Conservation. Springer Verlag, Berlin, pp. 149–158.

Hubold, G., 1992. Zur Ökologie der Fische im Weddellmeer. Ber. Polarforsch. 103, 1–157.

Hubold, G., Ekau, W., 1987. Midwater fish fauna of the Weddell Sea, Antarctica. In: Kullander, S.O., Fernholm, B. (Eds.), Proc. V Congr. Europ. Ichthyol. Stockholm 1985, 391–396.

Hubold, G., Ekau, W., 1990. Feeding patterns of post-larval and juvenile notothenioids in the Southern Weddell Sea (Antarctica). Polar Biol. 10, 255–260.

Hubold, G., Hagen, W., 1997. Seasonality of feeding and lipid content of *Pleuragramma antarcticum* (Nototheniidae) in the southern Weddell Sea. In: Battaglia, B., Valencia, J., Walton, D.W.H. (Eds.), Antarctic Communities: Species, Structure and Survival. Cambridge University Press, Cambridge, pp. 277–283.

Hubold, G., Tomo, A.P., 1989. Age and growth of Antarctic silverfish *Pleuragramma antarcticum* Boulenger, 1902, from the southern Weddell Sea and Antarctic Peninsula. Polar Biol. 9, 205–212.

Hudson, H.A., Brauer, P.R., Scofield, M.A., Petzel, D.H., 2008. Effects of warm acclimation on serum osmolality, cortisol and hematocrit levels in the Antarctic fish, *Trematomus bernacchii*. Polar Biol. 31, 991–997.

Hughes, J.E., Deegan, L.A., Wyda, J.C., Weaver, M.J., Wright, A., 2002. The effects of eelgrass habitat loss on estuarine fish communities of southern New England. Estuaries 25, 235–249.

Huntley, M.E., Sykes, P.F., Marin, V., 1989. Biometry and trophodynamics of *Salpa thompsoni* foxton (Tunicata: Thaliacea) near the Antarctic Peninsula in austral summer, 1983-1984. Polar Biol. 10, 59–70.

Hureau, J.C., 1970. Biologie compareé de quelques poissons antarctiques (Nototheniidae). Bull. Inst. Oceanogr. Monaco 68, 1–244.

Hureau, J.C., 1994. The significance of fish in the marine Antarctic ecosystems. Polar Biol. 14, 307–313.

Huston, M.A., 1979. A general hypothesis of species diversity. Am. Nat. 113, 81–101.

Iken, K., 1996. Trophische Beziehungen zwischen Makroalgen und Herbivoren in der Potter Cove (King George-Insel, Antarktis). Ber. Polarforsch. 201, 1–214.

Iken, K., Barrera-Oro, E.R., Quartino, M.L., Casaux, R.J., Brey, T., 1997. Grazing in the Antarctic fish *Notothenia coriiceps*: evidence for selective feeding on macroalgae. Antarct. Sci. 9, 386–391.

IPCC, 2007. Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Ishimatsu, A., Hayashi, M., Lee, K.S., Kikkawa, T., Kita, J., 2005. Physiological effects on fishes in a high-CO_2 world. J. Geophys. Res. 110, C09S09.

Ivany, L.C., Lohmann, K.C., Hasiuk, F., Blake, D.B., Glass, A., Aronson, R.B., Moody, R.N., 2008. Eocene climate record of a high southern latitude continental shelf: Seymour Island, Antarctica. Geol. Soc. Am. Bull. 120, 659–678.

Jacob, U., 2005. Trophic dynamics of Antarctic shelf ecosystems—food webs and energy flow budgets. PhD Thesis, University of Bremen, 141pp.

Jacob, U., Thierry, A., Brose, U., Arntz, W.E., Berg, S., Brey, T., Fetzer, I., Jonsson, T., Mintenbeck, K., Möllmann, C., Petchey, O., Riede, S., Dunne, J.A., 2011. The role of body size in complex food webs: a cold case. Adv. Ecol. Res. 45, 181–223.

Jacobs, S.S., Comiso, J.C., 1997. Climate variability in the Amundsen and Bellinghausen Seas. J. Clim. 10, 697–709.

Jacobs, S.S., Giulivi, C.F., Mele, P.A., 2002. Freshening of the Ross Sea during the late 20th century. Science 297, 386–389.

Janssen, J., Sideleva, V., Montgomery, J., 1991. Under-ice observation of fish behaviour at McMurdo Sound. Antarct. J. U.S. 26, 174–175.

Jarre-Teichmann, A., Brey, T., Bathmann, V.V., Dahm, C., Dieckmann, G.S., Gorny, M., Klages, M., Pagés, F., Plötz, J., Schnack-Schiel, S.B., Stiller, M., Arntz, W.E., 1995. Trophic flows in the benthic shelf community of the eastern Weddell Sea, Antarctica. In: Battaglia, B., Valencia, J., Walton, D. (Eds.), Antarctic Communities: Species, Structure and Survival. Cambridge University Press, Cambridge, pp. 118–134.

Johnson, K.H., 2000. Trophic-dynamic considerations in relating species diversity to ecosystem resilience. Biol. Rev. Camb. Philos. Soc. 75, 347–376.

Johnston, I., 2003. Muscle metabolism and growth in Antarctic fishes (suborder Notothenioidei): evolution in a cold environment. Comp. Biochem. Physiol. B 136, 701–713.

Johnston, I., Guderley, H., Franklin, C., Crockford, T., Kamunde, C., 1994. Are mitochondria subject to evolutionary temperature adaptation? J. Exp. Biol. 195, 293–306.

Kamler, E., Krasicka, B., Rakusa-Suszczewski, S., 2001. Comparison of lipid content and fatty acid composition in muscle and liver of two notothenioid fishes from Admiralty Bay (Antarctica): an eco-physiological perspective. Polar Biol. 24, 735–743.

Kashkina, A.A., 1986. Feeding of fishes on salps (Tunicata, Thaliacea). J. Ichthyol. 26, 57–64.

Kawaguchi, S., Kuruhara, H., King, R., Hale, L., Berli, T., Robinson, J.P., Ishida, A., Wakita, M., Virtue, P., Nicol, S., Ishimatsu, A., 2011. Will krill fare well under Southern Ocean acidification? Biol. Lett. 7, 288–291.

Kellermann, A., 1986a. Geographical distribution and abundance of postlarval and juvenile *Pleuragramma antarcticum* (Pisces, Notothenioidei) off the Antarctic Peninsula. Polar Biol. 6, 111–119.

