

Molecular adaptation and thermal plasticity in a cold-adapted Antarctic fish

**Untersuchungen zur molekularen Anpassungsfähigkeit an
den abiotischen Schlüsselfaktor Temperatur am Beispiel
der antarktischen Aalmutter**

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Abbreviations

3HBDH	3D-hydroxybutyrate dehydrogenase	GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
6-PGL	6-phosphoglucono-lactonase	GCS	glycine cleavage system
ACAC	acetyl-CoA carboxykinase	GHG	greenhouse gas
ACC	Antarctic Circumpolar Current	GYP	glycogen phosphorylase
ADP	adenosine 5'-diphosphate	GYS	glycogen synthase
AFP	antifreeze protein	h	hour(s)
AGFP	antifreeze glycoprotein	HADH	β -hydroxyacyl-CoA-dehydrogenase (also known as HOAD)
ANOVA	analysis of variance	HBDH	3-hydroxybutyrate-dehydrogenase
ATP	adenosine 5'-triphosphate	HIF	hypoxia inducible factor
β Act	beta-actin	HSP	heat shock protein
BLAST	Basic Local Alignment Search Tool	HSI	hepatosomatic index
BSA	bovine serum albumin	KOG/COG	categories of orthologous groups
CCA	canonical correspondence analyses	Ma BP	million years (<i>a</i>) before present
cDNA	complementary DNA	MCA	metabolic cold adaption
COX	cytochrome-c-oxidase	MCAD	medium chain acyl-CoA-dehydrogenase
CoA	coenzyme A	MDH	malate dehydrogenase
COX ₂	cytochrome-c-oxidase, subunit II	meNOGs	metazoan model sequences
COX ₄	cytochrome-c-oxidase, subunit IV	min	minute(s)
CPT	carnitine-palmitoyltransferase	mRNA	messenger RNA
CS	citrate synthase	MMR	maximum active metabolic rate
CSP	cold shock protein	MS-222	3-aminobenzoic acid ethyl ester
C _t	threshold cycle	MtPC	mitochondrial protein content
CTD	measurement of conductivity, temperature and depth with	MUFA	monounsaturated fatty acid
CytC	cytochrome-c	NAD	oxidized nicotinamide adenine dinucleotide
DMSO	dimethylsulfoxide	NADH	reduced nicotinamide adenine dinucleotide
dNTPs	deoxyribonucleoside triphosphates	NCBI	National Center for Biotechnology Information
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)	NRF	nuclear respiratory factor
DTT	dithiothreitol	NTC	no-target control (containing ultrapure water)
EA	enzyme activity	OCLTT	oxygen and capacity limited thermal tolerance
EDTA	ethylenediaminetetraacetic acid		
EST	expressed sequence tag		
fiNOGs	fish model sequences		
FW	fresh weight		

ABBREVIATIONS

OGT	optimal growth temperature	TAG	triacylglyceride
PC	protein content	TAGL	triacylglycerin lipase
PCA	perchloric acid	TBE	tris-borate-EDTA buffer
PCR	polymerase chain reaction	TCA	tricarboxylic acid cycle
PEPCK	phosphoenolpyruvate carboxykinase	THP	thermal hysteresis proteins
PGC	peroxisome proliferator activated receptor gamma cofactor	TRIS	2-amino-2-hydroxymethyl- propane-1,3-diol
PK	pyruvate kinase	tRNA	transfer RNA
pH _i	intracellular pH	UBI	ubiquitin
PPAR	peroxisome proliferator activated receptor	UCP	uncoupling protein
PPRE	peroxisome proliferator response element		
PRC	PGC1-related co-activators		
ppm	parts per million		
PSU	practical salinity units		
PUFA	polyunsaturated fatty acid		
Q ₁₀	temperature coefficient		
qRT-PCR	quantitative real-time polymerase chain reaction		
RACE	rapid amplification of cDNA ends		
RNA	ribonucleic acid		
ROS	reactive oxygen species		
rRNA	ribosomal RNA		
RT	negative control in PCR (without reverse transcription, containing RNA)		
RV	research vessel		
s	second(s)		
SCAD	short chain acyl-CoA- dehydrogenase		
SE	standard error		
SD	standard deviation		
SMR	standard metabolic rate		
SRA	sequence read archive		
SSH	suppression subtractive hybridization		
T	temperature in °C		
T _c	critical temperature		
T _p	pejus temperature		

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Summary

Evolutionary adaptation and the connected acclimation capacity of species to changing environmental conditions represents one of the key factors in the composition and dynamics of any ecosystem. In marine environments temperature is one of the major factors defining the aquatic fauna. In respect to progressing climate change the question arises how individual species react to higher temperatures and how this affects the entire ecosystem. Species that are highly specialized on stable environmental conditions seem to be especially vulnerable.

This thesis characterizes the Antarctic eelpout *Pachycara brachycephalum* (Pappenheim, 1912) in regards to its adaptation to low habitat temperatures as well as its capacity to acclimate to higher temperatures at the molecular level.

A cDNA library was established and subjected to high-throughput sequencing to provide a basis for sequence analyses. To characterize differences between a cold-adapted and a eurythermal species, an already existing cDNA library of the closely related congener *Zoarces viviparus* (Linnaeus, 1758) was included in large-scale comparisons.

The repertoire of functional genes reflected cold-adaptation features of cellular metabolism in *P. brachycephalum*, e.g. through a higher ratio of ubiquitin-related genes. These genes are of great importance in cold-adaptation and counter the cold denaturation of proteins.

Furthermore, the amino acid sequences of orthologous proteins displayed differences between the eury- and the stenotherm. The observed position-specific interchanges in *P. brachycephalum* highly conform with the flexibility hypothesis. According to this hypothesis a protein may be destabilized in its three-dimensional structure by minimal changes within the amino acid sequence. This destabilization sustains reaction kinetics in the cold. In addition, differences were noted in the encoding of amino acids at DNA level. Within homologous proteins of *P. brachycephalum* amino acids are encoded with a preference for AT-richer triplets on the third codon position. This trend promotes less stable transitional states at this level, too, as the base pairing of AT is less stable than that of GC. This may facilitate transcription and translation in the cold and thus constitute an adaptation to the habitat conditions of the Antarctic eelpout.

For studying the plasticity of this species in more detail, various acclimation experiments were conducted. At first, the chronology of warm acclimation was established by means of enzyme measurements as well as through expression analyses of various candidate genes. Through this the mechanisms that account for metabolic changes in the warmth could be identified at the protein as well as the transcriptomic level. The observed shift from lipid-based to carbohydrate-based metabolism provided evidence that the Antarctic eelpout

prepares for hypoxemic conditions. The shift to carbohydrate-fuels is advantageous under anaerobic conditions, which may be elicited by warmer temperatures, due to elevated oxygen demand insufficiently met by the limited capacity of the circulation/respiration system. Various transcriptomic factors of the PPAR family were identified as important mediators of the metabolic shift. These nuclear-located receptors „measure“ the energy status within a cell and regulate the expression of various genes involved in lipid- and carbohydrate metabolism. The expression profiles of all 26 genes examined, provided an acclimation time course that can be divided into 3 phases: acute, mid-term and long-term.

Another experiment was dedicated to the compilation of expression profiles of long-term acclimated specimens held at 6 different temperatures ranging from -1 to 9°C over a time period of 2 months. A total of 664 temperature-sensitive transcript sequences could be identified by using a custom microarray design based on the sequence information of the cDNA library. The growth optimum of the Antarctic eelpout was characterized by the smallest transcriptomic changes, i.e. the least regulatory effort.

The differently expressed functions revealed specific patterns for the temperature ranges „cold“, „intermediate“ and „warm“, with specific features in energy metabolism. Again, the aforementioned metabolic shift could be observed, with additional indicators for a potential use of amino acids as an energy reserve in the cold. Further differences between acclimation temperatures in processes like transcription, translation, protein degradation or the organization of the cytoskeleton are discussed with respect to their implications for metabolic energy consumption. Furthermore, regulatory mechanisms increasing the proliferation of blood vessels that obviously modify the cardio-vascular system to counter an oxygen limitation have been identified in the warmth. Beyond an acclimation temperature of 6°C an extreme energy deficit is indicated by a severe weight loss of the specimen. This was accompanied by a classic cellular stress response, signifying an essential threshold temperature of the thermal window of *P. brachycephalum*.

Overall, by combining various experimental approaches covering the entire thermal tolerance window and beyond, the correlation between the molecular and the whole animal level was established. The Antarctic eelpout displays capacities to acclimate to temperatures above the range of habitat temperatures. This thesis highlights the complexity of molecular adaptation at DNA, RNA and protein levels as well as various functional shifts in metabolism enabling life at various acclimation temperatures. The present findings help to substantiate the framework of the oxygen and capacity limited thermal tolerance at the molecular level.

Zusammenfassung

Die Anpassungsfähigkeit von Arten an sich verändernde Umweltbedingungen stellt einen entscheidenden Aspekt in der Zusammensetzung und Dynamik von Ökosystemen dar. In marinen Lebensräumen wirkt sich der Faktor Temperatur besonders stark auf die aquatische Fauna aus. Gerade im Hinblick auf den fortschreitenden Klimawandel stellt sich die Frage, wie einzelne Spezies auf eine Temperaturerhöhung reagieren und welche Konsequenzen sich daraus für das Ökosystem ergeben. Hochangepasste Organismen in Gebieten mit sehr konstanten Bedingungen sind dabei besonders gefährdet.

Im Rahmen dieser Doktorarbeit wurde die Antarktische Aalmutter *Pachycara brachycephalum* (Pappenheim, 1912) in ihrer Angepaßtheit an niedrige Lebensraumtemperaturen und Anpassungsfähigkeit an erhöhte Temperaturen auf molekularer Ebene charakterisiert.

Als Grundlage für verschiedene Sequenzanalysen wurde zunächst eine cDNA-Bank erstellt und anschließend im Hochdurchsatzverfahren sequenziert. Um Unterschiede zwischen einer kalt-adaptierten und einer eurythermen Art zu charakterisieren, wurde eine weitere, bereits bestehende cDNA-Bank der nah verwandten Art *Zoarces viviparus* (Linneaus, 1758) für einen groß-skaligen Vergleich herangezogen.

Verschiebungen im funktionellen Repertoire von Genen gaben dabei einen Hinweis auf einen kalt-angepassten Zellstoffwechsel in *P. brachycephalum* wie z. B. durch einen höheren Anteil von Ubiquitin-Genen, die im kalt-adaptierten Stoffwechsel von großer Bedeutung sind, um der Kältedenaturierung von Proteinen begegnen zu können. Des weiteren wurden Unterschiede in der Aminosäuresequenz von orthologen Proteinen festgestellt. Die beobachteten positionsspezifischen Austausche in *P. brachycephalum* sind in hohem Maße konform mit der Flexibilitätshypothese, laut welcher durch minimale Veränderungen in der Aminosäuresequenz ein Protein in seiner dreidimensionalen Struktur destabilisiert werden kann, um eine effiziente Reaktionsgeschwindigkeit in der Kälte zu gewährleisten. Darüber hinaus wurden Unterschiede in der Kodierung von Aminosäuren auf DNA Ebene festgestellt: Innerhalb von homologen Proteinen sind gleiche Aminosäuren mit einer Präferenz für AT-reichere Triplets auf der dritten Kodon-Position in *P. brachycephalum* kodiert. Da die Basenpaarung zwischen AT weniger stabil ist als die zwischen GC, begünstigt der beobachtete Trend auf dieser Ebene weniger stabile Übergangszustände. Damit werden die Transkription und Translation in der Kälte erleichtert und spiegeln die Anpassung an den kalten Lebensraum der Antarktischen Aalmutter.

Um die Anpassungsfähigkeit der Spezies genauer zu beleuchten, wurden verschiedene Akklimationsexperimente durchgeführt. Zuerst wurde der zeitliche Verlauf einer „Wärmeakklimation“ mit Hilfe von Enzymmessungen sowie anhand von Expressionsprofilen verschiedener Kandidatengene charakterisiert. Auf Protein- als auch auf transkriptomischer Ebene konnten dabei die Mechanismen nachvollzogen werden, die zu einem veränderten Stoffwechsel in der Wärme führen. Eine Verschiebung von einem fett-basierten zu einem kohlehydrat-basierten Speicherstoffwechsel gab einen Hinweis darauf, dass sich die Antarktische Aalmutter auf hypoxemische Bedingungen einstellt. Eine derartige Umstellung ist von Vorteil, da kohlenhydrat-basierte Speicherstoffe auch unter anaeroben Bedingungen nutzbar sind, welche in der Wärme, bedingt durch den höheren metabolischen Bedarf bei gleichzeitiger Kapazitätslimitierung des Zirkulations-/Respirationssystems, gehäuft vorkommen können. Darüber hinaus wurden verschiedene Transkriptionsfaktoren der PPAR-Familie als wichtige Mediatoren für eine Umstellung des Stoffwechsels identifiziert. Diese kern-lokalisierten Rezeptoren „messen“ den Energiestatus innerhalb der Zelle und regulieren die Expression verschiedener Gene, welche in den Fett- und Kohlehydrat-Stoffwechsel involviert sind. Durch das Expressionsprofil von allen 26 untersuchten Genen wurde ein mehrphasiger Akklimationsverlauf sichtbar, der sich in Akut-, Übergangs- und Langzeitphase einteilen läßt.

In einem weiteren Experiment wurden Expressionsprofile von „langzeit-akklimierten“ Tieren erstellt, die bei sechs verschiedenen Temperaturen im Bereich von -1°C bis 9°C für 2 Monate gehältert wurden. Hierfür wurde basierend auf den Sequenzinformationen der cDNA-Bank ein Mikroarray entworfen und eingesetzt. Insgesamt konnten 664 Sequenzen identifiziert werden, die temperaturabhängig reguliert wurden. Unabhängig von den kodierten Funktionen spiegelte die geringste transkriptomische Veränderung, sprich der geringste regulative Aufwand, das Wachstumsoptimum der Antarktischen Aalmutter wider. Die unterschiedlich modulierten Funktionen ergaben dabei ein spezifisches Muster für die applizierten Temperaturbereiche „kalt“, „intermediär“ und „warm“, deren Umsetzung unterschiedliche Kosten im Energiebudget verursachten. Die zuvor beschriebene metabolische Stoffwechsel-Verschiebung konnte erneut gezeigt werden, sowie auch eine mögliche Beteiligung von Aminosäuren als Energiereserve in der Kälte. Weitere Unterschiede in Prozessen wie Transkription, Translation, Proteinabbau oder dem Aufbau des Zytoskeletts wurden im Hinblick auf die metabolischen Kosten diskutiert. Darüber hinaus wurden Regulationsmechanismen für eine verstärkte Bildung von Blutgefäßen in der Wärme identifiziert, welche geeignet erscheinen, das Herz-Kreislauf-System zu modifizieren, um

einer Sauerstofflimitierung zu entgehen. Eine Temperatur jenseits von 6°C war dabei so kostenintensiv, dass eine Gewichtsabnahme beobachtet werden konnte, die mit einer permanenten klassischen zellulären Streß-Antwort auf transkriptomischer Ebene einhergeht. Diese Daten markieren somit einen kritischen Schwellenwert des Temperaturfensters von *P. brachycephalum*. Die Anpassungsfähigkeit der Antarktischen Aalmutter an höhere Lebensraumtemperaturen erscheint für einen begrenzten Temperaturbereich jenseits der realisierten Habitatterperatur möglich.

Diese Doktorarbeit beleuchtet die Komplexität der molekularen Anpassung auf DNA- und Proteinebene sowie funktionelle Besonderheiten im Zellstoffwechsel, die ein Leben in einem konstant kalten Habitat ermöglichen. An Hand der verschiedenen durchgeführten Laborexperimente konnten die Zusammenhänge zwischen der molekularen sowie der Ganz-Tier-Ebene über das gesamte Temperaturtoleranz-Fenster und darüber hinaus hergestellt werden. Mit diesen Befunden kann das Modell der sauerstoff- und kapazitätslimitierten Temperaturtoleranz auf der molekularen Ebene untermauert werden.

1 Introduction

The impact of key abiotic factors on species distribution and ecosystem structure has been increasingly studied during the last few decades in order to understand their roles as drivers for seasonal species-adaptability and evolutionary force. The progressive climate change augments concerns about a proper adaptability of vulnerable organisms to already altered and proposed future conditions.

A mechanistic understanding of correlations between environmental factors and physiological performances is vital to this examination. This thesis aims to characterize the thermal tolerance and potential acclimation strategies of an Antarctic fish species by illuminating the thermal plasticity and adaptability at the molecular level.

1.1 Temperature and thermal tolerance

Temperature plays an important role for all marine water breathers since it is permeant and influences physiological performance, reaction dynamics and the structural stability of biomolecules (Hochachka and Somero, 2002; Somero, 1997). Thermal gradients account for the biogeographic distribution of species along latitudinal clines, or across the costal and tidal zones (Somero, 2005). Thus, species-specific thermal tolerances are limiting and correlate with natural habitat temperature variations (Pörtner, 2001; Pörtner, 2012). Many temperature tolerance models were developed and refined over several decades (Elliot, 1981; Fry, 1971; Fry and Hart, 1948; Pörtner, 2001). The concept of oxygen- and capacity limited thermal tolerance (OCLTT, Fig. 1) implies that thermal limitation occurs through insufficient oxygen supply capacities at both sides of the thermal window (Pörtner, 2001; Pörtner, 2012). This restriction, which encompasses an aerobic energy conversion, shapes the aerobic scope of a species. The resulting thermal tolerance windows for poikilotherm species can differ in range and reflect the species' ability to maintain physiological functioning under altered conditions over short to long time scales (Pörtner and Farrell, 2008).

In fish, the capacity of the cardio-vascular system plays a critical role in limiting performance within the aerobic scope and thus, constitutes the „weakest link“ for the aerobic capacity (Lannig et al., 2004; Mark et al., 2002; Pörtner and Knust, 2007; Sartoris et al., 2003b). Subsequently, thermal tolerance is limited on the highest hierarchical level of systemic complexity, the intact organism (Pörtner, 2001).

When temperatures above the maximum aerobic scope are experienced, an increasing discontinuity between oxygen demand and supply is set by the cardiovascular system. This

thermal threshold is described as *pejus* temperature (lat. for “worse”, T_p) and marks a consecutive loss of aerobic performance. At critical temperatures (T_c) only a short period of time can be survived by relying on an anaerobic metabolism that becomes apparent in body fluids and/or tissues. This model was supported by findings in invertebrates like polychaetes, cephalopods and crustaceans (Frederich and Pörtner, 2000; Pörtner and Zielinski, 1998; Sommer et al., 1997) as well as in vertebrates such as teleost fish (Pörtner and Knust, 2007; van Dijk et al., 1999).

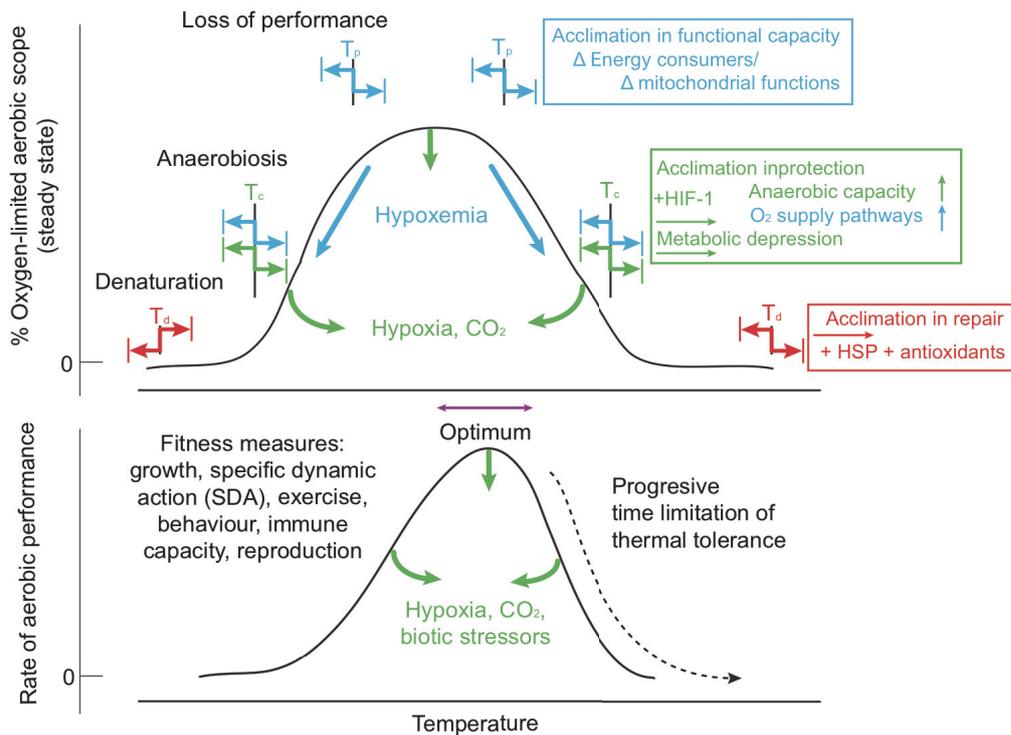


Figure 1: Concept of Oxygen and Capacity Limited Thermal Tolerance, after Pörtner, 2010.

The upper panel depicts the different temperature thresholds in the aerobic scope, dependent on oxygen supply. The maximal aerobic scope is characterized by an optimum energy conversion under ideal oxygen supply in tissues, resulting in a peak performance for various fitness parameters in the lower panel. This range is limited by *pejus* temperatures (T_p), which cause a discontinuity between oxygen supply and demand (hypoxemia) until critical temperatures (T_c) are reached. Hence, the organism relies on anaerobic energy production until the energy deficit leads to death after short time scales. Molecular signalling by the hypoxia inducible factor (HIF-1) becomes effective to mediate the transcription of genes that support hypoxic survival (Heise et al., 2007). Denaturation of single molecules (T_d) could largely exceed the thermal tolerance of the organism, which emphasizes the constraint of a limitation on a level of high complexity. During thermal stress heat shock proteins (HSPs) facilitate the molecular integrity of proteins by their chaperone function, refolding partial denatured proteins (Kregel, 2002) whereas antioxidants maintain the cellular redox balance (Heise et al., 2006). The threshold temperatures and corresponding aerobic scope can shift dynamically and within limits - as indicated by the arrows - due to seasonal acclimation. Further environmental disturbances, like hypoxia, CO_2 and biotic stressors are believed to cause a shrinking of the thermal tolerance window.

Further thresholds are based on the physico-chemical stability of proteins at certain temperatures. These set points specify temperatures of protein denaturation and structural disintegration (T_d) and are typically outside the scope of the organism. Nevertheless, in vertebrates and invertebrates the initiation of cellular repair mechanisms and detoxification

processes become increasingly activated beyond *pejus* temperatures (Heise et al., 2007; Heise et al., 2006; Tomanek, 2008). This ensures a time-limited survival under adverse conditions exceeding the aerobic scope. Furthermore, these features are essential for invertebrates inhabiting the intertidal zones (Abele et al., 1998; Hofmann and Somero, 1995; Tomanek and Sanford, 2003).

Aside from mechanisms sustaining functions on short time scales (e.g. diurnal) seasonal temperature fluctuations can cause a limited shift of the respective threshold temperatures in organisms (Brett, 1956; Pörtner and Farrell, 2008; Pörtner and Knust, 2007). Consequently, the aerobic scope is adjusted to a suitable energy production and conversion during seasonal variation. Furthermore, the composition of whole food webs relies on the thermal tolerance of phyto- and zooplankton, which also changes during seasons (Drinkwater et al., 2010; Winder and Schindler, 2004). This interrelation of species influences the ecosystem structure since all species are subjected to specific thermal tolerances.

Contrary to the ability of species to acclimate to a wide range of temperatures, i.e. eurythermy, different thermal tolerances can result from the specialisation on an extreme ecological niche. In stable environmental conditions, organisms undergo adaptation on evolutionary time scales, which can cause an uni-directional shift of the thermal tolerance window. The resulting stenothermy is characterised by much narrower thermal tolerance windows and thus higher temperature sensitivity.

1.2 Fishes of the Antarctic shelf

The Antarctic realm represents one of the most stable and cold environments on earth. The geographical isolation of Antarctica was completed ~31 Ma BP at the beginning of the Oligocene (Lawver et al., 1992). The appearance of the first sea ice led to a steady cooling trend (Lear et al., 2000), which was followed by the onset of the Antarctic Circumpolar Current (ACC) (for review see (Barker et al., 2007)). Annual temperature fluctuations in the most southerly waters near McMurdo Sound are found to vary by only 1.5°C annually (Hunt et al., 2003). The development of these extremely cold and well-oxygenated water conditions gave rise to many new ecological niches. These were occupied by some ancient species as well as by species which adapted to this environment after migration from lower latitudes (Clarke and Johnston, 1996).

Along the isolated Antarctic shelf a number of endemic fish species evolved - this characteristic Antarctic fish fauna was earlier referred to as “the occurrence of species flocks“ (Eastman and McCune, 2000). An unperturbed ecological diversification and niche-occupation resulted from the isolated conditions through the ACC accompanied by an absence

of further competition by other fish species (Eastman, 2000). The most successful and abundant fishes are the perciform groups of Notothenioidei and Zoarcidae, which together with the scorpaeniform family Liparidae, account for 87% of the fish species in this realm (Eastman and McCune, 2000).

1.3 Adaptation in cold stenothermal fish: from functional to molecular level

1.3.1 Systemic adaptations to cold environments

Various traits for functional cold adaptation were found on multiple levels of organization in stenothermic organisms. Many of the above-stated perciform fish share a benthic or demersal lifestyle and have low standard metabolic rates (SMRs) (Sidell, 2000). This counters the constraint of a rate reduction of biological and chemical reactions by a factor of 2-3 within $\sim 10^{\circ}\text{K}$ difference for a certain degree (Krogh, 1914).

However, early comparative studies of Arctic and tropical fish postulated higher SMRs in the cold when extrapolated to a standard-SMR curve (Scholander et al., 1953; Wohlschlag, 1960) which led to the hypothesis of metabolic cold adaptation (MCA). Through further experimentation and methodological refinement this hypothesis was seriously challenged (Brett, 1972; Holeyton, 1974). Nevertheless, the controversy encouraged a more detailed investigation of tissue structure in comparative studies and produced some evidence for MCA. Increased mitochondrial densities (number and size) in cold-adapted species contrasted those found in temperate ones (Clarke, 1983; Clarke and Johnston, 1999; Dunn, 1988; Guderley and St Pierre, 1996; O'Brien, 2011; O'Brien and Mueller, 2010). An increase of mitochondrial numbers and surface density is beneficial in cold environments, as it increases the concentration of aerobic metabolic enzymes relative to tissue mass (Johnston et al., 1998). This in turn, compensates for the Q_{10} effect and reduces diffusion distances for oxygen and metabolic intermediates (Sidell, 1991). Furthermore, metabolic enzyme activities in Antarctic fish, e.g., LDH and CS in brain tissue, are seen to be temperature-compensated in comparison to temperate fish (Kawall et al., 2002). However, a twofold lower total activity level indicates that a full compensation is not accomplished in the cold. From this point of view, Antarctic fish display lower capacities per mitochondrion, although a strict coupling of enzymes in the respiratory chain and low proton leakage rates (Hardewig et al., 1999a) support an efficient energy turnover at low SMR. However, a low SMR increases thermal sensitivity and highly accounts for the stenothermal lifestyle in a narrow (thermal) ecological niche (Pörtner, 2010). A major problem confronting the cardiovascular system at low temperatures is the increased

viscosity of body fluids. Due to the high oxygenation of the cold water, most Antarctic fish can afford to have lower haematocrit values in comparison to eurythermal which increases the fluidity of their blood (Egginton, 1996). One family of the Notothenoidei, the icefishes (Channichthyidae), have even lost the ability to express haemoglobin (Cocca et al., 1997; di Prisco et al., 2002) and in some cases even myoglobin (Sidell and O'Brien, 2006; Sidell et al., 1997). Instead, these fish also display modifications of their cardiovascular system, which compensates for the lack of an oxygen carrier molecule. They have considerably larger hearts (Sidell and O'Brien, 2006) with a cardiac output up to 5-fold higher than eurytherms (Hemmingsen et al., 1972). Moreover, increased capillary diameters and an augmented total blood volume (Fitch et al., 1984) distinguishes icefishes from red-blooded fish of similar size. Further cold adaptations are shared over a much broader bandwidth of species. One of the best characterized examples is the cellular protection by antifreeze proteins (AFP) and antifreeze glycoproteins (AGFPs) which was first discovered by DeVries in Nothothenoid fish (DeVries, 1971). Four different AFP types and one AGFP type have been identified among marine fishes either inhabiting high latitudes or facing temperatures close to 0°C seasonally (Fletcher et al., 2001; Harding et al., 2003; Hobbs et al., 2011). Along with several other forms discovered in bacteria, fungi, plants and terrestrial arthropods (Cheng, 1998; Duman, 2001; Duman and Olsen, 1993), these polypeptides and proteins were classified as thermal hysteresis proteins (THPs) due to their colligative effect on ice crystal growth. THPs therefore facilitate the survival of hypo-osmotic fish at temperatures below the freezing point of their extracellular fluids.

1.3.2 Lipids

The fluidity of cell membranes is largely affected by temperature as it alters the phase and static order of lipid bilayers. In the cold, gel-phase transition leads to an aggregation of rafts with similar acyl-chains due to the heterogeneity of utilized lipids in membranes. These temperature-induced phase separations alter the microenvironment for integrated protein complexes, whose functional properties may be severely impaired by protein delocalization and disassembly of 3D structures (Hazel, 1995).

However, organisms are able to counter this effect by altering the membrane composition. This ability is known as homeoviscous adaptation and was firstly recognized in *E. coli* cultured at different temperatures (Sinensky, 1974). Several cold adapted species are able to enhance membrane fluidity and maintain function by employing different head-groups in phospholipids to create a decreasing ratio of phosphatidyl choline to phosphatidyl ethanolamine, PC:PE (Hazel, 1995; Hazel and Carpenter, 1985; Williams, 1998). An

interspecies comparison of synaptosomal phospholipids showed that polyunsaturated fatty acids (PUFAs) were increased in both PC and PE phospholipids under cold conditions (Logue et al., 2000). Despite that monounsaturated fatty acids (MUFAs) showed little variation among species adapted to different temperatures. A further intrinsic modification involves the incorporation of cholesterol into membranes in order to increase the membrane stability in the warmth. Low cholesterol fractions discovered in ectotherms contrast higher portions found in homeotherms, thus giving further evidence to support cholesterol's stabilising function at higher temperatures (Robertson and Hazel, 1997). Together, these modifications provide an intact physical barrier to the extracellular environment as well as between reaction compartments of intra- and extracellular fluids. Therefore, homeoviscous adaptation generates an optimal microenvironment for membrane-bound proteins with functions for signalling, transportation (channels and pumps) as well as for membrane-coupled enzyme activity. As mitochondrial ATP production relies on transmembrane enzymatic functions and proton motive force, temperature dependent regulations can have huge implications on energy metabolism.

In general, cold adaptation as well as acclimation of eurythermal fish can be characterized by a higher utilization of lipids as stores and an augmentation of mitochondrial numbers and densities. Higher lipid fractions were found in liver and/or muscle tissues of several cold adapted fish species (Brodte et al., 2006a; Desaulniers et al., 1996; Lannig et al., 2003; Lucassen et al., 2006; Lund and Sidell, 1992; Sidell et al., 1995; Tocher, 2003).

1.3.3 *Proteins*

Membrane bound as well as soluble enzymes generally display temperature-dependent reaction dynamics and temperature-dependent adaptations in structural stability. Kinetic adaptations correlating to the species-specific habitat temperature have been found in homologous enzymes. Cold-adapted species typically have enzymes with higher k_{cat} -values (substrate turnover numbers) than homologous enzymes from warm-adapted species (Fields and Somero, 1998; Johnston and Walesby, 1977; Low et al., 1973). Comparisons of K_m -values (inverse measure for substrate affinity) of homologous enzymes among species from distinct habitats display similar values at natural body temperature. However, due to the specific thermal sensitivity of enzymes K_m -values increase with rising assay temperatures impairing protein stability and catalytic function. Subsequently, in inter-species comparisons of activities in enzyme homologues at any given temperature the lowest K_m is seen in the warm-adapted species whereas the highest is dedicated to a cold-adapted species (Fields and Somero, 1997; Hochachka and Somero, 2002; Holland et al., 1997). The correlation of high

turnover rates (K_{kat}) in cold-adapted species and a rapid loss of functional integrity (K_{m}) of enzymes are seen as a trade-off in thermal adaptation. The basis for this interrelation is the conformational flexibility of proteins, which is defined by the primary structure of proteins. In general, only very minor changes in amino acid sequences are required to adapt the kinetic and structural properties of proteins to different temperatures (Somero, 2009) and alter the global stability of proteins, e.g., the different stability of eye lens proteins of vertebrates from polar to desert environments (McFall-Ngai and Horwitz, 1990). However, catalytic centres of enzymes are highly conserved, and it is unlikely that amino acids involved in the reaction mechanism are altered by adaptations concerning flexibility at maintained substrate specificity (Deng et al., 1994).

Intrinsic alterations modifying the thermal stability were intensively studied by comparisons of orthologous proteins leading to the hypothesis of conformational flexibility by amino acid exchanges due to the habitat temperature (Závodszky et al., 1998). Further studies confirmed these findings by comparing enzyme homologues from extremophilic and mesophilic Archaea (Haney et al., 1999), respectively psychrophilic pro- and eukaryotes with meso- and thermophilic ones (Feller and Gerday, 1997; Feller and Gerday, 2003). These approaches were extended to a larger scale when considering multiple proteomes of thermally distinct species (Tekaia and Yeramian, 2006; Tekaia et al., 2002). The discovered global pattern of amino acid exchanges in homologous enzymes support the theory of protein destabilization in the cold in order to facilitate substrate binding, turnover and product release. The following trends were observed in cold adaptation: *a*) a decrease of charged residues (e.g., less R, L, E, D) to reduce ionic interactions and salt bridge formation; *b*) decreases of bulky hydrophobic side chains (e.g., A \Rightarrow G, I \Rightarrow L, L \Rightarrow M) for a looser molecular packing of the protein; *c*) a gain of uncharged polar residues (e.g., more S, T, N, D) to increase solvent interaction and *d*) an augmentation of the entropy (e.g., A \Rightarrow G, less P) to favour flexibility and decreasing hydrophobic effects (Feller and Gerday, 2003; Haney et al., 1999; Hochachka and Somero, 2002; Tekaia and Yeramian, 2006; Tekaia et al., 2002).

The best characterized examples for molecular cold adaptation on the protein level are lactate dehydrogenase and cytosolic malate dehydrogenase (for review see (Somero, 2004; Somero, 2009). These investigations included K_{m} , K_{kat} and amino acid sequence analyses among several ectotherm fish (stenotherm and eurytherm) as well as homeotherm species. A detailed knowledge of the reaction mechanism and the 3D structure permitted a correlation of the enzyme efficiency in relation to observed temperature dependent amino-acid exchanges.

A species' thermal tolerance at protein level is further influenced by temperature effects on

protein charge. The dissociation of histidyl residues (imidazole ring) depends highly on temperature. The pK of histidyl residues is close to the physiological pH, thus imidazole protonation and subsequent responsiveness is strongly affected by temperature. To maintain the protonated (reactive) state the intracellular pH (pH_i) is regulated from unicellular to complex metazoans, a mechanism termed as alaphastat-regulation (Reeves, 1977). To conserve the ionized state of proteins a down-regulation of ~0.019 pH units per °C temperature increase is essential to keep the responsiveness of enzymes.

Thus, the ability to regulate the pH_i can be critical as it contributes to limitations of thermal tolerance at the cellular level and implicated consequences for the whole animal performance. In Antarctic fish the ability to regulate the pH_i is critically limited under elevated temperatures (Mark et al., 2002; Pörtner et al., 1998; Sartoris et al., 2003; van Dijk et al., 1999) confining the thermal tolerance at the cellular level.

1.3.4 DNA, RNA and gene expression

The implications given by adaptive trends in the protein structure shaped by temperature dependent exchange patterns afford subsequent alterations on DNA and RNA levels. Moreover, up to six syntactic combinations of DNA bases are available for each amino acid.

It is a fact that encoding, transcription and translation are governed by the same thermodynamic and extrinsic constraints like other cellular processes. Thus, an optimization on these levels is likely due to the promotion of thermodynamic convenient adaptations.

Adaptation to high temperatures may comprise high GC content resulting from a preferred usage of G=C pairs with three hydrogen bonds over less stable A=T pairs with two hydrogen bonds (Wada and Suyama, 1986), whereas the reverse can be expected for cold adaptation. Genome-wide studies of thermophilic, mesophilic and psychrophilic prokaryotes have discussed the existence of an adaptation by a preferred GC usage in the warmth on a total genomic level (Hickey and Singer, 2004; Singer and Hickey, 2003; Tekaia and Yeramian, 2006; Tekaia et al., 2002). This trend is evident in coding sequences of orthologous proteins and for single-stranded structural RNAs in prokaryotes and vertebrates (Cruveiller et al., 1999; Galtier and Lobry, 1997; Varriale et al., 2008; Wang et al., 2006). Most poikilotherm organisms display a low variation among GC3 which is correlated to the GC1/2 content and indicates a systematic compositional variation across their genomes (Belle et al., 2002). Genomic studies in plants have revealed that genomic regions under strong selective pressures are more frequently recombined and display an increased GC3 content (Tatarinova et al., 2010). Possibly the evolutionary pressure upon optimized stability of nucleic acids in the cold combined with a relaxed functional selection promoted the occurrence of knock outs like

haemoglobin in icefish (Cocca et al., 1997; di Prisco et al., 2002) or the dysfunctional coding of heat shock protein hsp70 in *Trematomus bernacchii* (Buckley et al., 2004; Hofmann et al., 2000). Nevertheless, due to the lack of ancestral sequence information the evolutionary development and disintegration in these genes through low GC contents will remain obscure. GC rich sites in DNA are important targets for methylation to stably alter the gene expression pattern such as during cell differentiation and development (Straussman et al., 2009). These epigenetic modifications reflect the phenotypic differentiation of different life history traits (features of survivorship, growth, development and reproduction) in single organisms of a species.

Several studies have surveyed the expression of single candidate genes from eurythermal and stenothermal fish in response to altered temperatures. Thereby, rearrangements in energy metabolism became visible through differential expression of enzymes of mitochondrial respiration and uncoupling (Hardewig et al., 1999b; Lucassen et al., 2006; Mark et al., 2006). Microarray studies provided an even more comprehensive picture of responses to higher temperatures. For instance, patterns of a cellular stress response were found under thermal stress in the cold-adapted *Trematomus bernacchii* (Buckley and Somero, 2009), or the temperature dependent regulation of transcripts related to an acute inflammatory response and oxidative stress in *Harpagifer antarcticus* (Thorne et al., 2010).

To date, the increasing availability of sequenced transcriptomic libraries allow for large-scale comparisons of the deployed repertoires of genes and identify habitat-correlated meanderings. Comparisons of transcriptomic libraries between the notothenioid *Dissostichus mawsoni* and temperate/tropical fish revealed the contributions of specific protein families to the physiological fitness under constant cold conditions (Chen et al., 2008). An array of functions was found in this study including protein synthesis, folding and degradation, lipid metabolism, antioxidation, antiapoptosis, innate immunity as well as chorionogenesis. Recently, Shin and colleagues (Shin et al., 2012) further expanded the available transcriptomic data with three other Notothenioids: *Nothothenia coriiceps*, *Pleuragramma antarcticum* and *Chaenocephalus aceratus*. These data provided information of the above-stated overrepresented metabolic gene families in these fish and gave further indications of a high protein turnover as seen in proteo-biochemical studies (Todgham et al., 2007).

1.4 Fish model Zoarcidae

The monophyletic fish family of Zoarcidae comprises 284 species (Anderson and Fedorov, 2004) distributed in temperate, subpolar and polar waters. With this wide geographical range 24 species are endemic to the Antarctic waters (Anderson, 1990). Thus, this family provides a model system to study evolutionary adaptation to temperature and the resulting adaptability to thermal changes in a comparative manner.

The main focus of this thesis is the cold-adapted Antarctic eelpout, *Pachycara brachycephalum* (Pappenheim, 1912). This species is only distributed within Antarctic waters and inhabits the shelf regions at depths from 200 to 1,800 m (Anderson, 1990). The mean annual water temperature in the Southern Ocean fluctuates between $-1.5 - 2.0^{\circ}\text{C}$, due to the constant conditions of the ACC. However, *Pachycara brachycephalum* lives at temperatures between -0.4 and 1°C seemingly avoiding freezing conditions by preferring greater depths with slightly “warmer” temperatures (Brodte et al., 2006b). These scaleless fish reach on average a total length of ~ 35 cm and do not possess a swim bladder. The benthic lifestyle is characteristic for species of the genus *Pachycara*, which is suspected to have a deep-sea origin (Anderson and Peden, 1989; Brodte et al., 2006b).

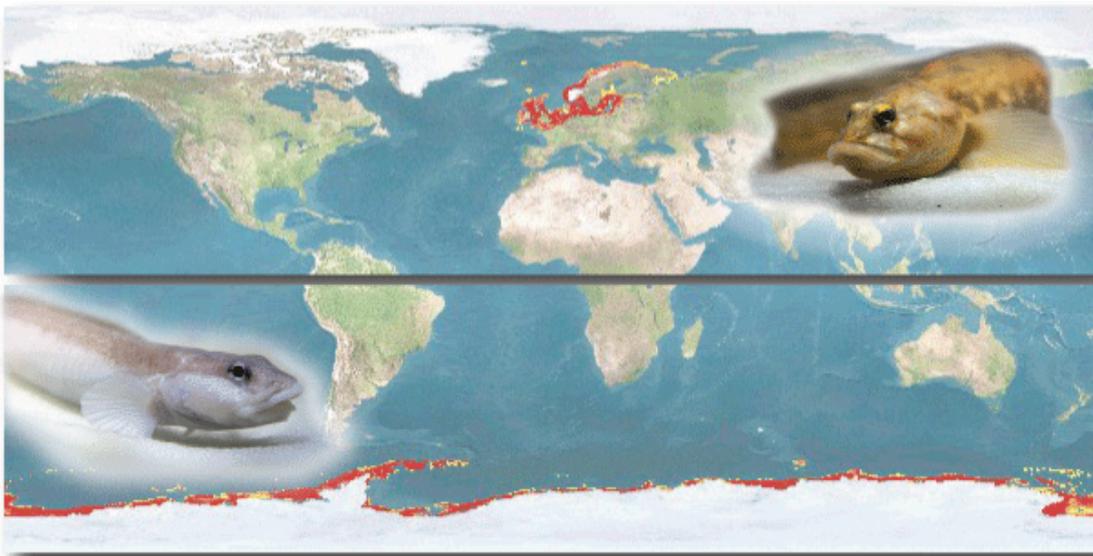


Figure 2: Geographical distribution patterns of zoarcid fish under study

Both maps show the species-specific distribution and an exemplary specimen. Upper panel: *Z. viviparus*. Lower panel: *P. brachycephalum*. In line with the distribution of the species maps were reduced to northern and southern hemisphere. Maps were generated and derived from FishBase (<http://www.fishbase.org>). Photographs were taken in the aquarium at the Alfred Wegener Institute in Bremerhaven.

In several studies *P. brachycephalum* has been compared to its temperate congener *Zoarces viviparus* (Linnaeus, 1758) at physiological and molecular levels. This species inhabits boreal coastal areas of the North, Baltic and White Sea and faces large seasonal temperature fluctuations from 0 to 20°C over the year (Zakhartsev et al., 2003). Temperature-dependent growth rates revealed a much narrower thermal niche for *P. brachycephalum* (Brodte et al., 2006a; Pörtner and Knust, 2007) compared to *Z. viviparus*. The narrow window of aerobic scope in *P. brachycephalum* is limited by oxygen supply (Mark et al., 2005) and pH; regulatory competence (Mark et al., 2002; van Dijk et al., 1999). Also, the Antarctic eelpout exhibits a higher degree of metabolic cold compensation evidenced by a faster recovery from bouts of anaerobic metabolism than the temperate species (Hardewig et al., 1998). Energy stores in this species are basically composed of lipids (Brodte et al., 2006a) and a characteristic membrane composition dominated by triacylglycerols (Brodte et al., 2008) distinguishing *P. brachycephalum* from its eurythermal congener at habitat temperatures. Cold-compensated mitochondrial capacities (Hardewig et al., 1999b; Lannig et al., 2005; Lucassen et al., 2003) and an increased uncoupling in the warmth (Mark et al., 2006) together with cold compensation of the transcription machinery (Storch et al., 2005) indicate residual warm acclimation capacities in the *P. brachycephalum*.

To fully assess the thermal plasticity of *P. brachycephalum*, the genomic and transcriptomic differences between congener species still needs to be investigated. In addition, the regulatory networks involved in the acclimation process, which promote the observed alterations in metabolic energy consumption and conversion, are not well characterized yet.

Although *P. brachycephalum* exhibits many characteristic features of cold adaptation at systemic and molecular levels, a high conservation of functional genes was observed when compared with *Z. viviparus* in studies of individual genes (Lucassen et al., 2003; Mark et al., 2006). This is a prerequisite for the investigation of small changes at the sequence level with respect to thermal tolerance and plasticity of the Antarctic species and therefore provides an excellent model for comparative studies at the molecular level.

1.5 Key abiotic factors and climate change

Since thermal adaptation and plasticity in marine ectotherms play a major role in species' vulnerability and biogeographic patterning, the ongoing climate change posts the question of consequences on individual species and the resulting effects on ecosystems.

Observations of increasing CO₂ concentration, rising temperatures and sea levels as well as the appearance of more extreme weather events increased the awareness of future scenarios. The changes belong to natural cycling and anthropogenic factors, whereas the latter are

caused by the consumption of fossil fuels since the beginning of the industrialization leading to increased atmospheric fractions of greenhouse gases (GHG). Forecasts from the Fourth Assessment Report of the United Nations Intergovernmental Panel on Climate Change predict an atmospheric increase of the CO₂ concentration from currently 390 ppm to 490-1,130 ppm as well as a global rise of the average surface temperature by about 2.4-6.1°C until the end of the 21st century, depending on future emission scenarios and atmospheric stabilization of GHG (IPCC, 2007).