Kellermann, A., 1986b. Zur Biologie der Jungstadien der Notothenioidei (Pisces) an der Antarktischen Halbinsel. Ber. Polarforsch. 31, 1–155.

Kellermann, A., 1987. Food and feeding ecology of postlarval and juvenile *Pleuragramma antarcticum* (Pisces, Notothenioidei) in the seasonal pack ice zone off the Antarctic Peninsula. Polar Biol. 7, 307–315.

Kellermann, A., 1989. Food and feeding of early stage *Chionodraco rastrospinosus* DeWitt & Hureau 1979 (Pisces; Notothenioidei) off the Antarctic Peninsula. Pesq. Antárt. Bras. 1, 25–30.

Kellermann, A., 1990. Identification key and catalogue of larval Antarctic fishes. Ber. Polarforsch. 67, 1–136.

Knox, G.A., 1970. Antarctic marine ecosystems. In: Holdgate, M.W. (Ed.), Antarctic Ecology, vol. 1. Academic Press, London, pp. 69–96.

Knust, R., Arntz, W.E., Boche, M., Brey, T., Gerdes, D., Gutt, J., Mintenbeck, K., Schröder, A., Starmans, A., Teixido, N., 2003. Iceberg scouring on the eastern Weddell Sea shelf (Antarctica). A benthic system shaped by physical disturbance. In: Huiskes, A.H.L., Gieskes, W.W.C., Rozema, J., Schorno, R.M.L., van der Vies, S. M., Wolff, W.J. (Eds.), Antarctic Biology in a Global Context. Backhuys Publishers, Leiden, pp. 96–101.

Kock, K.-H., 1992. Antarctic Fish and Fisheries. Cambridge University Press, Cambridge 359pp.

Kock, K.-H., 2005a. Antarctic icefishes (Channichthyidae): a unique family of fishes. A review, Part I. Polar Biol. 28, 862–895.

Kock, K.-H., 2005b. Antarctic icefishes (Channichthyidae): a unique family of fishes. A review, Part II. Polar Biol. 28, 897–909.

Kock, K.-H., 2007. Antarctic marine living resources—exploitation and its management in the Southern Ocean. Antarct. Sci. 19, 231–238.
Kock, K.-H., Everson, I., 1998. Age, growth and maximum size of Antarctic notothenioid fish—revisited. In: Di Prisco, G., Pisano, E., Clarke, A. (Eds.), Fishes of Antarctica—A Biological Overview. Springer Verlag Italia, Milano, pp. 29–40.
Kock, K.-H., Kellermann, A., 1991. Reproduction in Antarctic notothenioid fish. Antarct. Sci. 3, 125–150.
Kock, K.-H., Pshenichnov, L.K., DeVries, A.L., 2006. Evidence for egg brooding and parental care in icefish and other notothenioids in the Southern Ocean. Antarct. Sci. 18, 223–227.
Kock, K.-H., Reid, K., Croxall, J., Nicol, S., 2007. Fisheries in the Southern Ocean: an ecosystem approach. Philos. Trans. R. Soc. B 362, 2333–2349.
Kooyman, G.L., 1989. Diverse Divers: Physiology and Behaviour. Springer Verlag, Berlin.
Kooyman, G.L., Kooyman, T.G., 1995. Diving behaviour of Emperor penguins nurturing chicks at Coulman Island, Antarctica. Condor 97, 536–549.
Koubbi, P., Vallet, C., Razouls, S., Grioche, A., Hilde, D., Courcot, L., Janquin, M.A., Vacchi, M., Hureau, J.C., 2007. Condition and diet of larval *Pleuragramma antarcticum* (Nototheniidae) from Terre Adélie (Antarctica) during summer. Cybium 31, 67–76.
Kremer, P., Madin, L.P., 1992. Particle retention efficiency of salps. J. Plankton Res. 14, 1009–1015.
Kunzmann, A., 1991. Blood physiology and ecological consequences in Weddell Sea fishes (Antarctica). Ber. Polarforsch. Meeresforsch. 91, 1–79.
Kurihara, H., 2008. Effect of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar. Ecol. Prog. Ser. 373, 275–284.
La Mesa, M., Eastman, J.T., 2012. Antarctic silverfish: life strategies of a key species in the high-Antarctic ecosystem. Fish Fish. 13, 241–266.
La Mesa, M., Vacchi, M., 2001. Age and growth of high Antarctic notothenioid fish—review. Antarct. Sci. 13, 227–235.
La Mesa, M., Eastman, J.T., Vacchi, M., 2004. The role of notothenioid fish in the food web of the Ross Sea shelf waters: a review. Polar Biol. 27, 321–338.
La Mesa, M., Catalano, B., Russo, A., Greco, S., Vacchi, M., Azzali, M., 2010. Influence of environmental conditions on spatial distribution and abundance of early life stages of Antarctic silverfish, *Pleuragramma antarcticum* (Nototheniidae), in the Ross Sea. Antarct. Sci. 22, 243–254.
La Mesa, M., Catalano, B., Greco, S., 2011. Larval feeding of *Chioniodraco hamatus* (Pisces, Channichthyidae) in the Ross Sea and its relation to environmental conditions. Polar Biol. 34, 127–137.
Lannig, G., Storch, D., Pörtner, H.O., 2005. Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. Polar Biol. 28, 575–584.
Larsen, B.K., Pörtner, H.O., Jensen, F.B., 1997. Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. Mar. Biol. 128, 337–346.
Laws, R.M., 1977. Seals and whales of the Southern Ocean. Philos. Trans. R. Soc. B 279, 81–96.
Laws, R.M., 1985. The ecology of the Southern Ocean. Am. Sci. 73, 26–40.
Lea, M.-A., Nichols, P.D., Wilson, G., 2002. Fatty acid composition of lipid-rich myctophids and mackerel icefish (*Champsocephalus gunnari*)—Southern Ocean food web implications. Polar Biol. 25, 843–854.
Lee, J.E., Chown, S.L., 2007. *Mytilus* on the move: transport of an invasive bivalve to the Antarctic. Mar. Ecol. Prog. Ser. 339, 307–310.

Lewis, P.N., Riddle, M.J., Smith, S.D.A., 2005. Assisted passage or passive drift: a comparison of alternative transport mechanisms for non-indigenous coastal species into the Southern Ocean. Antarct. Sci. 17, 183–191.

Lewis, P.N., Bergstrom, D.M., Whinam, J., 2006. Barging in: a temperate marine community travels to the Subantarctic. Biol. Invasions 8, 787–795.

Liebsch, N., Wilson, R.P., Bornemann, H., Adelung, D., Plötz, J., 2007. Mouthing off about fish capture: jaw movement in pinnipeds reveals the real secrets of ingestion. Deep Sea Res. II 54, 256–269.

Lizotte, M.P., 2001. The contributions of sea ice algae to Antarctic marine primary production. Am. Zool. 41, 57–73.

Loeb, V., Siegel, V., Holm-Hansen, O., Hewitt, R., Fraser, W., Trivelpiece, W., Trivelpiece, S., 1997. Effects of sea-ice extent and krill or salp dominance on the Antarctic food web. Nature 387, 897–900.

Lowe, C., Davison, W., 2005. Plasma osmolarity, glucose concentration and erythrocyte responses of two Antarctic nototheniid fishes to acute and chronic thermal change. J. Fish Biol. 67, 752–766.

Madin, L.P., 1974. Field observations on the feeding behaviour of salps (Tunicata: Thaliacea). Mar. Biol. 25, 143–147.

Malzahn, A.M., Aberle, N., Clemmesen, C., Boersma, M., 2007. Nutrient limitation of primary producers affects planktivorous fish condition. Limnol. Oceanogr. 52, 2062–2071.

Mark, F.C., Bock, C., Pörtner, H.O., 2002. Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and 31P-MRS. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R1254–R1262.

Mark, F.C., Hirse, T., Pörtner, H.O., 2005. Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol. 28, 805–814.

Mark, F., Lucassen, M., Pörtner, H., 2006. Thermal sensitivity of uncoupling protein expression in polar and temperate fish. Comp. Biochem. Phys. D 1, 365–374.

Mark, F.C., Lucassen, M., Strobel, A., Barrera-Oro, E., Koschnick, N., Zane, L., Patarnello, P., Pörtner, H.O., Papetti, C., 2012. Mitochondrial function in Antarctic notothenioids with nd6 translocation. PLoS One 7, e31860.

Marshall, H.P., 1988. The overwintering strategy of Antarctic krill under the pack-ice of the Weddell Sea. Polar Biol. 9, 129–135.

Marshall, G.J., Orr, A., van Lipzig, N.P.M., King, J.C., 2006. The impact of a changing Southern Hemisphere Annular Mode on Antarctic Peninsula summer temperatures. J. Clim. 19, 5388–5404.

Matschiner, M., Hanel, R., Salzburger, W., 2011. On the origin and trigger of the notothenioid adaptive radiation. PLoS One 6, e18911.

McFarlane, G.A., King, J.R., Beamish, R.J., 2000. Have there been recent changes in climate? Ask the fish. Prog. Oceanogr. 47, 147–169.

Meerhoff, M., Teixeira-de Mello, F., Kruk, C., Alonso, C., González-Bergonzoni, I., Pacheco, J.P., Lacerot, G., Arim, M., Beklioğlu, M., Brucet, S., Goyenola, G., Iglesias, C., Mazzeo, N., Kosten, S., Jeppesen, E., 2012. Environmental warming in shallow lakes: a review of effects on community structure as evidenced from space-for-time substitution approach. Adv. Ecol. Res. 46, 259–350.

Melian, C.J., Vilas, C., Baldo, F., Gonzalez-Ortegon, E., Drake, P., Williams, R.J., 2011. Eco-evolutionary dynamics of individual-based food webs. Adv. Ecol. Res. 45, 225–268.

Melnikov, I.A., 1998. Winter production of sea ice algae in the western Weddell Sea. J. Mar. Syst. 17, 195–205.

Melzner, F., Göbel, S., Langenbuch, M., Gutowska, M.A., Pörtner, H.O., Lucassen, M., 2009. Swimming performance in Atlantic cod (*Gadus morhua*) following long-term (4-12 months) acclimation to elevated seawater pCO_2. Aquat. Toxicol. 92, 30–37.

Memmot, J., Martinez, N.D., Cohen, J.E., 2000. Predators, parasitoids and pathogens: species richness, trophic generality and body sizes in a natural food web. J. Anim. Ecol. 69, 1–15.

Meredith, M.P., King, J.C., 2005. Rapid climate change in the ocean west of the Antarctic Peninsula during the second half of the 20th century. Geophys. Res. Lett. 32, L19604.

Mianzan, H., Pajaro, M., Alvarez Colombo, G., Madirolas, A., 2001. Feeding on survival-food: gelatinous plankton as a source of food for anchovies. Hydrobiologia 451, 45–53.

Michaelidis, B., Spring, A., Pörtner, H.O., 2007. Effects of long-term acclimation to environmental hypercapnia on extracellular acid-base status and metabolic capacity in Mediterranean fish *Sparus aurata*. Mar. Biol. 150, 1417–1429.

Mihuc, T.B., Minshall, G.W., 1995. Trophic generalists vs. trophic specialists: implications for food web dynamics in post-fire streams. Ecology 76, 2361–2372.

Mincks, S.L., Smith, C.R., Jeffreys, R.M., Sumida, P.Y., 2008. Trophic structure on the West Antarctic Peninsula shelf: detritivory and benthic inertia revealed by $\delta^{13}C$ and $\delta^{15}N$ analysis. Deep Sea Res. II 55, 2502–2514.

Mintenbeck, K., 2008. Trophic interactions within high Antarctic shelf communities—food web structure and the significance of fish. PhD Thesis, University of Bremen, 137pp. http://nbn-resolving.de/urn:nbn:de:gbv:46-diss000109584.

Mintenbeck, K., Alarcón, R., Brodte, E., Vanella, F., Knust, R., 2003. Ecology, biodiversity, biogeography and evolution—demersal fish. In: Arntz, W.E., Brey, T. (Eds.), The Expedition ANTARKTIS XIX/5 (LAMPOS) of RV "Polarstern" in 2002, Ber. Polarforsch. Meeresforsch.462, 55–58.

Mintenbeck, K., Damerau, M., Hirse, T., Knust, R., Koschnick, N., Matschiner, M., Rath, L., 2012. Biodiversity and zoogeography of demersal fish. In: Knust, R., Mintenbeck, K. (Eds.), The Expedition of the Research Vessel "Polarstern" to the Antarctic in 2011 (ANT XXVII/3) (CAMBIO), Ber. Polarforsch. Meeresforsch.644, 43–54.

Moline, M.A., Claustre, H., Frazer, T.K., Schofield, O., Vernet, M., 2004. Alteration of the food web along the Antarctic Peninsula in response to a regional warming trend. Glob. Change Biol. 10, 1973–1980.