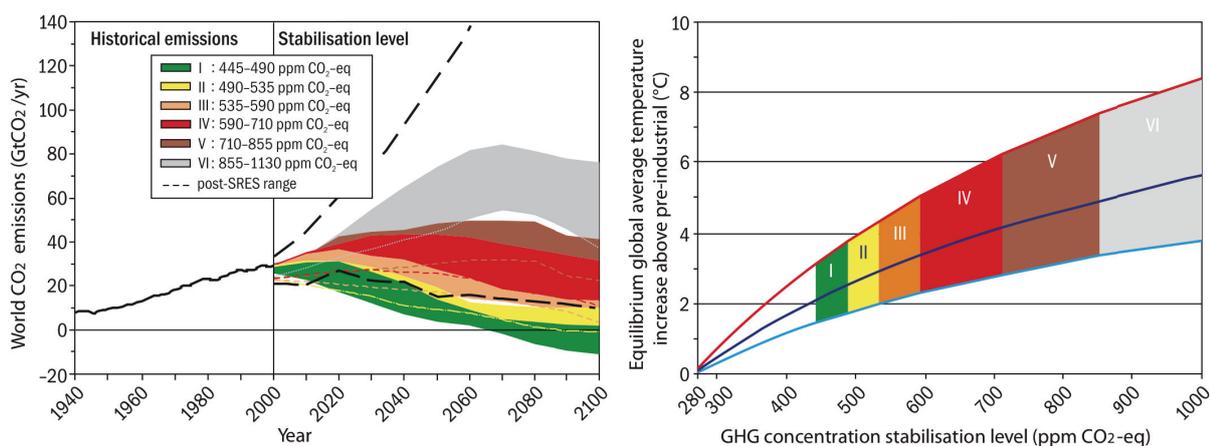


Figure 3: Projections of CO₂ emissions and corresponding average temperatures, after (IPCC, 2007)

The left panel shows the history of CO₂ emissions until the year 2000 as well as model-based scenarios of atmospheric CO₂ concentrations considering different CO₂ levels as maximum concentrations (colours of categories) reached in different time scales. The dashed line indicates the maximum estimates from the Special Report on Emission Scenarios (SRES) in 2000 (IPCC, 2000). The right panel shows the corresponding increase in global average temperature starting with the pre-industrial CO₂ concentration of 280 ppm and the potential progress dependent on the category-based scenarios. The confidence lines indicate different ranges of climate sensitivities towards elevated CO₂ concentrations (red line: 4.5°C, black line: 3°C, blue line: 2°C).

Alterations in both abiotic factors affect the water chemistry and temperatures of the global oceans. Higher concentrations of dissolved inorganic carbon (DIC) will lead to a decrease of pH, an effect which is termed ocean acidification (Caldeira and Wickett, 2003). A decrease in pH is particularly critical for calcifying organisms. Projections estimate that biogenic minerals like calcite and aragonite will dissolve in surface layers at higher atmospheric CO₂ concentrations (Cao and Caldeira, 2008). Both minerals form the shells of various species of phytoplankton, which are the very basis of the marine food web.

The correlated effect of surface warming is expected to be most striking in ice-covered regions, especially in the Arctic and Antarctic realm (IPCC, 2007). Surface temperatures at the Antarctic Faraday/Vernadsky station already increased at a rate of 0.56 °C per decade (Turner et al., 2005). Although the process of ocean warming is much slower than on the surface, a significant temperature rise by 0.17°C within the mid-depths water masses (~900 m) of the Antarctic Circumpolar Current has been measured between the years 1950 and 1980

(Gille, 2002).

The species inhabiting polar oceans are highly adapted to extremely cold temperatures close to the freezing point and have a questionable capacity to deal with the expected changes. It is most likely that ecological disturbances can be expected in this region with strong effects on the global food web. In the Northern hemisphere migrations of temperate fish species into higher latitudes were observed (Perry et al., 2005). Such migrations are expected to propagate into polar regions and implicate disturbances of the ecosystem structure and species diversity. Together with the direct effects of warming and acidification on species performance this would lead to the extinction of some of the most vulnerable and extraordinary species in the oceans.

1.6 Objectives of the thesis

This thesis aims to characterize the thermal sensitivity vs. plasticity of the cold-stenothermal species *P. brachycephalum* on a broad molecular scale by combining hypothesis-driven and comparative transcriptome-wide molecular studies. By linking the effects of thermal acclimation from the systemic and cellular level to the transcriptomic level, key functional traits as well as effective mechanisms defining climate sensitivity and adaptability were examined.

In detail the following questions are addressed:

1. How does the repertoire of the functionally relevant genome change with adaptation to an extremely (cold) but stable environment compared to adaptation strategies in environments in which the organisms are faced with large and often fast changes in abiotic key factors like temperature?
 - This question was addressed by comparative analyses of transcriptomic cDNA libraries of *Z. viviparus* and *P. brachycephalum*. With this approach alterations of the functional repertoire of genes were determined and comparisons of orthologous sequences at protein and RNA levels were used to reveal differences in the coding of the Antarctic species.
2. Which are the molecular networks that are involved in an acclimation process and how do they integrate acclimation responses of increasingly complex systems (from molecule, organelle, cell, tissue to organism)?
 - In this approach the acclimation response of *P. brachycephalum* to 5°C was examined in a time-dependent manner. Fitness parameters, enzyme measurements and the expression of potential candidate genes were quantified to characterize the course of the acclimation process.

3. How do gene expression patterns develop at various temperatures forming the thermal window of a species (optimum vs. limiting)?
 - Differentially temperature conditioned transcriptomes were examined by designing and employing a species-specific microarray for *P. brachycephalum*. The expression data were correlated with whole animal performance parameters to characterize the metabolic rearrangements within as well as at the borders of the thermal window of the Antarctic eelpout.
4. How does molecular plasticity define species success and contribute to setting climate sensitivity?
 - This final question will be discussed with respect to the conducted experiments and with existing literature in the final chapter of this thesis.

2 Materials & Methods

The following section summarizes methods applied within this work. Unless experimental procedures and protocols are described in the corresponding publications, the particulars are explained in detail as follows:

2.1 Animals

In all experiments specimens of the Antarctic eelpout, *Pachycara brachycephalum* (Pappenheim, 1912) were under examination. Fish were collected by means of baited traps at several positions near King George Island in depths between ~400 and 730 m (Fig 4, Tab. 1) during different cruises of RV “POLARSTERN”.

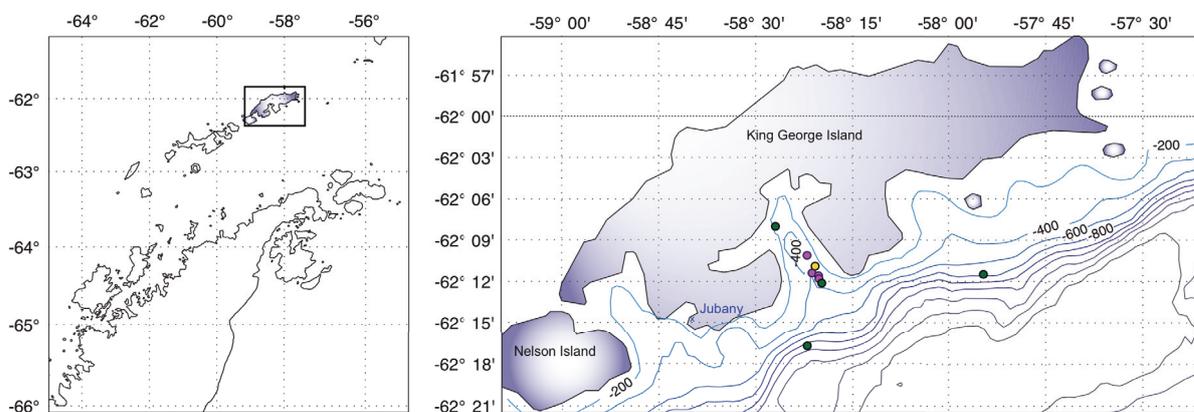


Figure 4: Sampling sites of *Pachycara brachycephalum*

Animals were caught with baited traps around King George Island during three cruises of RV “POLARSTERN”, as indicated by differently coloured labels. Fish from different expeditions were not mixed in the experimental incubation setups. Yellow: ANT-XV/3 in 1998, used for publication I+II (reference samples for enzyme measurements and cDNA library construction); green: ANT-XXIII/2-3 in 2006, used in publication I (time course experiment) and purple: ANT-XXV/4-5 used for publication III (microarray after long-term exposure to different temperatures). The maps were generated by PERPLEX version 4 (<http://perplex.awi.de>).

The animals were brought to the Alfred Wegener Institute at Bremerhaven where they were kept for at least six months for reacclimation at 0°C in recirculating seawater at 34 practical salinity units (PSU) before the beginning of any experimentation. The lighting conditions were set to equal photo- and scotophase with 12 h each. The fish were fed ad libitum with *Crangon crangon* once a week, which was terminated 5 days before sampling for all experiments.

Table 1: Catching data for *P. brachycephalum*

Expedition/ Cruise	Station number	Depth [m]	Temperature [°C]	Salinity [psu]	Caught animals	Bottom time [h]	Experimental usage
ANT-XV/3	PS48/314	-397	0.34	34.6	>500	48	Untreated (publication I+II)
ANT-XXIII/2-3	PS69/184-1	-606	-	-	20	48	Acclimation to 5°C in a time- dependent manner (publication I)
	PS69/184-2	-462			19		
	PS69/184-3	-444			63		
	PS69/184-4	-728			98		
ANT-XXV/4-5	PS73/255-1	-466	0.6	34.4	~300	24	Acclimation to six different temperatures for 2 months (publication III)
	PS73/255-2	-419			~300		
	PS73/255-3	-456			~200		
	PS73/255-4	-445			~200		

During different cruises of the RV “POLARSTERN” animals were caught with baited traps at different positions as denoted by the station numbers. The corresponding positions are listed in the Appendix Table Ap-1. The respective temperature and salinity data were gathered by CTDs close to the catching positions.

2.1.1 General sampling protocol

The experiments on live animals were approved by a competent German authority: Freie Hansestadt Bremen, Senatorin für Arbeit, Frauen, Gesundheit, Jugend und Soziales; Referat Lebensmittelsicherheit, Veterinärwesen, Pflanzenschutz; reference no. 522-27-11/02-00(93).

The recorded standard parameters for all samplings included length, weight, the hepatosomatic index (liver weight in relation to whole body weight in percent) as a fitness parameter, the grade of gut filling as well as the haematocrit.

During all samplings fish were anaesthetized by exposure to MS222 (0.2 g l^{-1}). Blood samples were taken from a caudal vessel before killing the fish by cutting the spine as close to the cranium as possible. Tissue samples were quickly excised and instantaneously frozen in liquid nitrogen. Sample material was taken from liver, heart, spleen, gill-filaments, brain, kidney and white muscle tissue. At least $50 \mu\text{l}$ of the full-blood were stored as sample, $\sim 10 \mu\text{l}$ were used for haematocrit determination (Compur Microspin 6500, Bayer Diagnostics, Munich/Germany) whereas the remaining sample was centrifuged for 10 min at 1,500 rpm at 4°C to extract serum samples (supernatant). All samples were stored at -80°C until further processing. In addition, the otoliths were excised and kept at -20°C .

2.2 Animal incubations and experimental setups

2.2.1 Untreated animals

Animals caught during ANT-XV/3 were held at 0°C and served as reference and template for molecular genetic approaches. Samples from 9 animals were utilized for cloning procedures as well as for evaluating the expression of potential candidate genes and transcription factors in a tissue panel (appendix). Liver samples were adopted as an additional control group for the enzyme measurements in publication I. Samples of liver and heart tissue were used for the construction of a normalized cDNA library (pub. II).

2.2.2 Time course of warm acclimation

Randomly chosen fish caught during the cruise ANT-XXIII/2-3 were exposed for six different time periods (1d, 2d, 4d, 7d, 14d and 42d) to a final temperature of 5°C reached by a heating regime of 1°C h⁻¹. Samples of control animals kept at 0°C were taken at the beginning (control 1, 0d) as well as at the end of experimentation (control 2, 43d). Each group comprised between 6 and 9 fish. One day before sampling each animal was placed in a separate basket allowing a stress-free transfer of the fish in portable chambers for anesthetization.

The resulting metabolic changes were characterized by measurements of enzyme activities in liver tissue and the quantification of 26 candidate genes by quantitative real-time polymerase chain reaction (qPCR) in publication I. Moreover, the osmolality and the concentration of 10 inorganic ions were determined in blood serum (appendix).

2.2.3 Long-term acclimation

Randomly chosen fish from the pool of *P. brachycephalum* caught during ANT-XXV/4-5 were incubated for a period of two months at different temperatures (-1°C, 0°C, 3°C, 5°C, 7°C, 9°C). The animals were held in swimming baskets with a single chamber for each fish to identify single individuals and reduce catching stress during the sampling procedures. Before the start of experimentation, the length and weight of individuals (a minimum of 12 fish per treatment) were determined under slight anaesthetisation (0.05 g l⁻¹ MS222) to track the growth performance during the experiment. The animals were warmed with a rate of 1°C d⁻¹. For the exposure to temperatures higher than 5°C an intermediate step was included. Those animals were preincubated at 5°C for a week before applying the final exposure temperatures of 7 and 9°C with a rate of 1°C d⁻¹.

The obtained liver samples were used in a microarray experiment to determine changes in the

expression profile caused by the exposure to different temperatures. Growth data, hepatosomatic index and haematocrit were used to link the expression data to whole animal levels.

2.3 Osmolality measurements

The osmolality was determined in undiluted serum samples using a Wescor 5500 Vapo pressure osmometer (ELITech, Logan, Utah/USA) with Wescor's Optimol osmolality standards. After temperature equilibration (prewarming) of the osmometer, 10 μ l serum samples were used to determine the total osmolality. All serum samples originating from one experiment were measured in sequence after calibration of the osmometer.

2.4 Molecular analytics

2.4.1 Enzyme measurements

Enzyme activities were determined for four enzymes involved in different metabolic pathways comprising the citric acid cycle (citrate synthase, CS), the respiratory chain (cytochrome-c-oxidase, COX), gluconeogenesis (phosphoenolpyruvate carboxykinase, PEPCK) and lipid oxidation (hydroxyacyl-Co-A-dehydrogenase, HADH) (Tab. 2).

All enzymes were measured in duplicates in the same crude extract. The total protein content was determined by the Bradford-method (Bradford, 1976) using bovine serum albumin as a standard. Enzyme activity measurements were conducted in a thermostatted spectrophotometer (Beckman, Fullerton, CA/USA) at the applied exposure temperatures of 5.0 °C (all enzymes) and 0.0 °C (COX and CS). The enzyme activities were calculated by the formulas:

$$A_{PC} = \frac{\Delta E * V_i}{\Delta t * \epsilon * d * V_s} * \frac{60}{PC_{tot}}$$

A_{PC} = protein specific enzyme activity [μ mol * h⁻¹ * mg⁻¹]
 $\Delta E/\Delta t$ = slope [min⁻¹]
 ϵ = extinction coefficient [mM⁻¹ * ml * cm⁻¹]
 d = cuvette diameter [cm]
 V_i = input volume [μ l]
 V_s = sample volume [μ l]
 V_{tot} = total volume of extract fraction [ml]
 PC_{tot} = protein content of extracted sample [mg * ml⁻¹]

$$A_{FW} = \frac{\Delta E * V_i}{\Delta t * \epsilon * d * V_s} * \frac{V_{tot}}{FW_{tot}}$$

A_{FW} = tissue specific enzyme activity [μ mol * min⁻¹ * g⁻¹]
 $\Delta E/\Delta t$ = slope [min⁻¹]
 ϵ = extinction coefficient [μ mol⁻¹ * ml * cm⁻¹]
 d = cuvette diameter [cm]
 V_i = input volume [μ l]
 V_s = sample volume [μ l]
 V_{tot} = total volume of extract fraction [ml]
 FW_{tot} = fresh weight of extracted sample [g]

For enzymes measured at different assay temperatures the Q_{10} –value was determined by the different specific activities:

$$Q_{10} = \left(\frac{A_1}{A_0} \right)^{\frac{10}{T_1 - T_0}}$$

Q = temperature coefficient
 A_1 = specific activity at high temperature
 A_0 = specific activity at low temperature
 T_1 = high temperature [in °C or K]
 T_0 = low temperature [in °C or K]

Table 2: Summary of enzyme activity measurements

Enzyme	Protocol after	Reaction mechanisms and measured substrates
CS	(Hardewig et al., 1999b; Sidell et al., 1987)	$\text{acetyl-CoA} + \text{oxaloacetate} + \text{DNTB} + \text{H}_2\text{O} \xrightarrow{\text{CS}} \text{citrate} + \text{DNTB-S-CoA}$ <p>The augmentation of product 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB)-S-CoA was quantified at $\lambda = 412 \text{ nm}$ ($\epsilon = 13.61 \text{ mM}^{-1} \text{ cm}^{-1}$).</p>
COX	(Hardewig et al., 1999b; Moyes et al., 1997)	$4 \text{ cytochrome-c}_{\text{red}} + \text{O}_2 + 4\text{H}^+ \xrightarrow{\text{COX}} 4 \text{ cytochrome-c}_{\text{ox}} + \text{H}_2\text{O}$ <p>The oxidation of the substrate, reduced cytochrome-c, was determined at $\lambda = 550 \text{ nm}$ ($\epsilon = 19.10 \text{ mM}^{-1} \text{ cm}^{-1}$)</p>
PEPCK	(Aas-Hansen et al., 2005)	$\text{phosphoenolpyruvate} + \text{CO}_2 + \text{GDP} \xrightarrow{\text{PEPCK}} \text{oxaloacetate} + \text{GTP}$ $\text{oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{malate} + \text{NAD}^+$ <p>The activity of PEPCK was determined indirectly by measuring the reduction of NADH, which was metabolized by MDH with oxaloacetate at $\lambda = 340 \text{ nm}$ ($\epsilon = 6.31 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of antimycin A.</p>
HADH	(McClelland et al., 2005)	$\text{acetoacetyl-Co-A} + \text{NADH} + \text{H}^+ \xrightarrow{\text{HADH}} \text{L-3-hydroxyacyl-Co-A} + \text{NAD}^+$ <p>HADH activity was quantified by measuring the reduction of NADH at $\lambda = 340 \text{ nm}$ ($\epsilon = 6.31 \text{ mM}^{-1} \text{ cm}^{-1}$) in the presence of antimycin A.</p>

Enzyme activities were determined after protocols given by the respective references. The reactions and the measured substrates are listed with the respective wavelengths and extinction coefficients of the substrates.

2.4.2 RNA extraction

RNA samples for all experiments were extracted by means of a standardized protocol with the Qiagen RNeasy kit according to the manufacturer's instruction (Qiagen, Hilden/Germany) including an additional step with a proteinase K digestion after homogenisation. Quantity and purity of the RNA were determined using the NanoDrop ND 1000 (Peqlab Biotechnologie, Erlangen/Germany). To ensure a high sample quality for further experimentation a minimum standard of ratios of $260\text{ nm}/280\text{ nm} \geq 2$ and $260\text{ nm}/230\text{ nm} \geq 1.8$ was preconditioned. Further quality assessment was done by capillary electrophoresis (Bioanalyser: Agilent, Waldbronn/Germany) to evaluate the integrity of RNA. A ratio of 28S/18S rRNA between 1.5 and 1.8 was a precondition for the construction of the cDNA library (pub. II) and the sample material for the microarray experiment (pub. III). In addition, to exclude any contamination of genomic DNA for further expression analyses RNA samples were treated with TurboDNase (TurboDNA-free Kit; Life Technologies, Darmstadt/Germany).

2.4.3 Cloning of candidate genes

For quantitative expression analyses (see below) cDNA sequences of transcription factors (PPAR1 α , PPAR1 β , PPAR1 γ , PGC1 α , NRF1) and functional genes (COX IV and GAPDH) were cloned from liver samples of untreated *P. brachycephalum*. On the basis of previously published sequences in other fish obtained from the NCBI database (for sequence survey see pub. I, Tab. 1), primer pairs in conserved sequence regions of the respective genes were designed with the MacVector software (version 10.0.2, MacVector Inc.).

For cloning, liver RNA samples of untreated *P. brachycephalum* were first reverse transcribed for the generation of cDNA. The reactions contained 0.5 μg sample RNA, 6 pmol of random-decamer primer, 0.625 mM deoxyribonucleoside triphosphates (dNTPs) (each) and 400 U Superscript RT III (Life Technologies, Darmstadt/Germany) in 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂ and 10 mM dithiothreitol (DTT). The reactions were incubated for 1 h at 42°C and reverse transcription (RT) was terminated by heating at 70°C for 20 min.

The following PCR reaction for the amplification of specific fragments was performed with 1 μl of the RT product, 20 pmol l⁻¹ specific primer (each), 25 mM dNTPs (each) and 1 U SuperTaq Plus polymerase (Life Technologies, Darmstadt/Germany) in 20 mM Tris/HCl (pH

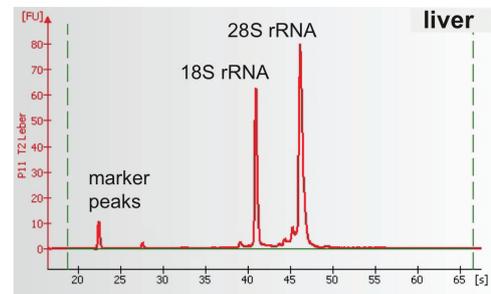


Figure 5: Quality control of RNA samples for further experimentation

Exemplary sample containing 0.688 $\mu\text{g}/\text{ml}$ liver RNA, separated by capillary electrophoresis. The peaks of 18S and 28S rRNA indicate an intact RNA by a ratio of ~ 1.5 and no degradation or contamination.

8.4), 50 mM KCl and 1.5 mM MgCl₂ in a total volume of 20 µl in a gradient cycler (T-gradient, Biometra, Göttingen/Germany). For each fragment 8 reactions were prepared which were amplified in a thermal gradient that was varied at the most by ± 4°C from the primer-specific melting temperature. After an initial denaturation at 94°C for 4 min, 32 cycles of denaturation (30 sec at 94°C), annealing at the primer-specific melting temperature ± 4°C (1 min) and synthesis for 30 sec at 72°C were carried out, followed by a final elongation phase at 72°C for 8 min. PCR fragments were separated by gel electrophoresis (1.5% agarose, 0.5 M tris-borate-EDTA buffer (TBE), pH 8.3) and purified after excision using the QiaquickGel extraction kit (Qiagen, Hilden/Germany). DNA fragments were cloned with the TOPO-TA cloning kit (Invitrogen, Karlsruhe/Germany) into the pCR4-TOPO vectors, which were thereafter transferred into chemically competent TOP10 *Escherichia coli* cells according to the manufacturers instructions. Transformed *E. coli* cells were cultivated on selective medium plates (100 µg/ml ampicillin, 80 µg/ml X-gal and 0.5 mM IPTG for blue/white screening) from which up to 5 clones were picked and grown over night in LB medium with ampicillin (100 µg ml⁻¹). Plasmids were extracted with the Qiaprep Spin Miniprep kit (Qiagen, Hilden/Germany) and analyzed for the presence and size of inserts by restriction-digestion with EcoRI (Invitrogen, Karlsruhe/Germany) and a following survey in an analytic agarose gel (1 % agarose in 0.5 M TBE). Plasmids containing fragments with the expected size were sequenced by MWGBiotech (Sanger sequencing: Ebersberg/Germany). Sequencing data were analyzed and assembled with the MacVector software (version 10.0.2). The sequence information was implemented in the NCBI database, together with fragments of the cDNA library, which were used for quantitative expression analyses (see pub. I, Tab. 2).

2.4.4 Quantification of transcripts via qPCR

Transcript amounts of specific genes were determined by the quantification of 1:1 reverse transcribed cDNA (High-Capacity cDNA Archive Kit; Applied Biosystems, Darmstadt/Germany) generated from DNA-free template-RNA. The analysis was performed with an ABI 7500 qRT-PCR cycler system (Applied Biosystems, Darmstadt/Germany). For each gene the efficiency of the selected primer pair was tested and adjusted if necessary to reach a continuous doubling of the transcript copies in each cycle. All samples were measured in triplicates (for detailed protocol and primer list see publication I, material and methods and Tab. 2) and expression data were evaluated as quantities calculated from mean Ct-values as follows:

$$Q = \left[10^{-1/e} \right]^{(Ct_{\min} - Ct_{\text{sam}})}$$

Q = quantity of transcript
 e = efficiency of reverse transcription, i.e. slope of standard curve (x = Log copy number; y = Ct-values)
 Ct_{min} = minimum threshold cycle for gene of interest
 Ct_{sam} = threshold cycle in sample

Quantities were normalized by a factor calculated from genes with the most stable expression using the geNorm Exel tool (Pfaffl et al., 2004; Vandesompele et al., 2002). β -actin and ubiquitin proved to be stable genes with low M-values (relative expression stability) and were used for normalization (for further details see pub. I, Materials and Methods).

2.4.5 cDNA library construction for *P. brachycephalum*

Pooled total RNA from liver and heart samples of untreated animals served as a template for the preparation of a random-primed and normalized cDNA library (Vertis, Freising/Germany; for details see Material and methods in pub. II). The 454 pyrosequencing was performed in high-density pico reactions by Eurofins-MWG (MWG-Biotech AG, Ebersberg/Germany) using GS FLX Titanium chemistry (MWG-Biotech AG) according to Margulies (2005). The sequence information of reads were assembled with MIRA (Version 2.9.43) with standard settings for stringency and homology (MWG-Biotech AG) in accordance to Chevreux (2004).

2.4.6 Sequence annotation of cDNA libraries

The assembled transcripts in the cDNA library of *P. brachycephalum* were annotated by using the Blast2GO tool (Conesa et al., 2005) for sequence similarity searches. Via BLASTx (Altschul et al., 1990; Altschul et al., 1997) annotations from the Swiss-Prot database (<http://www.uniprot.org/>) as well as from the non-redundant (nr) NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were obtained with a standard e-value cut-off of 10^{-3} and a minimum high-scoring segment pair (HSP) value of 33. Gene Ontology (GO) mapping of the nr-results revealed 14,112 sequences with 6,265 unique GOs. Via Interpro-scan (implemented in Blast2GO tool, (Conesa et al., 2005)) 4,119 unique protein motifs were identified in 31,754 sequences. Further annotation was obtained by assigning transcriptomic data with orthologous model sequences from the eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database (http://eggnoG.embl.de/version_3.0/index.html). Metazoan- (meNOGs) and fish-specific sequence models (fiNOGs) (Powell et al., 2011) were obtained by rpstBLASTn (reverse position specific BLAST of nucleotides, NCBI local BLAST tool version 2.2.25+) against the respective databases (meNOG/fiNOG) with an e-value cut-off of 10^{-20} .

For comparative analyses a cDNA library of *Z. viviparus* generated with the same construction protocol (Kristiansson et al., 2009) as used for *P. brachycephalum* was provided.

Between both approaches only the applied sequencing chemistry differed (for details see pub. II). The same annotation steps as described for *P. brachycephalum* were conducted for *Z. viviparus*, independently from the annotation of the original work. The GO mapping of the cDNA library of *Z. viviparus* resulted in 16,316 sequences with 7,448 unique GO terms. Using Interpro-scan 3,971 unique protein motifs in 24,340 sequences were identified.

2.4.7 Phylogenetic analyses

The relationship between *P. brachycephalum* and *Z. viviparus* was analyzed by sequence alignments of the genes COI and 16SrRNA. Comparing zoarcid sequences with species of other fish families a close relationship of the zoarcid fish became visible in phylogenetic trees. (generated with MacVector, version 10.0.2, MacVector Inc.). For detailed information see materials and methods in publication II.

2.4.8 Microarray – design of a test array

Independent from sequence annotation a set of contigs was chosen for the expression analyses via microarrays. Contigs with a minimum read length of 300 bases and an average coverage of 3.47 reads per contig were included in the design resulting in 17,024 transcripts. However, the orientation of contigs (5'-3' or 3'-5') was not explicit by the assembly. To encounter the ambiguity of the correct reading direction 3 probes were designed in 5'-3' direction as well as in 3'-5' direction

for each contig. Due to constraints for an optimal probe construction a yield of 6 probes per contig was not suitable for all considered contigs (Tab. 3). The final design was generated in eArray (Agilent Technologies, Waldbronn/Germany; online platform) targeting 16,904 contigs (Tab. 3). The aspired 6-fold probe coverage was successful for 87% of the transcripts (Tab. 3). Further control probes were included to provide an independent reporter system on the array (see below under 2.4.10). The arrays with a 2x105k probes per slide format (custom design identifier: AMADID 030564) were produced by Agilent (Agilent Technologies, Waldbronn/Germany). The experimental design was chosen to optimize and reduce the array

Table 3. Composition of the test array on a 2x105k probes per slide format

n numbers of probes in set	Number of different contigs	Total number of probes per set
1	413	413
2	763	1,526
3	791	2,373
4	855	3,420
5	822	4,110
6	13,260	79,560
unique	16,904	91,402

Due to the different lengths of contigs varying between 5,426 and 300 bases, the generation of 6 different probes was not feasible for all contigs. The resulting composition was used in a test array to validate the responsiveness of different probes.

format for further experimentation. To this end, the optimization process was combined with the aim to evaluate probably differing expression patterns of males and females in liver tissue. As the cDNA library of *P. brachycephalum* consisted of heart and liver the reference pool included both tissues of 12 control animals of the time course experiment (6 females + 6 males). Individual hybridization of liver samples revealed differences between males and females (see Appendix, section 6.5). The design is available in the database for Minimum Information About a Microarray Experiment (MIAME, ArrayExpress at the EBI <http://www.ebi.ac.uk/arrayexpress/>) under the ID A-MEXP-2249.

2.4.9 Microarray – compact design

Responsive probes of the 105k-array were selected by means of the “well above background” signal in the raw data file and sense/antisense selection. The redesigned 60K array comprised probes for 15,843 contigs of the cDNA library (for further details see Tab. 4). Providing 3 unique probes for each contig was successful for at least 62% of the represented transcripts. Arrays

Table 4. Composition of the compact array on a 8x 60k probes per slide format

n numbers of probes in set	Number of different contigs	Total number of probes per set
1	1,389	1,398
2	4,697	9,394
3	9,748	29,244
unique	15,843	40,036

The final design was generated based on the test array. The resulting composition was used in a test array to validate the responsiveness of different probes.

were produced by Agilent (Agilent Technologies, Waldbronn/Germany) with the custom design identifier AMADID 32359 on a 8x60k probes per slide format. The optimized array design was used to evaluate expression patterns in liver tissue of long term acclimated *P. brachycephalum* (Figure 6). The design is provided in the MIAME database ArrayExpress at the EBI; <http://www.ebi.ac.uk/arrayexpress/>, under ID A-MEXP-2248.

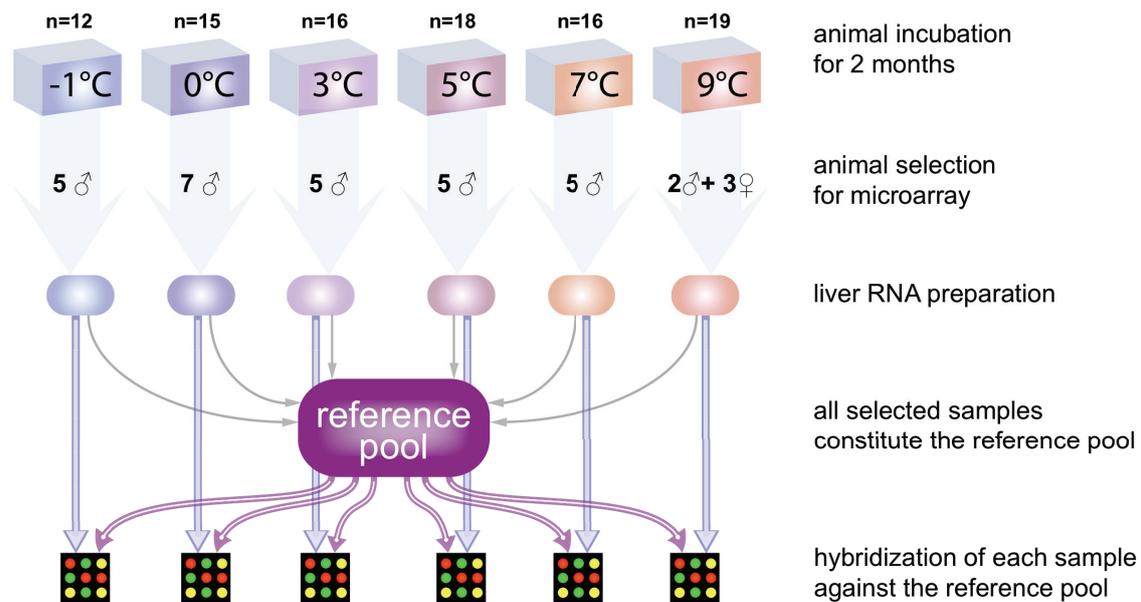


Figure 6: Experimental design of the microarray analyses after long-term thermal acclimation

Schematic overview of the workflow and pooling protocol applied for the microarray analyses of differentially incubated specimens of *P. brachycephalum* on the optimized 60k array format. In total 32 hybridizations were performed for the expression analyses.

2.4.10 Labelling, hybridization and scanning of microarrays

All RNA samples for hybridization were enriched with a positive control to monitor the procedure of sample amplification and microarray workflow (Agilent RNA Spike-In Kit). The preparation of samples was carried out according to the protocol for 2 colour arrays (Agilent, G4140-90050, version 6.5). cDNA containing a T7 promoter region was generated from samples and reference RNA (including spike RNA for a starting amount of 50 – 200 ng total RNA) and transcribed into antisense cRNA, incorporating dye-labelled cyanine (reference pool with spike A, labelled with Cy3; samples contained spike B and Cy5 labels). The labelled antisense cRNA was purified as recommended with a modified protocol for the RNeasy Kit (Qiagen, Hilden/Germany). Quantification of cRNA and specific activity were determined with the NanoDrop ND 1000 (Peqlab Biotechnologie, Erlangen/Germany).

All reactions were standardized for volume and yield depending on the used array format as recommended by the manufacturer. Dye-labelled samples were hybridized to the arrays after a fragmentation protocol. For a better handling accuracy not more than 4 slides were processed at once. Hybridizations were incubated at 65°C for 17 h while rotating with 10 rpm. Following the manufacturer's instructions, the arrays were washed and scanned with an Agilent Scanner (type B) using the AgilentHD_GX_2Color program (scan resolution 5nm). The generated *.tiff files were loaded to Agilent's Feature Extraction software and analyzed by means of protocol GE2_107_Sep09 and the corresponding Array design files (105k: AMADID 030564, 60k: AMADID 32359).

2.5 Statistical approaches

2.5.1 Univariate analyses

Physiological as well as molecular observations for publication I and II were tested group-wise whether the applied temperatures had effects on the analyzed measures with one-way ANOVA at a significance level of $p \leq 0.05$, followed by a Student-Newman-Keuls post hoc test using SigmaStat (version 3.5; Systat Software, Erkrath/Germany). Figures were generated using SigmaPlot (version 10; Systat Software) presenting mean values \pm SD or SEM.

2.5.2 Multivariate analyses

Expression data of the time course experiment (pub. I) were analyzed by canonical correspondence analyses (CCA) in R (R-Development-Core-Team, 2011) using the *ade4* package (Dray and Dufour, 2007) to reveal relationships between transcript abundances depending on temperature and exposure time as well as differences in the total expression profiles between individuals.

2.5.3 Comparative sequence analyses

The functional coverage of the zoarcid cDNA libraries was analyzed by superordinated functional categories of meNOGs (KOG/COG) with respect to the reference genome of *Gasterosteus aculeatus*, which proved to be a close relative of *P. brachycephalum* and *Z. viviparus* in the phylogenetic analyses. Genomic sequences of *G. aculeatus* were obtained from the EMBL database (http://www.ensembl.org/Gasterosteus_aculeatus/) and meNOGs were obtained in the same way as described above for the zoarcid fish. Position-specific amino acid usage patterns were analyzed on the basis of protein sequences gathered by BLASTx against the acquired corresponding fiNOGs. The correlated coding sequences were analyzed for codon usage patterns. Sequence alignments were analyzed in R (R-Development-Core-Team, 2011) with the *seqinR* and *ade4* package according to Charif (2005). Differential patterns in the codon usage were visualized by the application of a within canonical analyses. For further details see publication II.

2.5.4 Microarray analyses

Expression data obtained from the feature extraction (see above) were normalized in R (R-Development-Core-Team, 2011) using *limma* (Smyth and Speed, 2003). After background correction (“movingmin” method) normalization within arrays (“loess” method) and between arrays (“aquantile” method) was carried out (Figure 7).

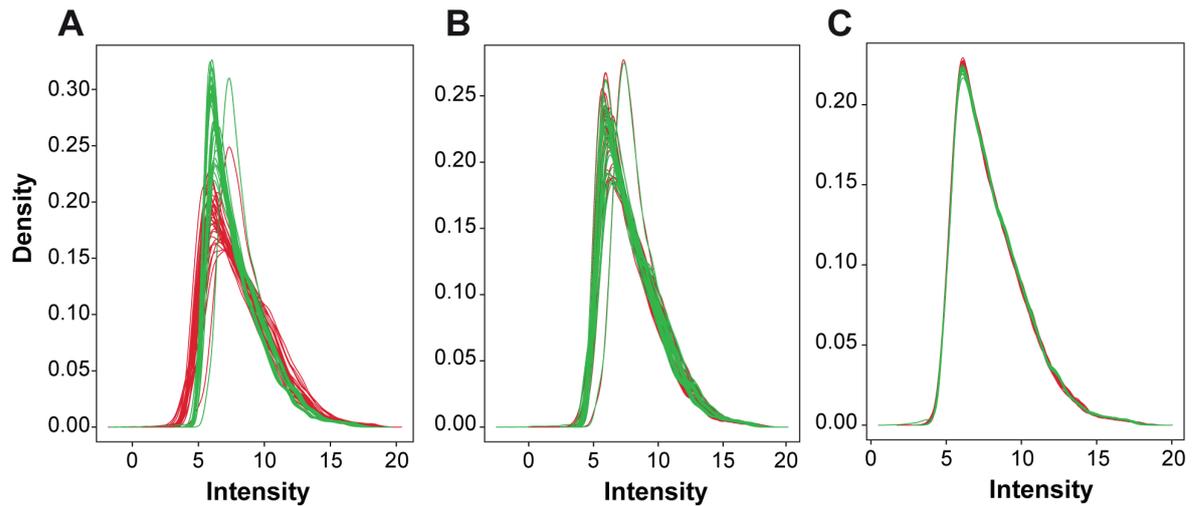


Figure 7: Normalization steps applied to microarray raw data

Normalization of the two-colour microarray data of the 60k array. Background corrected raw data (A) were normalized within (B) single arrays and between (C) all arrays before analysing expression data.

Normalized data were loaded to the MEV software (Saeed et al., 2006) and were further processed by significance analyses of microarray data (SAM).

To determine differential expression patterns between males and females (105k arrays) separate one-class tests (for males and females) against 0 were performed. The delta-values (females 7.192 and males 8.486) were adjusted to reduce the median false discovery rate to $q \leq 0.001\%$ and a 90th percentile of $FDR \leq 0.01\%$, followed by an adjusted Benjamini-Hochberg correction.

Different expression patterns induced by long-term exposure to different temperatures were addressed within the 60k arrays. The normalized data were analyzed by multi-class comparisons with a delta-value of 0.082 to reduce the median false positive rate to $q \leq 0.001\%$ and a 90th percentile of $FDR \leq 0.2\%$. For further details see material and methods in publication III.

3 Publications

List of publications and authors' contributions.

Publication I

Heidrun S. Windisch, Raphaela Kathöver, Hans-Otto Pörtner, Stephan Frickenhaus and Magnus Lucassen (2011).

Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways.

American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, **301** (5), R1453–R1466.

The concept of the study was elaborated by ML and myself. I performed the experiments with help of RK. The data analyses and interpretation was done by SF, ML, RK and myself. I drafted the manuscript, which was revised by HOP, ML and SF.

Publication II

Heidrun S. Windisch, Magnus Lucassen and Stephan Frickenhaus (2012).

Evolutionary force in confamiliar marine vertebrates of different temperature realms: adaptive trends in zoarcid fish transcriptomes.

BioMed Central Genomics **13**, 549.

The ideas for this study were developed by SF, ML and myself. I conducted the experiments and analyzed the data. The approach for a pair-wise comparative analyses was developed by SF. I wrote the manuscript, which was revised by ML and SF.

Publication III

Heidrun S. Windisch, Stephan Frickenhaus, Uwe John, Rainer Knust, Hans-Otto Pörtner and Magnus Lucassen (in preparation).

Transcriptomic features in *Pachycara brachycephalum* after chronic acclimation to various temperatures; a microarray study in Antarctic fish.

I developed the concept and design of the experiment in cooperation with ML, RK and SF, supported by UJ. I performed the experiments and analyzed the data with the help of SF and UJ. I wrote the manuscript, which was revised by HOP, ML, SF and UJ.

Publication I

Thermal acclimation in Antarctic fish: Transcriptomic profiling of metabolic pathways.

**HS Windisch, R Kathöver, H-O Pörtner,
S Frickenhaus and M Lucassen**

2011

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Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways

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Windisch HS, Kathöver R, Pörtner HO, Frickenhaus S, Lucassen M. Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways. *Am J Physiol Regul Integr Comp Physiol* 301: R1453–R1466, 2011. First published August 24, 2011; doi:10.1152/ajpregu.00158.2011.—It is widely accepted that adaptation to the extreme cold has evolved at the expense of high thermal sensitivity. However, recent studies have demonstrated significant capacities for warm acclimation in Antarctic fishes. Here, we report on hepatic metabolic reorganization and its putative molecular background in the Antarctic eelpout (*Pachycara brachycephalum*) during warm acclimation to 5°C over 6 wk. Elevated capacities of cytochrome *c* oxidase suggest the use of warm acclimation pathways different from those in temperate fish. The capacity of this enzyme rose by 90%, while citrate synthase (CS) activity fell by 20% from the very beginning. The capacity of lipid oxidation by hydroxyacyl-CoA dehydrogenase remained constant, whereas phosphoenolpyruvate carboxykinase as a marker for gluconeogenesis displayed 40% higher activities. These capacities in relation to CS indicate a metabolic shift from lipid to carbohydrate metabolism. The finding was supported by large rearrangements of the related transcriptome, both functional genes and potential transcription factors. A multivariate analysis (canonical correspondence analyses) of various transcripts subdivided the incubated animals in three groups, one control group and two responding on short and long timescales, respectively. A strong dichotomy in the expression of peroxisome proliferator-activated receptors-1 α and - β receptors was most striking and has not previously been reported. Altogether, we identified a molecular network, which responds sensitively to warming beyond the realized ecological niche. The shift from lipid to carbohydrate stores and usage may support warm hardiness, as the latter sustain anaerobic metabolism and may prepare for hypoxemic conditions that would develop upon warming beyond the present acclimation temperature.

warm acclimation; lipid and carbohydrate metabolism; mitochondria; PPARs; transcriptomic CCA

TEMPERATURE IS A CRUCIAL ABIOTIC factor due to its pervasive impact on all biological processes. On large latitudinal scales it determines and limits the geographical distribution of marine water-breathing animals. Thermal adaptation and phenotypic plasticity, which define the thermal niche and the responses to fluctuating environmental factors, are ultimately set by the genetic interior of the organisms. In the Antarctic realm studies focusing on the endemic fish suborder Notothenioidei provide evidence of novel physiological characters for coping with low temperatures like antifreeze glycoproteins (5). Some Antarctic fish have lost functional traits, like expression of hemoglobin and myoglobin in many icefishes (58, 59) and heat shock protein induction in *Trematomus bernacchii* (12, 23), indicating the absence of positive selection during evolution at

stable subzero temperatures (23). Although both examples may contribute to the high thermal sensitivity of these particular species, they do not represent general mechanisms in most cold-adapted fish species and their limitations toward high temperature.

Recent studies have demonstrated that several Antarctic fishes like the zoarcid, *Pachycara brachycephalum* (6, 11, 33), and the notothenioids *Pagothenia borchgrevinki* (6, 18), *Harpagifer antarcticus* (61), and even *T. bernacchii* (6, 9, 21) are able to acclimate to warmer temperatures. The Antarctic eelpout, *P. brachycephalum* ranged between high Antarctic and Subantarctic notothenioids with respect to heat tolerance and acclimation capacity (6). As a member of the cosmopolitan fish family Zoarcidae it allows comparisons between confamilial stenotherms and eurytherms from polar and temperate climates. While the species lives in a narrow natural thermal niche between approximately -1°C and $+1^{\circ}\text{C}$, a wider thermal tolerance range was found, associated with maximal growth at 4°C under conditions of optimal food supply (11).

Warm acclimation in *P. brachycephalum* includes altered liver composition and enhanced growth rates and therefore implies adjustments in central metabolism. Warm acclimation to 4°C caused an increase in the carbohydrate and a decrease in the lipid fraction of the liver (11) as well as a rise in whole organism heat tolerance (6). The higher growth performance in the warmth is likely supported by the altered composition of the liver tissue and an associated metabolic reorganization. Mitochondrial functioning and maintenance directly affect aerobic metabolism and standard metabolic rate and thereby constitute a key functional trait in thermal acclimation as well as evolutionary temperature adaptation following the concept of oxygen- and capacity-limited thermal tolerance (46). Acclimation to seasonal cold usually causes a rise in aerobic capacity in fish by increasing mitochondrial density or capacities (20, 47). Cold-adapted Antarctic fishes were shown to possess very high mitochondrial densities but at low capacities of individual mitochondria (per unit mitochondrial volume) (27, 44). Cold acclimation of the common eelpout (North Sea) caused elevated activities of citrate synthase (CS), while activities of cytochrome *c* oxidase (COX) remained constant (37). An even higher activity ratio of CS over COX was found in cold-adapted Antarctic eelpout at 0°C (37). This shift may support the anabolism of lipids, which typifies the metabolism of fish species in the cold (32, 36, 47).

While cold-compensated CS activities characterize both the cold-adapted and cold-acclimated zoarcids (33), warm acclimation may occur differently in polar and temperate zone fishes. Warm acclimation of Antarctic eelpout caused an up-regulation of a mitochondrial uncoupling protein (UCP-2), while it was downregulated in warm-acclimated temperate

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eelpout (40). Although the role of UCPs in ectotherms remains to be identified (25, 60), these contrasting data already indicate differences in the response to warmer temperatures between mitochondria in cold-acclimated eurytherms and cold-adapted stenotherms.

The regulatory factors and signaling pathways shaping metabolism and mitochondrial functioning may be important for thermal adaptation or sensitivity. Studies in mammalian models identified a family of nuclear receptors, the peroxisome proliferator-activated receptors (PPARs), sensing the energetic status of cells (2, 3, 29). The PPARs (α , β/δ , γ) regulate the expression of target genes by binding to DNA sequences [peroxisome proliferator response elements (PPREs)]. PPAR1 α and β were identified to regulate the β -oxidation of fatty acids (3, 67, 69). PPAR1 γ seems to be involved in the regulation of genes comprising the uptake of lipids, energy homeostasis, and mitochondrial uncoupling (28, 53, 54). Furthermore, the activation at peroxisome proliferator response elements needs cofactors the peroxisome proliferator-activated receptor- γ coactivator-1 (PGC1) and PGC1-related coactivators, which were also found to stimulate mitochondrial uncoupling effects (49). PGC1 family members also support the functioning of the nuclear respiratory factor 1 (NRF-1) (19), which was shown to regulate the capacities of respiratory chain components (17) and to coordinate the biogenesis of mitochondria in mammals (66).

For ectotherms PPAR1 α and β , PGC1 α , and NRF-1 expression was seen in fresh water fish of the family Cyprinidae (35, 41) in response to temperature and diet. Besides, PPARs from the pufferfish were shown to possess different ligand specificity compared with those from mammals (31). Recent analyses of various vertebrate PGC1 α sequences suggest that NRF-1 is not inducible by the cofactor in fish (34).

Warm acclimation in *P. brachycephalum* with an altered liver composition and enhanced growth rates imply adjustments of the energy metabolism and mitochondrial functioning. Its regulation may involve the signaling network of the PPARs and associated transcription factors. Since gene clusters and interactions between genes and their functions can hardly be detected under steady-state conditions, we monitored the changes in metabolic capacities of Antarctic eelpout upon exposure to 5°C over a time course of 6 wk together with the expression levels of receptors, transcription factors, cofactors, and numerous genes of lipid- and carbohydrate metabolism as well as the respiratory chain. By combining functional with explorative transcriptomic approaches this study suggests mechanisms effective in defining climate sensitivity and evaluates the potential thermal plasticity of cold-stenothermal fish.

MATERIAL AND METHODS

Animal collection. Specimens of *P. brachycephalum* were caught with baited traps at 62°16.74' S/58°22.05' W between 440- and 730-m depths during expedition ANTXXIII/2-3 in 2006 with RV Polarstern. The animals were brought to the Alfred Wegener Institute in Bremerhaven and kept at 0°C in recirculating seawater at 34 practical salinity units until the start of experimentation in 2008. The lighting conditions were set to equal photo- and scotophase with 12 h each. The fish were fed ad libitum with *Crangon crangon* once a week. During the experiment, feeding was terminated exactly 5 days before sampling, since the fish had almost digested the food but were not starving at that point in time. Feeding was successful at both 0°C

and 5°C. For enzymatic analyses, an additional independent control group, caught during the Polarstern cruise ANTXXV/3 in 1998 at 62°10.9' S/58°20.8' W, was established to exclude any population shifts or long-term effects by captivity. Handling and killing of the fish were conducted in line with the recommendations of the American Veterinary Medical Association. The work was approved by competent German authority [Freie Hansestadt Bremen, reference no. 522-27-11/02-00(93)].

Experimental setup and tissue collection. All animals were held in one large, recirculating tank (2.3 m³ total volume). Fish randomly chosen to be incubated at 5°C were moved to a separate tank and maintained in separate incubation groups (according to different exposure times) in swimming baskets. Control animals were exposed to the same handling procedures. For the study of thermal acclimation, animals were warmed at 1°C/h and exposed to 5°C for six different time periods (1 day, 2 days, 4 days, 7 days, 14 days, and 42 days). Two control groups were kept at 0°C: *group 1* was sampled on *day 0* and *group 2* after 43 days. Each group comprised between six and nine fish (see Table 4). The fish had a mean total body length of 23.51 \pm 3.63 cm (\pm SD) and a mean body weight of 46.53 \pm 24.79 g. One day before sampling, each animal was placed in a separate basket to minimize catching stress on the sampling day. For sampling, the fish were anesthetized by exposure to MS222 (0.2 mg/l) before being killed by cutting their spine as close to the cranium as possible. Tissue samples were quickly excised, frozen instantaneously in liquid nitrogen, and stored at -80°C until further processing.

Enzyme capacities. Functional capacities of key enzymes were determined in the same crude extract at the same day for all enzymes. Proteins were extracted from liver tissue following a method (modified from Ref. 36) by homogenizing frozen tissue in 10 volume ice-cold buffer (20 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 0.1% Triton X-100) with a glass homogenizer and in a second step with an Ultra Turrax. Cellular debris was removed by centrifugation for 10 min at 1,000 g and 0–2°C. The supernatant (crude extract) was transferred into a fresh tube without the upper lipid layer. Protein content was measured in the extracts according to Bradford (8), using BSA as standard. All enzyme measurements were conducted in a volume of 1 ml in a temperature-controlled spectrophotometer (Beckman, Fullerton, CA) at 5°C (all enzymes) and additionally at 0°C for COX and CS.

COX activity was determined according to a protocol modified from Moyes et al. (43) with 20–40 μ l of crude extract in 200 mM Tris-HCl containing 0.05% Tween 20 and 0.057 mM reduced cytochrome c (CytC) at pH 8.0. The decrease in absorbance at $\lambda = 550$ nm through oxidation of CytC ($\epsilon_{550} = 19.1$ mM/cm) was followed over time after adding the extract.

CS activity was determined following a modified protocol from Sidell et al. (56) using 20–40 μ l of homogenate in 75 mM Tris-HCl 0.25 mM DTNB, 0.4 mM acetoacetyl-CoA, and 0.5 mM oxaloacetate at pH 8.0. The reaction was started by adding oxaloacetate after thermal equilibration and stabilization of absorbance. The increase in absorbance at $\lambda = 412$ nm due to formation of the dye complex DTNB-S-CoA ($\epsilon_{412} = 13.6$ mM/cm) was measured over time.

Phosphoenolpyruvate carboxykinase (PEPCK) activity was determined after a protocol by Aas-Hansen et al. (1) using 30–60 μ l of homogenate with 0.05 mM acetoacetyl-CoA and 0.05 mM NADH in 50 mM imidazole at pH 7.4. Antimycin A, 2.5 μ g/ml, was added to prevent NADH oxidation through the respiratory chain. The reaction was initiated by adding NADH. The decrease in absorbance at $\lambda = 340$ nm due to oxidation of NADH ($\epsilon_{340} = 6.31$ mM/cm) was monitored over time.

Hydroxyacyl-CoA dehydrogenase (HADH, also known as HOAD) activity was determined according to McClelland et al. (42) using 30–60 μ l of homogenate in 0.1 mM acetoacetyl-CoA, 0.15 mM NADH, and 2.5 μ g/ml antimycin A in 50 mM imidazole/HCl at pH 7.2. After the reaction with homogenate was started, the decrease in absorbance was monitored over time at $\lambda = 340$ nm.

RNA extraction and cDNA synthesis. Total RNA was extracted from 20–40 mg tissue with the Qiagen RNeasy kit according to the manufacturer's instructions using the modified protocol with proteinase K digestion after homogenisation (Qiagen, Hilden, Germany). Quantity and purity of the RNA were determined using the NanoDrop ND 1000 (Peqlab Biotechnologie, Erlangen, Germany). Integrity of the RNA was analyzed in randomly chosen samples by capillary electrophoresis (bioanalyser; Agilent, Waldbronn, Germany). For cDNA synthesis 10 µg total RNA were exposed to 1 µl DNase (turbo DNase free kit, Applied Biosystems, Darmstadt, Germany). Then 0.4 µg of the DNase free RNA were transcribed into cDNA, utilizing the high-capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) in a 20 µl reaction.

Gene selection and primer design. Some of the investigated sequences were generated via reverse transcription, cloning, and sequencing (Table 1) according to a protocol described earlier (37). Primer design for cloning and the final sequences were generated with the MacVector software (version 10.0.2). Furthermore, 454 pyrosequencing (MWG-Biotech, Ebersberg, Germany) of a normalized cDNA library (Vertis, Freising, Germany) from liver and heart of *P. brachycephalum* under control conditions gave access to a number of further candidate genes (H. S. Windisch and M. Lucassen, unpublished observation). In this collection we selected probes for investigation of the genes relevant for lipid oxidation, glycolysis, gluconeogenesis, glycogen metabolism, and the pentosephosphate pathway. Sequences were identified using the NCBI Blast tool against the library with known sequences of related organisms. For the design of all real-time primers (Table 2) the Primer Express software (version 3.0; Applied Biosystems) was used.

Relative mRNA quantification via real-time PCR. Gene expression levels were determined by means of real-time PCR using the ABI 7500 qRT-PCR-system (Applied Biosystems). Primer concentrations were optimized to meet highest efficiency in the PCR reaction (Table 2). Each reaction contained 2 ng cDNA, the appropriate concentration of primers, and 0.5 volume of SYBR Green PCR

master mix (Applied Biosystems) in a 20 µl reaction. Baseline and the threshold cycle were always set to automatic in the sequence-detection software, version 1.3 (Applied Biosystems). All plates contained a no-template control, and each RNA sample was tested for DNA contaminations in RT-pools (without reverse transcription) before generation of cDNA.

Determination of reference genes and calculation of expression levels. Quantities for all genes and for each animal were calculated and used in the geNorm Excel sheet (45, 63) to find the most stable gene. β -actin (β -Act) and ubiquitin (Ubi) were identified to function as stable normalization genes with low M -values (relative expression stability; $M \leq 1.5$ for controls), namely with $M_{Act} = 0.54$; $M_{Ubi} = 0.59$. A normalization factor was calculated from both and quantities were recalculated for each gene under study.

Statistical analyses. Expression data were analyzed as normalized quantities with one-way ANOVA at a significance level of $P \leq 0.05$, followed by a Student-Newman-Keuls post hoc test using SigmaStat (version 3.5; Systat Software, Erkrath, Germany). For the enzyme measurements, the animals acclimated for 6 wk were additionally compared with controls via ANOVA alone to identify long-term effects rather than transient changes. Graphs of the time course of expression levels (see Fig. 3) and enzyme capacities (Fig. 1 and 2) were generated using SigmaPlot (version 10; Systat Software) showing means \pm SD.

To estimate the impact of transcription factors on metabolic gene expression we applied multivariate analyses [canonical correspondence analyses (CCA)] in the R environment (see Refs. 7 and 50), which is usually used to understand structures of ecological communities. The package generating a CCA [ade4 (16)] implements the duality diagram theory; here it was employed to clarify the relationships between different gene transcript abundances (variables) and the differences between individuals depending on temperature and exposure time (independent, independent variables). The levels of expression were also tested against each other to identify correlations in a linear model (see Table 5).

Table 1. Cloned genes with the related oligonucleotide

Gene	Design From	Primer Name	Sequence (5=3 \rightarrow)	Fragment Size
<i>NRF-1</i>	<i>Z. viviparus</i>	ZvNRF-F1 ZvNRF-B1	TGACGACACGGACATCCTCAAC AGAGACGGGTATGCCATCAATG	430
<i>NRF-1</i>	<i>Z. viviparus</i>	ZvNRF-F8 ZvNRF-B10	CGTGTTC AAGGTGTTGGTGC GGTTGGATTGTCATCTCGCC	719
<i>NRF-1</i>	<i>Z. viviparus</i>	ZvNRF-F16 ZvNRF-B12	CGTTGCGGACCATCGTAAAG CCAGACCTGTGCATCTTGAAC	584
<i>PGC1α</i>	<i>C. auratus</i>	CaPGC1a-F4 CaPGC1a-B4	GCTGCCTTGGTKGGYGAAGAC CCAGGGYTTCTGTRAGCATTGC	230
<i>PGC1α</i>	<i>C. auratus</i>	CaPGC1a-F4 CaPGC1a-B45	GCTGCCTTGGTKGGYGAAGAC GTGCGTTCARTGGTTTGTCTC	997
<i>PPAR1α</i>	<i>S. aurata</i>	SaPPARa-F1 SaPPARa-Bx	TCTTCCACTGCTGCCAGWGYAC TCAGTACATGTCCCTGTAGATYTC	590
<i>PPAR1α</i>	<i>S. aurata</i>	SaPPARa-F2 SaPPARa-B22	GTGGAGACGGTCACVGAGCTRAC GTCTGGGGAARAGGAAGGTGTC	430
<i>PPAR1β</i>	<i>S. salar</i>	SsPPAR1_b-HW-F1 SsPPAR1_b-HW-Bx	GCTTYGTRGAYCTTCTCAATG GTCTCYGACTCDGTCTTYTTGA	475
<i>PPAR1β</i>	<i>S. salar</i>	SsPPAR1_b-HW-F13 SsPPAR1_b-HW-Bx	GCTCACYGAGTTYGCCAAGWGC GTCTCYGACTCDGTCTTYTTGA	346
<i>COX4</i>	<i>Z. viviparus</i>	ZvCOX4_F1 Zv_COX4_B11	CMYTTGCTGTTGGWAAAC GCAARGAACWTAAGAGGAAGAGGC	636
<i>COX4</i>	<i>Z. viviparus</i>	ZvCOX4_F4 Zv_COX4_B11	GSRAGAGTGGAAATCTGTGGTTG GCAARGAACWTAAGAGGAAGAGGC	363
<i>GAPDH</i>	<i>Z. viviparus</i>	ZvGAPDH_F4 Zv_GAPDH_B17	GAAACCYGCCAAATAYGANGAC ACTCCATCTCAGACAACAGACAGG	485
<i>PPAR1γ</i>	<i>M. merluccius</i>	HW_MmPPAR1_g_F6 HW_MmPPAR1_g_Bx	ACAARGCCTCWGGGTTTCAYATG TCTTGCGGAACCTGTGCACTTC	664

Overview of genes cloned from *Pachycara brachycephalum* with the related primers for several fragments that were assembled after sequencing. Accession numbers for the full sequences can be found in Table 2.

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Table 2. Primer specification for real-time PCR

Gene	Accession No.	Primer Name	Primer Sequence (5=3=)	Slope	Efficiency
6PGL	HQ439154	Pb_6PGL_fw	TCTGTGACGAGCGAGTGGTT	-3.49	1.94
		Pb_6PGL_rev	TGACTCTGTACAGCCCGTAGGT		
ACAC	HQ439155	Pb_ACoACarb_fw	CATGTGGAGGATGAGGGAGAA	-3.36	1.99
		Pb_ACoACarb_rev	CAGCCCTTCATCTTTTCCAATG		
β -Act	AY227657	Pb_bAct_fw	GGAGAAGATGACCCAGATCATGTT	-3.49	1.93
		Pb_bAct_rev	AGTGGTACGACCAGAGGCATACA		
COX2	AY227659	Pb_COX2_fw	AGAGGCGGACCATCGAATAGTA	-3.39	1.97
		Pb_COX2_rev	CAGCGGAGACTAAGACTCGGATT		
COX4	HQ439156	Pb_COX4_fw	CTTTGACAGGCGGGAGAATC	-3.41	1.96
		Pb_COX4_rev	CTGGACTCAGGGTTGCACAT		
CPT-1	HQ439157	Zv_CPT_fw	ACTGGACCGAGCCAGATC	-3.35	1.99
		Zv_CPT_rev	AGGACCCGCCAGTCTTTG		
CS	AY382596	Pb_CiSy_fw	TGCACCCATGTCTCAGTTC	-3.38	1.98
		Pb_CiSy_rev	GCAAAGCTGCTCTCGCTGTT		
CytC	HQ439158	Pb_CytC_fw	GGCCGAGGGCTTCTCGTA	-3.51	1.93
		Pb_CytC_rev	GTGCCCTATCCACACAATA		
GAPDH	HQ439159	Pb_GAPDH_fw	TCCACAGACTCAACGGTGACT	-3.45	1.95
		Pb_GAPDH_rev	ATGCCAGCGCCAGCAT		
GYP	HQ439160	Pb_GYP_fw	ATGGATGCAAGAGGGTGAACA	-3.47	1.94
		Pb_GYP_rev	TCCGTTGACAGCGTGAGATC		
GYS	HQ439161	Pb_GYS_fw	GCGCTGGAAATGCTGACTTT	-3.47	1.94
		Pb_GYS_rev	CCCGCTCCTTGCAATGT		
HADH	HQ439162	Pb_HADH_fw	GGCTCGTCGACCAACTGTAG	-3.33	2.00
		Pb_HADH_rev	TATCGTTCTTCTCTGGACTTTC		
HBDH	HQ439153	Pb_HBDH_fw	GCCCACTTTGAGCACCTGAT	-3.33	2.00
		Pb_HBDH_rev	CCTTCTGTCGCTGTTGGA		
HIF1 α	DQ089691	Pb_HIF1a_fw	AAACACCGAGCGCAGCTT	-3.34	1.99
		Pb_HIF1a_rev	GGCCCTGTGGTGAGA		
MCAD	HQ439163	Pb_ACoDd_fw	GCATGGGAGCTTGGTCTGAT	-3.40	1.97
		Pb_ACoDd_rev	CAAGCCAGTCCACCATAGTC		
NRF-1	HQ439164	Pb_NRF1_fw	GCCATCGTGCTGTGCATCT	-4.25	1.72
		Pb_NRF1_rev	GGCACCAAACACCTTGAACA		
PEPCK	HQ439165	Pb_PEPCK_fw	CCAACTGGCCATGATGAAA	-3.30	2.01
		Pb_PEPCK_rev	TGTCGTCGCCACACACT		
PGC1a	HQ439166	Pb_PGC1a_fw	TCTGGCACCCGAAACTC	-3.37	1.98
		Pb_PGC1a_rev	TGTGCCTTGCCACCTGTGTA		
PK	HQ439167	Pb_PK_fw	TGGAGCATGGAGTCGACATG	-3.74	1.85
		Pb_PK_rev	CGTGGACGCTGCTGCTTT		
PPAR1 α	HQ439168	Pb_PPAR1a_fw	CCAATCACCCCGACAACCTCT	-3.41	1.97
		Pb_PPAR1a_rev	CCGGAGGTGACGCCAGTTTC		
PPAR1 β	HQ439169	Pb_PPAR1b_fw	CATGAGGCTATATTGGCCATGCT	-3.37	1.98
		Pb_PPAR1b_rev	TTGGCCACCAACAGTCCAT		
PPAR1 γ	HQ439170	Pb_PPAR1_g_fw	TGTCACACAACGCCATTCTG	-3.38	1.98
		Pb_PPAR1_g_rev	TCAGCCAGCAGTTTCTCTCTCT		
SCAD	HQ439171	Pb_ACoDu_fw	GGCCTGCATGGCGAAA	-3.40	1.97
		Pb_ACoDu_rev	TCGATGCATTAGATGTGGTTAGG		
TAG L	HQ439172	Zv_TAGL_fw	TCCCACCGCTCTGTTCTCA	-3.38	1.98
		Zv_TAGL_rev	AGGACCCGACGTCTTTG		
Ubi	HQ439173	Pb_Ubi_fw	GGGAAGACCATCACCCTTGA	-3.42	1.96
		Pb_Ubi_rev	GATCTTTGCCTTGACATTTTCGAT		
UCP2	AY625190	Pb_UCP2_fw	CGCCTCAGTCCGCATTG	-3.54	1.92
		Pb_UCP2_rev	TGATCAGAGCCTTTGGTGTAGAACT		

Primer list of all genes analyzed by real-time PCR. Primer conditions have been optimized by determining the slopes over at least 4 orders of magnitude and calculation of efficiency.

RESULTS

Enzyme activities. Capacities of key enzymes were investigated to assess functional shifts in metabolism. Total protein content of all extracts, including the three control groups was 42.9 ± 10.5 mg protein/g fresh wt, compared with 39.9 ± 9.0 mg protein/g fresh wt in incubated animals, and did not change significantly over the time course of incubation at 5°C. The activity of COX (Fig. 1A) was considered as a marker for the capacity of the respiratory chain. When assessed per gram fresh weight, a continuous increase to ~90% above control rates occurred within 7 days. Activity remained elevated there-

after until the end of the incubation period ($P = 0.014$). Protein-specific capacities (Fig. 1C) rose significantly to 80% above controls ($P = 0.004$). Quantifying enzyme capacity for the three control groups at 0°C and 5°C assay temperature resulted in a Q_{10} value of 1.21 for COX (Table 3).

The activity of the TCA cycle enzyme CS per unit fresh weight decreased from the onset of warm acclimation (Fig. 1B). Activity fell by ~29% during the first day and recovered slightly within 6 wk to a level 20% below controls. A different picture developed for protein-specific CS activities (Fig. 1D). While the course of activity was similar to the loss of fresh

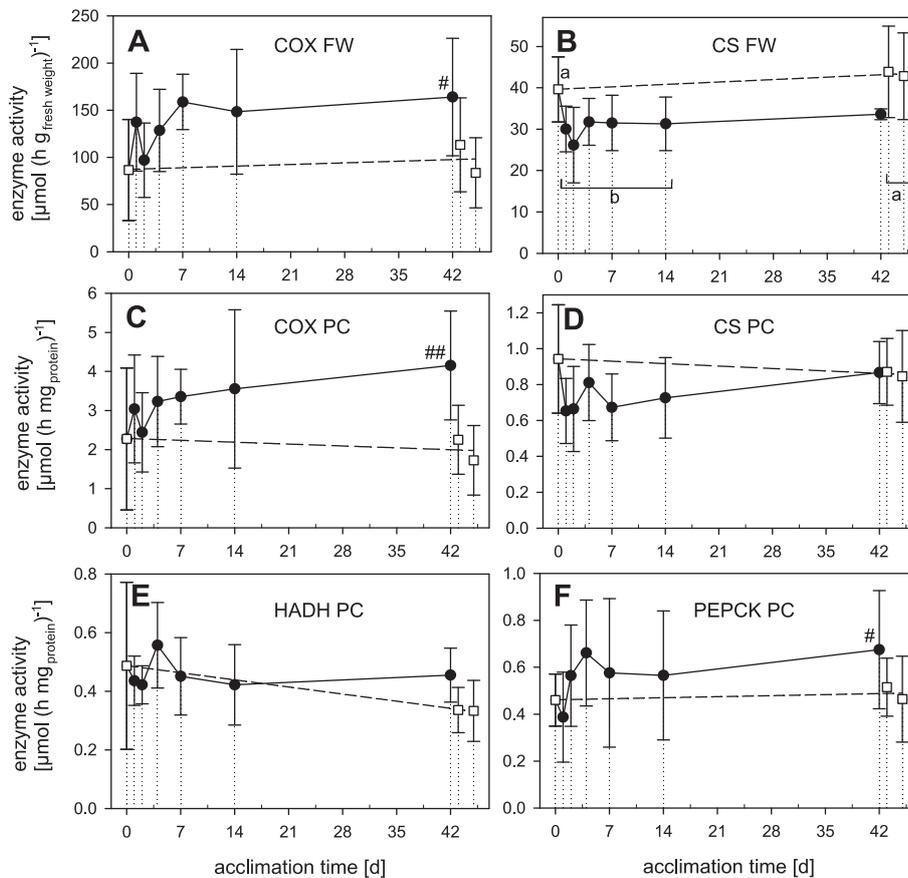


Fig. 1. Enzyme activities determined at 5°C under control conditions and over time during acclimation to 5°C. White squares represent controls at 0°C, filled circles are groups incubated at 5°C, the dashed line is obtained by fitting control data by linear regression, and the solid line connects the values (mean \pm SD; $n = 6-9$), including the start point of the 0°C control. Shown are cytochrome *c* oxidase (COX; A and C); citrate synthase (CS; B and D); hydroxyacyl-CoA dehydrogenase (HADH; E); and phosphoenolpyruvate carboxykinase (PEPCK) dehydrogenase (F). FW, activity in relation to fresh weight; PC, activity in relation to protein content. Letters indicate a statistically significant difference ($P < 0.05$). Significant differences between the data obtained after 6 wk of incubation and all controls are labeled with # ($P < 0.05$) or ## ($P < 0.005$).

weight-specific activity, no significant difference could be detected. A Q_{10} value of 1.96 was calculated for the control animals (Table 3).

As CS constitutes a central crossing point for different pathways and balances oxidative and biosynthetic pathways, normalization of enzymatic capacities to CS is useful to detect relative metabolic adjustments. The ratio of COX to CS activities showed a strong shift in the balance of metabolic pathways in liver mitochondria (Fig. 2A). The ratio doubled from 2.23 ± 1.30 to 4.43 ± 1.18 (day 1, $P < 0.001$), and reached a maximum after 1 wk at 5.13 ± 0.82 , remaining constant until the end of experimentation (4.85 ± 1.71).

HADH/CS activities may reflect a shift from lipid synthesis to catabolization (β -oxidation) during warm exposure (37, 47). While HADH activity remained constant over time (Fig. 1E), the ratio showed a significant increase during the first week,

from 0.49 ± 0.16 (control 1) to 0.72 ± 0.30 (day 7, $P = 0.032$) of acclimation (Fig. 2C). PEPCK activity (per milligram protein) as a marker for gluconeogenesis increased toward the end of warm exposure by $\sim 40\%$ above controls ($P = 0.031$) (Fig. 1F). PEPCK and CS are competing for the same substrate, oxaloacetate, and the ratio of PEPCK/CS seems useful to track the balance between lipid and carbohydrate anabolism. PEPCK/CS activities (Fig. 2B) increased significantly on the second day 0.89 ± 0.30 ($P = 0.048$), and the ratio was elevated in the end (0.78 ± 0.28), compared with controls (0.56 ± 0.15 , $P = 0.026$).

Expression profiles during warm acclimation. For assessing the regulation of the observed functional shifts we analyzed the mRNA expression levels of 24 genes of interest, grouped into four categories according to the respective pathways (Table 4). Overall, the extractable total RNA of all samples did not change significantly in liver tissue upon warm acclimation with means of 2.54 ± 0.91 $\mu\text{g}/\text{mg}$ (mean \pm SD) fresh water.

In the subset of transcription factors potentially influencing mitochondrial functioning, the expressions of all PPARs showed significant changes during the course of incubation (Table 4). The transcript levels of PPAR1 α dropped from the first day onward and remained at 40% of the initial levels (Fig. 3A). Conversely, PPAR1 β expression increased 1.8-fold (Fig. 3C). Both reached their maximum change from control levels on day 7 and stabilized below (PPAR1 α) or above (PPAR1 β) controls after 14 days. In contrast, PPAR1 γ expression in-

Table 3. Effect of temperature on enzyme activity

Enzyme and Assay Temperature	Means of Enzyme Activity per Protein [$\mu\text{mol} (\text{h mg}^{-1}) \pm \text{SD}$]	<i>P</i> Value	Means Q_{10}
CS 0°C	0.642 ± 0.195	<0.001	1.961
CS 5°C	0.882 ± 0.240		
COX 0°C	1.926 ± 1.165	0.672	1.214
COX 5°C	2.068 ± 1.190		

Means of Q_{10} values for citrate synthase (CS) and cytochrome *c* oxidase (COX) resulting from protein-specific activities were measured at 0°C and 5°C in liver extracts from control animals.

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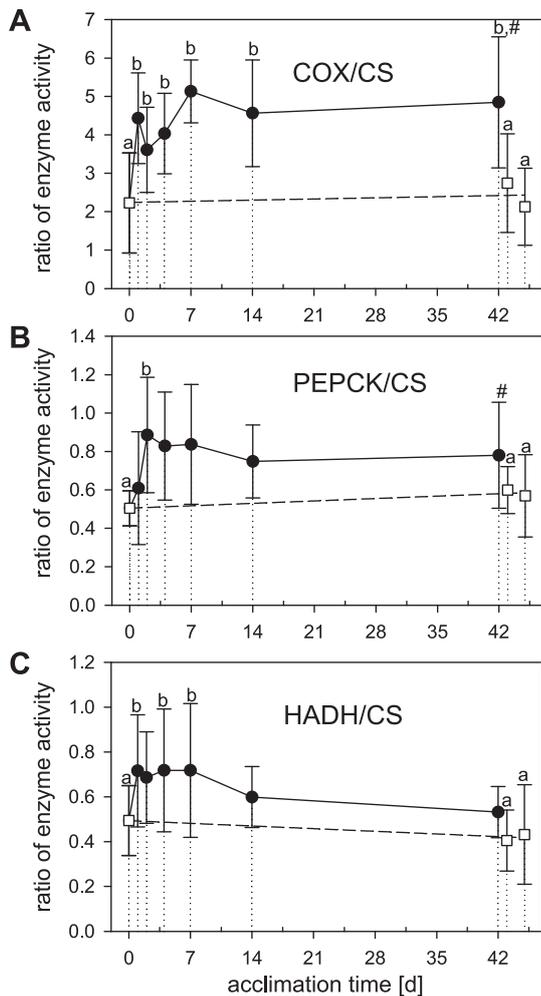


Fig. 2. Activity ratios of the enzymes COX (A), PEPCK (B), and HADH (C) over citrate synthase (CS) during acclimation to 5°C. Values are means \pm SD ($n = 6-9$) labeled with different letters to show significant differences ($P < 0.05$) according to one-way ANOVA. #Significant differences between the data obtained after 6 wk of incubation and all controls. White rectangles are 0°C controls, filled circles represent groups incubated at 5°C, the dashed line is the best fit of control data.

creased progressively, reaching 1.9-fold higher mRNA levels after 6 wk (Fig. 3D). NRF-1 levels decreased significantly and reached a minimum after 2 days of warming (Fig. 3B). Subsequently, expression rose and stabilized at a level 20% below controls. The expression levels of hypoxia inducible factor (HIF)1 α , which was monitored as an indicator of possible hypoxic stress in fish upon warming (48, 52), and of the coactivator PGC1 α did not change significantly over time.

Two subunits of COX were examined as markers of the respiratory chain COX 2, a subunit encoded in the mitochondria, and COX 4, a nuclear-encoded subunit of the enzyme. While COX 2 displayed constant expression, except for a transient drop to 40% on day 2, the expression of COX 4 decreased progressively leading to a long-term reduction of mRNA levels to 80% of controls after 6 wk. More drastic decrements were found for CytC, the transporter of electrons between complexes III and IV in the respiratory chain. After an

initial drop, levels stabilized at \sim 60% after 2 wk. The UCP-2 gene, which also characterizes mitochondrial membrane function, was apparently upregulated by a factor of 1.63, similar to previous findings (40), but this trend was not significant in the present study.

Expression levels of genes associated with lipid metabolism were significantly affected by warm acclimation (Table 4). Transcript levels of nearly all enzymes fell to 60–80% of control levels at 0°C, except for those of carnitine palmitoyl transferase-1 (CPT-1) and 3D-hydroxybutyrate dehydrogenase (3HBDH). As a gateway to lipid oxidation, triacylglycerate lipase (TAGL) catalyses the cleavage of triacylglycerides within the cytoplasm. TAGL mRNA levels decreased over time and leveled off at \sim 80% of control levels. Fatty acids are then channeled into mitochondria by CPT-1. The expression of the shuttle-protein transiently increased threefold between 7 and 14 days and returned to control levels within 6 wk. Fatty acids are processed further by acetyl-CoA dehydrogenases (SCAD and MCAD) catalyzing the fragmentation of different size classes of fatty acids. The expression of both short- and medium-chain dehydrogenases (SCAD and MCAD) decreased to \sim 75% of control levels. The step following hydration, catalyzed by HADH experienced a decrease in expression by half after 1 wk, followed by a recovery to 85% of control levels within 6 wk. 3HBDH is thought to balance the concentration of different ketone bodies according to oxidative status (NAD⁺/NADH ratio). Transcript levels of this enzyme rose twofold within 2 days and returned to control levels thereafter. The expression of acetyl-CoA carboxykinase (ACAC), which catalyses the initial step of lipid biosynthesis, fluctuated and stabilized finally at 60% of control levels.

Transcript amounts of CS, the initial enzyme of TCA, were reduced to 30% after 7 days and remained at 45% after 6 wk. In the subassembly of carbohydrate metabolism we traced six genes representing various pathways. The expression of glycolytic GAPDH remained unchanged, while pyruvate kinase (PK) expression decreased transiently to \sim 66% of control levels after 4 days and recovered to 90% thereafter. The expression of PEPCK, which catalyses the first step in gluconeogenesis, responded by increasing threefold over 4 to 7 days, but fell strongly to 70% of control levels after 6 wk. For the pentose phosphate-pathway 6-phosphogluconolactonase (6PGL) expression fell significantly to 60% of control levels within 1 wk. Thereafter, expression rose slowly and almost reached control mRNA levels after 6 wk.

Among two genes representing the metabolism of glycogen, glycogen phosphorylase catalyses the initial step in the degradation. The glycogen phosphorylase transcript peaked at 1.6-fold higher levels after 2 days, returning close to control levels during the remaining exposure time. In contrast, the transcript of glycogen synthase (GYS) increased rapidly and 4.8-fold during the first 2 days. Expression returned to control levels during the following acclimation period, but finally rose again and peaked at mRNA levels sixfold higher than controls.

A number of genes, especially all PPARs, as well as NRF-1, CytC, and CS, and five genes associated with lipid metabolism (TAGL, HADH, SCAD, MCAD, ACAC) displayed strong alternations in expression levels over time, predominantly during the first 4 days of acclimation. Finally, we observed that continued animal exposure to control conditions also had some

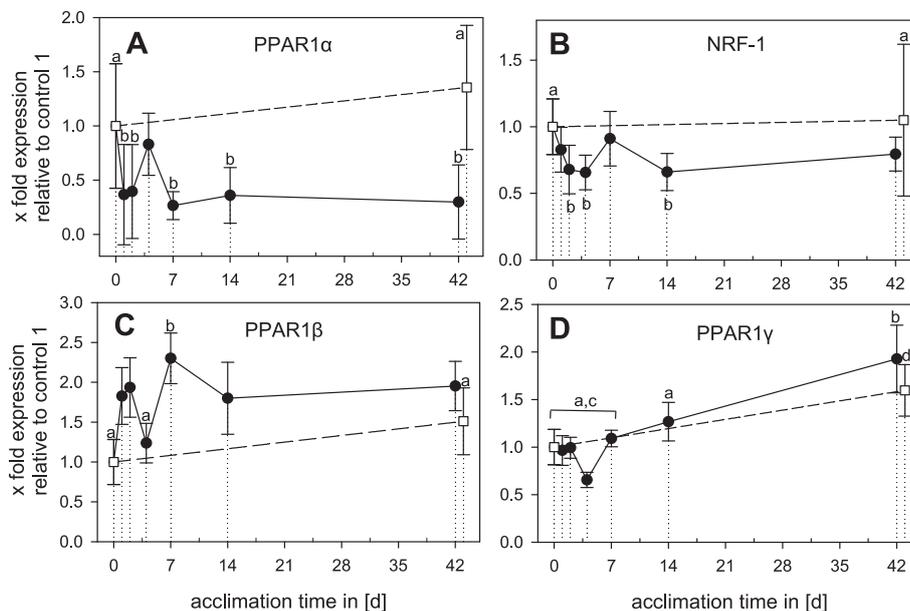


Fig. 3. Expression of transcription factors over the time course of acclimation to 5°C. Values are means \pm SD ($n = 6-9$), labeled with different letters to show significant differences according to one-way ANOVA ($P < 0.05$). White rectangles represent 0°C controls, filled circles are groups at 5°C, the dashed line is the best fit of controls. Shown are peroxisome proliferator-activated receptor 1 α (PPAR1 α ; A); nuclear respiratory factor-1 (NRF-1; B); PPAR1 β (C); and PPAR1 γ (D).

effect, seen in elevated levels of PPAR1 γ , COX4, CPT-1, and PK expression in control group 2.

Canonical correspondence of the RNA levels. In CCA (7) correlations between independent (usually environmental factors) and some dependent variables (usually species counts in ecological research) are plotted in a coordinate system of reduced dimension. In this study, CCA is applied to provide an overview of net effects of incubation time and temperature (as independent factors) on the RNA levels of various genes of interest (as dependent species counts, Fig. 4A). The position of each gene in relation to the center of the plot reflects the relative correlation with the variables temperature and acclimation time and defines the space of independent variables.

Temperature is more important for the ordination than incubation time, as indicated by the relative lengths of the arrows. The ordination along the variable time defines the velocity of a response to temperature, whether it is immediate, transient, or long term. The expression levels of GYS and PEPCK display the strongest correlations in the plot. Since PEPCK showed maximal transcription early on at 5°C (Table 4) and no long-term changes, expression is distributed more along the temperature than the incubation axis. The correlations of GYS also reflect the temperature effect, in addition to a strong offset by incubation time (see Table 4).

All ordinations of transcription factors constitute a frame for the remaining genes due to the more pronounced correlations detected. From the very beginning, PPAR1 α transcripts were significantly decreased and remained so for up to 6 wk (see Table 4). In the CCA they are found in the opposite direction of each of the independent variables spanning the plot as a result of the negative correlation of the two factors, time and temperature, and the respective mRNA levels. PPAR1 β is located in the opposite direction of PPAR1 α , indicating a contrasting role for both transcription factors (see Table 4). PPAR1 γ is positioned in the plot as a transcription factor that is mostly influenced by the factor of time. HIF1 α and PGC1 α

remained marginally unaffected (Table 4) but were shifted along the incubation axis, due to higher expression levels of the second control group. Most of the other investigated genes display a negative correlation along the vectors of both variables, indicating a decrease in transcript levels during incubation at elevated temperature.

Within this coordinate system of transcript level correlations it is possible to position each sample (animal) with the information of all 26 genes (including the two housekeeping genes) as affected by the treatments in a second plot (Fig. 4B, sample plot). The samples (animals) are given as dots and assigned by arrows pointing to different cluster-centers built from the means of the eight different groups of incubation times versus control. Only three main clusters are clearly resolved. Both controls fall into one cluster (*sector I*, Fig. 4B), showing steady-state expression levels according to maintained ambient conditions. The largest cluster, representing the short-term response, is mainly located in *sectors II* and *III*, reflecting the relative influence of both variables, temperature and time. Here, the time resolution between 1 day and 2 wk is lost, because of the scattering between individuals. The third cluster in *sector IV* is formed by the group acclimated for 6 wk with a respective shift along the time and temperature axes (but also comprising scattering according to variations between individuals). Altogether, the CCA visually separates the individuals into a control group, a group representing the short-term responses, and a third group displaying the long-term adjustments. The overlay of species and sample plot emphasizes the coordination between transcript-species and animal (cluster) so that genes can be classified according to their short-term and long-term importance.

DISCUSSION

Thermal acclimation in the metabolic pathways of ectothermic organisms supports the maintenance of their functional integrity and scope under changing environmental conditions.

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Table 4. Expression of various genes over time during acclimation (exposure to 5°C)

Gene	C1, n = 8	1 day, n = 8	2 days, n = 7	4 days, n = 7	7 days, n = 7	14 days, n = 7	42 days, n = 6	C2, n = 9	P Values
<i>Transcription factors</i>									
<i>PPAR1α</i>	1.00 ^a ± 0.57	0.37 ^b ± 0.46	0.40 ^b ± 0.43	0.83 ± 0.29	0.27 ^b ± 0.13	0.36 ^b ± 0.26	0.30 ^b ± 0.34	1.36 ^a ± 0.57	<0.001
<i>PPAR1β</i>	1.00 ^a ± 0.28	1.83 ± 0.36	1.93 ± 0.37	1.24 ^a ± 0.25	2.30 ^b ± 0.32	1.80 ± 0.45	1.95 ± 0.31	1.51 ^a ± 0.42	0.006
<i>PPAR1γ</i>	1.00 ^{a,c} ± 0.19	0.96 ^{a,c} ± 0.16	0.99 ^{a,c} ± 0.11	0.65 ^{a,c} ± 0.08	1.09 ^{a,c} ± 0.09	1.27 ^a ± 0.20	1.93 ^b ± 0.36	1.60 ^d ± 0.27	<0.001
<i>PGC1α</i>	1.00 ± 0.15	1.68 ± 0.29	1.88 ± 0.36	2.15 ± 0.19	0.86 ± 0.08	1.64 ± 0.28	2.83 ± 1.51	2.64 ± 0.37	0.214
<i>NRF-1</i>	1.00 ^a ± 0.21	0.83 ± 0.17	0.68 ^b ± 0.18	0.66 ^b ± 0.13	0.91 ± 0.21	0.66 ^b ± 0.14	0.79 ± 0.13	1.05 ^a ± 0.57	0.001
<i>HIF</i>	1.00 ± 0.13	1.05 ± 0.06	1.51 ± 0.07	1.13 ± 0.05	1.13 ± 0.06	1.38 ± 0.14	2.02 ± 0.05	1.29 ± 0.05	0.334
<i>Respiratory chain</i>									
<i>COX 2</i>	1.00 ± 0.60	0.79 ± 0.52	0.38 ± 0.30	1.12 ± 0.22	1.14 ± 0.16	0.99 ± 0.34	1.07 ± 0.75	1.18 ± 0.26	0.074
<i>COX 4</i>	1.00 ± 0.30	1.06 ± 0.24	0.82 ± 0.19	0.95 ± 0.29	0.85 ± 0.19	0.86 ± 0.29	0.78 ± 0.13	1.37* ± 0.40	<0.001
<i>CytC</i>	1.00 ^a ± 0.27	0.71 ± 0.18	0.28 ^b ± 0.07	1.01 ^a ± 0.31	0.33 ^b ± 0.07	0.59 ± 0.12	0.64 ± 0.16	1.13 ^a ± 0.47	0.001
<i>UCP2</i>	1.00 ± 0.28	1.38 ± 0.45	1.42 ± 0.58	0.79 ± 0.31	1.62 ± 0.36	1.15 ± 0.46	1.63 ± 0.66	2.33 ± 1.20	0.330
<i>Lipid metabolism</i>									
<i>TAGL</i>	1.00 ± 0.25	1.09 ± 0.24	0.87 ^a ± 0.39	1.21 ^c ± 0.21	0.68 ^{a,d} ± 0.18	0.92 ^a ± 0.21	0.83 ^a ± 0.18	1.33 ^b ± 0.32	<0.001
<i>CPT-1</i>	1.00 ± 0.05	1.15 ± 0.09	1.08 ± 0.04	1.77 ± 0.05	3.07 ± 0.14	3.08 ± 0.46	1.41 ± 0.09	1.93 ± 0.11	0.023
<i>HADH</i>	1.00 ± 0.26	0.97 ± 0.17	0.80 ± 0.32	1.06 ± 0.23	0.54 ± 0.18	0.67 ± 0.20	0.85 ± 0.34	1.48* ± 0.36	<0.001
<i>SCAD</i>	1.00 ± 0.35	0.87 ^a ± 0.23	0.72 ^a ± 0.21	0.94 ± 0.23	0.93 ± 0.26	0.80 ^a ± 0.23	0.77 ^a ± 0.36	1.20 ^b ± 0.29	0.006
<i>MCAD</i>	1.00 ^a ± 0.29	0.89 ^a ± 0.30	0.84 ^a ± 0.32	1.40 ± 0.16	0.88 ^a ± 0.23	0.86 ^a ± 0.20	0.79 ^a ± 0.31	1.73 ^b ± 0.34	<0.001
<i>3HBDH</i>	1.00 ^a ± 0.19	1.33 ^a ± 0.42	1.97 ^b ± 0.26	1.45 ± 0.11	1.27 ^a ± 0.34	1.22 ^a ± 0.18	1.42 ^a ± 0.36	1.48 ± 0.30	0.005
<i>ACAC</i>	1.00 ^a ± 0.40	0.84 ^a ± 0.33	0.57 ^a ± 0.36	1.43 ± 0.21	0.46 ^a ± 0.13	1.27 ± 0.40	0.61 ^a ± 0.37	1.96 ^b ± 0.52	<0.001
<i>Citric acid cycle</i>									
<i>CS</i>	1.00 ^a ± 0.46	0.86 ^a ± 0.20	0.74 ± 0.38	1.11 ^c ± 0.36	0.29 ^{b,d} ± 0.06	0.45 ^d ± 0.16	0.45 ^d ± 0.05	0.99 ^a ± 0.36	<0.001
<i>Carbohydrate metabolism</i>									
<i>GAPDH</i>	1.00 ± 0.49	0.87 ± 0.29	1.10 ± 0.20	1.39 ± 0.22	1.16 ± 0.33	1.34 ± 0.49	1.16 ± 0.33	1.08 ± 0.30	0.329
<i>PK</i>	1.00 ± 0.29	0.91 ± 0.26	0.77 ± 0.17	0.66 ± 0.17	0.70 ± 0.13	0.89 ± 0.31	0.94 ± 0.34	1.52** ± 0.22	<0.001
<i>PEPCK</i>	1.00 ± 0.23	1.50 ± 0.35	1.08 ± 0.32	2.99 ± 0.52	3.08 ± 0.39	1.89 ± 0.22	0.74 ± 0.19	2.32 ± 0.42	0.149
<i>6PGL</i>	1.00 ± 0.37	1.01 ± 0.35	0.97 ± 0.33	1.12 ^a ± 0.23	0.60 ^{b,c} ± 0.09	0.83 ^c ± 0.19	0.93 ± 0.35	1.28 ^d ± 0.24	0.002
<i>GYP</i>	1.00 ± 0.19	1.13 ± 0.37	1.64 ± 0.30	1.15 ± 0.14	0.96 ± 0.26	1.24 ± 0.20	1.18 ± 0.36	1.26 ± 0.24	0.172
<i>GYS</i>	1.00 ^{a,c} ± 0.10	3.02 ^c ± 0.31	4.80 ^b ± 0.25	1.15 ^{a,c} ± 0.03	0.88 ^{a,c} ± 0.05	1.06 ^{a,c} ± 0.04	6.09 ^d ± 0.58	0.88 ^{a,c} ± 0.06	<0.001

Means of the expression values (quantities relative to control 1 group) in the different exposed groups. P values show significant difference between groups using ANOVA at a significance level of $P \leq 0.05$, C1 and C2 are control groups 1 and 2. Letters show significant differences based on the Student-Newman-Keuls post hoc tests. * $P < 0.05$; ** $P < 0.005$.