Moline, M.A., Karnovsky, N.J., Brown, Z., Divoky, G.J., Frazer, T.K., Jacoby, C.A., Torres, J.T., Fraser, W.R., 2008. High latitude changes in ice dynamics and their impact on polar marine ecosystems. Ann. N.Y. Acad. Sci. 1134, 267–319.

Montgomery, J., Clements, K., 2000. Disaptation and recovery in the evolution of Antarctic fishes. Trends Ecol. Evol. 15, 267–270.

Morales-Nin, B., Garcia, M.A., Lopez, O., 1998. Distribution of larval and juvenile *Notothenios larseni* and *Pleuragramma antarcticum* off the Antarctic Peninsula in relation to oceanographic conditions. Cybium 22, 69–81.

Moran, D., Støttrup, J., 2010. The effect of carbon dioxide on growth of juvenile Atlantic cod *Gadus morhua* l. Aquat. Toxicol. 102, 24–30.

Moreno, C.A., 1980. Observations on food and reproduction in *Trematomus bernacchii* (Pisces: Nototheniidae) from the Palmer Archipelago, Antarctica. Copeia 1, 171–173.

Moreno, C.A., Zamorano, J.H., Duarte, W.E., Jara, H.F., 1982. Abundance of Antarctic juvenile fishes on soft-bottom substrates: the importance of the refuge. Cybium 6, 37–41.

Mueller, I.A., Grim, J.M., Beers, J.M., Crockett, E.L., O'Brien, K.M., 2011. Interrelationship between mitochondrial function and susceptibility to oxidative stress in red- and white-blooded Antarctic notothenioid fishes. J. Exp. Biol. 214, 3732–3741.

Munday, P.L., Dixson, D.L., Donelson, J.M., Jones, G.P., Pratchett, M.S., Devitsina, G.V., Doving, K.B., 2009. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc. Natl. Acad. Sci. U.S.A. 106, 1848–1852.

Murase, H., Matsuoka, K., Ichii, T., Nishiwaki, S., 2002. Relationship between the distribution of euphausiids and baleen whales in the Antarctic (35°E–145°W). Polar Biol. 25, 135–145.

Murphy, R.C., 1962. The oceanic life of the Antarctic. Sci. Am. 207, 186–210.

Murphy, J.M., Mitchel, J.F.B., 1995. Transient response of the Hadley Centre coupled ocean-atmosphere model to increasing carbon dioxide. Part II: spatial and temporal structure of response. J. Clim. 8, 57–80.

Murphy, E.J., Trathan, P.N., Watkins, J.J., Reid, K., Meredith, M.P., Forcada, J., Thorpe, S.E., Johnston, N.M., Rothery, P., 2007. Climatically driven fluctuations in Southern Ocean ecosystems. Proc. R. Soc. B 274, 3057–3067.

Naeem, S., 1998. Species redundancy and ecosystem reliability. Conserv. Biol. 12, 39–45.

Nicol, S., Allison, I., 1997. The frozen skin of the Southern Ocean. Am. Sci. 85, 426–439.

Nilsson, G.E., Crawley, N., Lunde, I.G., Munday, P.L., 2009. Elevated temperature reduces the respiratory scope of coral reef fishes. Glob. Change Biol. 15, 1405–1412.

North, A.W., White, M.G., 1987. Reproductive strategies of Antarctic fish. In: Kullander, S.O., Fernholm, B. (Eds.), Proc. V Congr. Europ. Ichthyol. Stockholm 1985, 381–390.

North, A.W., White, M.G., Burchett, M.S., 1980. Age determination of Antarctic fish. Cybium 8, 7–15.

Nöthig, E.-M., Bodungen, B.v., 1989. Occurrence and vertical flux of faecal pellets of probably protozoan origin in the southeastern Weddell Sea (Antarctica). Mar. Ecol. Prog. Ser. 56, 281–289.

Nöthig, E.-M., Bodungen, B.v., Siu, Q., 1991. Phyto- and protozooplankton biomass during austral summer in surface waters of the Weddell Sea and vicinity. Polar Biol. 11, 293–304.

O'Gorman, E., Emmerson, M., 2010. Manipulating interaction strengths and the consequences for trivariate patterns in a marine food web. Adv. Ecol. Res. 42, 301–419.

O'Brien, K., Sidell, B., 2000. The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. J. Exp. Biol. 203, 1287–1297.

O'Brien, K.M., Skilbeck, C., Sidell, B.D., Egginton, S., 2003. Muscle fine structure may maintain the function of oxidative fibres in haemoglobinless Antarctic fishes. J. Exp. Biol. 206, 411–421.

O'Grady, S.M., DeVries, A.L., 1982. Osmotic and ionic regulation in polar fishes. J. Exp. Mar. Biol. Ecol. 57, 219–228.

Öhman, M.C., Rajasuriya, A., 1998. Relationship between habitat structure and fish communities on coral and sand reefs. Environ. Biol. Fish. 53, 19–31.

Olbers, D., Gouretski, V., Seiss, G., Schroeter, J., 1992. Hydrographic Atlas of the Southern Ocean. Alfred Wegener Institute, Bremerhaven, Germany, hdl:10013/epic.12913.

Øresland, V., 1995. Winter population structure and feeding of the chaetognath *Eukronia hamata* and the copepod *Euchaeta antarctica* in Gerlache Strait, Antarctic Peninsula. Mar. Ecol. Prog. Ser. 119, 77–86.

Orsi, A.H., Whitworth III, T., Nowling Jr., W.D., 1995. On the meridional extent and fronts of the Antarctic Circumpolar Current. Deep Sea Res. I 42, 641–673.

Österblom, H., Casini, M., Olsson, O., Bignert, A., 2006. Fish, seabirds and trophic cascades in the Baltic Sea. Mar. Ecol. Prog. Ser. 323, 233–238.

Österblom, H., Olsson, O., Blenckner, T., Furness, R.W., 2008. Junk-food in marine ecosystems. Oikos 117, 967–977.

Ozaki, H., Obata, H., Naganobu, M., Gamo, T., 2009. Long-term bottom water warming in the north Ross Sea. J. Oceanogr. 65, 235–244.

Pakhomov, E.A., Froneman, P.W., Perissinotto, R., 2002. Salp/krill interactions in the Southern Ocean: spatial segregation and implications for the carbon flux. Deep Sea Res. II 49, 1881–1907.

Pankhurst, N.W., 1990. Growth and reproduction of the Antarctic nototheniid fish *Pagothenia borchgrevinki*. Polar Biol. 10, 387–391.