Recent studies have shown that the Antarctic eelpout *P. brachycephalum* displays a limited ability to acclimate to higher temperatures (11, 33). The present study demonstrates that some elements of the warm acclimation response in this species clearly differ from patterns that might be expected in analogy to a simple reversion of cold acclimation phenomena described in temperate zone fishes.

Under optimal food conditions maximized growth at 4°C indicates good whole animal performance of the Antarctic eelpout in the warmth. Alterations in fuel stores of the liver (11) suggest that the liver is actively involved in the acclimation process and that metabolic adjustments occur. We therefore aimed to unravel the alterations in metabolic pathways and the underlying molecular networks leading to a new steady state after acclimation.

Before doing so, it is necessary to distinguish the effects of an uncompensated stress response from mechanisms effective in acclimation. In the notothenioid *T. bernacchii*, temperature exposure to 3.8°C led to an acclimation response without signs of stress, as serum cortisol and hematocrit levels remained constant (24). The same species and *H. antarcticus* displayed transcriptomic responses of genes like HIF1 α , 6PGL, and Ubi detected in microarray-based studies of heat exposure (13, 61) and indicating involvement of hypoxia, oxidative stress, and other cellular stress responses. For the Antarctic eelpout, func-

tional hypoxemia during acute exposure to 5°C can be excluded (39). Although some stress response at the transcriptomic level may occur in the first hours, the stable expression profile of HIF1 α , 6PGL, and Ubi indicates that acclimation of the Antarctic eelpout to 5°C does not involve a stress response, a finding in line with the earlier observation of maximized growth and, thus, sustained fitness during warm acclimation.

Thermal impact on metabolic pathways. During warm exposure, the fastest response included functional modifications with significantly decreased activities of CS and elevated activities of COX. This indicates a change in respiratory capacity (through COX) as well as a change in the multiple functions of CS. The activity of CS in control animals assayed at 0°C was similar to that determined at 5°C in warm-acclimated animals, indicating perfect temperature compensation. The acute Q_{10} value of 1.96 (Table 3) was close to the value of 1.6 determined earlier in the same species (33). The slightly smaller Q_{10} value might be caused by assay temperatures higher than acclimation temperature, which may reduce the slope in the respective Arrhenius plot. CS activity in relation to protein content dropped in the beginning, but reached the original rate after 6 wk, indicating long-term modifications (Fig. 1D). Similarly, the mRNA level of CS was not tightly correlated with enzyme activity, as CS expression remained unchanged in the beginning of warm acclimation, while CS

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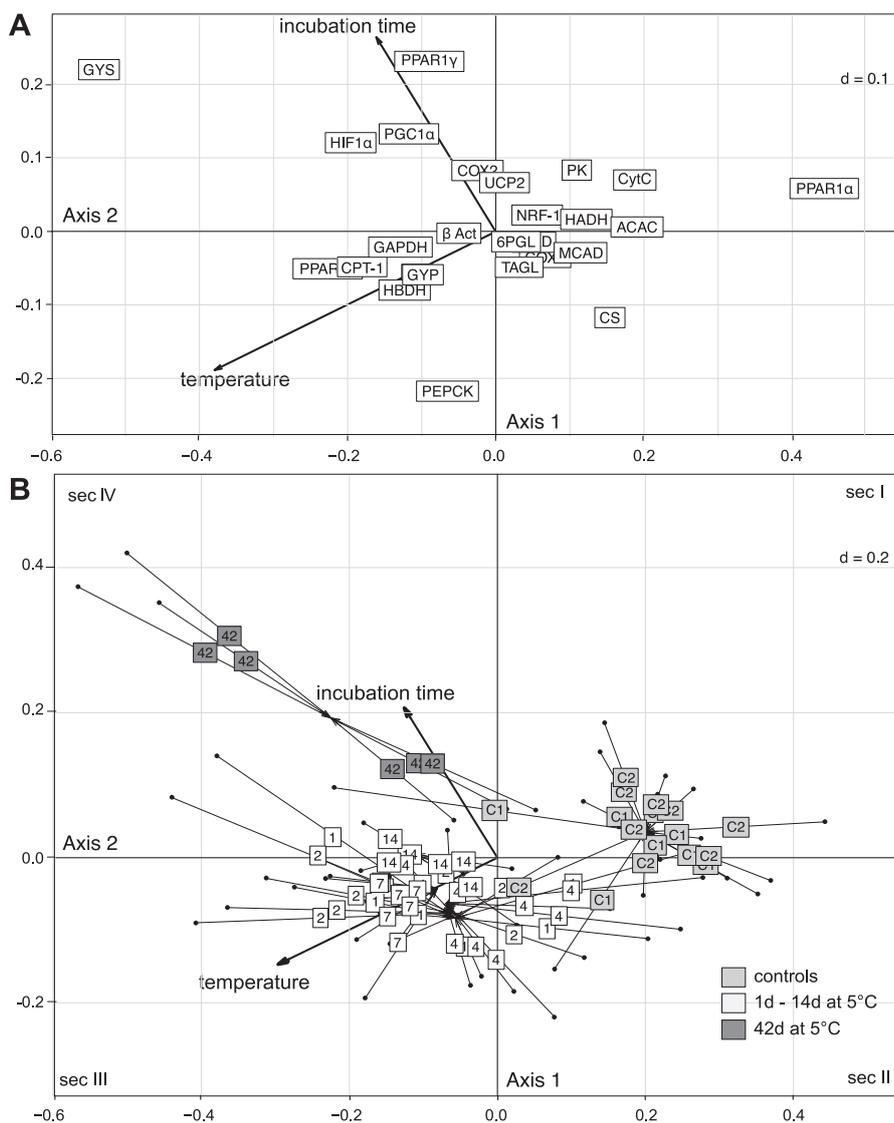


Fig. 4. Canonical correspondence analyses (CCA; ade4) including all animals. *A*: species plot. Map of gene distribution (dependent variables). Boxes show the changes of the gene expression shifted from the center along the factors time and temperature, dependent on the distribution of the measured samples. *B*: sample plot. Each shaded rectangle labels 1 animal with the chronologic sample number. Values of the animals are shown as a dot with a thin arrow pointing to the center of respecting group means. The dots are positioned on a matrix of all 26 investigated genes in a 2D plot. The arrows representing incubation and temperature are the independent variables defining the matrix. For identification purposes the graph is subdivided into 4 sectors (*sec I*: $x, y > 0$; *sec II*: $x > 0, y < 0$; *sec III*: $x < 0, y < 0$; *sec IV*: $x < 0, y > 0$). C1 and C2 are control groups 1 and 2.

activity was immediately reduced. The low expression levels (Table 4) together with improved protein-specific activities at the end of the acclimation period (Fig. 1B) indicate a long-term rise in translation efficiency. The findings on a fresh weight basis reflect a well-controlled reduction in the provision of intermediates from the TCA cycle, e.g., to lipid anabolism upon warming.

COX showed a continuous rise in activity from the beginning, which leveled off over time. An acute Q_{10} value of 1.21 (Table 3), similar to an earlier study with a $Q_{10} = 1.4$ (22), indicates a lower temperature dependence of activity than for CS. The gain in activity on top of the Q_{10} effect suggests that regulation may occur through higher translational efficiencies rather than through protein modifications.

CS constitutes a central crossing point for different pathways and represents the entrance of acetyl-CoA for final oxidation of carbohydrates and fatty acids, but it is also important for lipid syntheses, as excess acetyl-CoA from pyruvate oxidation (carbohydrates) is shuttled via citrate and malate from the mito-

chondrial matrix to the cytoplasm. Thus it balances oxidative and biosynthetic pathways, and normalization to CS seems appropriate to detect significant shifts in this balance. The increase in the COX-to-CS ratio (Fig. 2A) indicates a reduction in lipid biosynthesis relative to oxidative pathways. Comparable cooling experiments in eurythermal species led to a somewhat slower response for the induction of CS (37), so the fast response in *P. brachycephalum* visible in the COX-to-CS ratio is most likely due to the stimulating effect of warming on a cold-adapted species.

Further mitochondrial adjustments were found for HADH, with a lower expression (Table 4) at similar temperature-specific activities (Fig. 1E). This indicates a higher efficiency of translation or an enhanced half-life of transcripts in the warmth. Again, the latter would rather be expected in the cold and may explain why in *Danio rerio* (41) enzyme activities were higher at constant mRNA levels. The constancy of HADH activity may have supported the reduction of the lipid fraction described earlier (11). This may also involve reduced

lipid biosynthesis as indicated by the elevated ratio of HADH/CS activities (Fig. 2C) and the reduction of mRNA levels of ACAC, the key enzyme complex for biosynthesis (Table 4).

Both PEPCK and CS compete for the same substrate oxaloacetate, and their rising activity ratio (Fig. 2B) as well as elevated PEPCK activities (Fig. 1F) indicate an activation of gluconeogenesis during warm acclimation, associated with enhanced efficiency of the translation of PEPCK-mRNA.

The warm-induced increase in COX activity contrasts the general picture of cold-induced mitochondrial proliferation and its reversal in the warmth described for muscle tissue of many temperate species (26). In liver of eurythermal Atlantic cod and common eelpout this picture is modified but confirmed by rising CS at maintained COX activities after long-term cold acclimation (36, 37). An upregulation of aerobic capacity in warm-acclimated Antarctic eelpout was already indicated by an upregulation of UCP-2 expression in both liver and muscle (40), which contrasts the findings in temperate eelpout where rising mitochondrial capacities are mirrored in elevated UCP-2 transcripts and protein content in the cold. Together, rising mitochondrial capacities support improved whole animal performance (11), and may confirm the avoidance of early oxygen limitation upon warming in the whole animal in this range of temperatures.

Transcriptomic responses of functional genes. The emerging picture of cellular adjustments to the warmth becomes more integrative by taking a holistic view of the observed changes. For genes involved in the β -oxidation of lipids, warm acclimation caused transcript levels to decrease to $\sim 80\%$ of control levels, while enzyme capacities remained constant or even increased toward the end of the experiment, such as in case of HADH. Together with the observed drop in lipid levels and the threefold activation of the transcription levels of CPT-1 (Table 4) these findings indicate a higher demand for the transport of long-chain fatty acids into mitochondria for oxidation and a shift in lipid metabolism toward oxidation. The rising ratio of HADH over CS activities as well as the increased expression level of 3HBDH, involved in maintaining the pool of NAD^+ during mobilization of lipid stores in liver also support this conclusion.

The concomitant activation of gluconeogenesis during warm acclimation, is indicated by a reduction of PK, paralleled by higher PEPCK transcripts. A reduction of PK at most likely high ATP levels, seems reasonable at a functional level, followed by adjustment of mRNA amounts. The recovery of PK transcripts may then follow after the depletion of fatty acids and during the metabolic shift to carbohydrate fuels. Elevated PEPCK message (see CCA, Fig. 4A), activities (Fig. 1F) and rising PEPCK-to-CS ratios (Fig. 2B) would suggest a reorganization of high-energy stores to glucose and later glycogen. The putative shift to glycogen anabolism is supported by the sixfold higher expression levels of GYS after 6 wk. In the CCA it shows the largest deflection along the variables (Fig. 4A), in perfect agreement with the increased carbohydrate composition of liver after long-term warm acclimation (11).

For most of the genes with expression levels falling below those in controls, a trend to recover could be seen in the midphase of acclimation (*days 4 to 7*). Such oscillations in transcript levels have already been noticed in other time course studies, not only during thermal acclimation (37), but also

during salinity transfer or hypercapnic exposure (14, 15). Transiently elevated mRNA levels during short-term warming may push a process to a new steady-state level, and we suggest that alternations in expression reflect transcriptomic rearrangements under the constraints of more or less constant total amount of transcripts during the transition from medium- to long-term acclimation. On long time scales, the content of active metabolic enzyme proteins was likely adjusted using smaller amounts of transcripts and a higher transcription efficiency in the warmth.

The correspondence analysis divided the animals in three different groups with different time-dependent characteristics. After the onset of warm exposure the animals are clearly separate from controls and the short-term and mid-term groups clustered together. The long-term group is separated from the others and thus indicates a new steady state. This strong separation is due to differential developments of the time-dependent responses and partly due to the increase in time intervals between sampling. Future studies should time resolve the transient acclimation period involving partial transcriptomic recovery between mid-term and long-term phases by high-throughput methods and high resolution, i.e., to explore larger functional clusters on a finer temporal scale. Some of the genes showed an unexpected effect of time in the two controls during experimentation, although differences in handling and diet can be excluded. This effect was not consistent for all genes, and, moreover, the CCA (Fig. 4B) proved that both controls still cluster in one pool.

Overall, the current expression and functional data indicate an alteration from a lipid-based metabolic network to pathways associated with carbohydrate metabolism, which is in line with the observed changes in fuel composition in the liver tissue of Antarctic eelpout (11). Transcriptomic studies of *T. bernacchii* during short-term warm exposure (4 h), as well as for *H. antarcticus* incubated for 2 days, have shown large but short-term rearrangements in the transcriptome (13, 61). Although the timescale of thermal exposure was largely different from the present study, the early responses in the notothenioids also indicate wide rearrangements in lipid metabolism. Especially in *H. antarcticus* a significant shift to lipid catabolism was detectable. Thus, the picture emerging for *P. brachycephalum* may be common to a wider range of Antarctic fishes.

Impact of potential transcription factors. Successful cloning of the different PPARs demonstrated that *P. brachycephalum* expresses the same compounds of the PPAR signaling cascade as temperate eurythermal fish. Lipid accumulation in the cold found for the Antarctic eelpout (10) and for species in the suborder of the Notothenioidei (38) indicate the general preference for fatty acids as main energy stores in cold-adapted fishes. First, temperature-induced shifts in lipid degradation and synthesis pathways over time may provide intermediates sensed by the PPAR family of transcription factors. Strongly altered expression levels of PPAR1 α and - β , and a somewhat smaller response of PPAR1 γ then occur upon warm acclimation (Fig. 3, and Table 4).

In mammals PPAR1 α has a high affinity to saturated as well as to unsaturated fatty acids and functions as an activator of various enzymes involved in β -oxidation (3, 62). Changes in receptor transcription were positively correlated with those of various transcripts participating in lipid oxidation like TAGL, HADH, SCAD, and MCAD (Table 5). Similarly, ACAC as the

Table 5. Correlations in the expression of genes

Gene	Correlation	Slope	Intercept	R	P value
<i>Correlations with PPARα</i>					
TAGL	+	1.082	-0.469	0.635	<0.001
HADH	+	1.178	-0.309	0.747	<0.001
SCAD	+	1.193	-0.699	0.699	<0.001
MCAD	+	1.049	-0.306	0.728	<0.001
ACAC	+	0.886	0.012	0.762	<0.001
6PGL	+	1.170	-0.612	0.732	<0.001
PK	+	0.934	-0.151	0.56	<0.001
GYS	-	-0.763	0.750	0.429	<0.001
<i>Correlations with PPAR1β</i>					
GYS	+	0.499	0.82	0.371	0.004
PPAR1 α	-	-0.325	1.123	0.43	<0.001
TAGL	-	-0.448	1.369	0.348	0.007
HADH	-	-0.515	1.322	0.432	<0.001
ACAC	-	-0.281	1.115	0.319	0.014
PK	-	-0.377	1.229	0.299	0.021
6PGL	-	-0.584	1.527	0.484	<0.001
CS	-	-0.458	1.214	0.413	0.001
COX4	-	-0.431	1.361	0.334	0.01
CytC	-	-0.729	1.262	0.551	<0.001
<i>Correlations with PPAR1γ</i>					
GYS	+	0.376	0.518	0.421	<0.001
PGC1 α	+	0.246	0.522	0.288	0.027
<i>Correlations with PGC1α</i>					
NRF-1	+	0.285	0.039	0.296	0.023
HIF1 α	+	0.885	0.095	0.502	<0.001
TAGL	+	0.279	0.084	0.278	0.033
GYS	+	0.491	0.233	0.469	<0.001
GYP	+	0.378	0.083	0.336	0.009
3HBDH	+	0.276	0.082	0.289	0.026
<i>Correlations with HADH</i>					
COX4	+	0.719	0.008	0.693	<0.001
CS	+	0.642	0.357	0.69	<0.001
CytC	+	0.692	0.437	0.623	<0.001
TAGL	+	0.647	0.098	0.623	<0.001
MCAD	+	0.707	0.154	0.775	<0.001
6PGL	+	0.767	-0.030	0.757	<0.001
<i>Correlations with MCAD</i>					
SCAD	+	0.777	0.008	0.656	<0.001
6PGL	+	0.717	0.110	0.646	<0.001

Linear regression analyses of the correlation between the message levels of the investigated genes (see DISCUSSION).

enzyme for the initial step of lipid biosynthesis was induced in mammalian liver through transactivation by PPAR1 α (55). In the present study, receptor expression was positively correlated with that of ACAC (Table 5), indicating a similar reduction in expression accompanied by a reduction of lipid stores.

PPAR1 β is the least understood receptor of the PPAR-family, with a lower affinity to fatty acids compared with PPAR1 α (51, 69). In our study, we found an increased expression during warm acclimation, which perfectly mirrors the decrease in PPAR1 α . Thereby, the correspondence analyses indicate contrasting functions of both receptors (Fig. 4A). Fast responses of PPAR1 α and - β expression during the first 24 h indicate an immediate sensing of the altered situation and the beginning of the reorganization of lipid metabolism. Previous studies suggest that PPAR1 β also modulates glucose homeostasis (69). A significant positive correlation was found be-

tween expression levels of PPAR1 β and GYS; however, PPAR1 γ and the cofactor PGC1 α are also correlated with the expression of GYS (Table 5). The species plot in the CCA (Fig. 4A) specifies the picture of a potential induction of GYS transcription through PGC1 α and PPAR1 β , and furthermore, PPAR1 γ . The small angle between these factors in Fig. 4A indicates strong correlations. During long-term exposure to 5°C PPAR1 γ seems to become more important (shift along the incubation time axis) in supporting the storage of glycogen (Table 5) in association with PGC1 α .

These manifold results indicate that all of the investigated receptors become involved in the regulation of fatty acid-oxidation along the time axis of incubation, and thereby may sense different signals (intermediates) over time. The inverse patterns of PPAR1 α and - β may reflect a well-controlled sensing of initial, fast changes and indicate a direct interdependency of both receptors. In mammals, the message levels of PPAR1 β and - γ were found to act as the dominant repressors of PPAR α -mediated responses (30). Here, we observed inverse correlations (Table 5), i.e., suppression for nearly all genes that were positively correlated with the α -receptor. Hence, it is possible that PPAR1 α expression and PPAR α -mediated responses are also controlled by PPAR1 β in fish.

PGC1 α plays an unclear role in this network but held a central position in the correspondence analysis between all transcription factors (Fig. 4A). This coactivator of PPARs and NRFs is commonly described to be the master regulator of various mitochondrial processes (19, 35, 64, 65, 68), as well as of carbohydrate metabolism (70). The fact that temperature had no effect on its transcription level may emphasize the multifunctional role of PGC1 α for all transcription factors. In goldfish, temperature-dependent expression of PGC1 α were found in several tissues, albeit its expression increased both

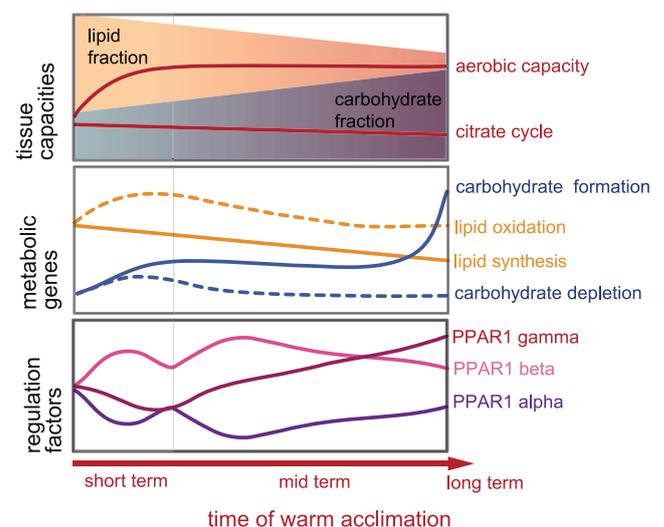


Fig. 5. Overview of the temperature-induced development over time in the metabolic pathways of the liver. A rising carbohydrate and a decreasing lipid fraction (11) is paralleled by increasing aerobic capacities. Curves of the respective metabolic pathways were extrapolated from the individual developments of the gene expression and the CCA, showing the general trends of rearrangements during the time course of acclimation. The transcription factors are shown in simplified curves to estimate interactions between the PPARs and their effects on the transcriptomic alterations shaping the observed shifts in metabolic pathways and finally, the fuel stores.

upon cooling or warming in liver (35). NRF-1 was correlated with PGC1 α (Table 5), as the expression of NRF-1 was significantly reduced. Besides, recent sequence analyses of PGC1 α indicate no interaction between NRF-1 and the cofactor in fish (34). More data from further fish species and from the corresponding binding domain on NRF-1 are necessary to clarify this issue.

NRF-1 is known to modulate mitochondrial proliferation (17) and binds to various promoters like that of CytC and of genes encoding subunits of COX. In line with lower NRF-1 levels CytC mRNA was drastically reduced; the functional subunits of COX also displayed a modest reduction, if at all. As our functional analyses indicated significant posttranscriptional regulation of COX, transcriptional regulation by NRF-1 may not be involved during warm acclimation. Earlier studies found clear evidence of a role of NRF-1 under cold stress (35, 41), when enzyme capacities were induced to compensate for the negative temperature effect. In the correspondence analyses NRF-1 is located close to the center of the variables, suggesting a minor role in the acclimation process to 5°C. These observations may be in line with the special pattern of warm acclimation in the Antarctic eelpout compared with temperate fishes.

Conclusions. Acclimation to warming involves a progressive shift in the metabolism of the Antarctic eelpout *P. brachycephalum*. Early alterations in enzyme activities of the respiratory chain and TCA cycle indicate a higher mitochondrial capacity in the warmth combined with a use of different fuel stores, which is likely linked to the chronology of altered pathway expression during the acclimation process (visualized in Fig. 5). The overall changes involve functional reorganization at different levels of organization, including posttranslational modifications and higher translational efficiencies of functional genes in the warmth.

While cold-acclimated animals are characterized by elevated lipid contents, rising temperatures caused a decomposition of lipids and a metabolic shift to carbohydrates. The shifts of the respective pathways may be controlled through sensing of the metabolic state via the PPARs. The strong dichotomy of PPAR1 α and - β receptors has not been reported in the literature; both are involved in β -oxidation, and a reciprocal (genetic) control seems possible. The correspondence analysis demonstrated the relevance of all three PPARs in the acclimation process, showing more extreme correlations, compared with other investigated gene transcripts in the species plot. This finding substantiates their importance for a fast response and reorganization under changing temperatures.

The expression profiles indicate that the acclimation process occurs in two different phases. The sensing of altered conditions and reorganization of the transcriptome takes place first, paralleled by changes in enzyme capacities and a rapid upregulation of transcription for some genes like PEPCK, glycogen phosphorylase, and synthase. Consecutively, the transcription of the initially upregulated genes is reversed. Energy demand is covered by energy-rich compounds like long-chain fatty acids, which are now mobilized and shuttled into mitochondria for oxidation. The final phase is characterized by a surplus of glucose fueling glycogen synthesis indicated by maximized glycogen synthase expression (Fig. 5).

Perspectives and Significance

A lipid-based metabolism is widely found in cold-acclimated as well as cold-adapted ectotherms (47, 57). Due to the higher energy content, lipids should always be the preferred nutrient, and the central questions remains, why *P. brachycephalum* (and most other fishes) switch to a carbohydrate-preferred metabolism in the warmth. The same pattern albeit in opposite thermal direction has been described in hibernating ectotherms like turtles and Crucian carp, which usually face anoxic conditions in ice-covered ponds (4). In these animals elevated glycogen stores, synthesized during aerobic periods, well ahead of anoxic exposures, indicate a general strategy to survive hypoxia by anaerobic energy production. Similarly, the shift observed here may support warm-hardiness, as, due to the oxygen limitation of heat tolerance (46), the development of functional hypoxemia is more likely in the warmth. Thus a change in oxygen availability and not in temperature per se seems to be the evolutionary driver for the shift in preferred fuel stores; however, the primary stimulus of this shift remains obscure. Altogether, the data support the view of an adaptive response and sustained well-being of this cold-stenothermal species under warmer (laboratory) conditions. The present study has identified a molecular network responding sensitively to warmer temperatures. Further efforts need to test the general relevance of these mechanisms in cold-stenothermal fishes including the notothenioids and to unravel the limitations of this response in both the laboratory and the natural environment of the Antarctic eelpout.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H. S. W. and R. K. performed experiments; H. S. W., R. K., S. F., and M. L. analyzed data; H. S. W., R. K., S. F., and M. L. interpreted results of experiments; H. S. W. and S. F. prepared figures; H. S. W., S. F., and M. L. drafted manuscript; H. S. W., R. K., H. -O. P., S. F., and M. L. edited and revised manuscript; H. S. W., H. -O. P., and M. L. approved final version of manuscript; M. L. conception and design of research.

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Publication II

Evolutionary force in confamiliar marine vertebrates of different temperature realms: adaptive trends in zoarcid fish transcriptomes.

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**Evolutionary force in confamiliar marine vertebrates
of different temperature realms:
adaptive trends in zoarcid fish transcriptomes**

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Abstract

Background:

Studies of temperature-induced adaptation on the basis of genomic sequence data were mainly done in extremophiles. Although the general hypothesis of an increased molecular flexibility in the cold is widely accepted, the results of thermal adaptation are still difficult to detect at proteomic down to the genomic sequence level. Approaches towards a more detailed picture emerge with the advent of new sequencing technologies. Only small changes in primary protein structure have been shown to modify kinetic and thermal properties of enzymes, but likewise for interspecies comparisons a high genetic identity is still essential to specify common principles. The present study uses comprehensive transcriptomic sequence information to uncover general patterns of thermal adaptation on the RNA as well as protein primary structure.

Results:

By comparing orthologous sequences of two closely related zoarcid fish inhabiting different latitudinal zones (Antarctica: *Pachycara brachycephalum*, temperate zone: *Zoarces viviparus*) we were able to detect significant differences in the codon usage. In the cold-adapted species a lower GC content in the wobble position prevailed for preserved amino acids. We were able to estimate 40-60% coverage of the functions represented within the two compared zoarcid cDNA-libraries on the basis of a reference genome of the phylogenetically closely related fish *Gasterosteus aculeatus*. A distinct pattern of amino acid substitutions could be identified for the non-synonymous codon exchanges, with a remarkable surplus of serine and reduction of glutamic acid and asparagine for the Antarctic species.

Conclusion:

Based on the differences between orthologous sequences from confamiliar species, distinguished mainly by the temperature regimes of their habitats, we hypothesize that temperature leaves a signature on the composition of biological macromolecules (RNA, proteins) with implications for the transcription and translation level. As the observed pattern of amino acid substitutions only partly support the flexibility hypothesis further evolutionary forces may be effective at the global transcriptome level.

Background

Marine ectotherms specialize to different thermal windows and undergo genomic changes under evolutionary forces seen as adaptation to environmental conditions. Temperature is a crucial abiotic factor causing seasonal variability in ecosystems and mainly distinguishing polar from temperate habitats, thereby determining the distribution of species on large scales [1].

Various molecular responses are described for seasonal temperature changes that range from alterations in the transcript amounts, enzyme activities and the resulting shifts in the energy- and housekeeping metabolism (for review see [2]). Furthermore, regulative mechanisms become effective in ectotherms, like the induction of specific heat shock proteins helping to adjust the metabolic functioning to seasonal warming [3]. In the cold, for example, glycoproteins are expressed to prevent freezing in subzero waters [4].

On longer time scales adaptation to temperature requires an effective housekeeping on the metabolic level (enzyme kinetics) as well as on the transcription level. An increase of protein numbers can only partly compensate for a loss in activity during seasonal acclimatization or permanent adaptation to cold, limited by constraints in cellular space and energy availability [5]. Hence, structural modifications at the protein level are necessary to improve enzyme operation in the cold. Different temperature adaptations and dependencies of the kinetics of single enzymes like the phosphoglycerate kinase [6, 7], chitobiase [7] or the lactate dehydrogenase [8] were studied intensively complemented by comparative analyses at the amino acid sequence level [9], discussing thermal acclimation up to adaptation at the molecular level. These studies described common principles of thermal adaptation in a limited number of enzymes and their three-dimensional structures. The flexibility hypothesis implies that thermal adaptation of enzymes is accompanied by shifts in catalytic turnover numbers (k_{cat}) and catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$). For cold adaptation this demands a higher structural flexibility of the protein sometimes at the expense of reduced thermal stability [5, 8, 10, 11]. For maintaining a certain degree of flexibility cold-adapted enzymes tend to have fewer salt links, less interactions within the hydrophobic core, a reduction in the number of proline and arginine residues, a reduction in the hydrophobicity of the enzyme, and improved solvent interactions with a hydrophilic surface via additional charged side chains. In most cases, the catalytic and binding centres are not changed, but mutations under adaptive pressure of temperature are changing the stability, the barriers of activation energy as well as the accessibility of the catalytic cleft through conformational changes in the structure [12].

Research on single enzymes demonstrated that several protein families adapt with different

strategies, which even contradict with other observed patterns to some extent. After all, single case studies are not sufficient to identify global adaptive patterns in sequences at the DNA and protein level due to environmental temperature.

Recent genomic and proteomic studies on distantly related extremophiles display coherence between the optimal growth temperature (OGT) and the composition of biological macromolecules (DNA, RNA and proteins), in which higher GC contents prevail on a large scale in warm-adapted species [12-16]. Moreover, similar GC-trends were detected in eukaryotic synonymous sequences, when comparing poikilotherm and homeotherm species [15-17]. Subsequently a modified amino acid composition [13-15, 17] of proteins can be observed, e.g., for non-synonymous mutations.

In the present study we aimed to analyze evolutionary trends in marine ectotherm vertebrates of the same family based on transcriptomic data obtained from two normalized cDNA libraries. Since 454 sequencing-techniques are developed and improved, a number of transcriptomic libraries of various species were published, giving insights in the molecular networks comprising various developmental stages and tissue types [18, 19]. The growing availability of sequence libraries allows for more specific inter-comparisons even in non-model organisms, e.g., by linking sequence data in closely related species in a pairwise manner.

The family of Zoarcidae (eelpouts) comprises 284 described species, distributed all over the global oceans [20]. Fishes of this cosmopolitan family are therefore appropriate to address the degree of adaptation to temperature in different realms by comparative methods. The eurythermal species *Zoarces viviparus* lives in the northern hemisphere and faces seasonal shifts of temperature ranging from 0°C in winter to above 20°C in summer [21]. Despite, the maximal growth rate for this species was determined at about 15 °C, indicating an optimal ecological and physiological performance at this temperature [22]. Kristiansson and colleagues sequenced a transcriptomic cDNA library of *Z. viviparus* under unstressed conditions [23]. Furthermore, this species is often compared in ecophysiological studies with its stenothermal Antarctic congener *Pachycara brachycephalum* [24] that is highly adapted to constant cold waters around 0°C and an optimal growth temperature at 4°C under laboratory conditions [25].

We constructed a transcriptomic cDNA library from liver and heart muscle tissue of the Antarctic eelpout *P. brachycephalum* under habitat conditions and compared it with a cDNA-library of *Z. viviparus* at functional level, their amino acid sequence translations and at RNA codon level to uncover differentiation, which we can explain at best by thermal adaptation.

Results

A classification of *P. brachycephalum* and *Z. viviparus* by molecular analyses of selected sequence data was used to determine the phylogenetic relationship with various bony fish species from widespread geographical regions. For two mitochondrial genes (*ribosomal 16S RNA (16SrRNA)* and *cytochrome-c-oxidase subunit I (COI)*) we computed phylogenetic trees of 13 selected species belonging to the class of Actinopterygii and the elasmobranch *Squalus acanthias* as outgroup. Both zoarcids formed a clade (Fig. 1A, B). The threespined stickleback *Gasterosteus aculeatus* appeared as their closest relative for both genes. The obtained phylogenetic trees mirror the grouping into the different families like Cyprinidae, Gadidae, Salmonidae, Tetraodontidae and other Percomorpha. Quantitatively, higher similarities across various species were found for the *16SrRNA*, i.e., smaller distances, than for the *COI* gene. From both trees a close relationship could be inferred among the zoarcid species, although *COI* is a functional gene and the *16SrRNA* is a structural RNA. We expect similar differences to be present also in sequences of functional genes. In this context a manual pre-selection of a limited subset of sequences would bias a comparative study. To this end a more complete approach is followed here based on all available transcriptomic sequences of the closely-related zoarcid fish.

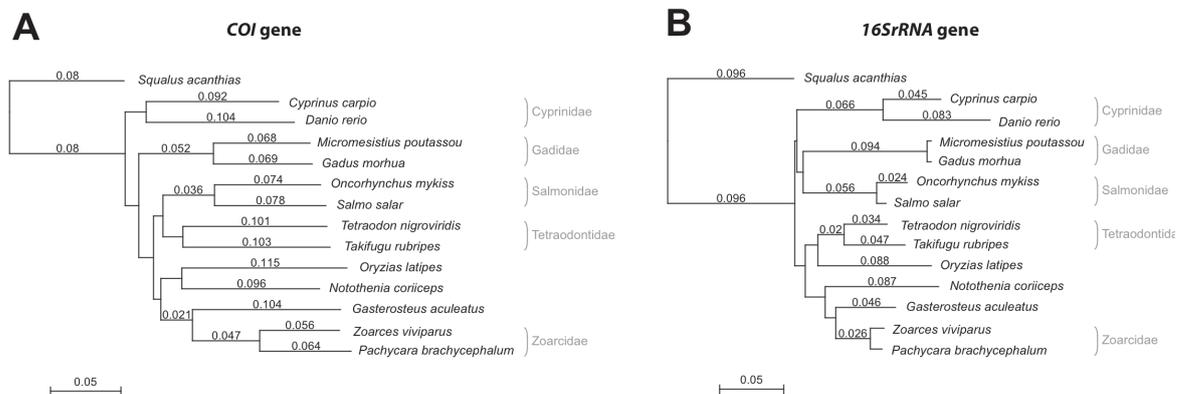


Figure 1 Phylogenetic trees of representative fish species inhabiting divergent temperature realms.

A: Sequence similarity based on the *COI* barcoding gene. B: Phylogenetic map based on the sequence information of the structural *16SrRNA* sequence. Both trees were generated by using the neighbour joining method. Node distances are calculated with Tamura-Nei algorithm. Numbers indicate relative nucleotide substitutions in which different substitution pathways occur with independent probabilities. Sequence gaps were distributed proportional. A summary of the used sequence information is summarized in material and methods Table 3.

Overall statistical comparison of zoarcid cDNA libraries

The setup comprises 454 sequencing data of two cDNA libraries, one prepared from liver and heart of *P. brachycephalum*, and one prepared from liver of *Z. viviparus* [23], generated with similar techniques and protocols (c.f. Material and Methods). The average read length in the library of *P. brachycephalum* was about 100 bases longer than that for

Table 1 Benchmark data of cDNA libraries

Database parameter	<i>P. brachycephalum</i>	<i>Z. viviparus</i>
Total Number of reads	481,802	~400,000
Number assembled	338,993	349,102
Average of read length	321	221
Number of all contigs	65,565	53,447
Number of non-redundant contigs	64,634	53,313
Percentage of redundancy	1.42	0.25

Overall statistical analyses of the sequencing runs of *Z. viviparus* data of the library generated by Kristiansson et al. 2009, in comparison to the library of the Antarctic eelpout *P. brachycephalum*. The resulting reads were set up to contigs with similar assembly techniques. Further analyses of the contig quality could be seen in Fig. 2.

Z. viviparus (Table 1), due to the usage of Titanium-chemistry for sequencing.

Subsequently, a higher yield of longer contigs with 487 bases in average for *P. brachycephalum* was found, compared to 342 bases in average per contig for *Z. viviparus* (Fig. 2A). Other basic parameters like number of reads per contig, (Fig. 2B), as well as the average coverage were comparable (Fig. 2C), constituting a suitable basis for alignment analyses of contigs.

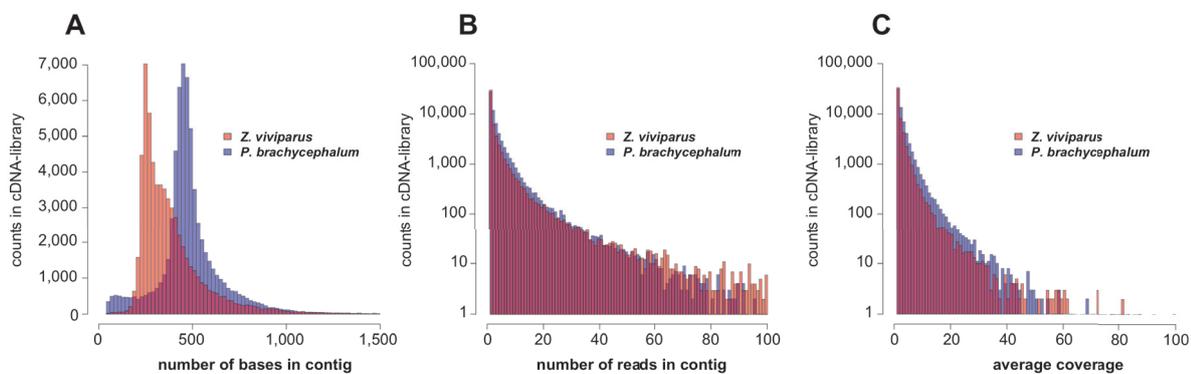


Figure 2 Statistical analyses of the sequencing runs and the associated assembly quality.

A: Distribution of the contig length of *P. brachycephalum* and *Z. viviparus*. B: Number of reads in the assembled contigs. C: Average coverage of reads in assembled contigs.

Using the BLAST2GO tool [26] for evaluating annotations of contigs, statistics of expect values (e-values) show qualitatively comparable profiles. But quantitatively, sequences of *P. brachycephalum* show higher similarity to known sequences (Fig. 3A) accompanied by higher coverages per hit of high-scoring segment pairs (HSP) (Fig. 3B). For the purposes of this study we filtered by BLAST [27] for sequences of known functions, i.e., showing known translations, resulting in 19,460 contigs for *P. brachycephalum* and 16,315 for *Z. viviparus*.

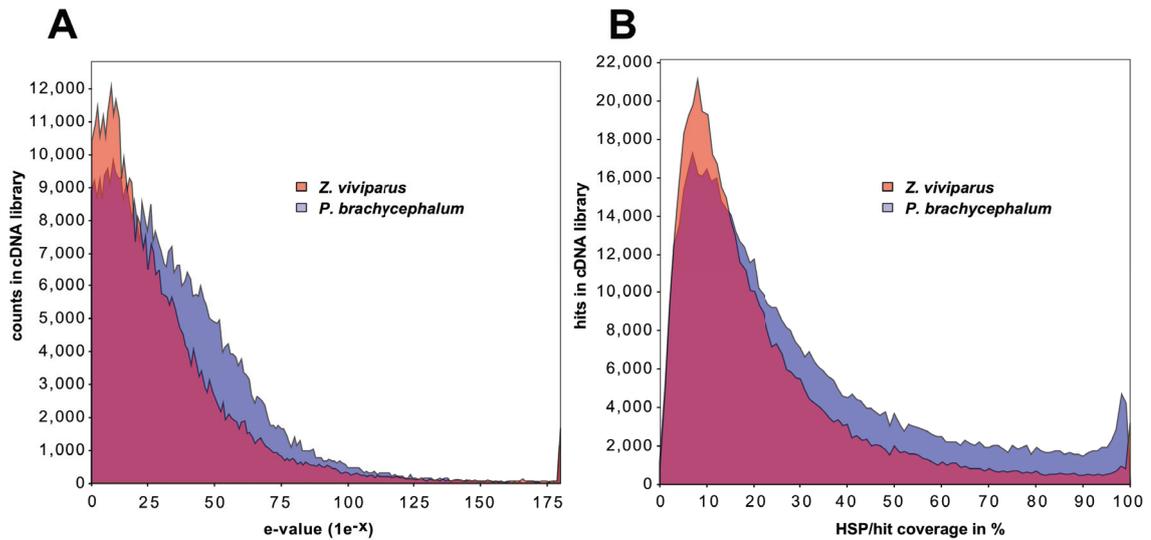


Figure 3 Overview of the distribution of BLAST results among the library assemblies with BLAST2GO. Overview of the distribution of BLAST results among the library assemblies with BLAST2GO. A: the e-value distribution shows the hit-reliability of the contigs B: The HSP (high-scoring segment pair) distribution gives a measure of the accuracy of the sequence alignment within the BLAST annotation.

Functional coverage compared to a phylogenetically related reference genome

The overall functional repertoires of the cDNA library sets were evaluated in terms of KOG/COG categories (clusters of orthologous groups) as obtained from the local alignment tool rpstBLASTn applied on the BLAST-annotated sequences against a database of metazoan-specific orthologies (meNOGs) [28, 29]. As a scale for the comparison, coding sequences from the closely related species *G. aculeatus* (Fig. 1A, B) were evaluated accordingly. From the reference genomic data (ENSEMBLE 61) 27,628 coding sequences were extracted to estimate the functional coverage of zoarcid libraries. To compare the appearance of functional terms quantitatively, the counts were pooled into superordinated categories of KOG/COGs (Fig. 4). The count data of both zoarcid transcriptomes were in a comparable range, but covered only a fraction of the reference genome. Strongest inter-zoarcid differences were seen for categories F (nucleotide transport and metabolism) and V (defense mechanisms), showing higher counts in *P. brachycephalum*. The overall coverage compared to the scales of *G. aculeatus* genome is estimated to be 40% to 60%, with a pronounced lesser coverage in categories U, V, D and B. Furthermore, a notable deviation is found in category N (cell motility), where both eelpouts show more counts than are present in the reference genome. Contrary, in category W (extracellular structures) *Z. viviparus* have more counts compared to the reference set, whereas the number of counts for *P. brachycephalum* nearly equals it.

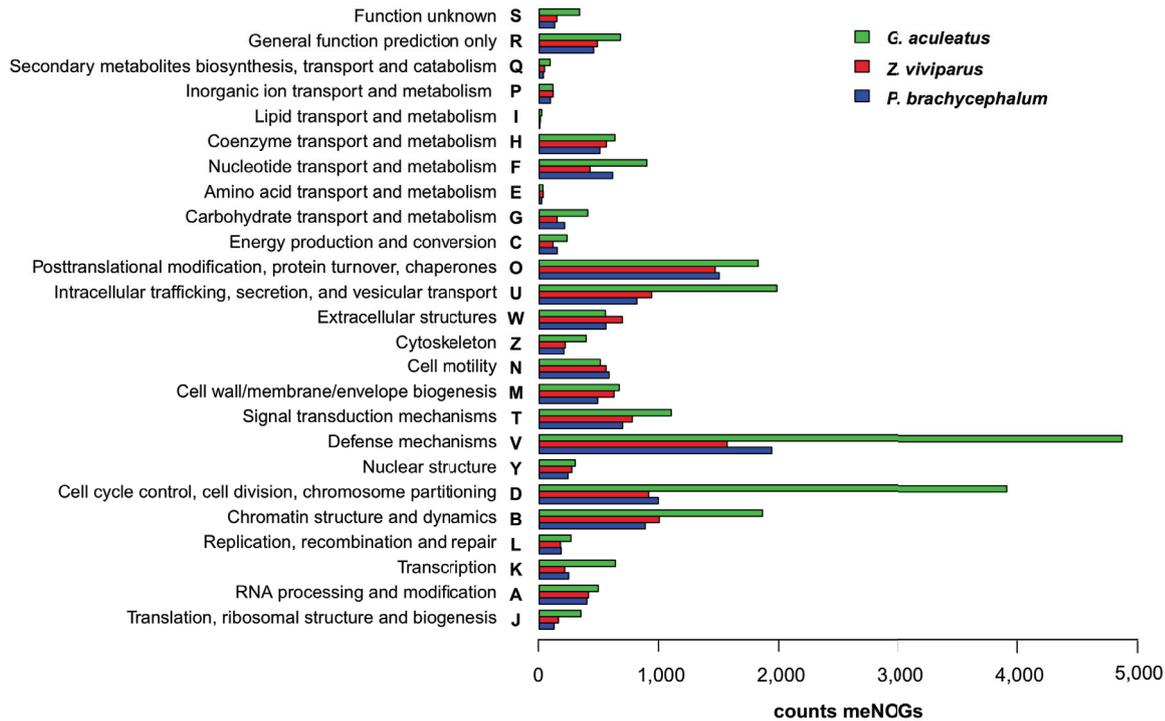


Figure 4 Distribution of metazoan orthologous terms in clusters of orthologous groups (COG).

COG distributions were determined by rpstBLASTn (e-value cut off 10^{-20}) in the transcriptomic libraries of *Z. viviparus* and *P. brachycephalum*. Complete genomic data of *G. aculeatus* representing a reference from a closely related fish were analyzed equally.

To identify species-specific functional patterns in gene expression, we searched for overrepresented meNOGs in each library set. The most exclusive terms in *Z. viviparus* are meNOG13752 coding for complement protein, meNOG11371 encoding for the cytochrome-*c*-oxidase (COI) and meNOG18150 for extracellular matrix constituent lubricant protein contributing to the structural extracellular matrix integrity. In *P. brachycephalum* the most specific term is a b-box-type zinc finger (meNOG24554) followed by meNOG15201 encoding for a tripartite motif protein and meNOG06310 which encodes a carboxylase function. Further terms in this species were ubiquitin (meNOG05057) and the heavy chain of myosin (meNOG 07153) (data not shown). The term for myosin (meNOG07153) is noticed as specific for *P. brachycephalum* consistent with the fact that we included heart RNA in the library construction.

Amino acid usage

Translations of coding sequence segments were generated based on fish-specific orthologies (fiNOGs) via rpstBLASTn for both cDNA libraries to exclude effects of sequencing artefacts and frame shifts on the subsequent analyses. From the identified orthologous sequences in each zoarcid fish, matching sequence segment pairs were obtained by harvesting best BLASTp hits per model orthology (i.e. fiNOG), providing a set of shared orthologous sequences in both fish. For a more stringent pairing, i.e., excluding probably false pairs, translated segment pairs were filtered for $\geq 80\%$ amino acid sequence identity. Accordingly, frequencies of amino acids were computed from 4,155 translated and realigned segment pairs, including non-identical amino acid positions, but excluding indels (insertions/deletions). Alterations in amino acid profiles became visible by assessing the total changes in the aligned non-synonymous codon positions (Fig. 5). For *P. brachycephalum* net losses for glutamic acid and asparagine, as well as a net gain of serine were most obviously.

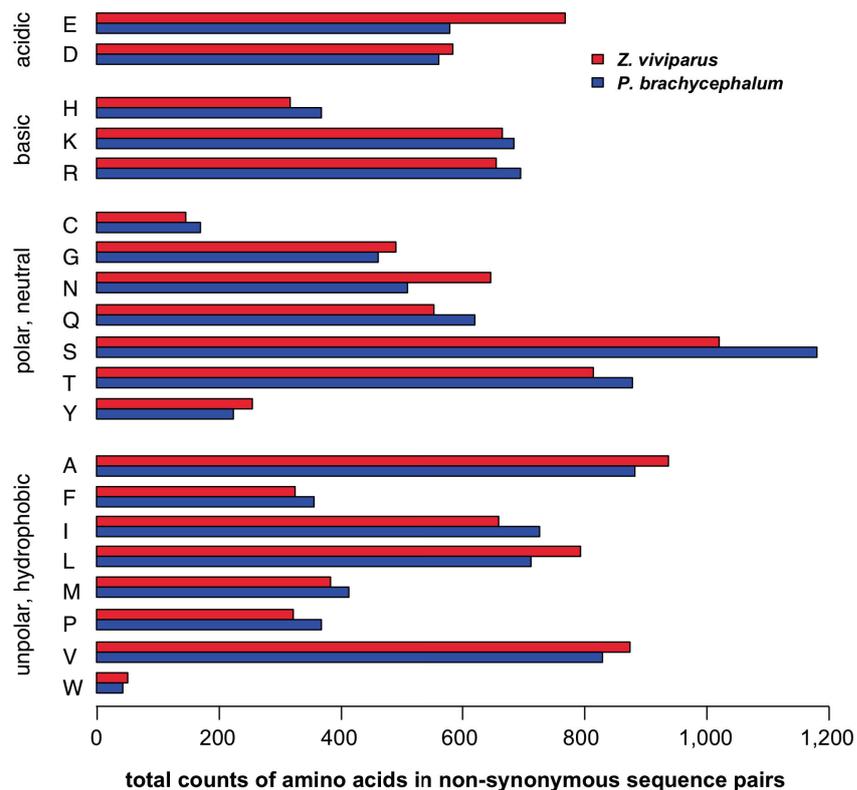


Figure 5 Frequency of differential amino acid usage based on the sequence alignments of the cDNA libraries with fish-specific orthologies.

Local alignments of resulting 4,155 sequence segment pairs were analyzed for net amino acid usage in non-synonymous translations.

To resolve the exchange patterns in more detail, a table of amino acid replacement counts was transformed to a measure of imbalance of amino acid replacements in *P. brachycephalum* compared to *Z. viviparus* (see Methods and Table 2). Similarly, the most striking difference is the loss of 85 glutamic acid in *P. brachycephalum* with a preferential usage of aspartic acid instead. Further patterns of prominent exchanges are the replacement of 31 leucine by alanine, likewise on a similar level the exchange of 30 glutamic acid residues by glutamine. Serine is used preferentially for almost all amino acids but only replaced by arginine (30 cases). The observed patterns for threonine are not only the preferential usage instead of 82 alanine and 41 asparagine but also replacements for 46 methionine and 31 serine. Beside an overall net loss, valine is preferred over alanine 51 times but also replaced by 65 isoleucine.

Table 2 Imbalances of amino acid usage in two zoarcid species.

		Amino acids preferred in <i>Z. viviparus</i>																			
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	
Amino acids preferred in <i>P. brachycephalum</i>	C	3																			
	D	-10	-1																		
	E	-19	-1	-																	
	F	-3	-3	85*																	
	G	-13	-8	7	5																
	H	0	-1	9	-3	-2	5														
	I	-5	-1	2	0	-15	0	-6													
	K	-11	1	7	15	-2	-2	-4	2												
	L	-31*	-3	3	-3	26	-4	-9	-25	2											
	M	5	-4	3	-2	-10	-1	-3	-20	-5	19										
	N	-18	1	25	-1	-8	-16	-21	-7	-23	2	2									
	P	3	0	1	2	6	4	0	-1	-3	-6	-5	5								
	Q	7	2	5	30*	0	20	7	1	-11	9	-4	18	-5							
	R	5	-8	0	18	-1	8	-10	0	-7	-5	-3	0	-25	22						
	S	5	11	2	26	19	8	1	9	20	11	2	15	3	6	30*					
	T	82*	12	21	-5	-2	-1	0	7	6	3	46*	41*	-7	-5	-4	31*				
	V	51*	-8	3	3	-19	1	-1	65*	7	-11	7	1	-6	-3	-7	-10	11			
	W	1	0	0	0	-4	-5	0	0	0	12	-1	0	0	-5	-3	-7	0	3		
	Y	3	-10	8	-1	-23	2	3	7	1	3	0	-6	0	-3	-2	-4	-4	-4	-1	

In 4,155 orthologous sequences amino acid usage was analyzed by aligning the fiNOG-orthologous sequences of *P. brachycephalum* and *Z. viviparus*. The algebraic sign shows the direction of the imbalance of differential usage. Negative signs show a loss, positive signs show a gain of the corresponding amino acid in the Antarctic species. Most evident changes were emphasized and labelled with an asterisk.

The observed imbalances were furthermore reanalyzed taking the ERK-proxy into consideration [30] to determine whether species-specific shifts are detectable within the observed pattern of differentially used amino acids. A significant preference for using E+R+K in *Z. viviparus* was detected when compared to the orthologous associate in *P. brachycephalum* (Wilcoxon test, $p=0.02$), analysing the 4,155 translated sequences and taking only exchanged positions into account.

Furthermore, with codon usage differences from 4,155 sequence segment pairs (filtered for >80% identical translated positions, depicted in Fig. 7), found significantly more positive shifts (23 in the left panel) of codons ending with A/T (AT3, marked “>”) were detected compared to the amount of negative shifts (7 in the right panel). For this analysis, non-synonymous codon positions were not taken into account. A Fisher-test for the distribution of a contingency table of AT3 vs. GC3 codons in the left and right panel of this graph reveals a p-value of $5 \cdot 10^{-4}$, indicating a significantly non-random distribution of shifts.

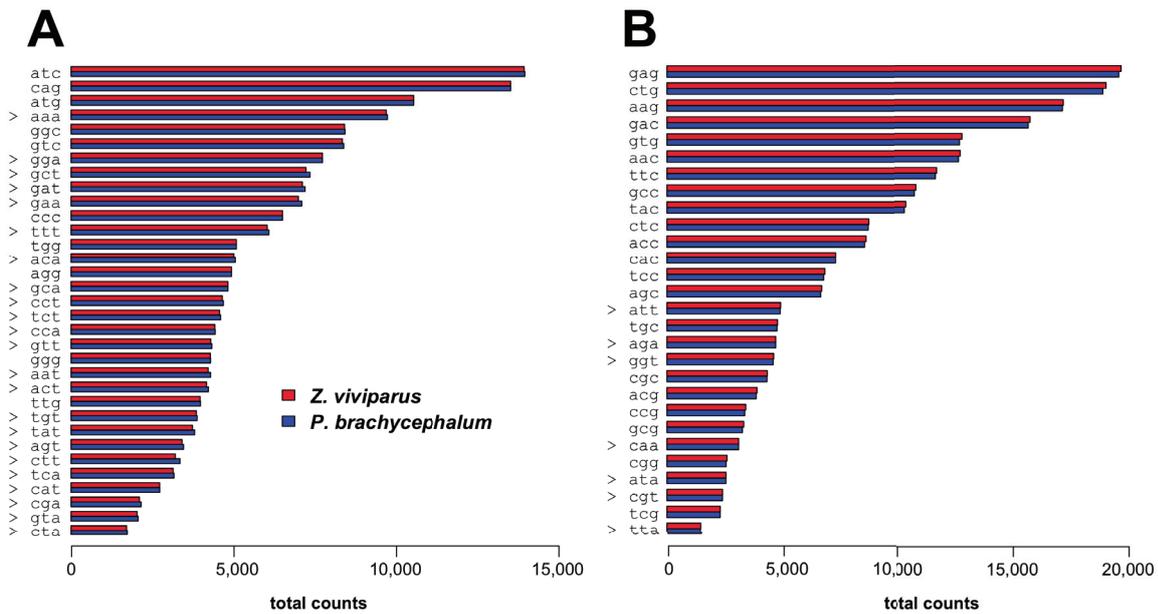


Figure 7 Differential usage of amino acids in the zoarcid species. 4,155 homologue sequence pairs were used for the analyses. All codons with A/T in the third position are labelled with ‘>’; A: The panel displays codons with higher usage frequencies in the Antarctic species; B: Complementary plot for higher usage frequencies in the temperate species.

The overall number of aligned amino acid residue pairs in this set was 414,978, constituting an average of 100 aligned codon pairs per segment with a mean of 96 conserved residues. Comparing 402,219 synonymous codon pairs with conserved nucleotides in positions 1 and 2, *P. brachycephalum* shows a net GC3-loss of 1,030, re-confirming an imbalance towards a preferential A/T in the third position of the codons in this cold-adapted fish. The analysed coding sequence segments showed a mean GC-fraction of 53.196% for *Z. viviparus* and 53.094% for *P. brachycephalum* mirroring the observed pattern on the third codon position.

Discussion

In the present study we profiled transcriptomic libraries of two confamilial fish species from different temperature realms for signatures of thermal adaptation at the sequence level. The phylogenetic analyses (Fig. 1A, B) indicate a close relationship between *P. brachycephalum* and *Z. viviparus*, which is supported by transcriptomic sequence comparisons finding a pool of 4,155 comparable sequence segments with a mean identity of 96%. As only minor sequence changes are sufficient to adapt kinetic properties of orthologous proteins, this tight relationship is essential for identifying a potential thermal signature at the genomic and transcriptomic level in species from thermally distinct habitats. Habitat temperature may be an important trigger for the observed patterns. As *Z. viviparus* faces similar cold temperatures in winter like *P. brachycephalum*, a signature for thermal shifts may appear. As optimal growth performance constitutes an important marker for maximal protein synthesis, which was determined for the upper third of the temperature window [22, 25], we postulate that the transcriptome is optimized to this temperature range and not to the cold edge, where metabolic activity and performance of the eurythermal species is comparably low.

It is important to note the assumptions we made to argue that the observed changes are adaptive. Garland and Adolph [32] generally argued against comparisons of only two species for making appropriate conclusions about adaptation, since the probability for finding a difference for any trait between two species is always 50% and no valid null hypothesis can be made. According to their critical statement [32], the limitation could be overcome by analysing several traits (about 5 independent) as this will reduce the type I error rate to the expected rate ($p < 0.05$). As we used several thousand mRNA/protein sequences for the analyses from the beginning, we can assume that there are still enough independent traits left to reach a $p < 0.05$. This is essentially true for the analyses of the synonymous codon exchanges, which have no impact on function and can be stated as “neutral” at best. For the non-synonymous codon exchanges we can postulate the same, as the exchange of one specific amino acid in one protein will not cause a definite amino acid in another protein, even if both proteins belong to the same trait. If only a genetic drift has taken place after separation of the two species from the common ancestor, no change in codon or amino acid usage would be expected when analyzing thousands of traits. Since we found a clear signal, we have to conclude that this change is adaptive. The question remaining to be answered is whether the driving force is temperature (alone) or a combination with further factors (see below).

Codon and amino acid usage were found highly correlated with expression levels at least in fast growing bacteria and yeast [33]. Specific needs of an organism may shape a specific codon-usage-profile with a concomitant optimization in the translation apparatus (tRNA amount, stability of secondary structures of RNA, amino acid levels, etc.). Here, we compared qualitatively the profiles of two (related) species. The normalized cDNA libraries were synthesized with the same protocol by the same laboratory, but sequenced with different procedures. Nevertheless, both libraries proved to be comparable on the level of transcript diversity (Fig. 2) as well as qualitatively on the level of attributable functions (c.f. Fig. 3). Comparable subsets of transcriptomic sequences comprising reliable sequence information for alignments on amino acid and RNA level were generated based on the BLAST results, covering 40 to 60% of functions seen in a related reference genome. In summary, shifts in codon usage between the species seem not biased by expression level per se, but possibly by evolutionary forces requiring specific expression levels in a certain (thermal) environment. This aspect is not further addressed within this study.

Functional coverage compared to a reference genome

It is noteworthy, that translated coding sequences of *G. aculeatus* are part of the eggNOG orthologies. Therefore, we expect that a comparison in a subset of these orthologies, i.e., metazoan (meNOGs), is useful to serve as a scale for inter-comparisons of the present cDNA libraries. Nevertheless, comparisons with *G. aculeatus* genomic coding sequences might show an overestimation of coverage due to the unresolved multiplicities by splice variants present in the assemblies of the transcriptomes. This fact might explain the observed higher coverage of functional terms in the KOG/COG categories N (cell motility) and W (extracellular structures) of the zoarcid fish compared to the reference genome.

Screening for occurrence of library-specific terms in principle allows for detection of signatures of the environmental conditions under which the transcriptomes were obtained, and not necessarily indicates presence or absence of genes in one of the species genomes. As a result transcripts related to *COI* were detected in elevated proportion in *Z. viviparus*, which is against the expectation that the normalization step in library preparation should reduce over-abundant RNA-variants in both libraries to a comparable degree. The same holds for the Antarctic species, for which the frequency of ubiquitin annotation appears in a higher rank. Discovering library specific functional terms, which would be expected to be included in similar ranks in both transcriptomes, reflects differences in the efficiency of normalization. Other terms, specific for the cDNA library of *Z. viviparus* are related to complement protein

(meNOG13752) and extracellular matrix constituent protein (meNOG18150). These terms summarize functions for immune response and integrity of the tissue, comprising cell communication, growth factors and wound healing. The term for tripartite motif (TRIM) proteins, which is predominantly found in the *P. brachycephalum* library, summarizes sequences of a protein family associated to pathogen-recognition, regulation of the concomitant transcriptional responses, constituting an important part of the immune system [34]. These differences may indicate adaptive forces acting on the immune system or simply different life histories of the sampled specimens. Responses of the innate immune system in fish were apparently more robust and diverse than in higher vertebrates [35]. Similarly, e.g., Atlantic cod comprises a unique immune system with substantial gene losses in adaptive components without being exceptionally susceptible to disease under natural conditions [36]. Consequently specific adaptations in the immune systems in the two eelpout species due to the different habitat conditions seem possible.

Another overrepresented term in *P. brachycephalum* is a carboxylase, which belongs to the KOG/COG category I - lipid transport and metabolism, as listed in the meNOG tables. The lipid fraction in liver of *P. brachycephalum* kept at 0°C is threefold higher than in *Z. viviparus* at habitat temperature [37], in line with the general finding that Antarctic species have high capacities for catabolism of fatty acids [38]. Furthermore, a recent study demonstrated relationships between preferred energy fuels and transcript levels of respective genes in *P. brachycephalum* [39]. Although the overrepresentation of the carboxylase fits into the current picture, further genes could have been expected to occur substantiating this evidence. This mismatch may diminish when the entire genome sequence data will become available. In summary, some of the library specific functions identified in our study may point to transcriptomic and possibly genomic contrasts due to the different environmental conditions of the discrete habitats of the two zoarcid fish under study.

Amino acid usage

With the advent of more genomic data from species from various habitats analyses of temperature effects on amino acid usage profiles and the correlated GC content became possible [13, 40]. Gu and Hilser [41] analyzed intra-protein interaction energies on the level of primary and secondary structural sequence segments in thermophilic and psychrophilic proteomes, supporting the flexibility hypothesis of cold adaptation. Furthermore, local and global adaptation patterns were differentiated, depending on different protein families.

By comparing the amino acid composition of a comprehensive set of orthologous sequence

pairs globally we discovered a distinct pattern of replacements, which may result in structurally important changes in the functional structure of proteins supporting cold adaptation of primary protein structures (Fig. 5, Table 2). In the following section all observed changes of amino acid usage are discussed in respect to the Antarctic species when compared to the *Z. viviparus* transcriptome.

The most obvious shifts found for *P. brachycephalum* are the net loss of glutamic acid and asparagine and the gain of serine (compare values of Table 2 and Fig. 5). The frequency of all acidic residues is reduced and contrasts with slightly higher frequencies for the basic amino acids lysine and arginine as well as histidine. Due to the eminent loss of glutamic acid, the total amount of charged residues for stabilizing salt bridges is reduced. This may contribute to weaker interaction involved in substrate binding and protein interaction. The net loss of glutamic acid and a preferential exchange with 85 aspartic acid residues preserves the acidic function at reduced flexibility of the side chain. These exchanges may cause increments in charge density at the surface favouring increased solvent interaction as postulated earlier [11, 42]. A reduction of salt links results from the exchange of the charged glutamic acid by polar glutamine (30 Q over E), in line with earlier assumptions drawn from analyses of individual proteins [11, 42]. Arginine as a bulky amino acid was identified to be less abundant in cold-adapted species [11, 42]. However, replacements of 30 serine into arginine and a slight increase of arginine on a global level contrasts with both former assumptions.

Based on genomic comparisons Wang and Lercher [30] developed a simple, reduced predictor based on replacement frequencies of the charged amino acids glutamine (E), arginine (R) and lysine (K), which were found most often exchanged in a thermal cline. Higher values for the ERK-proxy are found to be characteristic for hyperthermophiles compared to thermophiles and mesophiles. However, the ERK signal in our study is purely based on the large E change (Fig 5), as a minor change of R+K in the reverse direction does not confirm the ERK-hypothesis perfectly. We interpret this partial contrasting outcome as a consequence of extrapolation, i.e., application of a thermophile-mesophile hypothesis below the mesophile scope seems to be appropriate only for the E signal. Furthermore, we are aware of the finding of Lobry and Necsulea [40] that thermophile-mesophile signatures of cold adaptation are more prominent [43] than mesophile-psychrophile signatures, at least in analyses of codon-usage trends in coding sequences of complete genomes.

Within the group of polar amino acids the picture is heterogeneous due to the large gain of serine and the net loss of asparagine. Comparing the total counts of gains and losses of polar with unpolar residues a gain of 102 polar residues remains. This indicates that despite this

pooling unpolar to polar shift -supporting increased solvent interaction- cannot be resolved as a strong signal within our study.

By comparing a limited set of prokaryotic proteome sequences amino acid frequencies canonically discriminating psychrophilic, mesophilic and thermophilic species were determined after modelling of candidate genes on existing three-dimensional structures [43]. In this way the analysis of particular orthologous sequences were extended to part of the proteomes, and amino acid frequencies were assessed separately in structural categories 'buried' and 'surface'. In general, a trend towards polar residues (in particular serine) resemble the finding of a (solely significant) preferential usage of serine and a net gain of polar residues for a cold-adapted species. Our study confirm these findings as a cold induced positive serine shift is one of the dominating signals in our study. Therefore an increased protein surface-solvent interaction in cold-adapted proteins can be hypothesized, at least on the basis of the net serine signal. However, for a confirmation, further sequence analyses are required to gain structural discrimination of solvent exposure. A diverse pattern is detectable for threonine, accounting to a small net gain similar to serine: the surplus of 82 exchanges over alanine significantly increases the polarity, possibly contributing to less hydrophobic interactions within and improved solvent interactions at the surface of proteins. Similarly, the net gain of threonine over asparagine contributes to the overall reduction of the latter and may support better solvent interaction through increased polarity. A surplus of 30 serine exchanges for threonine was detected. At preserved polarity this exchange is likely to increase the accessibility of the hydroxyl group. In contrast, methionine is preferred over threonine (46 M over T), decreasing the polarity. In summary, the multitude and diversity of exchanges within the polar category of amino acids together with the strong signal found in an earlier study [43] point to their importance for thermal adaptation of proteins.

Within the group of unpolar residues no prominent net gains or losses were detected (Figure 5). It should be noted that a moderate gain in the usage of proline for *P. brachycephalum* is detectable, contrasting conclusions of existing views [11, 42], as this amino acid causes a large negative impact on structural flexibility. Several amino acid exchanges with conserved functions became apparent in the hydrophobic domain: net gains of 31 alanine in exchange for leucine reflect the preference of shorter residues, i.e., reducing entropic and enthalpic net contributions of side chains to structural stability of protein cores [8, 10, 11]. The finding of 51 valine over alanine and 65 isoleucine over valine, contrast with the global pattern of reduced hydrophobic interaction. However, it is likely that an enlargement in unpolar residue length may enhance the protein-lipid interaction, e.g., within membranes, and must not be in

contradiction with the flexibility hypothesis.

As only minor changes in sequence seem to be necessary to adapt kinetic properties, adaptive changes may be difficult to be identified unless proteins of closely related species from different thermal habitats are compared [5, 8]. Furthermore, studies, based on structural attribution, are restricted to proteins with available 3D structures, which is possibly biased towards soluble proteins with enzymatic function as these proteins are overrepresented in common databases. The approach of our study considers all types of expressed proteins within the transcriptome including membrane proteins, structural proteins, etc. without further assumptions.

We claim that our study is less distorted by a probable bias from a specific selection of species, in that we restrict our analyses on model orthologous sequences from two comparable transcriptomes. Furthermore, we focussed on aligned segments, excluding insertions, i.e., we hypothesize that adaptation can be analysed on a purely local basis at single amino acid positions.

In summary, the observed amino acid substitutions represent a global net pattern of molecular shifts in the local, i.e., position-specific amino acid composition for two species of the same family, inhabiting thermally very distinct sites.

Codon usage

A holistic approach for studying temperature-dependent evolutionary profiles on genetic up to proteomic levels was initially subjected to archaea, bacteria and only some eukaryotes on a large thermal scale [14, 44], uncovering trends of a higher GC usage for species with a higher OGT. As the GC content reflects the degree of hydrogen bonding in nucleic acid chain molecules, higher GC contents lead to an increased thermal stability in (deoxy-) ribonuclein acid chains [45, 46]. Thermal adaptation was analyzed in structural ribosomal RNAs for prokaryotic *16SrRNA* finding rising GC contents in species with higher OGTs [47]. In vertebrates similar observations were detected in the *18SrRNA* [48] with a trend for higher GC content in endotherm animals compared to poikilotherms showing a correlation to the environmental habitat temperature of the species under study. Similarly, the coding parts of genomes of cold blooded vertebrates and mammals are proposed to be separated by a “major compositional transition” in the GC content, resulting in nearly 100% GC3 levels in mammals [15]. In contrast, genomes of poikilotherm vertebrates are characterized throughout by lower GC3 levels.

Based on the identified segment pairs on the coding sequence level, we focused on a subset of

transcript sequences of equal size in both libraries to uncover temperature-related patterns in the codon usage. This filter is supposed to allow for a more specific statistical comparison in that it is not distorted by effects of insertions. We furthermore filtered for highly similar and aligned sequences (80% minimum translated sequence identity) to reduce false pairing. Admittedly, total codon frequencies are on comparable levels for the two species (Fig. 7). However, by aggregating all pairwise comparisons in a single Fisher's test we were able to detect a significant signal ($p=5*10^{-4}$) of increased AT3 for the Antarctic eelpout (Fig. 7). A set of biochemically meaningful hypotheses may be proposed to explain the latter findings. Firstly, under energetically constrained conditions such as in polar environments, proof reading of DNA might be too cost-inefficient and an increment of A/T in the last codon position encoding for synonymous amino acids might be advantageous without having any impact on protein function. A subsequently lowered GC content is therefore discussed for the cold-adapted diatom *Fragilariopsis cylindrus* (Thomas Mock, personal communication). Secondly, translation itself can be repressed kinetically through increased GC in codons, over-stabilizing enzymatic transition states within the chain of reaction steps of translation in the cold. Thirdly, to avoid over-stabilized secondary structure elements in messenger RNA in the cold lowering GC content could be an evolutionary resort. For example, the molecular function of cold shock proteins (CSPs) has been described to prevent mRNA from frozen, i.e., over-stabilized hairpin conformations in the cold [49]. An adaptation to the cold by means of reducing GC in the mRNA would imply a reduced pressure to express CSPs permanently at high levels, with implications for metabolic cost.

Conclusion

So far, many studies have analyzed subsets of orthologous genomic sequences from phylogenetically diverse species to study principles of thermal adaptation on the sequence level. As adjustments in kinetic and thermal properties of enzymes only need subtle changes in primary structure, high genetic identity is pivotal to uncover possible common principles. The present approach includes a comprehensive set of transcriptomic sequence pairs from confamiliar species inhabiting thermally distinct realms differing by about 10°C OGT. Furthermore, the filtering and alignment procedures allow for local analyses on the biomolecular level.

The analysis of synonymous codons uncovers a significant pattern of a higher A/T content for the Antarctic eelpout *P. brachycephalum* compared to the North Sea species *Z. viviparus*. This finding supports the view that cold-adaptation at the DNA/mRNA level took place even

on a small thermal gradient in line with results from large scale approaches of distantly related psychrophilic up to hyperthermophilic species [13]. The question arose whether general patterns of cold-adaptation can be identified on the primary structure of proteins. The observed dominating position-specific differences in the primary structure of proteins are pointing to differences in the secondary structure and dynamics, affecting stability and kinetics. Our findings may support the flexibility hypothesis of cold-adaptation, but differences to the existing literature became visible. The former studies revealed some trade-offs for optimization of cold-adapted enzymes like less salt-bridges and more charged side chains for improved solvent interactions. Consequently, cold-adaptation of single proteins uses only a subset of possible alterations. For an understanding of general evolutionary trends in primary protein structure, we provide a more global approach resolving differences between species with similar lifestyles but different thermal adaptation. Protein flexibility at different environmental temperatures may be one adaptation goal whereas for the whole transcriptome other requirements may prevail. The present study should motivate subsequent research with suitable species pairs/groups and full genome coding sequence data to uncover further details of sequence adaptation to the cold.

Material and methods

Phylogenetic analyses of fish

Multiple alignments and phylogenetic trees were generated with MacVector software (Version 10.0.2, MacVector Inc.). For tree construction the neighbour joining method was used, because it avoids assumptions of constant divergence rates among sequences. Node distances were calculated using the Tamura–Nei model, assuming that nucleotide substitutions in different pathways occur with independent probabilities. The sequence data of the genes *COI* and *16SrRNA* were retrieved using the taxonomy browser of the NCBI webpage (<http://www.ncbi.nlm.nih.gov/>). Detailed information of the used sequences are summarized in Table 3.

Table 3. Summary of sequence data for phylogenetic trees

species	Accession numbers <i>COI</i>	intercept/ fragment size	Accession numbers <i>16SrRNA</i>	intercept/ fragment size
<i>Cyprinus carpio</i>	NC_001606	6,399:7,949	NC_001606	2,021:3,701
<i>Danio rerio</i>	NC_002333	6,425:7,975	AF036006	1,623
<i>Gadus morhua</i>	GU324197	652	AM489716	1,093:2,757
<i>Gasterosteus aculeatus</i>	EU524639	652	NC_003174	1,087:2,776
<i>Micromesistius poutassou</i>	HQ882656	579	NC_015102	1,090:2,753
<i>Notothenia coriiceps</i>	EU326390	652	NC_015653	16,581:18,273
<i>Oncorhynchus mykiss</i>	GU324178	652	NC_001717	2,088:3,767
<i>Oryzias latipes</i>	AP004421	5,455:7,011	AP004421	1,084:2,757
<i>Pachycara brachycephalum</i>	HQ713113	652	Z32732	542
<i>Salmo salar</i>	GU324184	652	NC_001960	2,093:3,770
<i>Squalus acanthias</i>	EU074608	652	NC_002012	1,093:2,768
<i>Takifugu rubripes</i>	HM102315	639	NC_004299	1,090:2,755
<i>Tetraodon nigroviridis</i>	AP006046	5,457:7,016	NC_007176	1,088:2,764
<i>Zoarces viviparus</i>	EF208064	1,102	FJ798757	588

List of species used for generating the phylogenetic trees in Fig. 1 with the mitochondrial sequences for the *COI* gene and structural *16SrRNA* with the according accession numbers. For some species both sections were extracted from the whole mitochondrial genome, due to the availability of the sequence information on the NCBI browser.

Animal collection and sample preparation of Pachycara brachycephalum

Specimens of *P. brachycephalum* were caught with baited traps at the position 62°10.9' S 58°20.8' W during expedition ANTXV/3 with the RV “Polarstern” in 1998. The animals were brought to the Alfred Wegener Institute in Bremerhaven and kept at 0°C in re-circulated seawater at 34 PSU (practical salinity units). The fish were fed *ad libitum* with *Crangon*

crangon once a week; feeding was terminated exactly one week before sampling.

For sampling, the fish were anaesthetized by exposure to MS222 ($0.2 \text{ mg} \cdot \text{l}^{-1}$) before being killed. The fish ($n=9$) had a mean body length of $26 \pm 0.83 \text{ cm}$ ($\pm \text{SEM}$) and a mean body weight of $69.77 \pm 5.78 \text{ g}$. Tissue samples were quickly excised, frozen instantaneously in liquid nitrogen and stored at -80°C until further processing.

Handling and killing of the fish were conducted in line with the recommendations of the American Veterinary Medical Association (AVMA). The work was approved by a competent German authority (Freie Hansestadt Bremen, reference number 522-27-11/02-00(93)).

*cDNA synthesis, normalization and pyrosequencing of *Pachycara brachycephalum**

Total RNA was extracted from 20-40 mg tissue with the Qiagen RNeasy kit with a modified protocol (proteinase K digestion after homogenisation) according to the manufacturer's instruction (Qiagen, Hilden, Germany). Quantity and purity of the RNA were determined using the NanoDrop ND 1000 (Peqlab Biotechnologie, Erlangen, Germany). Integrity of the RNA was analysed by capillary electrophoresis (bioanalyser: Agilent, Waldbronn, Germany). Liver- and heart RNA samples of fish were pooled in equal amounts and used for the synthesis of cDNA.

Preparation of a random-primed and normalized cDNA for pyrosequencing was done with 100 μg of pooled total RNA from liver and heart (Vertis, Freising, Germany). Poly(A)+ RNA was prepared and first strand cDNA was amplified with a N6 randomized Primer. 454 sequencing adapter A (5'-GCCTCCCTCGCGCCATCAG-3') and B (5'-CTGAGCGGGCTGGCAAGGC-3') were ligated to the 5' and 3' ends of the cDNA, followed by an amplification for 21 cycles with a proof-reading enzyme. Normalization was carried out by one cycle of denaturation and reassociation of the cDNA, resulting in N1-cDNA. Reassociated ds-cDNA was subtracted from the remaining ss-cDNA by separation with a hydroxylapatite column. The purified (and normalized) ss-cDNA was amplified with 8 PCR cycles.

The resulting cDNA product was loaded to a preparative agarose gel (1.5%) and fragments in a size range of 450 – 650 bp were eluted for sequencing. The application was performed at Eurofins-MWG, Germany according to [50] using the GS FLX Titanium-technique (MWG-Biotech AG, Ebersberg, Germany). High-density pico reactions were performed on a half of a sequencing run.

Quality assessment and assembly of cDNA of P. brachycephalum:

The sequencing of the normalized cDNA library of *Pachycara brachycephalum* resulted in 481,802 reads with an average read length of 321 bases (Table 1).

The reads were preprocessed first with a base calling quality control, followed by a polyA-clipping and a screening for remaining sequencing primer adapters in the 5' as well as in the 3' ends that were truncated. The assembly was done with standard settings for stringency and homology by the Mira Assembler Version 2.9.43 (MWG-Biotech AG, Ebersberg, Germany) according to the instructions by MIRA [51, 52].

The high quality reads (338,993) were assembled into 65,565 contigs while 123,038 singlets could not be matched against any other reads. The contigs have a mean length of 487 bases and 22,651 sequences were larger than 500 bases (cf. Fig. 2A). The larger contig length compared to *Zoarces viviparus* may be due to different sequencing protocol, as we also used the FLX sequencer with a protocol including Titanium chemistry.

Functional annotation and comparisons of zoarcid cDNA libraries

The assembled contigs of *Pachycara brachycephalum* were loaded into the free software tool BLAST2GO [26] and BLASTed against the NCBI non-redundant database (BLASTx) [53] with an e-value cut-off of 1.0^{-3} and a HSP cut-off length of 33 bases. In total 47,584 Sequences had a BLAST hit, while the e-value distribution (Fig. 3A) and the HSP/hit distribution (Fig. 3B) reflects the good quality of the *P. brachycephalum* library. We processed the library of contigs of *Zoarces viviparus* in the same way [23] and found in the 53,459 sequences 35,133 with a BLAST result. After the GO-mapping step in BLAST2GO we processed both libraries for annotation with no cut-off for the HSP/hit -coverage, resulting in 19,460 sequences for *P. brachycephalum* and 16,315 sequences for *Z. viviparus*.

The latter sets of sequences were used for evaluating amino acid usage differences and for synonymous/non-synonymous codon usage comparisons. For this, we generated partial sequence translations by rpstBLASTn against a collection of fish-specific orthologies (protein sequences from fiNOG from eggNOG version 3) [54] with e-value below 10^{-9} , which yields 52,729 (*P. brachycephalum*) and 34,733 (*Z. viviparus*) hits in a total set of 7,374 fiNOG-orthologies. Pairwise alignments between sequences of the two libraries in translated form were then obtained from BLASTP of the obtained fiNOG-compatible translations. 5,352 pairwise alignments (best filtered HSP per fiNOG) with percent-identity above 80% and e-value below 10^{-9} were obtained. Coding sequences for the aligned translated segments were

aligned accordingly to the protein alignments. Removal of sequence pairs containing stop-codons and/or ambiguity nucleotide-codes yielded a set of 4,155 segments for further analyses. GC3 comparisons are based on aligned synonymous codon positions with unchanged nucleotides in the first and in the second codon positions.

Amino acid usage has been analysed from these alignments, as well as synonymous codon usage (Within Canonical Analysis), both in R [55] with the packages seqinr and ade4 (after [31]). The re-aligned sequence segments contain pairwise information that can be compared in a position specific way, allowing to quantify preferred amino acid usage, e.g., by counting amino acid specific replacement frequencies $f_{i(P),j(Z)}$ for amino acid i in *P. brachycephalum* to amino acid j in *Z. viviparus* at the same position in the alignment. A non-synonymous replacement imbalance is computed from the matrix $M = f_{i(P),j(Z)} - f_{i(Z),j(P)}$ after filtering for sequence pairs of $\geq 75\%$ identity.

For a functional characterisation of the libraries, rpstBLASTn was used against the database of metazoan orthologies computed from eggNOG-alignments (version 2, [29]). For comparison with a reference genome of a related fish, we analysed the coding sequences from the genome of *G. aculeatus* (BROADS1, ENSEMBLE Version 1.63 at EMBL) in the same way. Best hits to meNOG orthologies with e-value below 10^{-20} were kept, pooling functional annotations into COG/KOG categories.

Access to data

The transcriptome of *P. brachycephalum* is published in the sequencing-read archive (SRA) at NCBI under Accession SRA049761.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HSW participated in the concept and experimental design, carried out the preparation of samples, the annotation of contigs, participated in the interpretation of data and drafted the manuscript. ML conceived the concept and designed the experiment, participated in the interpretation of data and helped to draft the manuscript. SF contributed the idea of sequence-based signatures to this study, developed the concept and implementation of the sequence analyses and helped to draft the manuscript. All authors read and approved the final manuscript.

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Publication III

Stress response or beneficial temperature acclimation: Transcriptomic signatures in Antarctic fish (*Pachycara brachycephalum*)

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In preparation.

**Stress response or beneficial temperature acclimation:
Transcriptomic signatures in Antarctic fish (*Pachycara
brachycephalum*)**

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Abstract

Research on the thermal biology of Antarctic marine organisms has increased awareness of their vulnerability to climate change, as a flipside of their adaptation to life in the permanent cold and their limited capacity to acclimate to variable temperatures. Within this study on Antarctic eelpout, *Pachycara brachycephalum*, we employed a species-specific microarray with 40,036 unique probes for 15,843 ESTs of a normalized cDNA library to identify the patterns of steady-state gene expression after 2 months of acclimation to six temperatures between -1°C and 9°C. Changes in cellular processes comprised signalling, post-translational modification, sub-cellular organization, metabolic rearrangements and alterations in the transcription as well as translation machinery. The magnitude of responses was reflected in whole animal fitness parameters. An optimal growth performance at 3°C was paralleled by a minimum in altered transcript diversity and expression levels. The up-regulation of ribosomal proteins at 5°C and above was accompanied by differential protein degradation pathways. From 7°C upwards an incipient cellular stress response became visible through an increase of transcripts for heat shock proteins and an acute inflammatory response. Together, these patterns reflect cellular rearrangements as a consequence of cold and warm acclimation and/or the progressive development of functional imbalances. A temperature-dependent energy deficit and a shift to autophagy became visible in the warmth by linking physiological performance to liver gene expression. The observed temperature-specific expression profiles reveal the molecular basis of thermal plasticity and refine the understanding of the outline and location of the thermal window of this cold-adapted species.

Introduction

Temperature plays a crucial role for all poikilotherm animals, since this factor is pervasive and affects cellular components by altered viscosity, fluidity of membranes and enzyme kinetics (Hochachka and Somero, 2002). Exceedingly affected are poikilotherm water-breathers since they are fully subjected to the temperature regime of their habitats.

The concept of oxygen and capacity limited thermal tolerance (OCLTT) provides a model to characterize the performance of a species within its thermal tolerance window (Pörtner, 2010). Indicators of performance and thermal limitation such as growth, arterial blood flow, heart rate as well as aerobic and anaerobic metabolites reflect the aerobic scope and shape of the temperature-dependent performance window of poikilotherm species (Pörtner and Knust, 2007).

Thermal adaptation and phenotypic plasticity in response to fluctuating environmental temperatures are ultimately set by the genetic repertoire of organisms and their regulation. Eurythermal fish have been studied excessively with respect to thermal acclimation as they are exposed to varying thermal regimes, which is reflected in their wide range of biogeographical distribution (Pörtner, 2002a; Somero, 2005). In-depth expression analyses through microarrays in numerous eurythermal fish revealed extensive expression profiles changing concomitantly in response to acute and long-term exposures to temperatures above and below the thermal optimum: (*Ictalurus punctatus* (Ju et al., 2002), *Austrofundulus limnaeus* (Podrabsky and Somero, 2004), *Cyprinus carpio* (Gracey et al., 2004) and *Onocorhynchus mykiss* (Vornanen et al., 2005). *Gillichthys mirabilis* was under study in various experiments (Buckley et al., 2006; Logan and Somero, 2010; Logan and Somero, 2011), and the same array design was employed in further heterologous hybridization experiments for other species (*Pomacentrus moluccensis* (Kassahn et al., 2007) and *Thunnus orientalis* (Castilho et al., 2009)).

Exceedingly critical is the thermal limitation in cold-adapted species since their specialization is thought to have evolved as a trade-off at the expense of their thermal flexibility (Pörtner and Farrell, 2008). Such traits to cope with life close to the cold edge are, among others, antifreeze glycoproteins to prevent freezing of body fluids, or the loss of haemoglobin seen in the family of the Channichthyidae possibly with the benefit of a reduction in blood viscosity at high oxygen saturation in cold water and at low metabolic rates (for review see (Cheng and Detrich, 2007).

Due to the local influence of global climate change these highly adapted stenothermal fish

currently experience rising water temperatures around the Antarctic Peninsula (Gille, 2002; Turner et al., 2005). Furthermore, predictive models indicate that the warming trend is especially strong in polar regions (IPCC, 2007). The capacity for acclimation may therefore become especially critical for species survival in those regions.

To our knowledge, the transcriptomic response of Antarctic fish to warming has been characterized in two species by monitoring broad sets of genes in microarray studies upon acute warming. In a heterologous hybridization experiment, employing the established array of *G. mirabilis* (Gracey et al., 2001), (Buckley and Somero, 2009) analyzed the transcriptomic response of *Trematomus bernacchii* to 4 °C for 4 hours and after recovery. Although this species is known to lack the classical heat-shock response (Clark et al., 2008; Hofmann et al., 2000), the microarray revealed an altered expression of hundreds of genes connected to the evolutionarily conserved cellular stress response (CSR) (Kültz, 2005) indicating remnant capacities to respond to thermal stress.

The acute heat-response of *Harpagifer antarcticus* was characterised through the application of a species-specific array based on an EST library while being exposed to 6°C over 24 hours (Thorne et al., 2010). Responsive transcripts, such as induced components of the classical vertebrate acute inflammatory response and genes related to the oxidative stress response, displayed similar patterns as found in eurythermal fish (Thorne et al., 2010). Nevertheless, holistic studies on the mechanisms of warm acclimation in Antarctic fish and their endurance beyond the acute response are absent.

The Antarctic eelpout *Pachycara brachycephalum* is a cold-adapted stenothermal species showing signatures of cold adaptation at the codon usage- and amino acid sequence level, when compared to a eurythermal congener (Windisch et al., 2012). Nevertheless, this species exhibits acclimation capacities to warmer temperatures, reaching optimal physiological performance between 4°C and 6°C (Brodte et al., 2006a; Lannig et al., 2005). At these temperatures, rearrangements in liver tissue composition indicate a metabolic shift from a lipid- to a carbohydrate-based metabolism (Brodte et al., 2006a). Nuclear receptors of the PPAR-family were found to trigger the differential usage of fuels that could also be metabolized under anaerobic conditions supporting a “warm hardiness” at 5°C (Windisch et al., 2011).

Within the present study we examined the consequences of long-term exposure of *P. brachycephalum* to six different temperatures ranging from -1°C up to 9°C, to identify the temperature range of successful warm acclimation in connection with the changes in the transcriptome at the respective acclimation temperatures and beyond. To this end, we

designed a microarray experiment based on sequence information of a cDNA library (Windisch et al., 2012) comprising 40,036 unique probes for 15,843 ESTs. During the exposure, we recorded whole animal performance indicators to link transcriptomic signals to changes at different levels of physiological organisation and to discuss potential mechanisms that constitute the basis of thresholds within the thermal window.

Methods

Animal collection and incubation

Specimens of *P. brachycephalum* were caught with baited traps at positions 62°19.01' S 58°35.49' W; 62°16.86' S 58°36.75' W; 62°19.33' S 58°33.80' W; 62°19.69' S 58°33.68' W during expedition ANT-XXV/4 of RV “Polarstern” in April 2009. The animals were brought to the Alfred Wegener Institute at Bremerhaven and kept at a temperature of 0°C in recirculated seawater at 34 PSU (practical salinity units) in one single tank (2.3m³ total volume) until the start of experimentation.

Fish were randomly chosen to be incubated at different temperatures and were transferred to separate tanks (one for each temperature) in swimming baskets with a single chamber for each fish to monitor individual parameters. Control animals held at 0°C were exposed to the same handling procedures. Five different temperatures (-1°C, 3°C, 5°C, 7°C, 9°C) were applied to groups of a 12 fish minimum per treatment for a total duration of nine weeks. All fish were weighted under a slight anaesthesia (0.05 g l⁻¹ MS222) before being exposed to different temperatures. To adjust the temperature in the different setups, animal groups were warmed at 1°C d⁻¹. Animals exposed to 7°C and 9°C were warmed in a stepwise procedure, first being incubated for 1 week at 5°C before being exposed to 7°C for the entire duration of the experiment. The group of fish intended to be studied at 9°C were held at 7°C for another week before being exposed to the final experiment temperature.

The fish were fed *ad libitum* with *Crangon crangon* once a week; feeding was terminated exactly one week before sampling. For sampling, the fish were anaesthetized by exposure to MS222 (0.2 g l⁻¹) and blood samples were taken with a heparinised syringe from the caudal vessel before being killed. Syringes were coated by being purged with a sodium heparin solution (2500 U ml⁻¹) and dried at room temperature thereafter. Liver samples were excised quickly, frozen instantaneously in liquid nitrogen and stored at -80°C until further processing. Further tissue samples were taken for follow-up studies.

Handling and killing of the fish was conducted in line with the recommendations of the American Veterinary Medical Association (AVMA). The animal experiment was approved by the responsible veterinary department (Freie Hansestadt Bremen, reference number 522-27-11/02-00(93)).

Animal performance

Growth performance (GP) was calculated as percentage of the weight difference between the starting and end point of the acclimation period. The hepatosomatic index (HSI) was calculated by the 100-fold ratio of liver weight in g over the total weight of the fish in g. The haematocrit was recorded in fresh blood samples by means of a haematocrit centrifuge (Compur Microspin 6500, Bayer Diagnostics Munich, Germany).