Papetti, C., Lio, P., Ruber, L., Patarnello, T., Zardoya, R., 2007. Antarctic fish mitochondrial genomes lack nd6 gene. J. Mol. Evol. 65, 519–528.
Pearson, P.N., Foster, G.L., Wade, B.S., 2009. Atmospheric carbon dioxide through the Eocene–Oligocene climate transition. Nature 461, 1110–1113.
Peck, L.S., Brockington, S., Vanhove, S., Beghyn, M., 1999. Community recovery following catastrophic iceberg impacts in a soft-sediment shallow-water site at Signy Island, Antarctica. Mar. Ecol. Prog. Ser. 186, 1–8.
Perissinotto, R., Pakhomov, E.A., 1998a. Contribution of salps to carbon flux of marginal ice zone of the Lazarev Sea, southern ocean. Mar. Biol. 131, 25–32.
Perissinotto, R., Pakhomov, E.A., 1998b. The trophic role of the tunicate *Salpa thompsoni* in the Antarctic marine ecosystem. J. Mar. Syst. 17, 361–374.
Permitin, Y.Y., 1977. Species composition and zoogeographical analysis of the bottom fish fauna of the Scotia Sea. J. Ichthyol. 17, 710–726.
Perry, A.L., Low, P.J., Ellis, J.R., Reynolds, J.D., 2005. Climate change and distribution shifts in marine fishes. Science 308, 1912–1915.
Plötz, J., 1986. Summer diet of Weddell seals (*Leptonychotes weddelli*) in the eastern and southern Weddell Sea, Antarctica. Polar Biol. 6, 97–102.
Plötz, J., Gerdes, D., Gräfe, M., Klages, N., Reijnders, P., Steinmetz, R., Zegers, K., 1987. Weddell seals and emperor penguins in the Drescher Inlet. In: Schnack-Schiel, S. (Ed.), The Winter Expedition of RV "Polarstern" to the Antarctic (ANT V/1-3), Ber. Polarforsch.39, 222–227.
Plötz, J., Ekau, W., Reijnders, P.J.H., 1991. Diet of Weddell seals *Leptonychotes weddellii* at Vestkapp, eastern Weddell Sea (Antarctica), in relation to local food supply. Mar. Mamm. Sci. 7, 136–144.
Plötz, J., Bornemann, H., Knust, R., Schröder, A., Bester, M., 2001. Foraging behaviour of Weddell seals, and its ecological implications. Polar Biol. 24, 901–909.
Plötz, J., Bornemann, H., Liebsch, N., Watanabe, Y., 2005. Foraging ecology of Weddell seals. In: Arntz, W.E., Brey, T. (Eds.), The expedition ANTARKTIS XXI/2 (BENDEX) of RV "Polarstern" in 2003/2004, Ber. Polarforsch.503, 63–67.
Podrabsky, J.E., Somero, G.N., 2006. Inducible heat tolerance in Antarctic notothenioid fishes. Polar Biol. 30, 39–43.
Pörtner, H.O., Farrell, A.P., 2008. Physiology and climate change. Science 322, 690–692.
Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315, 95–97.
Pörtner, H.O., Peck, M.A., 2010. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. J. Fish Biol. 77, 1745–1779.
Pörtner, H.O., van Dijk, P.L.M., Hardewig, I., Sommer, A., 2000. Levels of metabolic cold adaptation: tradeoffs in eurythermal and stenothermal ectotherms. In: Davison, W., Williams, C.W. (Eds.), Antarctic Ecosystems: Models for a Wider Understanding. Caxton Press, Christchurch, pp. 109–122.
Pörtner, H.O., Bock, C., Knust, R., Lannig, G., Lucassen, M., Mark, F.C., Sartoris, F.J., 2008. Cod and climate in a latitudinal cline: physiological analyses of climate effects in marine fishes. Clim. Res. 37, 253–270.
Ptacnik, R., Moorthi, S.D., Hillebrand, H., 2010. Hutchinson reversed, or why there need to be so many species. Adv. Ecol. Res. 43, 1–44.
Purcell, J.E., 1985. Predation on fish eggs and larvae by pelagic cnidarians and ctenophores. Bull. Mar. Sci. 37, 739–755.
Purcell, J.E., 2005. Climate effects on formation of jellyfish and ctenophore blooms: a review. J. Mar. Biol. Assoc. U.K. 85, 461–476.
Purcell, J.E., Decker, M.B., 2005. Effects of climate on relative predation by scyphomedusae and ctenophores on copepods in Chesapeake Bay during 1987-2000. Limnol. Oceanogr. 50, 376–387.

Pusch, C., Hulley, P.A., Kock, K.H., 2004. Community structure and feeding ecology of mesopelagic fishes in the slope waters of King George Island (South Shetland Islands, Antarctica). Deep Sea Res. I 51, 1685–1708.

Quartino, M.L., Boraso de Zaixso, A.L., 2008. Summer macroalgal biomass in Potter Cove, South Shetland Islands, Antarctica: its production and flux to the ecosystem. Polar Biol. 31, 281–294.

Radtke, R.L., Hubold, G., Folsom, S.D., Lenz, P.H., 1993. Otolith structural and chemical analysis: the key to resolving age and growth of the Antarctic silverfish, *Pleuragramma antarcticum*. Antarct. Sci. 5, 51–62.

Ralph, R., Everson, I., 1968. The respiratory metabolism of some Antarctic fish. Comp. Biochem. Physiol. 27, 299–307.

Riebesell, U., Schloss, I., Smetacek, V., 1991. Aggregation of algae released from melting sea ice: implications for seeding and sedimentation. Polar Biol. 11, 239–248.

Rignot, E., Bamber, J.L., van den Broeke, M.R., Davis, C., Li, Y., van de Berg, W.J., van Meijgaard, E., 2008. Recent Antarctic ice mass loss from radar interferometry and regional climate modelling. Nat. Geosci. 1, 106–110.

Robertson, R., Vosbeck, M., Gordon, A.L., Fahrbach, E., 2002. Long-term temperature trends in the deep waters of the Weddell Sea. Deep Sea Res. II 49, 4791–4806.

Robinson, E.E., 2008. Antarctic fish: thermal specialists or adaptable generalists? PhD Thesis, University of Canterbury, Christchurch, 229pp.

Robinson, E., Davison, W., 2008. The Antarctic notothenioid fish *Pagothenia borchgrevinki* is thermally flexible: acclimation changes oxygen consumption. Polar Biol. 31, 317–326.

Robinson, E., Egginton, S., Davison, W., 2011. Warm-induced bradycardia and cold-induced tachycardia: mechanisms of cardiac and ventilatory control in a warm-acclimated Antarctic fish. Polar Biol. 34, 371–379.

Rodhouse, P.G., White, M.G., 1995. Cephalopods occupy the ecological niche of epipelagic fish in the Antarctic Polar Frontal Zone. Biol. Bull. 189, 77–80.

Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K., Bullister, J.L., Wanninkhof, R., Wong, C.S., Wallace, D.W.R., Tilbrook, B., Millero, F.J., Peng, T.H., Kozyr, A., Ono, T., Rios, A.F., 2004. The oceanic sink for anthropogenic CO_2. Science 305, 367–371.

Sahade, R., Tatián, M., Kowalke, J., Kühne, S., Esnal, G.B., 1998. Benthic faunal associations on soft substrates at Potter Cove, King George Island, Antarctica. Polar Biol. 19, 85–91.

Sahade, R., Tarantelli, S., Tatían, M., Mercuri, G., 2008. Benthic community shifts: a possible linkage to climate change? In: Wiencke, C., Ferreyra, G., Abele, D., Marenssi, S. (Eds.), The Antarctic Ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo), Ber. Polarforsch. Meeresforsch. 571, 331–337.

Scharek, R., Nöthig, E.M., 1995. Das einzellige Plankton im Ozean der Arktis und der Antarktis. In: Hempel, I., Hempel, G. (Eds.), Biologie der Polarmeere. Gustav Fischer Verlag, Jena, pp. 116–127.

Scharek, R., Tupas, L.M., Karl, D.M., 1999. Diatom fluxes to the deep sea in the oligotrophic North Pacific gyre at Station ALOHA. Mar. Ecol. Prog. Ser. 182, 55–67.

Schloss, I.R., Ferreyra, G.A., González, O., Atencio, A., Fuentes, V., Tosonotto, G., Mercuri, G., Sahade, R., Tatián, M., Abele, D., 2008. Long-term hydrographic conditions and climate trends in Potter Cove. In: Wiencke, C., Ferreyra, G., Abele, D., Marenssi, S. (Eds.), The Antarctic Ecosystem of Potter Cove, King-George Island (Isla 25 de Mayo), Ber. Polarforsch. Meeresforsch. 571, 382–389.

Schoener, T.W., 1989. Food webs from the small to the large. Ecology 70, 1559–1589.

Schröder, A., Artigues, B., Gonzales, J., Mintenbeck, K., Knust, R., 2001. Biodiversity and biogeography. Demersal fish fauna. In: Arntz, W., Brey, T. (Eds.), The Expedition ANTARKTIS XVII/3 (EASIZ III) of RV "Polarstern" in 2000, Ber. Polarforsch. Meeresforsch.402, 70–74.

Schwarzbach, W., 1988. The demersal fish fauna of the eastern and southern Weddell Sea: geographical distribution, feeding of fishes and their trophic position in the food web. Ber. Polarforsch. 54, 1–93.

Seebacher, F., Davison, W., Lowe, C.J., Franklin, C.E., 2005. A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. Biol. Lett. 1, 151–154.

Sidell, B.D., 1991. Physiological roles of high lipid content in tissues of Antarctic fish species. In: Di Prisco, G., Maresca, B., Tota, B. (Eds.), Biology of Antarctic Fish. Springer Verlag, Berlin, pp. 221–231.

Sidell, B.D., O'Brien, K.M., 2006. When bad things happen to good fish: the loss of hemoglobin and myoglobin expression in Antarctic icefishes. J. Exp. Biol. 209, 1791–1802.

Siegel, V., Skibowski, A., Harm, U., 1992. Community structure of the epipelagic zooplankton community under the sea-ice of the northern Weddell Sea. Polar Biol. 12, 15–24.

Sinensky, M., 1974. Homeoviscous adaptation—a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 71, 522–525.

Sirabella, P., Giuliani, A., Colosimo, A., Dippner, J.W., 2001. Breaking down the climate effects on cod recruitment by principal component analysis and canonical correlation. Mar. Ecol. Prog. Ser. 216, 213–222.

Sloman, K.A., Mandic, M., Todgham, A.E., Fangue, N.A., Subrt, P., Richards, J.G., 2008. The response of the tidepool sculpin, *Oligocottus maculosus*, to hypoxia in laboratory, mesocosm and field environments. Comp. Biochem. Physiol. A 149, 284–292.

Slósarczyk, W., 1986. Attempts at a quantitative estimate by trawl sampling of distribution of postlarval and juvenile notothenioids (Pisces, Perciformes) in relation to environmental conditions in the Antarctic Peninsula region during SIBEX 1983-84. Mem. Natl. Inst. Polar Res. 40, 299–315.

Smale, D.A., Brown, K.M., Barnes, D.K.A., Fraser, K.P.P., Clarke, A., 2008. Ice scour disturbance in Antarctic waters. Science 321, 371.

Smetacek, V., Scharek, R., Nöthig, E.M., 1990. Seasonal and regional variation in the pelagial and its relationship to the life history cycle of krill. In: Kerry, K.R., Hempel, G. (Eds.), Antarctic Ecosystems. Ecological Change and Conservation. Springer Verlag, Berlin, pp. 103–114.

Smith Jr., W.O., Nelson, D.M., 1986. Importance of ice edge phytoplankton production in the Southern Ocean. Bioscience 36, 251–257.

Smith Jr., W.O., Sakshaug, E., 1990. Polar phytoplankton. In: Smith Jr., W.O. (Ed.), Polar Oceanography, Part B: Chemistry, Biology, and Geology. Academic Press, San Diego, pp. 477–525.

Smith, C.R., Mincks, S., DeMaster, D.J., 2006. A synthesis of bentho-pelagic coupling on the Antarctic shelf: food banks, ecosystem inertia and global climate change. Deep Sea Res. II 53, 875–894.

Smith, C.R., Grange, L.J., Honig, D.L., Naudts, L., Huber, B., Guidi, L., Domack, E.A., 2012. A large population of king crabs in Palmer Deep on the west Antarctic Peninsula shelf and potential invasive impacts. Proc. R. Soc. B 279, 1017–1026.

Snyder, W.E., Snyder, G.B., Finke, D.L., Straub, C.S., 2006. Predator biodiversity strengthens herbivore suppression. Ecol. Lett. 9, 789–796.

Somero, G.N., 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. J. Exp. Biol. 213, 912–920.

Somero, G.N., DeVries, A.L., 1967. Temperature tolerance of some Antarctic fishes. Science 156, 257–258.