All performance parameters were analyzed by applying one-way ANOVA at a significance level of $p \leq 0.05$, followed by a Student-Newman-Keuls post hoc test using SigmaStat (version 3.5; Systat Software, Erkrath, Germany). This was also applied to changes in GP, HSI and haematocrit, tested in pooled sets of “cold” (-1; 0°C), “intermediate” (3; 5°C) and “warm” (7; 9°C). The graphs showing GP, HSI and haematocrit showing means \pm SEM were generated using SigmaPlot (version 10; Systat Software).

Experimental design

Based on the quality (ratios of 260nm/280nm ≥ 2 ; 260nm/230nm ≥ 1.8) and integrity (ratio of 28S/18S rRNA between 1.5 and 1.8) of liver RNA (extraction protocol below), analysed by capillary electrophoresis (Bioanalyser: Agilent Technologies, Waldbronn, Germany), 5 fish of each treatment and 7 fish of the control group were selected for expression analyses. Only male specimens were selected for the hybridizations to reduce sex-specific observations. However, at 9°C only 2 males and 8 females survived the duration of the exposure. Accordingly, for the expression analyses this group contained samples of mixed genders (2 males, 3 females). All selected fish had a mean body length of 21 ± 0.94 cm (\pm SEM) and a mean body weight of 35.61 ± 5.06 g. Liver RNA samples of all selected fish were pooled in equal amounts as reference pool. Each single sample representing one animal was hybridized against the reference pool on a single array.

Array design

The array is based on a transcriptomic cDNA library (SRA049761) of *P. brachycephalum* and encompasses 40,036 unique probes for a subset of 15,843 contigs in the library.

For a compact and valid design we optimized the probes with a test array (custom design from the cDNA library comprising 91,402 probes for 17,024 contigs, unpublished data) in advance to check probe efficiency as well as to achieve a high coverage of the available transcriptomic data with the given array capacity. Based on the results, custom design arrays were produced

by Agilent (Agilent Technologies, Waldbronn, Germany). The design data are available in the database for Minimum Information About a Microarray Experiment of the European Bioinformatics Institute (MIAME, ArrayExpress at the EBI <http://www.ebi.ac.uk/arrayexpress/>) under ID A-MEXP-2248 (8x60k format) and A-MEXP-2249 (2x105k format).

Sample preparation and labelling

Total RNA was extracted from 20-40 mg liver tissue of all incubated animals with the Qiagen RNeasy kit with a modified protocol (proteinase K digestion after homogenisation) according to the manufacturer's instruction (Qiagen, Hilden, Germany). Quantity and purity of the RNA were determined using the NanoDrop ND 1000 (Peqlab Biotechnologie, Erlangen, Germany). Labelling reactions for samples and the reference pool were prepared with a starting input amount of 200 ng total RNA and a 1:16 dilution of a positive control RNA (Agilent RNA Spike-In Kit) to monitor the procedure of sample amplification and microarray workflow. As recommended, the treatment samples contained the RNA spike-B-mix and were labelled with Cy5. The reference pool containing the RNA spike-A-mix was labelled with Cy3. cDNA was generated and amplified according to manufacturer's instructions. The labelled and amplified cRNA containing dye-labelled cyanine was purified with Qiagen RNeasy kit with modifications as described in Agilent's protocol for two colour arrays (Agilent, G4140-90050_GeneExpression_Two_Color_v6.5). The labelling products were quantified by using the NanoDrop ND 1000 microarray measurement protocol.

Hybridization and feature extraction

All reactions were standardized (volume and yield) according to the used 8x60K array format by following the instructions of the above-stated protocol. Dye-labelled samples containing 300 ng sample and reference each were hybridized to the arrays for 17 h at 65°C while rotating the hybridization chambers with 10 rpm. Disassembly and washing steps followed manufacturer's instructions. To reduce washing artefacts, the detergent TritonX-102 was added to both wash buffers (GE Wash Buffer 1+2 (Agilent, 5188-5325; 5188-5326)) with a final concentration of 0.005%. After washing procedures the slides were immersed in acetonitrile (VWR, Darmstadt, Germany) for 10 s, and finally incubated in a stabilization and drying solution (Agilent, 5185-5979) for 30 s. The arrays were directly scanned with the Agilent Scanner (type B) under utilization of the AgilentHD_GX_2Color program (scan resolution 5nm).

Data processing and interpretation

Scanned data were extracted with the Feature Extraction Software version 9.0 (Agilent) applying the GE2_107_Sep09 protocol. Data were normalized within and between arrays after background correction by application of locally weighted scatterplot-smoothing (LOWESS) as implemented in Limma (Smyth and Speed, 2003) in R (R-Development-Core-Team, 2011).

At first, t-tests against the control group (0°C) were performed (Welch) to gather significant features. This analysis revealed transcripts which, when compared to the averaged reference, were down- or up-regulated at all temperatures (data not shown). The focus of this study was to find differently expressed genes among the applied temperatures. Significance analyses for microarray data (SAM) were performed (Tusher et al., 2001) within the MeV software environment (Saeed et al., 2006). For the Multi-class comparisons of all six groups, the delta value (tuning parameter to set the cutoff for significance) was adjusted to 0.082 for a median false positive rate $q \leq 0.001\%$ and a 90th percentile FDR $\leq 0.2\%$. The resulting data, comprised of 1,120 responsive probes representing 664 contigs, were further analyzed to characterize gene expression patterns. Group-median values were calculated per probe for each incubation group, and probes related to the same contig were averaged arithmetically.

Contig annotations were obtained by BLASTx-searches (Altschul et al., 1990) with an e-value cutoff 10^{-3} against the SwissProt database (<http://www.uniprot.org/>) as well as the non-redundant database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) within the Blast2GO tool (Conesa et al., 2005). Furthermore, contigs were assigned to gene models (protein sequences from fiNOG within eggNOG, version 3 (Powell et al., 2011)) based on a rpstBLASTn against the fish-specific orthologous sequences, i.e. fiNOG, with an e-value cutoff of 10^{-20} (see (Windisch et al., 2012) for the assignment of transcripts to orthologous sequences).

Contigs with significantly altered expression levels obtained from SAM analyses were filtered by group (each temperature separately) for an absolute fold change (FC) ≥ 1.5 to examine temperature-specific functional terms by means of fiNOGs. Superordinated categories of fiNOGs, i.e. clusters of orthologous groups (COG/KOG), were used for a weighted diagram to show in detail functional characteristics of responsive genes within treatments (cold, intermediate, warm) in percent per treatment (Fig. 3A) as well as per category (Fig. 3B).

To identify further details inside the selected categories, the corresponding transcripts were arranged by hierarchical clustering with Pearson correlation distance metrics and complete linkage clustering under utilization of the the MeV software.

It is noteworthy that the SwissProt annotation was available for nearly all transcripts with fiNOG-hits and the functional descriptions were highly congruent (data not shown). SwissProt annotation was preferred for the discussion of the detailed examination of altered expression patterns. However, to increase functional sequence information, 8.4% of the transcripts in this analysis were complemented by descriptions of BLASTx results gathered from the non-redundant database (labelled with an asterisk in the supplementary figures).

Due to the lack of function information, responsive transcripts inside the categories R (general function prediction only), S (function unknown) and X (no result) were postponed.

Based on the significant sets of contigs with an absolute FC ≥ 1.5 , GO enrichments were calculated with Blast2GO (Conesa et al., 2005) for the three major acclimation groups.

To determine whether GO terms of the corresponding subsets were over- respectively underrepresented in comparison to the remaining terms of the array, Fisher's Exact tests were performed at an alpha-level of 0.05 and a subsequent correction for multiple testing by FDR ≤ 0.05 (Benjamini and Hochberg).

Results and Discussion

Whole animal performance

Successful thermal acclimation of poikilotherms may only be accomplished within the boundaries of the physiological tolerance window. Since thermal thresholds of individual components of functional networks (e.g. enzymes) would likely be found outside of the whole organism thermal window (Pörtner, 2012), analyzing the molecular constituents would have to consider changes in whole animal performance when evaluating their contribution to warm acclimation. In a natural population of *P. brachycephalum*, the upper critical thermal limit was determined to be between 9°C and 10°C by measuring oxygen consumption, pH_i and the onset of anaerobic end products in various tissues (Mark et al., 2002; van Dijk et al., 1999). In the present study, similar critical limits were detected by assessing the mortality rate during experimentation. All animals of the 0°C, 3°C, and 5°C incubations survived the experiments. At -1°C and 7°C one animal died in each group, whereas at 9°C ~50% of the incubated animals died, indicating that this temperature is beyond the long-term upper thermal tolerance limit.

Not only aerobic exercise but also growth performance (GP) (Fig. 1A) reflects aerobic scope, since this parameter comprehends the net quantity of the total energy turnover for poikilotherm organisms (Pörtner, 2010). At low incubation temperatures (“cold”) animals displayed a gain in total body mass of $3.87\% \pm 2.35\%$ at -1°C and $7.23\% \pm 0.83$ at 0°C. Although these temperatures represent the normal thermal range of the natural population of *P. brachycephalum*, the highest weight gain was determined at 3°C with $11.17\% \pm 4.73\%$. At 5°C the weight gain of $8.31\% \pm 2.14\%$ was still higher than at cold temperatures (both groups are further referred to as “intermediate”).

In contrast, the animals exposed to the two highest temperatures (assigned as “warm”) showed weight losses with $6.23\% \pm 2.95\%$ at 7°C turning into a dramatic decline of body weight by $26.03\% \pm 4.28\%$ at 9°C.

Overall, the present growth data result in a bell-shaped GP-curve, similar to a former study for this species, where the maximum growth temperature was documented at 4°C and growth rates were similar at 6 and 0°C (Brodte et al., 2006a).

As a corollary, a distinct thermal point between 6°C and 7°C where long-term GP of *P. brachycephalum* gets lost resembles the upper pejus temperature in the OCLTT concept at which only a short period of worse conditions could be survived.

The hepatosomatic index (HSI) represents an important fitness parameter in fish (Busacker et al., 1990). We observed (Fig. 1B) a decrease of the mean HSI to 1.55 ± 0.11 in the warm-exposed group indicating a massive loss of total liver weight, compared to a HSI of 2.7 ± 0.16 in the cold group or a mean HSI of 2.41 ± 0.15 in the intermediate one.

GP and HSI data indicate an elevated energy demand at 7°C and 9°C, which could not be covered by normal food intake. As feeding was successful in all groups, the deficiencies caused through warming obviously have been balanced by mobilization of body-own reserves.

Higher energy requirements in the warm-exposed group were paralleled by a rise in haematocrit levels (Fig. 1C). In the cold- and intermediate-acclimated groups the parameter remained inconspicuous ($12.15 \pm 1.11\%$, respectively $12.58\% \pm 1.1\%$). However, a remarkable increase of 38% resulting in haematocrit levels of $17.04 \pm 1.10\%$ was observed after warm exposure.

Elevated haematocrit levels in connection with chronic warm exposure may be due to a limited compensation for functional hypoxemia (sensu OCLTT; Pörtner, 2010) resulting in elevated oxygen demand (Mark et al., 2002; van Dijk et al., 1999).

The existing knowledge on aerobic scope together with the fitness parameters at whole animal and tissue levels provide evidence for thermal constraints becoming visible below -1°C and above 7°C as well as a thermal optimum at around 3°C.

Functional outline of temperature-conditioned transcriptomes

The performance patterns observed after chronic exposure to various temperatures indicate that thermal acclimation has been successfully completed within the thermal optimum range but also that it had reached capacity limits at temperatures beyond. Constraints in performance may be paralleled by the onset of thermal stress such that a species within its thermal optimum would display a homeostatic response that turns progressively into a cellular stress response CSR (Kültz, 2005) at temperatures beyond the optimum range bordered by pejus temperatures. Stressors like temperature, hypoxia, contaminants, pollutants and handling stress affect the organisation of the cytoskeleton, the induction of chaperones accompanied by changes in the metabolism, DNA repair mechanisms as well as alterations in replication/cell cycle, transcription and translation (Kassahn et al., 2009).

SAM analyses revealed temperature-dependent changes in expression of 4.3% of the genes represented on the array. When examining the median expression of transcripts among the applied temperatures with a minimum $|FC| \geq 1.5$, an imbalance of up and down-regulation became visible (Fig. 2A). The extent of up- and down-regulation is similar at cold temperatures, whereas an inclination of approx. 88% down-regulation is observed in the intermediate group. The strongest down-regulation could be observed for warm temperatures, but also a gain of responsive transcripts. Intersections of unique transcripts within exposure groups indicate that a unique/specific expression is rare for the intermediate group (Fig. 2 B), as most of the transcripts are subsets of both remaining groups. The transition from cold to intermediate temperatures led to reduced transcript diversity followed by an increase during transition to warm temperatures. In conclusion, most of the thermally sensitive genes have different expression profiles outside of the optimum range (Fig.1A) Yet, the profile of Fig. 2A also leads to the question, whether homeostatic acclimation or stress responses were involved in the different exposure groups.

Information-storage and processing

To identify the putative cellular reorganisation initiated by the transcriptomic changes, we attributed the responsive transcript sets of Fig. 2 B to fish-specific orthologous functions (fiNOGs) classified in COG/KOG categories (Fig. 3 A). The itemization of the responses inside individual categories provides information on the relative effects of temperature treatments in these categories (Fig. 3 B). In addition, GO enrichments were performed to specify and emphasize regulated processes and functions within COG/KOG categories (c.f. Table 1&2), as discussed in the following sections.

All acclimation groups revealed responsive transcripts within the functional COG-categories of translation (J), RNA processing and modification (A) as well as transcription (K). In contrast, DNA-organization mechanisms (L, and B) were affected only in the cold and warm groups (Fig. 3 A). Within all categories (Fig. 3 B), the intermediate group was least affected. Only the mechanisms related to translation (J) displayed a progressive increase with temperature. This was confirmed by GO enrichments, as all of the up-regulated transcripts in the intermediate group belong to this category (Table 1, GO:0022625, GO:0003735, GO:0003723, GO:0006414, GO:0042254). Consequently, transcripts related to translation were significantly down-regulated in the cold (Table 2, GO:0005852) and up-regulated in the warmth (Fig. 3A+B)

In greater detail, several regulators of transcription as well as transcripts of mitochondrial ribosomal proteins were induced on the cold side, whereas less regulatory but more ribosomal protein transcripts were seen in the warmth (Suppl. cluster 1). The conspicuous up-regulation of mitochondrial transcripts in the cold might relate to the cold-induced proliferation of mitochondrial densities, which are discussed below (see metabolism, C). Subunits of the eukaryotic translation initiation factor and ribosomal proteins of the 60S and 40S subunits were induced by intermediate temperatures and above. This may indicate a higher turnover of these transcripts (or proteins) in the warmth or a higher stability of the protein synthesis machinery in the cold. Reduced translational capacities in the warmth as seen in functional studies (Storch et al., 2005), might result from increased protein destabilization and a shorter half-life, especially of large protein complexes, where correct assembly underlies optimal functioning. Excess transcripts may be produced to maintain a functional protein synthesis machinery. Our observations suggest that modifications of the translational apparatus are implemented *in vivo* through cold-repression and warm-induction of transcripts in this category.

Within RNA processing and modification (A), a variety of different small nuclear ribonucleoproteins, like u6/u4 snRNA proteins, are induced in the cold (Suppl. cluster 1). These proteins are involved in the splicing mechanism indicating cold-compensated RNA processing. However, the target genes are not yet known. At warm temperatures, proteins containing WD repeat domains (tryptophan-aspartate repeat) are induced, which are known to function as scaffolds for many protein-protein interactions. Their contribution to the formation of the transcriptional initiation complex has been described in detail (Smith, 2008) and their induction in *P. brachycephalum* may be important for sustaining the translation process in the warmth. Furthermore, particular RNA helicases are induced at cold and warm

temperatures, but a temperature-dependent utilization of distinct functional types is not yet described and remains speculative at this stage.

A similar picture emerges for the category of transcription (K) with transcripts that were exclusively induced in the warm or cold groups. This array of mediators of transcription (e.g. transcription initiation factor 2b, or TATA-box binding protein) also show a clear temperature-dependency (Suppl. cluster 1). Candidate genes induced in the cold seem to be directly involved in transcription, whereas those expressed in the warmth are rather related to signal transduction. This interpretation is in line with observations regarding categories C, Z, and U (see below), in the sense that higher transcription rates must compensate for sustained performance in the cold or for cellular reorganisation in the warmth, respectively (see category T below).

Transcripts for replication, recombination and repair (L) appear up-regulated in the cold in terms of methylation, DNA replication and nuclear migration (Table 1, GO:0008168, GO:0045740, GO:0007097), and down-regulated at higher temperatures (Table 2, GO:0007097, GO:0045740). Chromatin structure and dynamics (B) displayed more transcripts for histones as well as an ATP-dependent helicase in the warmth, and were reduced in the cold (Suppl. cluster 1). A replacement of thermally damaged proteins (i.e. histones) is likely as well as partial reprogramming of the transcriptome by chromatin remodelling. The latter mechanism is described in yeast to promote the transcription of a different set of genes accompanied by alterations of the condensation state (hetero- and euchromatin) of DNA by histone-density and the provision of methylation sites to trigger the expression of target genes (Shivaswamy and Iyer, 2008).

Cellular processes and signalling

Since temperature affects the solubility and stability of proteins at large, changes/modifications should also occur in the basic cellular structure during acclimation. The according responses comprise regulated transcripts of cell cycle control (D), signal transduction (T), cytoskeleton organisation (Z) intracellular trafficking (U) and posttranslational modification (O) (Fig. 3 A). The onset of higher temperatures causes an arrest in cell division, which matches reduced growth rates. More specific, cell cycle control is basically stronger controlled in the warmth as the progression from G2 to M phase in response to DNA damage is blocked (Table 1, GO:0000079, GO:0007095). In contrast, the same or similar terms are repressed at cold (Table 2, GO:0007095, GO:0000079) and intermediate temperatures (Table 2, GO:0022402, GO:0000278). Notable transcripts are cyclin-g1 and programmed-cell-death-1-ligand 1, which are gradually induced by higher

temperatures (Suppl. cluster 2). Cyclin-g1 is known for its role to arrest cells after damage and silence cell growth at the G2/M checkpoint in higher vertebrates (Kimura et al., 2001). Since the growth and HSI data indicate strong weight loss and consumption of energy fuels, it seems possible that cells are not only arrested, but rather than absorbed after apoptosis or by autophagy. The latter process is known to become effective during starvation and metabolic stress causing intracellular damage (Lum et al., 2005). However, no alterations in transcript levels of the corresponding “classical” signal–transduction components of mTOR- or AMPK-signalling were detected.

Changes in transcript levels associated with signal transduction mechanisms (T) (Fig. 3 A) are summarizing contributions from GTP binding, MAPK- and JNK- signalling. These were induced in the cold and repressed in the warmth (Table 1&2, GO:0030742, GO:0031435, GO:0046330; further repression in warmth: GO:0005525, GO:0003924). GTP binding is involved in various signalling processes (Hamm, 1998; Neves et al., 2002) including MAPK- and JNK- cascade, which are involved in cell proliferation, differentiation and inflammation (Goldsmith and Dhanasekaran, 2007; Ip and Davis, 1998). A closer inspection of single transcripts involved in signalling revealed respective candidates for the cold response: chemokines, ras suppressor protein-1 and GTP-binding protein-8 (Suppl. cluster 2). In the warmth a structural reorganization through signalling between the cytoskeleton and the nucleus is supported by the induction of the four-and-a-half-lim-domains protein 1 (Kadmas and Beckerle, 2004). Furthermore, angiogenesis (angiopoetin-related protein 3) and the regulation of development and homeostasis of neuronal differentiation mediated by neogenin (Wilson and Key, 2007) became visible. In addition, elevated levels of transcripts of organic solute transporter(s) support an increased supply with nutrients. Thus, mechanisms involving the extracellular matrix organisation by vascularisation and tissue remodelling, together with elevated haematocrit, became effective upon warm exposure, suitable to counteract more likely hypoxemic events in the warmth (Pörtner, 2010).

Nearly the same profiles appeared in the COG/KOG analyses for the closely connected categories Z and U (Fig. 3). Several GO terms approve that transcripts of proteins involved in cellular integration experienced an adjustment in different temperature regimes. Cell communication is induced in the cold (Table 1, GO:0006949, GO:0031274, GO:0046847, GO:0034332), but repressed in intermediate and warm incubations (Table 2, GO:0034332, GO:0046847, GO:0031274). In addition, transcripts for structural proteins, facilitating microtubule formation and microtubule-based movement were repressed at higher temperatures (Table 2, GO:0005839, GO:0007018). The solubility and viscosity of the

cytosol is affected by temperature and its maintenance may require cytoskeleton remodelling. In this respect, it is hypothesized that WD-repeat-domain proteins play a role by facilitating multi-protein assemblies as scaffolding molecules. Dynein, myosin and tubulin as well as the channel protein sec61 (gamma subunit) are induced in the cold to promote protein shuttling from the ER to the cytosol (Suppl. cluster 2). Expression levels of all these components are reduced in the warmth. Instead, anchor and structure-maintaining proteins like spectrin (associated protein), dystrobrevin (alpha) and desmoplakin are induced upon warming. These proteins stabilize the inner side of the plasma membrane, link the cytoskeleton to the extracellular matrix and connect neighbour-cells more closely. From these observations it seems that cells are losing shape and volume in the warmth, which also suggests a connection to autophagy of cellular components.

In the category posttranslational modification, protein turnover and chaperones (O) significant GO terms were correlated with an induced proteasomal protein-degradation in the cold (Table 1, GO:0005839, GO:0004298) and a repression in the warmth (Table 2, same terms and GO:0071822). Multiple components of the proteasome, i.e. regulatory proteins and ubiquitin-conjugating enzymes, are up-regulated in the cold. A similar finding was described in *Austrofundulus limnaeus* by (Podrabsky and Somero, 2004) with higher transcript levels for the 26S ubiquitin subunit after chronic cold exposure accompanied by constant levels of de-novo-synthesized proteins. Furthermore, higher levels of ubiquitin-conjugated proteins are observed in Antarctic fish (Todgham et al., 2007) and are discussed as major constraint in the cold-adaptation process, since high rates of “non-productive” folding in the cold results in high levels of degradation and high costs for ubiquitination. Nevertheless, it is remarkable that protein degradation differs in different thermal treatments. Whereas damaged proteins seem to be degraded by proteasomal depletion in the cold, the lysosomal pathway seems to be preferred in the warmth (in line with autophagy). The different use of degradation pathways in the cold and warmth may have implications for the energy budget. A higher cost of protein degradation via ubiquitination in the cold may represent a trade-off between cellular maintenance and growth. In addition to cold-denaturation of proteins, an indication for oxidative stress is given by induced transcripts for the glutathione-s-transferase (Suppl. cluster 2), whereas a repression of transcripts of the redox-system was significant in the warmth (Table 2, GO:0004364). Excess of oxygen availability and high mitochondrial densities in the cold could lead to a higher development of ROS species (Abele and Puntarulo, 2004). The induction of redox-counteracting transcripts is seen here mainly in the cold. Despite that, ROS formation is primarily discussed for this species in the warmth (Mark et al., 2006), when

oxygen-supply is limited and an insufficient entry into the respiratory chain causes ROS by a disturbed electron-transfer and too high membrane potentials. Nevertheless, it is likely that a cold-compensation (i.e. higher levels in the cold at maintained functionality) occurs for the glutathione redox system.

Further induced transcripts within this category comprise coagulation factors and acute phase proteins of the complement system which were detected in the warm groups and indicate a similar reaction of the innate immune system as seen for chronic responses in temperate or acute responses in other Antarctic fish (Buckley and Somero, 2009; Podrabsky and Somero, 2004; Thorne et al., 2010). These marker proteins are responsible for the labelling of damaged cells. Since fish depend more on the robust innate instead of the adaptive immune system (Tort et al., 2003), this mechanism is effective to clear damaged cells and to organize inflammation and healing processes. One potential mediator of wound healing is fibronectin, an integrin that mediates cell adhesion and phagocytosis of opsonised cells (Grinnell, 1984). Fibronectin is found in the responsive transcripts of *P. brachycephalum* at warm temperatures and may contribute as chemo attractant for the maintenance of the connective tissue after autophagy.

The induction of chaperones like heat-shock protein 71 and 105 revealed that *P. brachycephalum* activates only a set of the HSPs represented on the array after long-term exposure. Several probes targeting different subunits of 5 HSP types, including low and high molecular weight species, were present on the array (data not shown). HSPs are essential components in the CSR attesting in this experimental setup that a cellular homeostasis is not reached after 2 months. In the warmth, high molecular weight HSPs seem to be permanently required at higher levels due to their function for conformational maintenance of proteins. Also, high turnover rates for themselves are likely since HSPs may also experience a loss of stability and a subsequent degradation at higher temperatures.

Metabolism

A first inspection of this cluster revealed that cold and warm incubation affected all categories with mixed patterns (Fig. 3 A). Despite this, no up-regulated COG/KOGs were noticeable for the intermediate group, whereas down-regulation occurred in 50% of the categories.

Key functional traits of the metabolism are dependent on mitochondrial processes through provision of ATP and the intermediates of the citric acid cycle. In Antarctic fish, mitochondria are characterized by high densities, strict coupling of the respiratory chain and lower capacities than in temperate species (Hardewig et al., 1999; Johnston et al., 1998; Pörtner, 2002b).

After chronic exposure, energy conversion and production (C) in the cold was linked to up-regulated molecular functions connected with reductive functions of the NADP-dependent isocitrate dehydrogenase (Table 1, GO:0004450, GO:0019643, GO:0006102, Suppl. cluster 3). The latter enzyme catalyzes the reaction from isocitrate to 2-oxoglutarate by generation of NADP(H). A similar pattern was seen for the cytoplasmic aconitase hydratase, which is also involved in the provision of substrates in the citric acid cycle. Together with the elevated expression of respiratory chain components (subunits of complexes I, III, IV) and ATP-synthase, the array of induced transcripts indicates a high capacity of mitochondria in the cold. On the warm side, different subunits of complex I and -IV were induced. It is likely that proteins of the respiratory chain either need to be modified (e.g. via alternative splicing) or exchanged due to unfolding or damage in the warmth. Compared to 0°C, a higher enzyme capacity of complex IV was reported in this species after six weeks of acclimation to 5°C at constant transcript levels (Windisch et al., 2011). The higher activity was discussed to contribute to an elevated aerobic capacity, which may promote the enhanced energy allocation into growth (Brodte et al., 2006a). The extension of expression profile even to higher temperatures indicates, that higher transcript amounts are required due to higher rates of protein damage at 7°C and above. Another indication for functional constraints of mitochondria is given by the higher expression of uncoupling proteins in the warmth pointing to the critical state of too high membrane potentials and ROS formation at 7°C and 9°C. Mark and colleagues (Mark et al., 2006) described the role of uncoupling protein 2 in this species by facilitating a fast and flexible regulation of the electron transport chain for specimen acclimated to 5°C. An increased uncoupling and proton leakage in mitochondria due to constraints of higher temperatures finally results in lower energy efficiency, which may exert its effect up to the level of GP.

In general, the metabolism in cold-adapted fish is lipid-based (Crockett and Sidell, 1990). This is confirmed by high triacylglycerol lipase activities in various Antarctic fish (Sidell and Hazel, 2002). Moreover, high lipid content facilitates oxygen supply and covers the high energetic demand in cold-adapted fish species (Pörtner et al., 2005). Acclimation of *P. brachycephalum* to higher temperatures led to a metabolic shift, which is characterized by a carbohydrate-based metabolism through the establishment of glycogen stores (Brodte et al., 2006a; Windisch et al., 2011). These alterations were discussed in the context of facilitated energy allocation at higher temperatures under more likely hypoxemic conditions as well as a strategy promoting “warm hardiness”, as carbohydrates in contrast to lipids allow for energy production at anaerobic conditions.

The observed pattern in the fiNOG analyses confirms the earlier reported observations at a broader transcriptomic level. Carbohydrate (G) and lipid metabolism (I) showed a reversed regulation at cold and warm temperatures. High expression levels of enzymes involved in lipid metabolism prevail only in the cold (Table 1& 2, GO:0043552), whereas high transcript levels of components related to the carbohydrate metabolism are found in the warmth (Suppl. cluster 3). The induction of the glycogen branching enzyme supports the previously reported glycogen uptake at 5°C (Windisch et al., 2011) and at higher temperatures.

A new aspect in connection with metabolic fuelling is the high amino acid transport and metabolism in the cold (E). An up-regulation of transcripts affiliated to the glycine cleavage system (GCS) in the cold (Suppl. cluster 2) is confirmed by enriched GO-terms (Table 1, GO:0005960, GO:0051603, GO:0019464), whereas a repression was noticeable at higher temperatures (Table 2, GO:0005960, GO:00015171, GO:0019464, GO:0015807, GO:0051603). Subsequently, free amino acids are not only used as compatible solutes (see below, category P) or to provide new building blocks for protein synthesis but also seem to play an important role as an energy-rich form of fuel (especially glycine). Kikuchi and colleagues (2008) reviewed the composition and reaction mechanism of the mitochondrial located GCS protein complex. The products of the enzymatic reaction are $\text{NADH}+\text{H}^+$, CO_2 , NH_3 and 5,10-methylene-tetra-hydrofolate, which is an intermediate for the synthesis of purines, thymidylate and methionine. This pathway is widely distributed from bacteria to plants and animals. In many vertebrate tissues the system is abundant and utilized for serine- and glycine catabolism with the highest turnover rates in kidney, brain and liver. It remains to be clarified whether the induction of the GCS in the cold-conditioned transcriptomes of *P. brachycephalum* is similar in other marine organisms.

Transcripts correlated with the regulation of inorganic ion transport (P) were repressed in the cold, whereas an induction was seen in the warmth. To support freeze protection at sub-zero temperatures, it is likely that amino acids are involved to a higher degree than inorganic ions in the cold. A wide variety of compatible solutes that account for anti-oxidation, redox-balance and freezing prevention are derived from amino acids serving as osmolytes and energy reserve (Yancey, 2005). However, their contribution to cellular homeostasis in cold-adapted fish is not well characterized and remains to be investigated. The induction of transcripts of the inorganic ion transport in the warmth could indicate that the content of organic compatible solutes is reduced due to the need for metabolites and energy. Therefore, it would be beneficial to regulate the osmotic homeostasis with inorganic solutes instead.

Transcripts within the category Q, summarizing functions of secondary metabolites,

contribute to cellular organization and detoxification. For example, 3-hydroxyacyl-dehydrogenase type-2 is involved in mitochondrial tRNA maturation (UniProt, by similarity) and was induced in the cold, like many other mitochondrial transcripts (see J, C, E). Another mitochondrial transcript, beta-beta-carotene-9-10-oxygenase, was induced in the warmth. This non-heme iron oxygenase was recently discovered to degrade carotenoides during oxidative stress and protection against carotenoid-induced mitochondrial dysfunction (Amengual et al., 2011). It is likely that this mechanism complements the observed induction of uncoupling proteins to prevent ROS formation.

Within this category a heat-responsive transcript was detected which was exclusively expressed at the critical temperature of 9°C. Haptoglobin, which binds free haemoglobin has been discussed to be relevant for microbial protection by reducing the availability of metal ions (Bayne et al., 2001). The induction of this transcript might be an indication for a beginning hemolysis and a mechanism to scavenge haemoglobine molecules. Together with the rise of haematocrit levels in warm temperatures (Fig. 1C) as well as signalling for vascularisation (see T), this transcript accounts for the high oxygen demand and constitutes a mechanism for damage limitation at the upper thermal limit.

Transcripts with unknown function

A bulk of differently expressed genes was observed in the last block of poorly characterized genes. When analyzing the distribution of the associated functional terms across the total response (Fig 3 A), it became evident that these transcripts contribute to a great extent to the acclimation process. It remains to be investigated whether these responsive transcripts point to species-specific mechanisms or more general, still unknown pathways. It is likely that within this set potential new candidate genes are hidden, which may contribute to responses to other environmental disturbances, thereby essential for a general CSR.

Conclusion

To our knowledge, this is the first approach to monitor chronically temperature-conditioned transcriptomes of an Antarctic fish to characterize the acclimation success and the shape of the thermal window on a broad molecular basis. A comprehensive picture of the temperature response became visible by linking expression data to physiological performance parameters.

The magnitude of expression levels and the functional outline of temperature-conditioned transcriptomes combined with growth performance of *P. brachycephalum* indicate a remarkable capacity to acclimate successfully to temperatures up to 6°C. Although the natural thermal niche of *P. brachycephalum* ranges from temperatures of -0.4°C to 1°C (Brodte et al., 2006b), a growth maximum at 3°C indicates a well-being and optimal physiological performance at this temperature. At the transcriptomic level this was paralleled by the lowest regulative effort through smallest expression levels of various metabolic genes.

Differently expressed functions above and below this optimum revealed cellular mechanisms that are temperature-sensitive and require adjustment for maintenance at cold and warm temperatures. Thereby, the regulatory effort increases to the edges of the thermal tolerance window indicating a transition from a homeostasis into a cellular stress response, which is more evident in the warmth through the induction of a broad array of signalling cascades and rescue molecules.

The up-regulated transcripts and correlated functions at low temperatures indicate a high energy-demand to cover the housekeeping metabolism caused by high protein turnover rates and ATP-dependent proteasomal degradation, possibly due to cold-denaturation or damage caused by ROS. This is reflected by lower growth rates than at the optimum temperature. Nevertheless, positive GP and the highest HSI levels reflect a covered energy demand and a surplus of free energy that can still be allocated into growth.

With increasing temperatures alterations in the energy fuel stores became visible. Transcript amounts indicate the previously described shift from a lipid-based fuelling in the cold to a carbohydrate-based fuelling in the warmth (Brodte et al., 2006a; Windisch et al., 2011). However, excessive expression of the GCS indicate also the utilization of amino acids as fuels in the cold.

At warm temperatures different constraints of the cellular maintenance became visible. The massive induction of ribosomal RNAs at temperatures of 5°C and above indicate a compensatory response to counter the functional impairment of temperature-induced damage and increasing instability of the protein synthesis machinery. High ribosomal protein

induction was described similarly for Antarctic and temperate fish in acute warming experiments (Buckley and Somero, 2009; Logan and Somero, 2011).

A strong decrease of the total body and liver weight is noted for temperatures between 6°C and 7°C. The activation of an inflammatory response as well as lysosomal degradation processes connected to autophagy and cell cycle arrest point to critical cellular damages and energetic deficiencies at temperatures from 7°C upwards. Together, these data indicate a thermal threshold at the molecular level that coincides with the upper pejus temperature earlier determined by physiological studies in this species. Mark and colleagues (2002) found that temperatures above 6°C lead to limitations of the cardiovascular system by a disturbed blood flow, and at the intracellular level to an interfered alphasat–regulation.

It is likely that the main trigger for thermal limitation is the restricted oxygen supply in the warmth, in line with the concept of the OCLTT (Pörtner, 2010). Accordingly, the demand of oxygen increases with higher temperatures at tissue levels, whereas the cardiovascular system seems to be limited for a proper supply (Mark et al., 2002). At the tissue level Potential mechanisms to cope with the imbalance were noted through the augmentation of the hematocrit and at the transcriptomic level by the induction of gene expression involved in tissue remodelling through the induction of angiogenesis and the recycling of haemoglobin by haptoglobin at ultimate temperatures.

Farther, it is likely that a fundamental constraint may limit the acclamatory capacity in *P. brachycephalum*, since multiple performances at various levels of systemic organization seem to be affected at the same temperature. As proteins in this species display signatures of cold adaptation through a compositional bias of amino acids that increase their flexibility (Windisch et al., 2011) it could be hypothesized that large protein complexes limit the scope of a thermal acclimation at the molecular level due to higher instabilities of functional three-dimensional protein structures at warmer temperatures. Such disturbances would strongly increase the metabolic cost for a functional maintenance and subsequently increase the energy demand. Broad scaled studies correlating the molecular size and number of subunits of large native enzyme complexes to their thermal sensitivity (i.e. functional three dimensional structure) would be required to address a potential limitation at this level.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HSW participated in the concept and experimental design, carried out the experiments, data-analyses, interpretation, online-implementation of array design and experiments and drafted the manuscript. SF arranged the compact array design, contributed to the data analyses and interpretation in terms of molecular functions, and helped to draft the manuscript. UJ participated in experimental design, supervised the laboratory procedures for the hybridizations and helped to draft the manuscript. HOP contributed to the concept, data interpretation and helped to draft the manuscript. ML conceived the concept and designed the experiment, participated in the interpretation of data and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figures:

Figure 1:

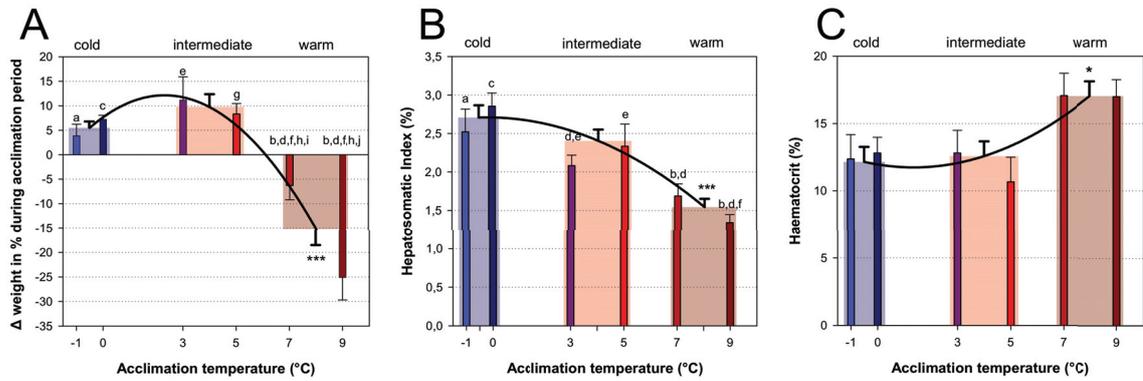


Figure 1: Animal performance parameters.

A: Growth performance. B: HSI, hepatosomatic index. C: Haematocrit. Values are means ± SD labeled with different letters to show significant differences (P < 0.05) according to one-way ANOVA.

Figure 2:

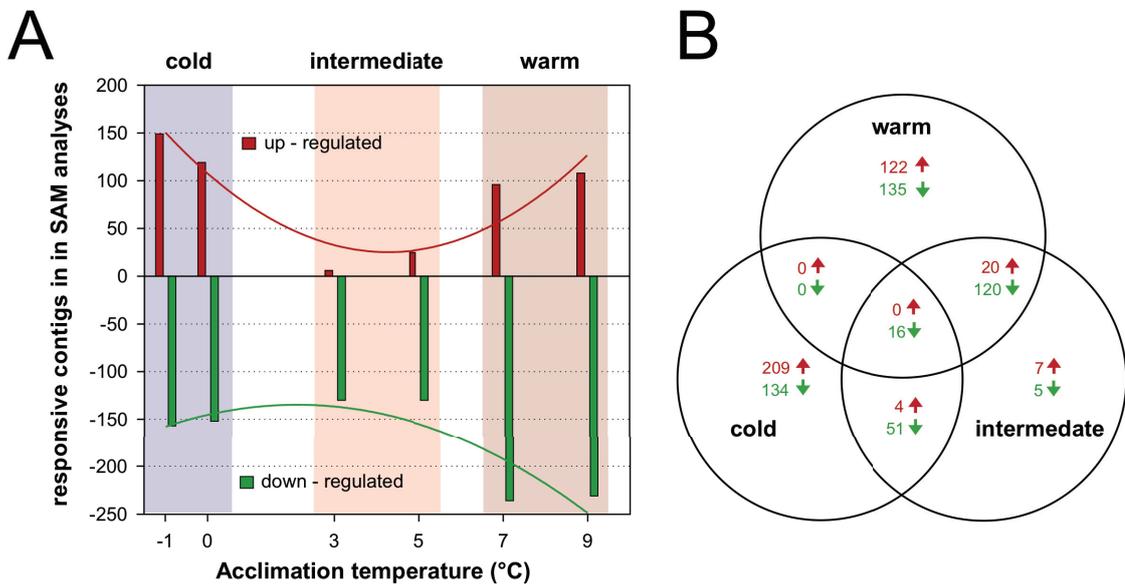


Figure 2: Expression of regulated genes.

A: Absolute counts of unique transcripts among the applied temperatures with a minimum |FC| ≤ 1.5. B: Venn diagram of pooled unique transcripts inside acclimation groups.

Figure3:

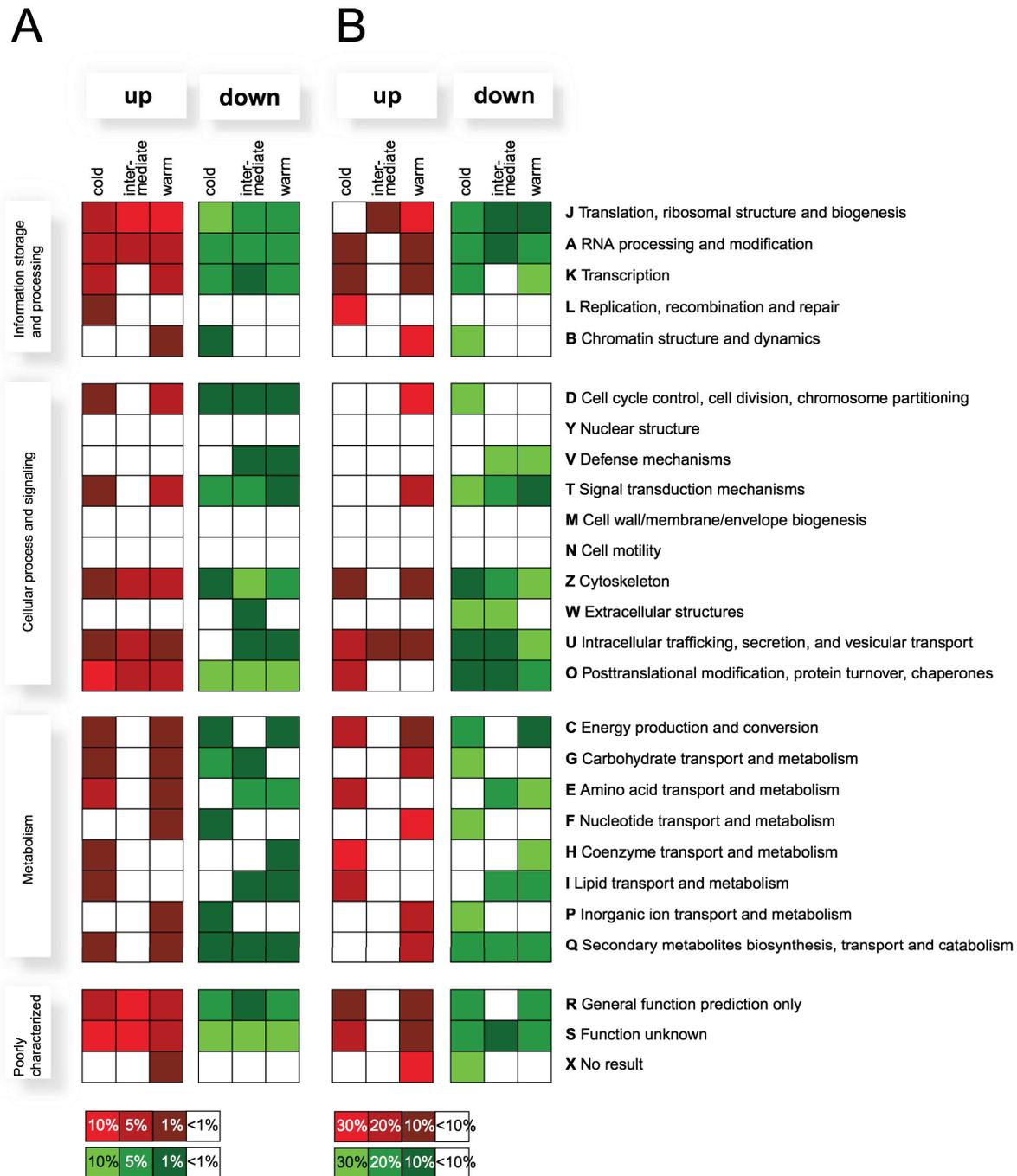


Figure 3: Functional overview of regulated genes.

Significant transcripts determined by SAM with a minimum fold change ≤ 1.5 were included in a functional overview of regulated genes in COG/KOG categories. Transcript abundance in categories was analyzed by fiNOG- annotation. The different maps represent the frequencies of functions within a treatment (A) or the temperature dependent recurrence within a category (B).

Tables:*Table 1:***Table 1: GO enrichments of up-regulated transcripts**

GO Term	Name	Type	Cold	Inter- mediate	Warm	CR
GO:0035770	ribonucleoprotein granule	C	4,90E-05			A
GO:0005839	proteasome core complex	C	2,00E-04			O
GO:0005960	glycine cleavage complex	C	2,50E-05			E
GO:0005852	eukaryotic translation initiation factor 3 complex	C			4,00E-05	J
GO:0022625	cytosolic large ribosomal subunit	C		2,90E-06	2,90E-08	J
GO:0004298	threonine-type endopeptidase activity	F	1,70E-04			O
GO:0003735	structural constituent of ribosome	F		1,50E-07	6,20E-10	J
GO:0019843	rRNA binding	F			1,40E-04	J
GO:0003723	RNA binding	F		9,20E-05		J
GO:0031435	mitogen-activated protein kinase kinase kinase binding	F	8,50E-05			T
GO:0008168	methyltransferase activity	F	1,30E-05			JLQ
GO:0004450	isocitrate dehydrogenase (NADP+) activity	F	2,00E-04			CG
GO:0030742	GTP-dependent protein binding	F	2,00E-04			TO
GO:0006414	translational elongation	P		1,80E-05	8,10E-11	J
GO:0006949	syncytium formation	P			5,60E-05	ZU
GO:0042254	ribosome biogenesis	P		3,60E-07	4,20E-09	J
GO:0000079	regulation of cyclin-dependent protein kinase activity	P			8,80E-06	D
GO:0019643	reductive tricarboxylic acid cycle	P	2,40E-04			C
GO:0051603	proteolysis involved in cellular protein catabolic process	P	8,30E-05			E
GO:0031274	positive regulation of pseudopodium assembly	P	8,50E-05			Z
GO:0043552	positive regulation of phosphatidylinositol 3-kinase activity	P	4,90E-05			I
GO:0046330	positive regulation of JNK cascade	P	4,90E-05			T
GO:0045740	positive regulation of DNA replication	P	2,80E-04			L
GO:0007097	nuclear migration	P	1,10E-06			L
GO:0048664	neuron fate determination	P	1,00E-05			X
GO:0007095	mitotic cell cycle G2/M transition DNA damage checkpoint	P			1,70E-06	D
GO:0030225	macrophage differentiation	P	2,80E-04			X
GO:0006102	isocitrate metabolic process	P	2,00E-04			C
GO:0019464	glycine decarboxylation via glycine cleavage system	P	2,50E-05			E
GO:0046847	filopodium assembly	P	1,30E-04			U
GO:0034332	adherens junction organization	P	2,50E-05			U

Results were generated by means of a two-tailed Fischer's exact test in Blast2GO at a significance level of $p \leq 0.05$. Data and were filtered with a maximum FDR ≤ 0.05 to exclude false discovery. All terms were overrepresented. The last column indicates a cross-reference to the COG/KOG categories.