Somero, G.N., Giese, A.C., Wohlschlag, D.E., 1968. Cold adaptation of the Antarctic fish *Trematomus bernacchii*. Comp. Biochem. Physiol. 26, 223–233.

Stammerjohn, S.E., Martinson, D.G., Smith, R.C., Iannuzzi, R.A., 2008a. Sea ice in the western Antarctic Peninsula region: spatio-temporal variability from ecological and climate change perspectives. Deep Sea Res. II 55, 2041–2058.

Stammerjohn, S.E., Martinson, D.G., Smith, R.C., Yuan, X., Rind, D., 2008b. Trends in Antarctic annual sea ice retreat and advance and their relation to El Niño-southern oscillation and southern annular mode variability. J. Geophys. Res. 113, C03S90.

Stroeve, J., Holland, M.M., Meier, W., Scambos, T., Serreze, M., 2007. Arctic sea ice decline: faster than forecast. Geophys. Res. Lett. 34, L09501.

Suchman, C.L., Daly, E.A., Keister, J.E., Peterson, W., Brodeur, R.D., 2008. Feeding patterns and predation potential of scyphomedusae in a highly productive upwelling region. Mar. Ecol. Prog. Ser. 358, 161–172.

Tada, S., Sato, T., Sakurai, H., Arai, H., Kimpara, I., Kodama, M., 1996. Benthos and fish community associated with clumps of submerged drifting algae in Fildes Bay, King George Island, Antarctica. Proc. NIPR Symp. Polar Biol. 9, 243–251.

Takahashi, M., Nemoto, T., 1984. The food of some Antarctic fish in the western Ross Sea in summer 1979. Polar Biol. 3, 237–239.

Takeuchi, I., Watanabe, K., 2002. Mobile epiphytic invertebrates inhabiting the brown macroalga, *Desmarestia chordalis*, under the coastal fast ice of Lützow-Holm Bay, East Antarctica. Polar Biol. 25, 624–628.

Tatián, M., Sahade, R., Esnal, G.B., 2004. Diet composition in the food of Antarctic ascidians living at low levels of primary production. Antarct. Sci. 16, 123–128.

Tavares, M., De Melo, G.A.S., 2004. Discovery of the first known benthic invasive species in the Southern Ocean: the North Atlantic spider crab *Hyas araneus* found in the Antarctic Peninsula. Antarct. Sci. 16, 129–131.

Teixidó, N., Garrabou, J., Gutt, J., Arntz, W.E., 2004. Recovery in Antarctic benthos after iceberg disturbance: trends in benthic composition, abundance and growth forms. Mar. Ecol. Prog. Ser. 278, 1–16.

Tetens, V., Wells, R.M.G., Devries, A.L., 1984. Antarctic fish blood: respiratory properties and the effects of thermal acclimation. J. Exp. Biol. 109, 265–279.

Thatje, S., Anger, K., Calcagno, J.A., Lovrich, G.A., Pörtner, H.O., Arntz, W.E., 2005. Challenging the cold: crabs reconquer the Antarctic. Ecology 86, 612–625.

Thatje, S., Hall, S., Hauton, C., Held, C., Tyler, P., 2008. Encounter of lithodid crab *Paralomis birsteini* on the continental slope off Antarctica, sampled by ROV. Polar Biol. 31, 1143–1148.

Tierney, N., Hindell, M.A., Goldsworthy, S., 2002. Energy content of mesopelagic fish from Macquarie Island. Antarct. Sci. 14, 225–230.

Tomczak, M., Godfrey, J.S., 1994. Regional Oceanography: An Introduction. Pergamon, Oxford.

Torres, J.J., Donnelly, J., Hopkins, T.L., Lancraft, T.M., Aarset, A.V., Ainley, D.G., 1994. Proximate composition and overwintering strategies of Antarctic micronektonic Crustacea. Mar. Ecol. Prog. Ser. 113, 221–232.

Tranter, D.J., 1982. Interlinking of physical and biological processes in the Antarctic Ocean. Oceanogr. Mar. Biol. Annu. Rev. 20, 11–35.

Turner, J., Colwell, S.R., Marshall, G.J., Lachlan-Cope, T.A., Carleton, A.M., Jones, P.D., Lagun, V., Reid, P.A., Iagovkina, S., 2005. Antarctic climate change during the last 50 years. Int. J. Climatol. 25, 279–294.

Tyler, S., Sidell, B., 1984. Changes in mitochondrial distribution and diffusion distances in muscle of goldfish upon acclimation to warm and cold temperatures. J. Exp. Zool. 232, 1–9.

Urschel, M.R., O'Brien, K.M., 2009. Mitochondrial function in Antarctic notothenioid fishes that differ in the expression of oxygen-binding proteins. Polar Biol. 32, 1323–1330.

Urschel, M.R., O'Brien, K.M., 2008. High mitochondrial densities in the hearts of Antarctic icefishes are maintained by an increase in mitochondrial size rather than mitochondrial biogenesis. J. Exp. Biol. 211, 2638–2646.

Vacchi, M., La Mesa, M., Dalu, M., MacDonald, J., 2004. Early life stages in the life cycle of Antarctic silverfish, *Pleuragramma antarcticum* in Terra Nova Bay, Ross Sea. Antarct. Sci. 16, 299–305.

Vallet, C., Beans, C., Koubbi, P., Courcot, L., Hecq, J.H., Goffart, A., 2011. Food preferences of larvae of Antarctic silverfish *Pleuragramma antarcticum* Boulenger, 1902 from Terre Adélie coastal waters during summer 2004. Polar Sci. 5, 239–251.

Van Franeker, J.A., Bathmann, U.V., Mathot, S., 1997. Carbon fluxes to Antarctic top predators. Deep Sea Res. II 44, 435–455.

Varsamos, S., Nebel, C., Charmantier, G., 2005. Ontogeny of osmoregulation in postembryonic fish: a review. Comp. Biochem. Physiol. A 141, 401–429.

Vaughan, D.G., Marshall, G.J., Connolley, W.M., Parkinson, C., Mulvaney, R., Hodgson, D.A., King, J.C., Pudsey, C.J., Turner, J., 2003. Recent rapid regional climate warming on the Antarctic Peninsula. Clim. Chang. 60, 243–274.

Voss, J., 1988. Zoogeography and community analysis of macrozoobenthos of the Weddell Sea (Antarctica). Ber. Polarforsch. 45, 1–145.

White, M.G., 1991. Age determination in Antarctic fish. In: Di Prisco, G., Maresca, B., Tota, B. (Eds.), Biology of Antarctic Fish. Springer Verlag, Berlin, pp. 87–100.

Whitehead, P.J.P., 1985. FAO species catalogue. Vol. 7. Clupeoid fishes of the world (suborder Clupeioidei). An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf-herrings. Part 1-Chirocentridae, Clupeidae and Pristigasteridae. FAO Fish. Synop. 125, 1–303.

Wienecke, B., Robertson, G., Kirkwood, R., Lawton, K., 2007. Extreme dives by free-ranging emperor penguins. Polar Biol. 30, 133–142.

Wilson, R.P., Quintana, F., 2004. Surface pauses in relation to dive duration in emperial cormorants; how much time for a breather? J. Exp. Biol. 207, 1789–1796.

Windisch, H.S., Kathöver, R., Pörtner, H.O., Frickenhaus, S., Lucassen, M., 2011. Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R1453–R1466.

Wohlschlag, D.E., 1963. An Antarctic fish with unusually low metabolism. Ecology 44, 557–564.

Wöhrmann, A., 1996. Antifreeze glycopeptides and peptides in Antarctic fish species from the Weddell Sea and the Lazarev Sea. Mar. Ecol. Prog. Ser. 130, 47–59.

Wöhrmann, A., 1997. Freezing resistance in Antarctic and Arctic fishes: its relation to mode of life, ecology and evolution. Cybium 21, 423–442.

Woodward, G., Ebenman, B., Emmerson, M., Montoya, J.M., Olesen, J.M., Valido, A., Warren, P.H., 2005. Body-size in ecological networks. Trends Ecol. Evol. 20, 402–409.

Woodward, G., Benstead, J.P., Beveridge, O.S., Blanchard, J., Brey, T., Brown, L., Cross, W.F., Friberg, N., Ings, T.C., Jacob, U., Jennings, S., Ledger, M.E., Milner, A.M., Montoya, J.M., O'Gorman, E.O., Olesen, J.M., Petchey, O.L., Pichler, D.E., Reuman, D.C., Thompson, M.S., Van Veen, F.J.F., Yvon-Durocher, G., 2010a. Ecological networks in a changing climate. Adv. Ecol. Res. 42, 72–138.

Woodward, G., Blanchard, J., Lauridsen, R.B., Edwards, F.K., Jones, J.I., Figueroa, D., Warren, P.H., Petchey, O.L., 2010b. Individual-based food webs: species identity, body size and sampling effects. Adv. Ecol. Res. 43, 211–266.

Yeager, L.A., Layman, C.A., Allgeier, J.E., 2011. Effects of habitat heterogeneity at multiple spatial scales on fish community assembly. Oecologia 167, 157–168.

Yvon-Durocher, G., Montoya, J.M., Trimmer, M., Woodward, G., 2011. Warming alters the size spectrum and shifts the distribution of biomass in freshwater ecosystems. Glob. Change Biol. 17, 1681–1694.

Zhou, M., Dorland, R.D., 2004. Aggregation and vertical migration behaviour of *Euphausia superba*. Deep Sea Res. II 51, 2119–2137.

Zhuang, X., Cheng, C.H., 2010. Nd6 gene "lost" and found: evolution of mitochondrial gene rearrangement in Antarctic notothenioids. Mol. Biol. Evol. 27, 1391–1403.

Zwally, H.J., Comiso, J.C., Parkinson, C.L., Campbell, W.J., Carsaey, F.D., Gloersen, P., 1983. Antarctic sea ice 1973-1976: satellite passive-microwave observations. NASA SP 459, Washington, 206pp.

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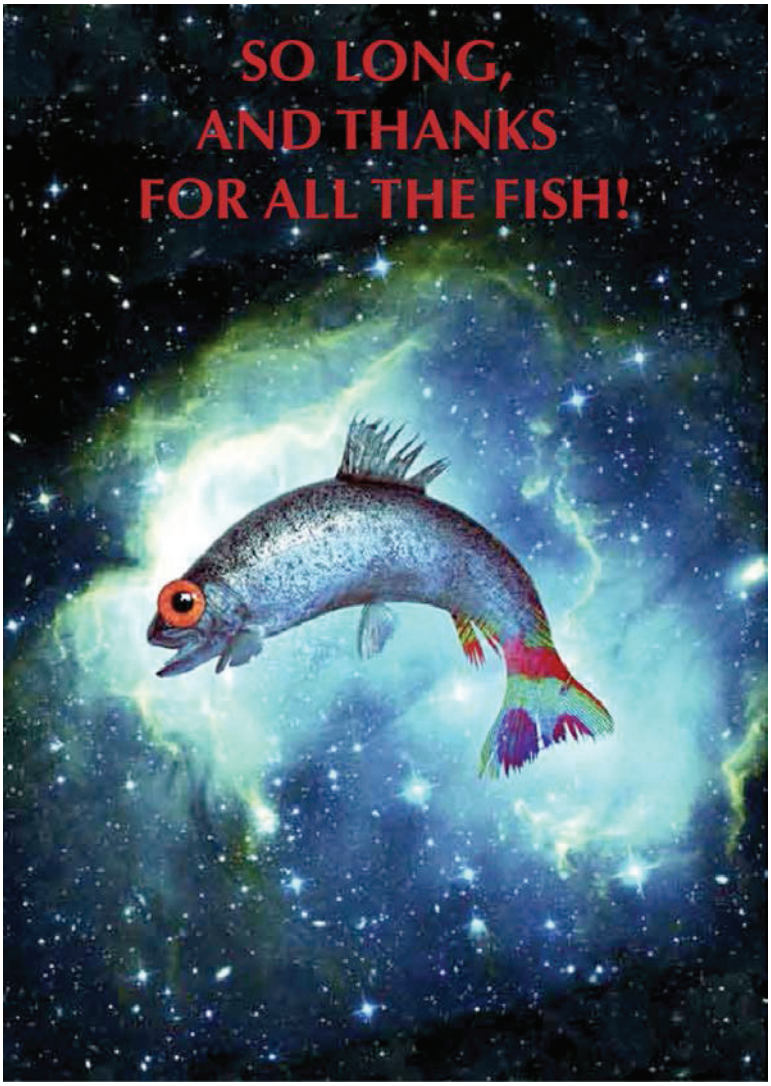

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Erklärung gemäß § 6 (5) der PromO der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche
(vom 14. März 2007)

Hiermit erkläre ich, Anneli Strobel, dass ich die Arbeit mit dem Titel:

‘Mitochondrial plasticity in response to changing abiotic factors in Antarctic fish and cephalopods’

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