Table 2:

Table 2: GO enrichments of down-regulated transcripts

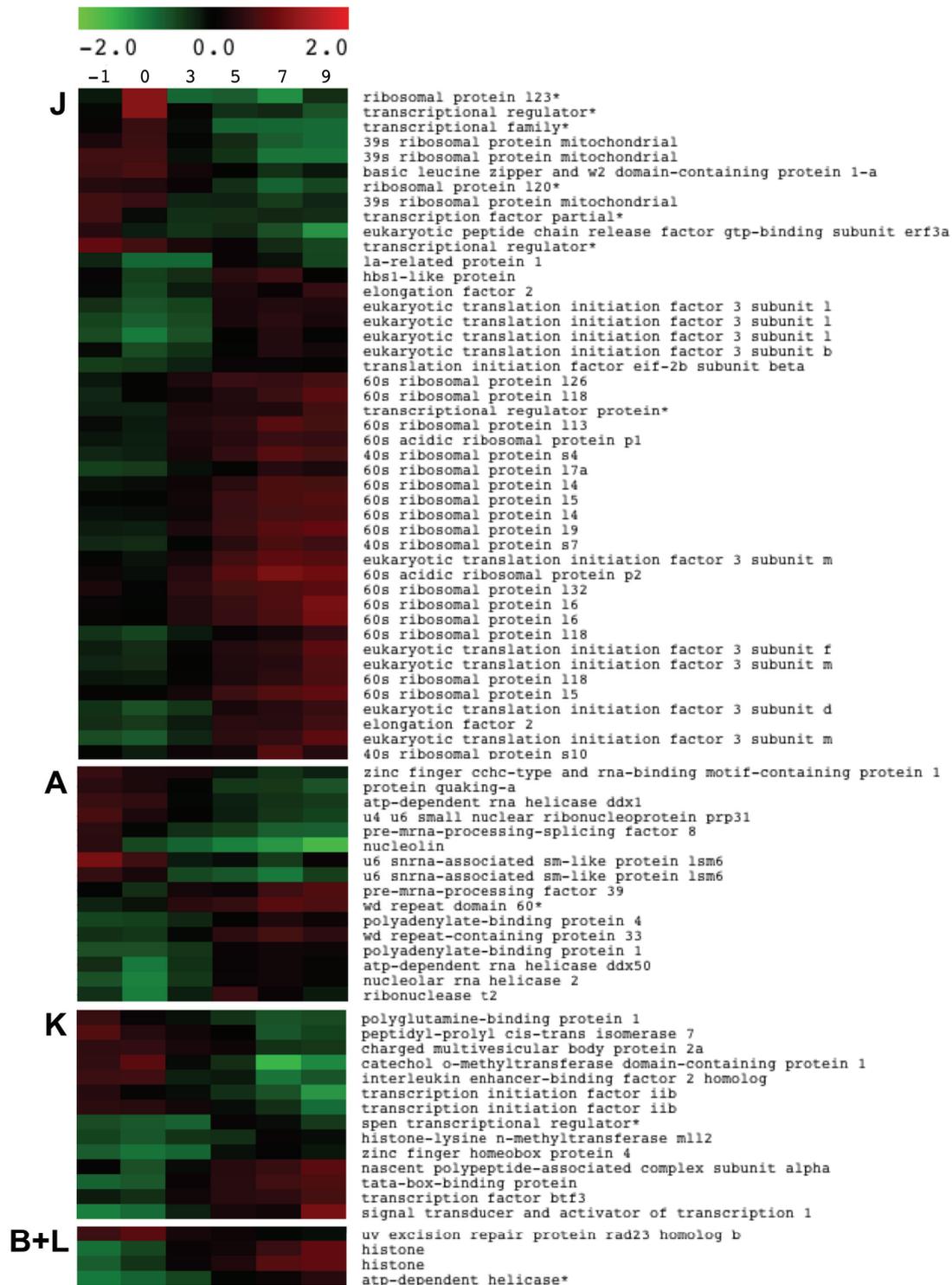
GO Term	Name	Type	Cold	Inter- mediate	Warm	CR
GO:0005852	eukaryotic translation initiation factor 3 complex	C	2,90E-06			J
GO:0001650	fibrillar center	C	5,50E-05			A
GO:0005960	glycine cleavage complex	C			4,90E-05	E
GO:0005874	microtubule	C		1,60E-04	2,00E-04	Z
GO:0005839	proteasome core complex	C			5,40E-05	O
GO:0015171	amino acid transmembrane transporter activity	F		6,90E-05		E
GO:0004364	glutathione transferase activity	F		2,10E-05	9,60E-05	O
GO:0005525	GTP binding	F		5,00E-05		T
GO:0030742	GTP-dependent protein binding	F		8,80E-05		T
GO:0003924	GTPase activity	F		3,50E-06	2,10E-05	T
GO:0031435	mitogen-activated protein kinase kinase kinase binding	F		3,70E-05	1,70E-04	T
GO:0016903	oxidoreductase activity, acting on the aldehyde or oxo group of donors	F	2,30E-05			X
GO:0004298	threonine-type endopeptidase activity	F			4,70E-05	O
GO:0034332	adherens junction organization	P		1,10E-05	4,90E-05	U
GO:0022402	cell cycle process	P		7,00E-05		D
GO:0035088	establishment or maintenance of apical/basal cell polarity	P		2,20E-04		U
GO:0046847	filopodium assembly	P		5,90E-05	2,60E-04	U
GO:0006803	glutathione conjugation reaction	P		2,10E-05	9,60E-05	X
GO:0019464	glycine decarboxylation via glycine cleavage system	P			4,90E-05	E
GO:0015807	L-amino acid transport	P		1,70E-04		E
GO:0030225	macrophage differentiation	P		1,20E-04		X
GO:0007018	microtubule-based movement	P		2,40E-06	2,90E-05	U
GO:0000278	mitotic cell cycle	P		1,50E-05		D
GO:0007095	mitotic cell cycle G2/M transition DNA damage checkpoint	P	2,90E-06			D
GO:0048664	neuron fate determination	P		4,30E-06	2,00E-05	X
GO:0007097	nuclear migration	P		3,70E-05	1,70E-04	L
GO:0045740	positive regulation of DNA replication	P		1,20E-04		L
GO:0046330	positive regulation of JNK cascade	P		2,10E-05	9,60E-05	T
GO:0043552	positive regulation of phosphatidylinositol 3-kinase activity	P		2,10E-05	9,60E-05	I
GO:0031274	positive regulation of pseudopodium assembly	P		3,70E-05	1,70E-04	Z
GO:0071822	protein complex subunit organization	P			8,90E-05	O
GO:0051603	proteolysis involved in cellular protein catabolic process	P			2,50E-05	E
GO:0000079	regulation of cyclin-dependent protein kinase activity	P	1,80E-05			D

Results were generated by means of a two-tailed Fischer's exact test in Blast2GO at a significance level of $p \leq 0.05$. Data and were filtered with a maximum FDR ≤ 0.05 to exclude false discovery. All terms were overrepresented. The last column indicates a cross-reference to the COG/KOG categories.

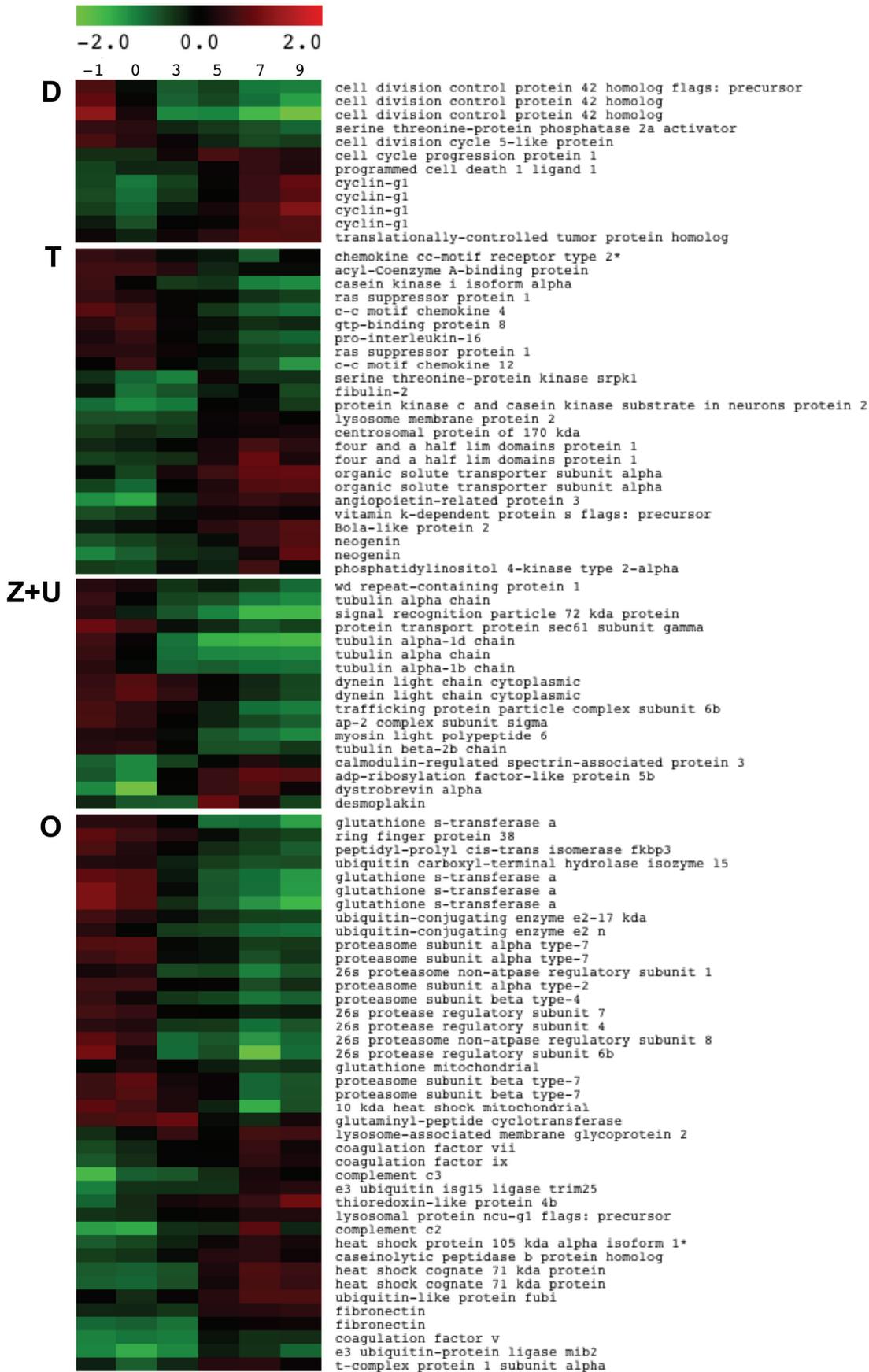
Supplementary figures:

Significant transcripts with known functions identified by SAM are clustered by means of their expression levels in respective COG categories, indicated by capital letters. Functional descriptions were obtained by BLASTx from SwissProt or from the non-redundant database indicated by asterisks.

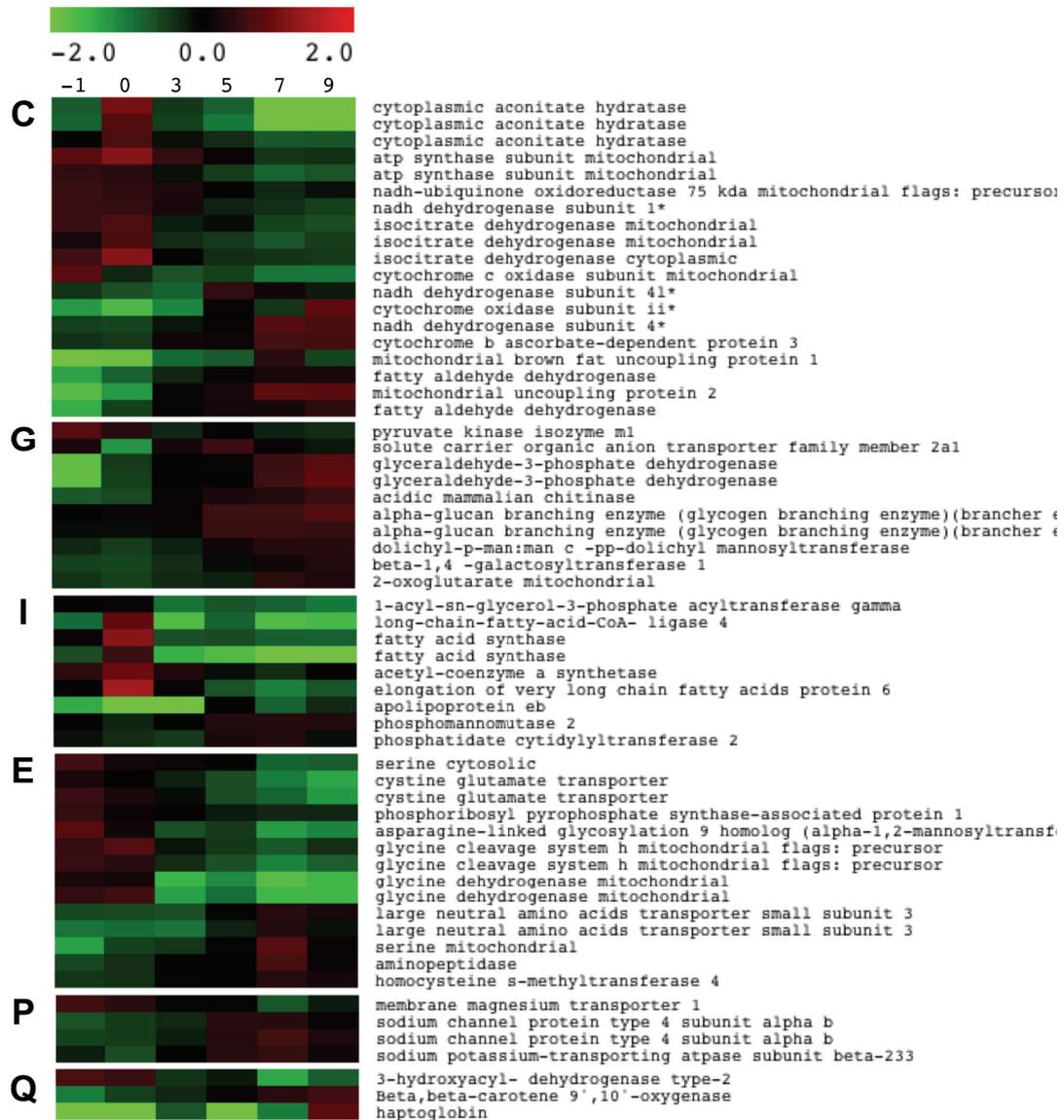
Cluster 1: Information storage and processing



Cluster 2: Cellular processes and signalling



Cluster 3: Metabolism



4 Discussion

This thesis addresses the thermal plasticity of the Antarctic eelpout *Pachycara brachycephalum* (Pappenheim, 1912) at the molecular level. With publications I-III single aspects like the species' adaptation to its habitat and the adaptability to the key factor temperature were elucidated. The following chapter will compile striking results and complement them with additional data to address the objectives of this thesis (chapter 1.6) through an integrative discussion as well as a consolidation of the data with corresponding literature.

4.1 Molecular adaptations in *Pachycara brachycephalum*

Endemic fish families in Antarctic waters inherit a distinct genetic repertoire resulting from their phylogenetic history representing their contemporary state of adaptation as described for e.g. Antarctic Notothenioidei (Montgomery and Clements, 2000). However, adaptation is apparently not only reflected by the development or degeneration of genes but also through thermodynamically beneficial adjustments at the molecular level. Numerous studies have illuminated trends of molecular evolution with respect to habitat temperature and characterized the adaptation of species at the level of proteins (Feller and Gerday, 2003; Marx et al., 2004; Tekaiia and Yeramian, 2006), RNA (Varriale et al., 2008; Wang et al., 2006) and at the genomic level (Bernardi, 2004). Thereby, different lifestyles of prokaryotes ((hyper-) thermophilic, mesophilic and psychrophilic) as well as between vertebrates (endothermic and poikilothermic) were distinguishable by signatures at the respective levels.

In order to characterize the molecular thermal plasticity of two poikilotherm species from distinct habitats, it is mandatory to exclusively compare closely related congeners. Only the absence or near absence of sequence changes not related to temperature adaptation would reveal differences that are adaptive (Somero, 1997). These preconditions were fulfilled by the Antarctic eelpout *P. brachycephalum* and its temperate congener *Z. viviparus*, since these species are closely related (pub. II, Fig 1) and inhabit thermally distinct habitats with a difference in the mean annual temperature of $\sim 10^{\circ}\text{C}$.

4.1.1 Selective pressure on codon usage

The stability of DNA and RNA increases at low temperatures as the strength of ionic interactions and hydrogen bonds is augmented in the cold. The base composition decisively contributes to the molecular stability since G=C pairs have an additional hydrogen bond

compared to A=T pairs. Due to the degeneration of the genetic code, a certain amount of variability is given at the nucleic acid level to encode synonymous amino acids and allowing for an adjustment of DNA and RNA stability through a differing base composition.

Several groups (Galtier and Lobry, 1997; Varriale et al., 2008; Wang et al., 2006) have demonstrated a significant correlation between the GC-content of structural RNAs and growth temperature. Especially in double-stranded stem regions of RNA secondary structures a higher GC-content is prevailing in species with higher optimal growth temperature (OGT).

Variations in nucleotide composition can significantly affect the patterns of codon usage (Bernardi, 2000; Duret et al., 1995; Frank and Lobry, 1999; Kanaya et al., 2001; Lynn et al., 2002) as well as the amino acid composition of the encoded proteins (Knight et al., 2001; Lobry, 1997; Lobry and Necsulea, 2006; Tekaiia and Yeramian, 2006). Lynn (2002) and colleagues have shown a consistent difference at transcriptomic level in the synonymous codon usage between mesophilic and thermophilic prokaryotes resulting from a selection linked to thermophily.

The codon usage of *P. brachycephalum* and *Z. viviparus* was compared in order to test the hypothesis that adaptation to different habitat temperatures leaves a signature on the transcriptomic level in poikilotherm vertebrates. For *P. brachycephalum* a preference for codons ending with A/T became visible by means of fish-specific orthologous sequence models (fiNOGs) (pub. II, Fig. 7). This pattern was confirmed through a species-specific difference in the usage of GC3 and AT3 among synonymous amino acids by a p-value of $5 \cdot 10^{-4}$ (pub. II, Fig. 6). Due to the large number of 4,155 position-specific sequence segment pairs of translated sequences, a sufficient number of independently evolved traits can be assumed. Thus, the observed AT3 preference in *P. brachycephalum* is not to be considered an effect of genetic drift as this should result in random exchanges with no pattern. This finding indicates an adaptive promotion of thermodynamically convenient mRNA even on a small thermal gradient. A lower GC-content diminishes over-stabilized transition states in transcription and translation and promotes cold compensation at this level. Furthermore, the need for cold shock proteins (CSPs) would be reduced since these chaperones prevent over-stabilized hairpin formations of RNA (Graumann and Marahiel, 1998; Schindelin et al., 1993).

Nevertheless, the full genomic sequences are required to characterize the zoarcid gene landscapes since intronic and regulative CpG-islands are not projected in mRNA. For instance, Jeffares (2008) have shown that the density of introns is reduced in GC-rich vertebrate sequences correlating to higher rates of transcription. Moreover, genes involved in

cellular metabolism display a higher richness in GC3 than genes in other functional gene classes (D'Onofrio et al., 2007). It has been hypothesized that an acute selective pressure on genes has driven codon usage to a higher GC-content in frequently transcribed genes, like housekeeping genes (Tatarinova et al., 2010). This would be in line with a case study comparing two housekeeping genes (A_4 -lactate dehydrogenase and α -actin) in multiple vertebrate species including Antarctic fish (Ream et al., 2003). Throughout different species no difference in the total GC- or in the GC3-content was detected in these genes.

Subsequently, the nucleic acid composition at the genomic and transcriptomic level has to be balanced between transcription rates and the thermodynamic constraints connected to thermal adaptation. Since in the comparative analyses of zoarcid fish no pre-selections of gene classes were made the results of publication II exemplify the prevailing trends of codon usage for several functional genes with different transcription rates.

4.1.2 *Proteomic cold adaptation*

The very basis of protein flexibility/stability rests upon the primary structure, i.e. the amino acid sequence. Only small changes at this level are necessary to alter the half-life, substrate affinity or the catalytic activity (Feller and Gerday, 2003; Hochachka and Somero, 2002; Marx et al., 2004). Differences in the composition of orthologous proteins became visible through proteomic comparisons of mesophilic, thermophilic and hyperthermophilic prokaryotes (Kreil and Ouzounis, 2001; Singer and Hickey, 2003; Tekaia and Yeramian, 2006; Wang and Lercher, 2010). In the latter studies the frequencies of charged amino acids, glutamic acid (E), arginine (R) and Lysine (K) were noted to be correlated to higher habitat temperatures to support an increased stability. Based on this general pattern Wang and Lercher (2010) developed a reduced predictor proxy (ERK-proxy) to illustrate the adaptation of orthologous proteins in thermal gradients. With this proxy even proteins of poikilotherm and endotherm vertebrates could be distinguished in comparative analyses. Nevertheless, among mesophilic and psychrophilic organisms this predictor becomes less effective (Wang and Lercher, 2010) since only the ionic interactions are considered. The amino acid usage in cold-adapted species includes multiple features to promote a higher flexibility (see chapter 1.3.3). For instance, Methé et al., (2005) found a lower frequency of glutamic acid and a higher usage of serine in the psychrophilic bacterium *Colwellia psychrerythraea* when compared to mesophilic and thermophilic ones. Metpally and Reddy (2009) confirmed these observations for several psychrophilic bacteria through comparison with mesophilic species and found an overrepresentation of threonine. These exchanges indicate a reduction of charge

and an augmentation of solvent interaction in the cold. Similar shifts in the amino acid usage were observed in orthologous sequence pairs of zoarcid fish. A distinct pattern for *P. brachycephalum* became visible when considering the total frequencies of all amino acids in both species (pub. II, Figure 5) or position specific exchanges (pub. II, Tab. 2). The most striking features for *P. brachycephalum* were a massive loss of glutamic acid (E) and asparagine (N) as well as a gain of serine (S) (pub. II, Figure 5). Altogether the observed exchange patterns cumulate in a loss of charge and a gain of polarity among orthologous proteins in *P. brachycephalum*.

Analyzing the ERK-proxy in zoarcid sequences revealed a significant difference between both species with higher values for *Z. viviparus*. Nevertheless, in *P. brachycephalum* the ERK-values were purely based on the loss of E in protein sequences. Together with a slightly higher usage of basic amino acids the observations for *P. brachycephalum* are not fully in line with the ERK-hypothesis. Therefore, it would be useful to adopt the concept of this proxy reconsidering it with respect to the inclusion of further patterns modulating protein flexibility like frequencies of polar residues. A new model predictor should likewise reflect the thermal plasticity of proteomes of mesophilic, thermophilic and hyperthermophilic organisms.

The differential net pattern of the amino acid usage in *P. brachycephalum* and *Z. viviparus* reflects characteristic adaptations that correlate to the respective habitat temperature. The usage of more polar and less charged amino acids in *P. brachycephalum* is in line with former studies of cold-adapted organisms (Methé et al., 2005; Metpally and Reddy, 2009; Tekaiia and Yeramian, 2006). Further cold-adaptive patterns in amino acid usage like a decrease of bulkiness and an augmentation of entropy, were seen for the most part in the position specific exchanges (pub. II, Tab. 2). As a consequence, proteomic cold adaptation affects the stability of the three-dimensional structure and subsequently the functionality of proteins. Although a higher flexibility is beneficial in the cold to facilitate reaction dynamics and kinetics, it also increases the susceptibility of proteins at higher temperatures. This may especially be critical for large enzyme-complexes, which assemble from multiple subunits. As the interaction between single components is highly dependent on the affinity of surface residues, elevated temperatures may impair such interactions and will subsequently limit the thermal tolerance.

4.1.3 Functional repertoire of genes

Cold adaptation affords an array of different adjustments of molecular functions. Thereby the modulation of biological macromolecules plays a central role for the cellular maintenance. Besides the above-stated differences in the use of nucleic and amino acids a functional comparison of transcriptomes was performed to comprehend differences in the repertoire of

genes between a cold-adapted and a eurythermal species. To abstract general gene expression patterns in the respective cDNA libraries, transcriptomic sequences were analysed by means of functional categories. These are correlated with the annotations of sequence models of metazoan genes, i.e. meNOGs (pub. II, Fig. 4). Zoarcid model sequences were mapped to a reference genome of another closely related species (*Gasterosteus aculeatus*) to provide a genomic reference as scale for the functional coverage. The differences in categories of orthologous groups (COG) between the zoarcid fish provide a qualitative overview of the expressed functions under habitat conditions. All overproportionally represented categories (further referred to as “overrepresented”) of *P. brachycephalum* are listed in Table 5 in summary and references were added if these functions were already described in an Antarctic fish species.

Table 5: Summary of overrepresented COG categories in *P. brachycephalum*

COG	COG Description	Associated function in Reference	References
C	Energy production and conversion	Mitochondrial capacity	(van Dijk et al., 1999)#, (Clarke and Johnston, 1996)*, publication III#
D	Cell cycle control, cell division, chromosome partitioning	Cell division control	publication III#
F	Nucleotide transport and metabolism	-	-
G	Carbohydrate transport and metabolism	-	-
K	Transcription	Zinc fingers	(Chen et al., 2008)*
L	Replication, recombination and repair	-	-
N	Cell motility	Lipid composition of membranes	(Brodte et al., 2008)#, (Sidell et al., 1995)*
		ER-associated shuttling	(Römisch et al., 2003)*
		Microtubule assembly	(Detrich et al., 2000)*, publication III#
O	Posttranslational modification, protein turnover, chaperones	Chaperones/HSPs	(Place et al., 2004)*
		Ubiquitination	(Shin et al., 2012; Todgham et al., 2007)*, publication III#
V	Defence mechanisms	Immune response	(Chen et al., 2008; Shin et al., 2012)*

Overrepresented functions of *P. brachycephalum* were determined in comparison to *Z. viviparus* by means of metazoan sequence models. COG descriptions were obtained from eggNOG (see material and methods section 2.4.6). References were labelled with asterisks when an associated function within a category was found in Notothenioids, rhombi symbols label zoarcid species.

The overrepresented functions in *P. brachycephalum* do highly reflect knowledge about single mechanisms concerning the housekeeping metabolism among Antarctic fish species. However, the overrepresentation of the categories F (nucleotide transport and metabolism), G (carbohydrate transport and metabolism) and L (replication, recombination and repair) seem questionable in respect to a specific pattern connected to cold adaptation. Further studies should address these patterns to clarify if these functions are connected to cold adaptation in

other species. Most conspicuous is category G – “carbohydrate transport and metabolism”, since Antarctic fish were shown to rely more on a lipid-based metabolism (Brodte et al., 2006a; Lund and Sidell, 1992; Sidell et al., 1995). However, the overview signifies the imbalance of *P. brachycephalum* and *Z. viviparus*, and a broader functional repertoire for carbohydrate metabolic genes in the Antarctic species could not be excluded by this analyses. Another approach was used to find single overrepresented functional terms by means of meNOGs (Fig. 8). Characteristic individual terms were discussed separately in publication II. However, it is likely that a closer interconnection between meNOG terms 1, 2 and 4 (Fig. 8) for *P. brachycephalum* does exist with respect to protein degradation and defence mechanisms. One of the identified candidates, TRIM (tripartite motif protein), compiles several of the other overrepresented terms in *P. brachycephalum*. TRIM proteins (meNOG15201) have a typical structure including a B-box type zinc finger (meNOG24554). These proteins are associated with multiple functions like ubiquitination (meNOG07153) (Short and Cox, 2006) and have a regulatory function within immune responses (Ozato et al., 2008), which matches the observation of an overrepresentation in COG category O (posttranslational modification, protein turnover, chaperones) and V (defence).

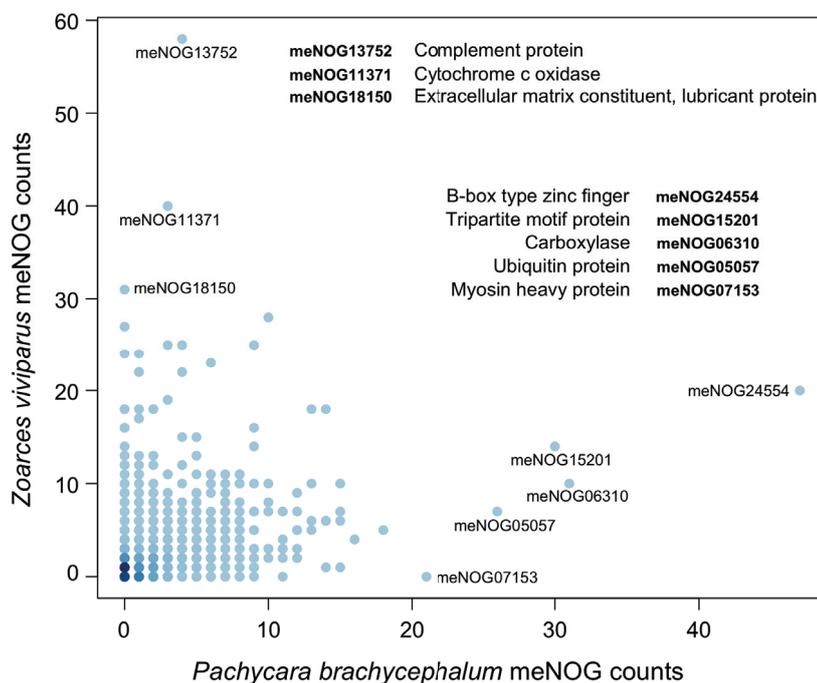


Figure 8: Overrepresented functional terms in cDNA libraries of *Z. viviparus* and *P. brachycephalum*
Frequencies of metazoan orthologous sequence models dedicated to both libraries were plotted in a density map. The most species-specific terms were labelled with the respective IDs. Descriptions of single terms were obtained from the meNOG description file (see Material & Methods section 2.4.6).

Moreover, when analyzing the 100 most abundant sequence motifs obtained from Interproscan in nucleotide sequences of both species (See Appendix, Figure AP-3 and Tab. AP-1) multiple zinc finger motifs as well as an immunoglobulin-like motif were found in sequences of *P. brachycephalum*.

The same functions were shown to be overrepresented in the Antarctic toothfish *Dissostichus mawsoni* due to gene duplication events (Chen et al., 2008). The latter study also described duplications of genes involved in protein folding and ubiquitin-dependent protein degradation as well as for zinc finger containing potential transcription factors. The overrepresented term for ubiquitination in *P. brachycephalum* (meNOG05057) was at first discussed in publication II as a bias resulting from normalization procedures of the cDNA library. It is likely that gene duplications concerning cellular functions occurred also in *P. brachycephalum*, which would be beneficial in the cold. Further evidence for a higher utilization of e.g. ubiquitin in the cold was given through the microarray study detailed in publication III. Several functions detected in the meNOG analyses (Tab. 5) could be proven by high expression levels of corresponding genes at -1 and 0°C in that experiment.

Through the comparison of a comprehensive set of transcriptomic sequences a differential evolutionary adaptation of zoarcid fish became visible. Adaptation to constant cold conditions has apparently resulted in remarkable shifts in the composition of proteins and nucleic acids in *P. brachycephalum* with respect to its temperate congener *Z. viviparus*. The different utilization of functional genes may indicate gene duplication events similar to those seen in Antarctic Notothenioids (Chen et al., 2008; Shin et al., 2012). It is likely that augmented functions in *Pachycara brachycephalum* promote a cold compensation for the respective mechanisms. The resulting phenotypic plasticity enables this species to encounter physiological challenges posed by low temperatures and high oxygen saturation.

4.2 Thermally sensitive molecular networks in *P. brachycephalum*

The capacity for acclimation is determined by the scope of the existing repertoire of regulative mechanisms in a species. According to the concept of the OCLTT (Pörtner, 2001; Pörtner, 2012), Antarctic fish have a narrow thermal window to cover their energy demands. Acute warm exposures in Antarctic fish have demonstrated their small thermal tolerance range determining incipient lethal temperatures for e.g. Antarctic notothenioid fish species at only 6°C (Somero and DeVries, 1967). Nevertheless, remaining acclimation capacities of Antarctic fish became visible when they were allowed to acclimate to higher temperatures within the *pejus*-range for a certain time (Bilyk et al., 2012; Bilyk and DeVries, 2011; Hudson

et al., 2008; Seebacher et al., 2005). Similarly, the Antarctic eelpout possess a remaining capacity for warm acclimation (Brodte et al., 2006a; Lannig et al., 2005; Storch et al., 2005). A remarkable alteration in the liver composition became visible after long-term acclimation to higher temperatures (Brodte et al., 2006a). The characteristic high lipid content in the cold contrasted with a prevailing carbohydrate fraction in the warmth. Moreover, the optimum growth temperature was determined at 4°C which is notably higher than the natural habitat temperature of *P. brachycephalum* (Brodte et al., 2006a).

The central question that arises from these observations is, which mechanisms become effective to adjust the energy metabolism for a maintained and even increased performance.

A detailed survey comprising enzymatic and transcriptomic analyses was used to characterize the mechanisms of an acclimation response to 5°C in *P. brachycephalum* in a time-dependent manner (pub. I). The analyzed metabolic pathways illustrate the development of metabolic rearrangements with respect to energy metabolism and possible signalling cascades.

4.2.1 Energetic and metabolic adjustments during acclimation

Cold-adapted Antarctic fishes display very high mitochondrial densities, which are characterized by lower capacities when compared to eurythermal species under habitat conditions (Johnston et al., 1998). At the same time, the adjustment of mitochondrial functions by cold compensation is a key functional trait to cover the energy demand in the cold (Guderley, 2004; Pörtner, 2002). This is essentially true for both, thermal acclimation in eurytherm fish as well as evolutionary cold adaptation in stenothermal species (Lucassen et al., 2006; Lucassen et al., 2003). However, elevated mitochondrial densities and capacities correlate with a high oxygen demand, which causes problems in warm acclimation due to the capacity limitation of the oxygen supply system (Pörtner, 2001; Pörtner, 2012). This links cellular functional capacity to the whole organism level, since aerobic scope depends on mitochondrial energy production and conversion.

In order to characterize the acclimation capacities and the related metabolic rearrangements of *P. brachycephalum*, several mitochondrial enzyme activities were analyzed during exposure to 5°C. Together these enzymes represent metabolic pathways comprising the citric acid cycle (citrate synthase, CS), the respiratory chain (cytochrome-c-oxidase, COX), lipid oxidation (hydroxyacyl-CoA dehydrogenase, HADH) and gluconeogenesis (phosphoenol carboxykinase, PEPCK) (pub. I, Fig. 1). In general, the citric acid cycle constitutes the central hub for the provision of metabolites and energy. As a proxy, CS was used to monitor the connected pathways by employing its activity as a reference (i.e. denominator in a ratio) to track the balance between the considered pathways.

Elevated COX/CS ratios indicated high mitochondrial respiratory capacities since the beginning of the time-course of warm acclimation to 5°C. This observation contrasted with the existing picture of a more restricted aerobic capacity of cold-adapted fish when exposed to higher temperatures (see above). Lucassen (2003) and colleagues showed similar activities for both enzymes under habitat conditions in this species. It was discussed that the high ratio of CS over COX supports lipid anabolism in the cold, which was confirmed in a later study analyzing the liver composition (Brodte et al., 2006a). The latter study showed a differential use of stores with a higher lipid fraction at habitat conditions shifting to a larger carbohydrate fraction in the warmth. The respective observations were proven through measured enzyme activities in publication I. An augmented baseline for gluconeogenesis (by means of PEPCK/CS) as well as a higher baseline for lipid oxidation (by means of HADH/CS) reveals the underlying metabolic processes for the compositional shift (pub. I, Fig. 2). Together, these observations indicate increasing mitochondrial capacities in the Antarctic eelpout at higher temperatures and contrast with elevated mitochondrial capacities in cold acclimated temperate zone eurytherms, e.g. *Z. viviparus* (Lucassen et al., 2003).

Further evidence for this contrasting picture was provided by analyzing mitochondrial uncoupling proteins (UCP) in both species (Mark et al., 2006). Cold acclimated *Z. viviparus* displayed high UCP levels similar to those detected in warm acclimated *P. brachycephalum* (RNA and protein). However, when considering the total mitochondrial protein content, both species showed classical cold compensation (Lannig et al., 2005).

In summary, *P. brachycephalum* adjusts mitochondrial capacities in a different way than eurytherm species, possibly by mechanisms connected to posttranslational modifications as hypothesized in publication I. A metabolic shift and differential utilization of fuel stores indicate a remarkable capacity for warm acclimation. A carbohydrate-based metabolism is beneficial in the warmth since these stores can be mobilized even during hypoxic events, which are more likely to occur in the warmth. Other ectoderms like crucian carp or turtles prepare for possible hypoxic metabolism before hibernation by similar metabolic shifts building up glycogen fuel stores in advance (Bickler and Buck, 2007). Subsequently, *P. brachycephalum* displays a tendency to “warm hardiness” promoting metabolic maintenance at higher temperatures.

4.2.2 Time dependent regulation of warm acclimation

Further details of the warm induced tissue remodelling were revealed by relative quantification of changes in the mRNA amount of measured enzymes and additional candidate genes involved in the respective pathways (pub. I, Tab. 4). Interestingly, transcript

levels of nearly all enzymes showing elevated (COX, PEPCK) or reduced (CS) activities were constant or reduced during warm acclimation (besides PEPCK). A higher translation efficiency at 5°C or posttranslational modifications may contribute to these findings. The effect that mRNA levels did not directly correlate with enzyme activity was described in a former study of this species (Lucassen et al., 2003).

The relevance of single genes in the interplay of all 26 investigated candidates (pub. I, Tab. 2) became visible by canonical correspondence analyses (CCA; pub. I, Fig. 4A). The strong induction of glycogen synthase mRNA (GYS) by a prolonged exposure to 5°C as well as the immediate repression of e.g. the acyl-CoA carboxykinase (ACAC) reflect the mechanisms involved in tissue remodelling at the transcriptomic level. The individual expression profiles of all single specimen (comprising 26 measures each) were visualized in a factorial map in relation to time and temperature and represent the state of acclimation of each individual under study (pub. I, Fig. 4B). As a result, the incubated animals clustered in two different groups reflecting two phases in the acclimation process representing the transition state (white labels) and the long-term state of acclimation (dark gray labels). A similar biphasic acclimation process was reported in *Z. viviparus* in response to reduced temperatures (Lucassen et al., 2003) as well as in connection with high CO₂ concentrations (Deigweier et al., 2008).

In order to determine potential regulators of metabolic shifts several transcription factors were cloned in *P. brachycephalum* and surveyed during the acclimation process (cloning summary: pub. I, Tab. 1). These factors are known to be involved in energy homeostasis of homoeotherm and poikilotherm vertebrates. An overview of all included factors and their potential roles in signalling is summarized in Figure 9.

The PPARs were identified to play a major role in acclimation and tissue remodelling of *P. brachycephalum*. The different expression levels (up- and down-regulation) of all surveyed PPARs ($\alpha, \beta/\delta, \gamma$) was most obvious in the CCA as all isoforms surrounded the metabolic genes through different correlations to the factors temperature and time (pub. I, Fig. 4A). Similar to findings in mammals (Schoonjans et al., 1995), PPAR1 α and $\sim\beta$ expression were found to be highly correlated with the expression of lipid metabolic enzymes (pub. I, Tab. 5). PPAR1 β perfectly mirrored the expression of PPAR1 α in a reverse direction (pub. I, Fig. 3A, C + Fig. 4A). Moreover, PPAR1 β was positively correlated to the expression levels of glycogen synthase (pub. I, Tab. 5). This is in line with a former study reporting the contribution of the β -receptor in glucose homeostasis (Xu et al., 1999). Similarly, PPAR1 γ was found to be involved in modulating glycogen uptake (pub. I, Tab. 5) in a late phase of the

acclimation (pub. I, Fig. 3D + 4A). As a corollary, different sets of transcription factors are required in each phase of the biphasic acclimation process (pub. I, Fig. 3). In addition, the strong dichotomy of PPAR α and β indicate a similar regulation of the PPARs in *P. brachycephalum* as seen in mammals (Kliwer et al., 1994). The latter study discovered PPAR1 β and γ as dominant repressors of PPAR α .

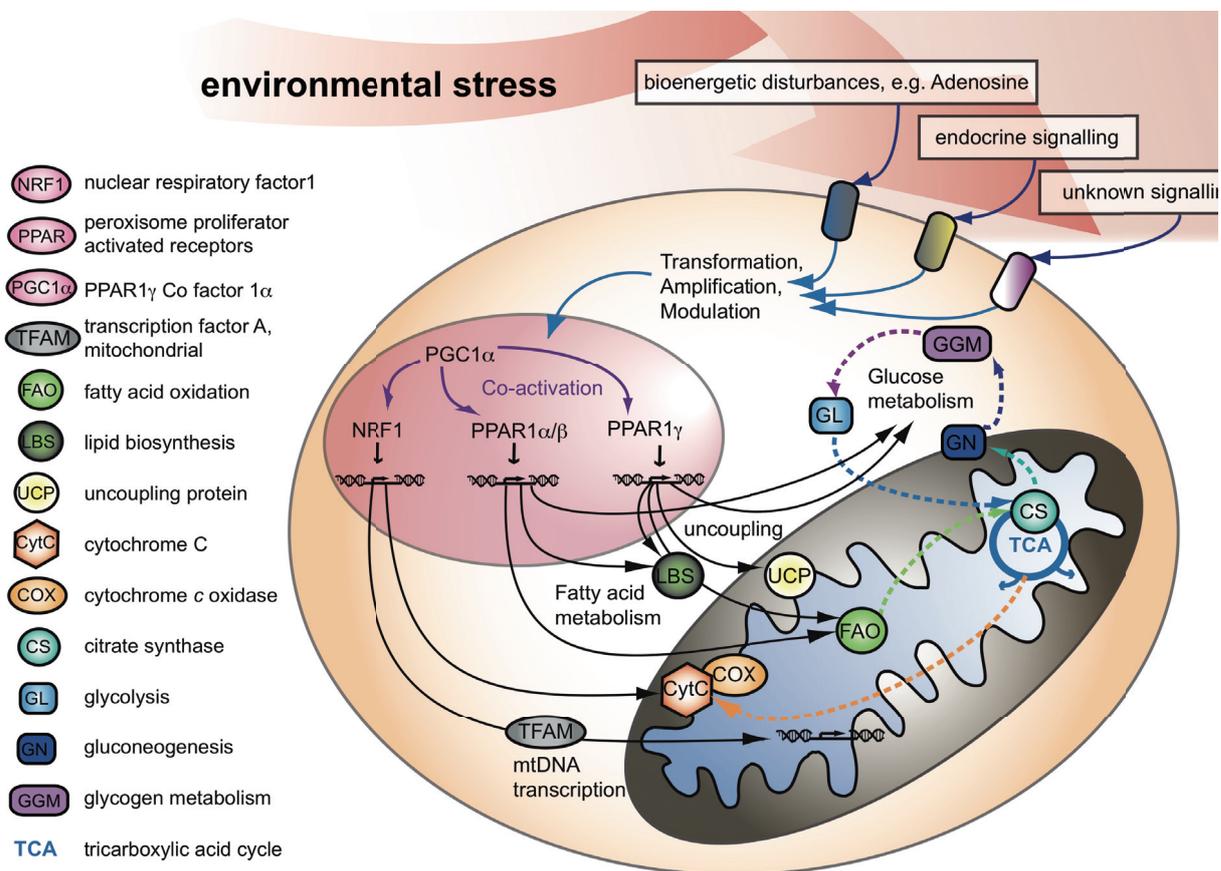


Figure 9: Cartoon illustrating potential regulators for tissue remodelling during warm acclimation

Isoforms of the PPAR family comprise nuclear receptors involved in the regulation of energy metabolism and serve as sensors of the energetic status of the cell (reviewed by Berger and Moller, 2002). Different isoforms of PPARs ($\alpha, \beta/\delta, \gamma$) are inducible transcription factors that modulate the transcription of metabolic genes by binding to specific peroxisome proliferator response elements (PPREs) of various target genes solely or by further activation through co-activators like PGC1 α . PGC1 family members also support the functioning of NRF-1 (Gleyzer et al., 2005) which was shown to regulate the capacities of respiratory chain components (Evans and Scarpulla, 1990) and coordinate the biogenesis of mitochondria in mammals (Virbasius and Scarpulla, 1994). PPAR1 α and β were identified to regulate the metabolism of fatty acids (Berger and Moller, 2002; Wang et al., 2003; Xu et al., 1999). In addition, PPAR1 β was shown to be involved in glucose homeostasis (Xu et al., 1999). PPAR1 γ holds multiple roles and was shown to be involved in the regulation of genes comprising mitochondrial uncoupling (Kelly et al., 1998), lipid metabolism and glucose homeostasis (Berger and Moller, 2002). Black arrows indicate targets of transcription factors, blue arrows symbolize signalling pathways, purple arrows indicate potential co-activation of transcription and dashed coloured arrows indicate crosslinks between metabolic pathways.

Albeit a significant reduction of NRF-1 expression was detected in the early phase of acclimation (pub. I, Fig. 3B), no clear contribution of the transcription factor to warm acclimation became visible in the CCA. The expression of NRF-1 showed no significant correlation to its former described targets of the respiratory chain (see Fig. 9). Although this

transcription factor was described to play a role in adjusting mitochondrial capacities in fish during cold exposure (LeMoine et al., 2008; McClelland et al., 2006), it seems not to be likewise involved in a warm acclimation process. Nevertheless, the reduction of NRF transcripts as well as of its targets indicate either remaining capacities to regulate mitochondrial density or is an artefact evoked by the general warm compensation of the transcription machinery, i.e. less transcript is sufficient to sustain protein synthesis.

The potential role of the co-activator PGC1 α in the acclimation process is versatile as it is a co-activator of all considered transcription factors (Gleyzer et al., 2005; LeMoine et al., 2008; Vega et al., 2000; Vercauteren et al., 2006; Wu et al., 1999). The PGC1 α expression correlated with glycogen metabolism, lipid oxidation and transcription factors (pub. I, Tab. 5). However, PGC1 α occurred in the CCA only after prolonged exposure to a higher temperature. Thus, it can be supposed that co-activation occurs only in the late phase of acclimation to promote a durable tissue remodelling.

In summary, the investigated PPARs seem to control tissue remodelling during warm acclimation in *P. brachycephalum*. Yet, it becomes evident that certain thresholds exist for the factor ‘incubation time’ indicating different phases of an acclimatory response. The late induction of the co-activator PGC1 α may indicate the transition from an acute and intermediate acclimatory response into a chronic phase. Such trigger would be convenient to promote robust long-term metabolic adjustments, in contrast to mechanisms that become effective to cope with short-term disturbances.

4.2.3 Systemic effects during warm acclimation

During the time course of acclimation animal performance was recorded by means of the fitness parameter HSI (Fig. 10 A). A remarkable reduction since the first day complies well with the immediate mobilization of energy stores in liver (Brodte et al., 2006a) by increased baseline rates of mitochondrial respiration, lipid oxidation and gluconeogenesis (pub. I, Fig. 2). When considering the six-week HSI values as long-term observations, these data match the findings after long-term exposure to 5°C in publication III (Fig. 1B).

The blood parameters total serum osmolality and haematocrit (Fig. 10 B, C) decreased during warm acclimation and levelled off at control levels after a period of six weeks of acclimation. These properties, together with the personal observation of easier access to blood samples during this time, led to the impression of a diluting effect on blood.

In general, serum osmolalities of *P. brachycephalum* at 0°C resemble more those of temperate fish (~330 mosmol kg⁻¹) than those of Antarctic species (~575 mosmol kg⁻¹) (O’Grady and DeVries, 1982). The latter study suggested that a decrease of the seawater-to-extracellular

fluid osmotic gradient is advantageous in Antarctic teleosts through a reduction of the energy demand of the Na^+/K^+ -ATPase to excrete NaCl . An increasing hypo-osmolality during warm acclimation of Antarctic fish was previously reported and discussed in the context of a positive temperature compensation of the Na^+/K^+ -ATPase activity (Gonzalez-Cabrera et al., 1995; Guynn et al., 2002; Hudson et al., 2008). The activity of the Na^+/K^+ -ATPase was shown in the present data set to remain uncompensated (Lucassen, Windisch and Sun, unpublished) resulting in elevated capacities (approximately by 1/3, data not shown) at 5°C that may contribute to the reduction of osmolality. However, restored osmolalities after 6 weeks of thermal acclimation contrast with the latter findings indicating the contribution of further mechanisms that affect the composition of blood serum.

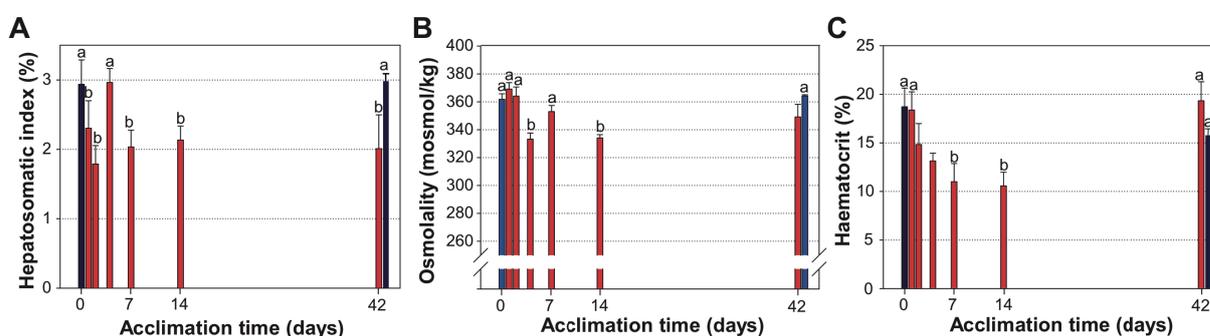


Figure 10: Physiological performance indicators of animals acclimated to 5°C .

A: hepatosomatic index, B: osmolality and C: haematocrit of blood serum samples. Red bars label animals acclimated to 5°C , blue bars label controls. Values are means \pm SEM. Letters indicate significant differences with $p < 0.05$ in one way ANOVA.

Temperature-dependent higher “drinking rates” paralleled by reduced serum osmolalities were found in Antarctic fish (Petzel, 2005) as well as in temperate species (Carroll et al., 1995; Raymond, 1993). Membrane fluidity and permeability are also highly dependent on temperature. In addition, membrane remodelling in fish were reported to occur through increased cholesterol-to-phospholipid ratios after long-term warmth exposure (Robertson and Hazel, 1995). The latter study documented a homeoviscous acclimation response reducing membrane fluidity and permeability. It is likely that reduced osmolality in *P. brachycephalum* is caused by compensated enhanced ion exchange combined with increased drinking rates possibly followed by long-term membrane modifications.

Assuming that increased drinking rates correlate with an increase of the total blood volume a transient reduction of the haematocrit seems likely. So far only modest, if any, changes in haematocrit have been reported in response to higher temperatures in Antarctic fishes (Hudson et al., 2008; Lowe and Davison, 2005; Qvist et al., 1977; Tetens et al., 1984; Wells et al., 1984). A decline of the haematocrit may be an alarm signal indicating reduced capacities of oxygen supply and possibly triggering tissue remodelling. This is in line with

observations of altered mitochondrial enzyme capacities promoting a metabolic shift to fuel stores that can be metabolized during functional hypoxemia.

Together, the monitored systemic parameters reflect early alterations of enzyme activities of the liver energy metabolism and to some extent in ion regulation, influencing the oxygen transport system. Similar to observations at the transcriptomic level blood parameters display a transition and a long-term adjustment during acclimation. The primary stimulus for the overall changes at different levels of systemic organization may be a combination of temperature and oxygen availability. But also the duration of exposure seems crucial for long-term adjustments in warm acclimation. Since glycogen uptake appear to be initialized after a prolonged exposure by the induction of additional Co-factors it remains to be investigated if such mechanism is a general strategy to mediate between short-term endurance or long-term adjustments.

4.3 Changes of transcriptomic signatures at different temperatures

Since large-scale transcriptomic and genomic approaches become affordable even for non-model species more comprehensive approaches become available today. Custom designed microarrays based on library sequence information allow for the simultaneous analyses of the expression of thousands of target genes. Through this application distinct patterns were revealed in eurytherm fish in response to various environmental stressors like temperature, hypoxia or pathogens (reviewed by Douglas, 2006). In Antarctic fish, only acute responses to temperature were characterized at such a broad scale so far (Buckley and Somero, 2009; Thorne et al., 2010). The latter studies show the induction of elements of the evolutionary conserved stress response (Kültz, 2005) combined with increased energy requirements in *Trematomus bernacchii* and *Harpagifer antarcticus* after acute heat stress. However, long-term studies especially in cold-adapted species are required to gain basic knowledge of cellular trade-offs set by cold adaptation and to evaluate the capability of species to acclimate to altered temperatures which will likely occur in the Southern Ocean during the next century due to climate change.

Since *P. brachycephalum* has shown remarkable metabolic rearrangements when exposed to higher temperatures (Brodte et al., 2006a) the central question remains which transcriptomic responses are maintained after prolonged acclimation and how these patterns reflect whole animal performance. To this end, a microarray for broad transcriptomic analyses was constructed based on the sequence information of the normalized cDNA library of *P. brachycephalum* (see material and methods 2.4.8 and 2.4.9). Liver expression profiles of specimens incubated at different temperatures for two months (-1°C, 0°C, 3°C, 5°C, 7°C and

9°C) were analyzed with this species-specific compact array design (for details of the experimental design see Figure 6, section 2.4.9). Linking temperature-specific transcriptomic patterns to physiological performance indicators, characteristic metabolic changes became visible at the respective temperatures, revealing the thermal plasticity of *P. brachycephalum* (pub. III).

4.3.1 Gene regulation and animal performance

According to the concept of the OCLTT certain thermal thresholds for poikilotherm species that shape the thermal tolerance window exist (Pörtner, 2001; Pörtner, 2012). Such specific set points in fish have either been determined with physiological (e.g. aerobic exercise), ecological (e.g. abundance) or biochemical (e.g. onset of anaerobic end products) measures (Pörtner and Farrell, 2008; Pörtner and Knust, 2007). In *P. brachycephalum* the upper critical temperature was determined to be between 9°C and 10°C by measuring oxygen consumption, pH_i and the onset of anaerobic metabolism in various tissues (Mark et al., 2002; van Dijk et al., 1999). A similar upper critical temperature limit became visible by means of mortality during experimentation (pub. III). Nearly all animals survived exposure up to a temperature of 7°C (one specimen died at -1°C and one at 7°C, respectively). At a temperature of 9°C a mortality rate of nearly 50% showed that the upper limit of long-term thermal tolerance was exceeded.

The corresponding growth data resulted in a bell-shaped curve with a maximum growth rate visible at 3°C (pub. III, Fig 1A) similar to a former study documenting the maximum growth temperature for this species at 4°C (Brodte et al., 2006a). Despite excessive food supply under laboratory conditions, incubation at higher temperatures (7°C and upwards) resulted in an increasing weight loss representing a thermal threshold between 6°C and 7°C manifested through a disturbed energy allocation. This is in line with a drastic reduction of the HSI (pub. III, Fig. 1B) at temperatures of 7°C upwards showing fuel stores being mobilized to prolong survival under adverse conditions. The set-point matches the earlier observation of a threshold temperature of 6°C, at which disturbances in the alpha-stat homeostasis were induced under acute thermal stress (Mark et al., 2002). Together these findings indicate the thermal threshold for long-term survival (*pejus*-temperature).

Interestingly, the temperature-dependent transcript levels determined in liver tissue seem to be negatively correlated with growth performance. Regardless of their encoded function the regulated genes reflect a permanently higher transcriptomic effort at cold (-1°C, 0°C) and warm (7°C, 9°C) temperatures. At “intermediate” temperatures (3°C, 5°C) a minimum of transcript diversity was paralleled by optimal growth performance (Fig. 11). The inflexion

points of the fitted growth rate and expression curves match at approximately 3°C. The absolute numbers of altered transcripts may differ when using another array design. However, the observed coherence between growth rates and gene expression should develop similarly when using array designs with a certain coverage of metabolic genes. The latter result suggests that chronic thermal exposure causes transcriptomic adjustments below and above the optimum temperature that reflect imbalances in homeostatic mechanisms and, at the same time, are paralleled by unfavourable shifts in energy budget. Some of the transcriptomic changes may be responses to such unfavourable shifts (e.g. the preferred usage of carbohydrate-based fuels with increasing temperatures to prepare for hypoxemic events), or be causative in stimulating baseline energy demand (e.g. enhanced protein turnover via ubiquitin-related enzymes in the cold).

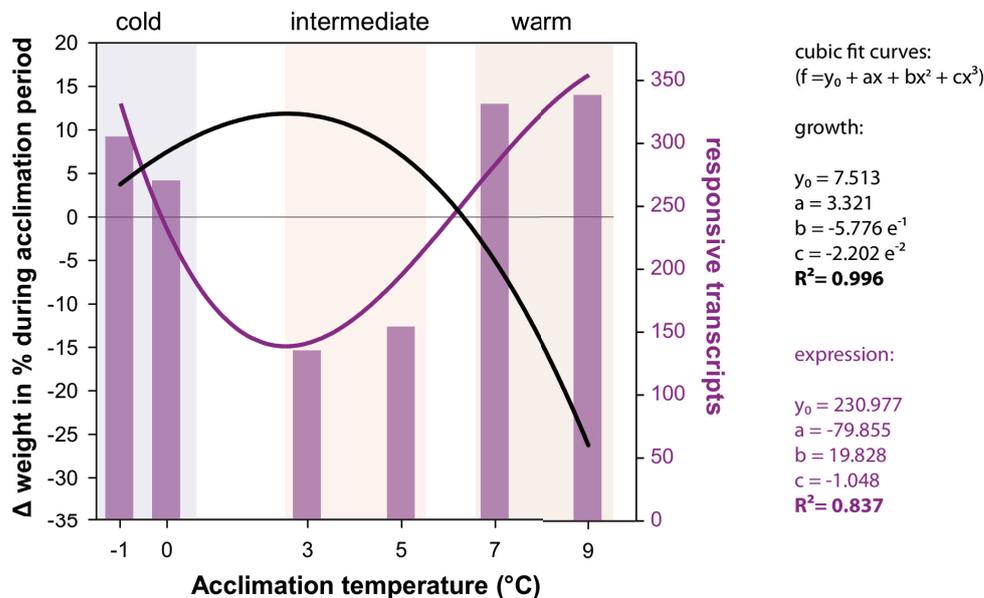


Figure 11: Growth performance and transcriptomic efforts at different temperatures

Purple bars show temperature sensitive transcript amounts with a minimum altered expression of 1.5 fold (up- and down regulation). The corresponding fit was calculated using a cubic function. The black curve represents the best fit (also cubic) of growth performance calculated from original data (measures from all six temperatures, for bar plot see pub. III, Fig 1A).

Despite a higher effort to regulate genes at cold temperatures, energy is still allocated to growth, whereas the attempt to acclimate to warm temperatures is apparently futile and characterized by a strong energetic deficiency. Although the observation of optimum performance at a temperature of 3°C to 4°C argues against a strictly cold-stenothermal lifestyle, the uneven energy allocation along with the maximum growth performance illustrates that *P. brachycephalum* is well-suited to adjust metabolic functioning at the cold end of the thermal window.

4.3.2 Characteristic transcriptomic profiles at different temperatures

The encoded functions of the regulated transcripts reflect complex patterns that developed in response to a prolonged exposure to the respective temperatures (pub. III, Fig. 3 and Suppl. clusters). No specific pattern appeared for intermediate treatments, rather a transition state between cold and warm temperatures (pub. III, Fig. 2B). An overview of the most prominent functions at cold and warm temperatures is summarized in Table 6. In general, the entire pattern observed at cold temperatures reflects functions that were found to be characteristic in Antarctic fish (Chen et al., 2008; Shin et al., 2012) and also to some extent matches inducible functions of cold-stressed eurytherm common carp (Gracey et al., 2004).

Table 6: Transcriptomic functions developing at different temperatures after long-term thermal incubation

Function	Cold temperatures	Warm temperatures
Transcription	Zinc finger mediated transcription	Contribution of scaffolding proteins (WD-repeat motifs)
Translation	Mitochondrial ribosomal proteins	Ribosomal proteins
Protein degradation	Ubiquitin - proteasome pathway	Lysosomal pathway
Cytoskeleton	High density of structural organisation: dynein, myosin, tubulin	Anchor proteins: spectrin, dystrobrevin and desmoplakin
Cellular protection	Compatible solutes (mainly amino acids), glutathione	HSPs, scaffolding proteins (WD-repeat motifs), thioredoxin
Energy metabolism	Elements of TCA, glycine cleavage system mitochondrial ATP production	Uncoupling proteins and respiratory chain components
Metabolized fuels	Lipids and amino acids	Carbohydrates (glycogen), body-own reserves indicated by autophagy
Signalling	Chemokine signalling (cell homeostasis and innate immune system), components of the Ras/MAPK signal transduction pathway	Inflammatory response, angiogenesis, cytoskeleton remodelling
Cell cycle	Arrested cell division	Damage-mediated cell cycle arrest

Summarized are the most striking up-regulated functions of cold and warm temperature-conditioned transcriptomes. The overview was extrapolated from observations in COG/KOG categories of the finOG analyses (see pub. III, Fig. 3), from detailed expression profiles (see pub. III, suppl. clusters) as well as from GO-enrichment analyses (pub. III, Tab. 1 and 2).

The observed high utilization of zinc finger mediated transcription in the cold was also found in publication II when comparing the cDNA libraries of zoarcids (see Tab. 5, category K; Fig. 8 and Fig. AP-3). However, the benefits of such binding motifs at low temperatures are still unclear but seem to be a common overrepresented function in Antarctic fish (Chen et al., 2008). At warm temperatures scaffolding proteins containing WD-repeat domains (tryptophan-aspartate) were found in connection with transcriptional functions (category K, pub. III, suppl. Cluster 1). Such proteins were previously shown to contribute to the formation of the transcriptional initiation complex (Smith, 2008). Their induction may be important for maintaining the functionality of the transcription machinery at higher temperatures.

Elevated mitochondrial functions were found in connection with translation as well as energy

metabolism. These patterns reflect high mitochondrial densities in *P. brachycephalum* under habitat conditions, similar to findings in other cold-adapted poikilotherm species (Clarke, 1983; Clarke and Johnston, 1999; Dunn, 1988; Guderley and St Pierre, 1996; O'Brien, 2011; O'Brien and Mueller, 2010). However, some mitochondrial components of the respiratory chain (subunits of complex I and IV) were induced at high temperatures. The functionality of the respective complexes depends on the correct assembly of multiple enzyme subunits (complex I: 45 subunits (McKenzie and Ryan, 2010), complex IV: 13 subunits (Lazarou et al., 2009)). Since proteins in *P. brachycephalum* are cold-adapted, i.e. more flexible (pub. II), such elaborate enzyme architectures are especially temperature-sensitive and may constitute a molecular bottleneck in warm acclimation.

As the expression profiles of subunits from complex IV in publication I did not reveal any changes at 5°C but displayed elevated expression levels at temperatures of 7°C upwards during the experiments of publication III, it might be argued that the functionality of an enzyme complex is limited only by single subunits due to their respective half-life or temperature sensitivity.

Another observed mitochondrial modification in the warmth relates to increased uncoupling mechanisms of the proton motive force established by the respiratory chain. Elevated mRNA and protein levels of UCPs described earlier and verified here, seem suitable to counteract high mitochondrial membrane potentials that are likely to occur through increased energy demand at limited oxygen availability (Mark et al., 2006).

Cellular maintenance seems severely disturbed at warm temperatures clearly indicated by a cellular stress response (Kültz, 2005) including high levels of heat shock proteins (HSPs) and signalling factors for an acute inflammation. The latter observations were accompanied by excessive transcription of ribosomal proteins. The protein synthesis machinery (ribosom) is one of the largest multi-enzyme-complexes in cells involving 50 to 80 structural protein subunits (for review see Korobeinikova et al., 2012). The aforementioned higher thermal sensitivity of multi-subunit-enzyme-assemblies also seems to apply in this case and implicates high metabolic costs to maintain protein synthesis.

Interestingly, the protein degradation pathways differed largely at cold and warm temperatures. The ubiquitin-proteasome pathway prevailing in the cold contrasted with a preferred lysosomal pathway in the warmth. As the proteasome is a large protein complex assembled from at least 50 single subunits (Lander et al., 2012) it might therefore be highly temperature-sensitive due to molecular thermal adaptation.

High levels of ubiquitin-conjugated proteins in Antarctic fish are discussed as a major

constraint in the cold adaptation process since high rates of “non-productive” folding in the cold result in high levels of degradation (Todgham et al., 2007). Sufficient energy seems to be available for the application of an ATP-dependent process for protein degradation at low temperatures. A shift to the lysosomal pathway is possibly caused by a combination of loss of function of the proteasome and a constrained oxygen supply at higher temperatures (Pörtner, 2001; Pörtner, 2012), severely reducing energy available for performance.

The lysosomal pathway has been described by Lum and colleagues (2005) as a mechanism for autophagy in response to starvation and metabolic stress causing intracellular damage. This feature promotes the mobilization of remaining reserves and is reflected in the dramatic weight loss of the animals as well as the observations concerning cell cycle control. At tissue level an increased haematocrit further indicates an oxygen limitation at warm temperatures (pub. III, Fig. 1C). At the transcriptomic level mediators for angiogenesis were detected pointing to an induced vascularisation for a better oxygen supply.

Another warm-induced signalling pathway concerns the reorganisation of the cytoskeleton mediated by four-and-a-half-lim-domains protein 1 (Kadmas and Beckerle, 2004). Despite a high structural organization in the cold, cytoskeleton remodelling resulted in a completely different cellular organisation in the warmth. Under the latter conditions tubulin, myosin and dynein transcripts were down-regulated accompanied by the induction of mainly anchor and structure-maintaining proteins. Such a reorganisation possibly involves the disintegration of cells by autophagy, and it may be postulated that cells are losing shape and volume in the warmth.

Finally, the previously described metabolic shift from a lipid-based metabolism in the cold to a carbohydrate-based metabolism in the warmth (Brodte et al., 2006a, pub. I) became visible through altered transcripts of respective metabolic genes. Moreover, a new aspect in connection with metabolic fuelling in the cold became apparent through high transcript levels of the glycine cleavage system (GCS) indicating enhanced catabolism of glycine and serine at low temperatures. This respective mechanism has recently been identified to be present in bacteria, plants and animals (Kikuchi et al., 2008). Other than the fact that free amino acids are known to be important cytoprotectants in fish (Yancey, 2005), amino acids could also play an important role as energy reserves. It remains to be investigated if the GCS does significantly contribute to energy production in Antarctic fish.

The in-depth characterization of long-term thermal acclimation of *P. brachycephalum* illustrates the complex metabolic and cellular rearrangements that limit and determine physiological performance at higher levels of systemic organization. Within a thermal range

from -1°C to 6°C the savings in cellular maintenance costs allow excess energy to be allocated into growth delineating the optimal performance range. Metabolic costs are minimal at the optimum growth temperature and increase at high and low temperatures due to the different mechanisms that are required to support a prolonged endurance at respective temperatures (Tab. 6), severely reducing available energy. Better cellular maintenance at cold temperatures is based on the molecular cold adaptation of proteins, which in turn may limit the functional integrity of large enzyme complexes at higher temperatures. Subsequently, thermal limitation at the molecular level concerns particular processes involving large enzyme complexes for processes like protein synthesis, protein degradation or catalytic steps in the respiratory chain. Due to oxygen limitation at warm temperatures energy production and allocation are severely restricted causing the exhaustion of fuels finally leading to death under continuous warmth exposure. Nevertheless, the ability to survive transient warm conditions that exceed the upper *pejus*-temperature of the organism would promote a time-limited survival of the species. These findings are well in line with the concept of the OCLTT (Pörtner, 2001; Pörtner, 2012) substantiating it at the molecular level.

4.4 Conclusions

Several aspects of molecular cold adaptation as well as acclimation capacity of the Antarctic eelpout *P. brachycephalum* could be addressed within this thesis. This chapter integrates the above-detailed results in a broader context with respect to the molecular plasticity and the resulting climate sensitivity of Antarctic fish.

Since species distribution and abundance in marine ecosystems are mainly driven by the key abiotic factor temperature (Pörtner, 2001; Pörtner, 2012; Somero, 2012), it is important to profoundly understand the mechanisms shaping thermal plasticity of organisms to estimate their ability to cope with ongoing climate change. Specialization on a particular environmental factor can only occur if evolutionary processes develop at a higher rate than the change of environmental conditions (Seebacher et al., 2005). In this case, adaptation opens the prospect of a sustained and successful niche occupation. Such specialization on niches is thought to have developed at the expense of a reduced or even lost ability (“trade-off”) to respond to changing environmental factors (Somero et al., 1996).

The closely related congeners *P. brachycephalum* and *Z. viviparus* of the cosmopolitan fish family Zoarcidae inhabit thermally distinctly differing environments and therefore provide an excellent model for comparative studies of evolutionary temperature adaptation. Signatures of molecular cold adaptation of *P. brachycephalum* became apparent through comparisons of

zoarcid transcriptomic sequences and their respective translations (pub. II). The identified exchanges in the Antarctic eelpout thereby promote a more flexible design of biological macromolecules resulting in a relaxed thermostability.

An adaptation at DNA/RNA and protein levels could largely affect the capacity to acclimate to higher temperatures, as processes like replication, transcription and translation of DNA may be impaired by a higher instability of DNA and RNA. Similar disturbances are likely to occur at protein level upon warming. Cold-adapted proteins typically possess higher natural flexibilities that would cause destabilization and possibly disassembly or unfolding of functional enzymes upon warming. The results from the microarray study also suggest that large protein complexes suffer first from thermally induced disturbances, as indicated by the futile expression of e.g. components of the protein synthesis machinery at warm temperatures. A similar hierarchy as proposed for whole animal level thermal tolerance becomes visible at the molecular level also, as structures of higher organisational complexity are impaired first.

Moreover, thermal adaptation involves a different functional repertoire of genes. Characteristic patterns of cold adaptation (e.g. ubiquitin, zinc finger proteins or proteins involved in the innate immunity) were found in the comparative transcriptomic analyses of *P. brachycephalum* with *Z. viviparus* (pub. II). The same cold adaptation genes were identified in in Antarctic Notothenioids (Chen et al., 2008; Shin et al., 2012). These studies strongly suggest that evolution in the cold has produced genomic expansions and/or up-regulations of specific gene families by duplication events. Such amplified functions may not be critical for Antarctic species experiencing higher temperatures. However, the disintegration of regulative sites in genes as seen in case of HSPs in *Trematomus bernacchii* (Buckley et al., 2004; Hofmann et al., 2000) or even the loss of genes, like haemoglobin in the family of the channichthyidae (Cocca et al., 1997; di Prisco et al., 2002) dramatically limits their capacity to acclimate to higher temperatures. Hence, the functional repertoire of genes and the ability to modulate their expression limits the physiological performance of a species building on the molecular level. Modelling approaches may be useful to link the development or loss of functional traits to species abundance via physiological performance and fitness parameters.

Characteristic gene expression profiles developed under constantly altered thermal conditions reveal the acclimatory capacity in *P. brachycephalum* at the transcriptomic level (pub. III). By linking the identified functional patterns to the respective growth performances, cause and effect relationships became visible connecting the molecular level to the physiological scope. In doing so, it became evident that particular cellular functions are differentially modulated below and above the optimum growth temperature of 3°C. Thereby the maximum growth rate

is paralleled by the lowest regulative effort to modulate gene expression. Multiple cold-associated functions demonstrated by transcriptome comparisons (pub. II) were substantiated through the expression profiles of *P. brachycephalum* under habitat conditions. Moreover, along with the well-known lipid-based metabolism indications were found for a contribution of amino acid catabolism in the cold (pub. III). Large rearrangements of the cytoskeleton, different protein degradation pathways as well as a differential usage of fuels were found upon warming (cf. Tab. 6).

The metabolic shift from lipid- to carbohydrate-based fuels is triggered at the transcriptomic level by nuclear receptors. This shift occurs in different phases comprising sensing, short-term response and a durable long-term reorganization promoted through additional transcriptional co-factors (pub. I). From the holistic expression analysis (publication III) and considering the large fraction of uncharacterized proteins in transcriptomes it seems likely that further functional networks exist contributing to an effective warm response.

The loss of growth performance at temperatures above 6°C indicates a thermal threshold – the upper *pejus*-temperature. The correlated expression patterns emphasize the term “*pejus*” since the observed changes indicate worsening conditions. Several rescue mechanisms like an inflammatory response, autophagous energy scavenging combined with increased vascularisation and elevated haematocrit levels contribute to a time-limited survival at high metabolic cost above this temperature. Hence, if warming above this *pejus* temperature occurs only in short seasonal peaks a survival of originally cold-adapted Antarctic species through remnant acclimation capacities seems possible.

This threshold temperature coincides with the upper *pejus* temperature of this species sensu Pörtner determined in thermal ramp experiments, which is set by limitations at systemic levels. This equivalence differentiates the Antarctic eelpout from its temperate congener *Z. viviparus*, where positive growth is detectable beyond the upper *pejus* temperature (Pörtner and Knust, 2007) and may be substantial for the higher thermal sensitivity of *P. brachycephalum*. The general applicability of this hypothesis has to be tested in Antarctic fish like Notothenioids where such combined molecular to organismic studies are absent so far. In summary, the findings of this thesis substantiate the concept of the OCLTT (Pörtner, 2001; Pörtner, 2012) at a molecular level revealing cellular mechanisms that modify oxygen supply and energy availability, thereby shaping the thermal plasticity of cold-adapted *P. brachycephalum*. Nevertheless, a clear hierarchy from high-level organisation (the intact organism) down to molecular level remains obscure as organismic, systemic and molecular thresholds coincide in this species.

The question remains as to why *P. brachycephalum* naturally occurs below its thermal growth optimum. As the maintenance at low temperatures is connected with a high energy demand to cover the housekeeping metabolism and higher growth rates not necessarily define an optimal competitiveness under natural conditions (Pörtner et al., 2010), these findings suggest that the thermal optimum is not subsequently reflected in the realized thermal niche, but may reflect an evolutionary past at warmer temperatures.

4.5 Future perspectives

While it has been possible to highlight several aspects of molecular adaptation in *P. brachycephalum*, further work is required to extend the knowledge base on the structure and architecture of genomic landscapes in this species. A genomic backbone would offer a fundamental basis to address gene duplication events as well as identify new non-redundant genes in comparative approaches. Translations of sequence data would also allow for broader proteomic analyses. As mentioned before, it would be useful to reconsider the proteomic proxy “ERK” (characterizing protein stability) with respect to the inclusion of more cold adaptive amino acid exchange patterns to resolve protein flexibility dependent on OGT on a cold thermal scale. The degree of molecular adaptation and subsequently species vulnerability to rising temperatures could be assessed through the utilization of such a new predictor.

Since many transcripts in the conducted analyses (pub. II+III) have no functional assignment, further in-silico analyses are required to characterize genes with unknown function. A combination of sequence validation by similarity searches in other recently published sequence collections of Antarctic fish (Chen et al., 2008; Shin et al., 2012) and a following sequence-motif analysis would provide a new model database similar to the fiNOGs (fish orthologous sequences) for Antarctic fish.

Finally, it would be important to address the effects of further climate-change related abiotic stressors to characterize the species vulnerability to expected conditions in greater detail. Synergistic effects of temperature and CO₂ result from both factors influencing the same physiological performances. It should be verified if this is also true at the molecular level.

This would provide a more comprehensive view of the species' response to projected future scenarios in the Southern Ocean. It would also be enlightening to include early live stages in such analyses since these are thought to be more sensitive to altered environmental conditions (Pörtner and Farrell, 2008) and represent a critical bottleneck in species distribution.

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6 Appendix

6.1 Geo-coordinates of catching positions

Table Ap-1: GPS positions of the catching positions

Expedition/Cruise	Date of Recovery	Station Number	GPS Position
ANT-XV/3	16.03.1998	PS48/314	62°11,00 S 58°20,70 W
ANT-XXIII/2-3	29.01.2006	PS69/184-1	62°11,48 S 57°54,63 W
		PS69/184-2	62°12,14 S 58°19,77 W
		PS69/184-3	62°8,02 S 58°26,95 W
		PS69/184-4	62°16,74S 58°22,05 W
ANT-XXV/4-5	03.04.2009	PS73/255-1	62°19,01 S 58°35,49 W
		PS73/255-2	62°16,86 S 58°36,75 W
		PS73/255-3	62°19,33 S 58°33,80 W
		PS73/255-4	62°19,69 S 58°33,68 W

Additional data to Table 1 and Figure 3. Exact GPS positions are given in degree and decimal minutes.

6.2 Tissue-specific expression of candidate genes

During cloning procedures of candidate genes for publication I single targets were tested to characterize their constitutive expression in different tissues of *P. brachycephalum*. The respective genes contribute to regulatory mechanisms of the energy metabolism and signalling pathways.

Four transcription factors were included in the preliminary test. Targets under examination were PPAR1 α and β , the Co-factor PGC1 α as well as NRF-1 (roles of all factors are summarized in Figure 9, section 4.2.2). In addition, metabolic genes were quantified representing components of the aerobic (CS) and anaerobic metabolism (LDH, muscle-specific isoform), the respiratory chain (CytC and COX2) and an ubiquitous cellular structure protein (β -actin).

The transcription factors displayed a more distinct pattern of expression between different tissues than the metabolic genes (Fig. AP-1). Most conspicuous were the high expression levels of PPARs in liver tissue. In contrast, the metabolic genes mostly displayed similar levels among different organs. The only exceptions were the muscle-specific LDH, which was solely found in white muscle tissue as well as β -actin that was found in spleen at high levels.

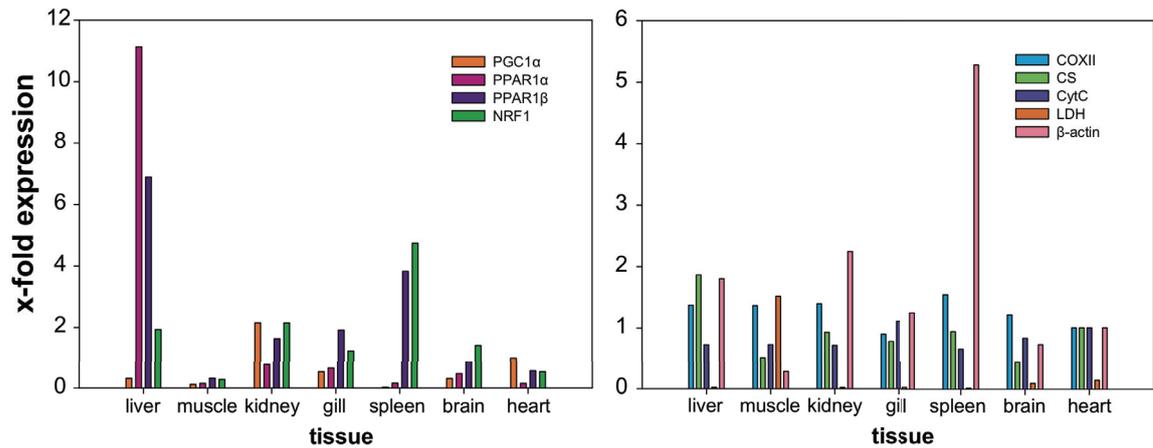


Figure AP-1: Tissue-specific expression levels of some candidate genes of *Pachycara brachycephalum*

Both panels show the x-fold expression levels of transcription factors (left side) and metabolic genes (right side), which were determined by means of quantities in different tissue samples of untreated animals. Quantities from 3 technical replicates were normalized by a factor calculated from the most stable genes - here COXII and Cyt-C (relative expression stability of $M=0.563$).

The high expression of PPARs in liver tissue emphasizes the strong involvement of this organ in energy production and conversion. With the focus on metabolic changes caused by thermal acclimation liver tissue constitutes a suitable monitor to analyze cellular responses. However, a contribution of the monitored genes in an acclimation process in other tissues is also likely.

6.3 Characteristic sequence motifs of zoarcid cDNA libraries

Overrepresented protein motifs were analyzed by means of the top 100 Interpro IDs found in sequences of zoarcid cDNA libraries. Counts for each sequence motif were plotted in a density map to evaluate exclusive or overrepresented protein motifs dedicated to one of both fish species.

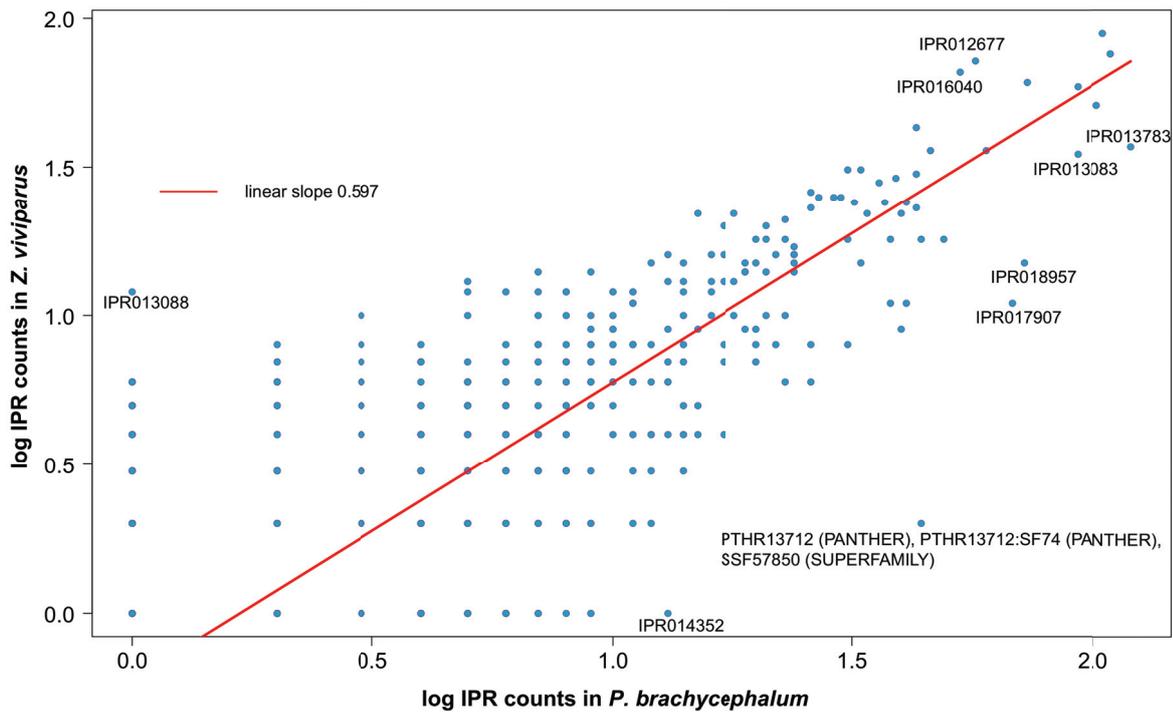


Figure AP-3: Analyses of overrepresentation of sequence motifs in sequences of zoarcid cDNA libraries
 Conspicuous protein sequence motifs overrepresented in one species are labelled with the respective Interpro ID. The descriptions of the most prominent motifs are summarized in Table Ap-1.

Table Ap-1: Summary of most abundant sequence motifs in zoarcid cDNA libraries

Overrepresented motifs	Description
<i>Z. viviparus</i>	
IPR013088	Zinc finger, NHR/GATA-type http://www.ebi.ac.uk/interpro/IEntry?ac=IPR013088
IPR016040	NAD(P)-binding domain http://www.ebi.ac.uk/interpro/IEntry?ac=IPR016040
IPR012677	Nucleotide-binding, alpha-beta plait http://www.ebi.ac.uk/interpro/IEntry?ac=IPR012677
<i>P. brachycephalum</i>	
IPR014352	FERM/acyl-CoA-binding protein, 3-helical bundle http://www.ebi.ac.uk/interpro/IEntry?ac=IPR014352
PTHR13712(Panther), PTHR13712:SF74 (Panther), SSF57850 (Superfamily)	RING/U-box superfamily http://supfam.cs.bris.ac.uk/SUPERFAMILY/cgi-bin/scop.cgi?sunid=57850
IPR017907	Zinc finger, RING-type, conserved site http://www.ebi.ac.uk/interpro/IEntry?ac=IPR017907
IPR018957	Zinc finger, C3HC4 RING-type http://www.ebi.ac.uk/interpro/IEntry?ac=IPR018957
IPR013083	Zinc finger, RING/FYVE/PHD-type http://www.ebi.ac.uk/interpro/IEntry?ac=IPR013083
IPR013783	Immunoglobulin-like fold http://www.ebi.ac.uk/interpro/IEntry?ac=IPR013783

Descriptions of species-specific protein motifs identified by the Interpro-scan depicted in Figure AP-4.

6.4 Sequence diversity in the cDNA library of *P. brachycephalum*

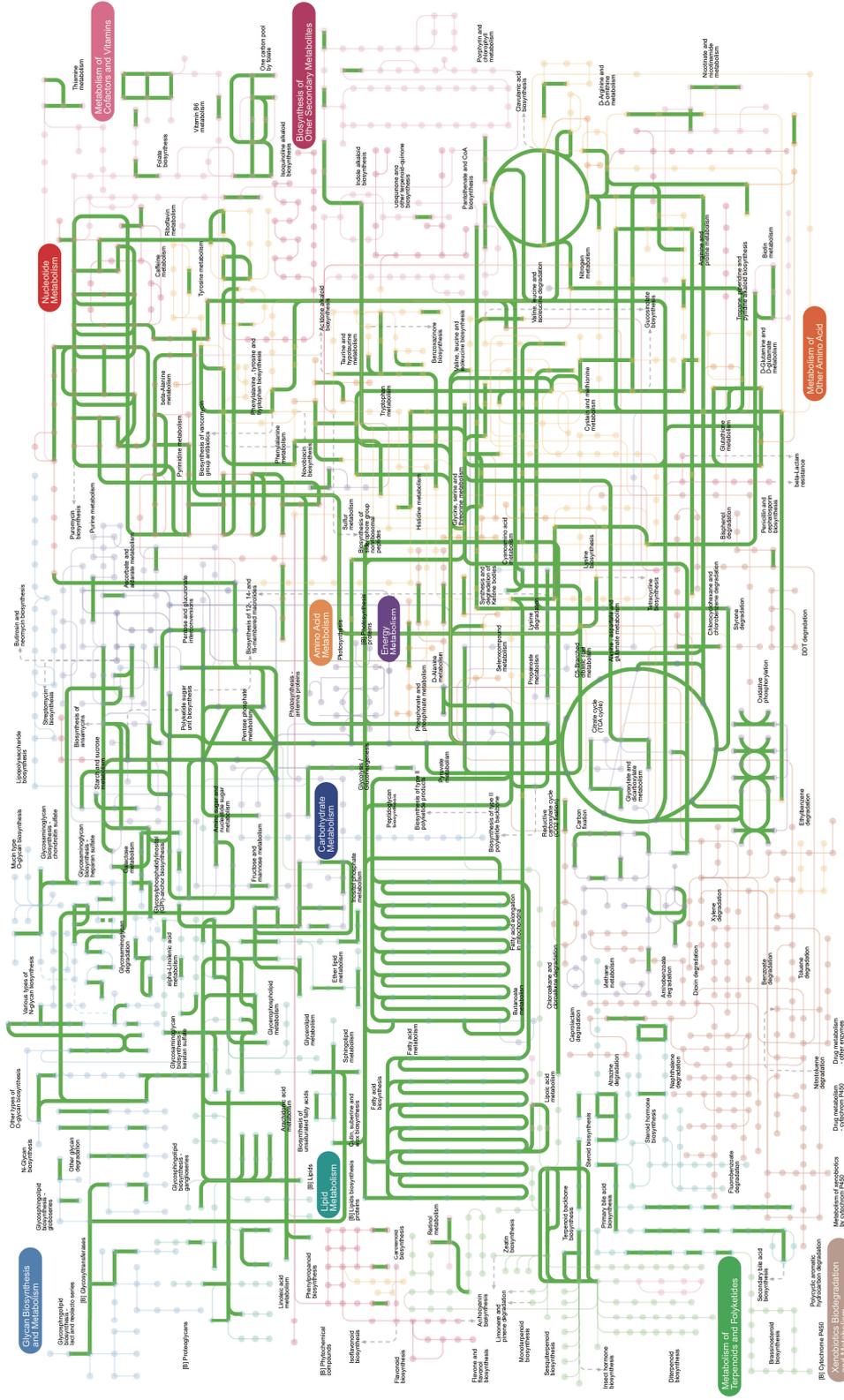


Figure AP-4: Mapping of the *P. brachycephalum* cDNA library on the global map of metabolic pathways

KEGG Orthologies (KOs) of library sequences were obtained from the KEGG automatic annotation server (KAAS) (<http://www.genome.jp/tools/kaas/>). KOs were loaded to the Interactive Pathway Explorer (iPath) version 2 (<http://pathways.embl.de/iPath2.cgi>) to visualize the coverage of metabolic pathways (green lines).

6.5 Sex-dependent gene expression in liver tissue – 105k array

Sex-specific differences were previously found and discussed to promote gonad growth especially in females ((Brodte, 2001) R. Kathöver and M. Lucassen, personal communication; own observations). Within the test array (see Material and Methods) the optimization of microarray probes and the differential gene expression between males and females were addressed on a format with 2x105k probes per slide. A differing gene expression was found for transcripts mostly of unknown functions. In total 1,391 transcripts were identified for females and 1,280 transcripts for males (testing groups against zero, see Material and Methods section 2.5.4). Both sets displayed an intersect of 1,111 sequences. Exclusively dedicated to a sex were 169 male-specific and 278 female-specific transcripts. As a consequence, for the following study with an optimized 60K array preferably males were used. No difference between males and females became visible in the 9°C incubation group during the study of publication III.

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Erklärung gemäß § 6 (5) der PromO der Universität Bremen

(Stand: 14. März 2007)

Ich erkläre hiermit, dass ich die Arbeit mit dem Titel
„Molecular adaptability to thermal challenges in a cold-adapted Antarctic fish“

1. ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe
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