Thermal acclimation in Antarctic fish: transcriptomic profiling of metabolic pathways

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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-1α 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 hardness, as the latter sustain anaerobic metabolism and may prepare for hypoxic 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 of 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 Pagotheria 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°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 upregulation of a mitochondrial uncoupling protein (UCP-2), while it was downregulated in warm-acclimated temperate...
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)]. PPARα and -β were identified to regulate the β-oxidation of fatty acids (3, 67, 69). PPARγ 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 PPARα and -β, PGC1α, 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 vetebrate PGC1α 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 starved 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 ANTXV/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 I was sampled on day 0 and group II 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 ± 3.63 cm (± SD) and a mean body weight of 46.53 ± 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 anaesthetized 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 µl 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 A650 nm through oxidation of CytC (ε650 = 19.1 mM/cm) was measured over time after adding the extract.**

**CS activity was determined following a modified protocol from Sidell et al. (56) using 20–40 µl 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 λ = 412 nm due to formation of the dye complex DTNB-S-CoA (ε412 = 13.6 mM/cm) was measured over time.**

Phosphoenolpyruvate carboxykinase (PEPKC) activity was determined after a protocol by Aas-Hansen et al. (1) using 30–60 µl 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 µg/ml, was added to prevent NADH oxidation through the respiratory chain. The reaction was initiated by adding NADH. The decrease in absorbance at λ = 340 nm due to oxidation of NADH (ε340 = 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 µl of homogenate in 0.1 mM acetoacetyl-CoA, 0.15 mM NADH, and 2.5 µg/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 λ = 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 μl of total RNA was exposed to 1 μl DNase (turbo DNase free kit, Applied Biosystems, Darmstadt, Germany). Then 0.4 μl of the DNAfree 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 concentrations 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 ≤ 1.5 for controls), namely with Mβ-Act = 0.54; MUbi = 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 ≤ 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 ± 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 3).

<table>
<thead>
<tr>
<th>Table 1. Cloned genes with the related oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
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<td>NRF-1</td>
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<td>PPARβ</td>
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<tr>
<td>COX4</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td>PPARγ</td>
</tr>
</tbody>
</table>

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.
Table 2. Primer specification for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Name</th>
<th>Primer Sequence (5′-3′)</th>
<th>Slope</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
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<td>6PGGL</td>
<td>HQ439154</td>
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<td></td>
<td>HQ439155</td>
<td>Pb_6PGGL_fw</td>
<td>AATGGACCGACGGACGTGTT</td>
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<td>1.94</td>
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<td>ACAC</td>
<td>HQ439156</td>
<td>Pb_ACoAcarb_rev</td>
<td>TGCAGTACGACGGACGTGTT</td>
<td>−3.47</td>
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<tr>
<td></td>
<td>HQ439157</td>
<td>Pb_ACoAcarb_fw</td>
<td>AATGGACCGACGGACGTGTT</td>
<td>−3.49</td>
<td>1.94</td>
</tr>
<tr>
<td>β-Act</td>
<td>HQ439158</td>
<td>Pb_CytC_rev</td>
<td>TCCAGACTTACGACGGACGTGTT</td>
<td>−3.45</td>
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<tr>
<td>GAPDH</td>
<td>HQ439159</td>
<td>Pb_CytC_fw</td>
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<td>−3.51</td>
<td>1.93</td>
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<tr>
<td>GYPS</td>
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<td>PPARα</td>
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<td>PPARβ</td>
<td>HQ439170</td>
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<td>TCCAGACTTACGACGGACGTGTT</td>
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<tr>
<td>PPARγ</td>
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<td>TAGL</td>
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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 thereafter 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 Q10 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

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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 and balances oxidative and biosynthetic pathways. The ratio of COX to CS activities (Fig. 2A) increased significantly on the first day onward and remained at 40% of the initial levels (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. PEPCCK activities (Fig. 2B) increased significantly on day 3 (Table 4). PEPCK activity (per milligram protein) as a marker for gluconeogenesis increased toward the end of warm exposure by ~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. PEPCCK activities (Fig. 2B) increased significantly on the second day $0.89 \pm 0.30$ ($P = 0.048$), and the ratio was elevated in the end ($0.78 \pm 0.28$), compared with controls ($0.56 \pm 0.15$, $P = 0.026$).

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 $2.54 \pm 0.91 \mu g/mg$ (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 PPAR1α dropped from the first day onward and remained at 40% of the initial levels (Fig. 3A). Conversely, PPAR1β expression increased 1.8-fold (Fig. 3C). Both reached their maximum change from control levels on day 7 and stabilized below (PPAR1α) or above (PPAR1β) controls after 14 days. In contrast, PPAR1γ expression in-

Table 3. Effect of temperature on enzyme activity

<table>
<thead>
<tr>
<th>Enzyme and Assay</th>
<th>Temperature</th>
<th>Means of Enzyme Activity per Protein</th>
<th>Means</th>
<th>$Q_{10}$</th>
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<tr>
<td></td>
<td></td>
<td>[µmol (h mg)$^{-1}$] ± SD</td>
<td>$P$ Value</td>
<td></td>
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<tr>
<td>CS 0°C</td>
<td>0.642 ± 0.195</td>
<td>&lt;0.001</td>
<td>1.961</td>
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<tr>
<td>CS 5°C</td>
<td>0.882 ± 0.240</td>
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</tr>
<tr>
<td>COX 0°C</td>
<td>1.926 ± 1.165</td>
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<tr>
<td>COX 5°C</td>
<td>2.068 ± 1.190</td>
<td>0.672</td>
<td>1.214</td>
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</table>

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.
increased 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)1α, which was monitored as an indicator of possible hypoxemic stress in fish upon warming (48, 52), and of the coactivator PGC1α 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 mitochondrion, 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 ~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, tricarlyclycerol lipase (TAGL) catalyzes the cleavage of triacylglycerides within the cytoplasm. TAGL mRNA levels decreased over time and leveled off at ~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 ~75% of control levels. The step following hydration, catalyzed by HADH experienced a decrease in expression by half after 1 wk, followed by an 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 glycogenic GAPDH remained unchanged, while pyruvate kinase (PK) expression decreased transiently to ~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. 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 ± 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.](http://ajpregu.physiology.org/DownloadedFrom/R1458THERMALRESPONSEINCOLDADAPTEDFISH)
effect, seen in elevated levels of PPAR1γ, 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, PPAR1α 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. PPAR1β is located in the opposite direction of PPAR1α, indicating a contrasting role for both transcription factors (see Table 4). PPAR1γ is positioned in the plot as a transcription factor that is mostly influenced by the factor of time. HIF1α and PGC1α 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.
other cellular stress responses. For the Antarctic eelpout, functional hypoxemia during acute exposure to 5°C can be excluded (39). Although some stress response at the transcriptional level may occur in the first hours, the stable expression profile of HIF1α, 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
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 mitochondrial 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 ~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 PPARα and -β, and a somewhat smaller response of PPARγ then occur upon warm acclimation (Fig. 3, and Table 4).

In mammals PPARα has a high affinity to saturated as well as 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...
envelope for the initial step of lipid biosynthesis was induced in mammalian liver through transactivation by PPAR\(\alpha\) (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.

PPAR\(\beta\) is the least understood receptor of the PPAR-family, with a lower affinity to fatty acids compared with PPAR\(\alpha\) (51, 69). In our study, we found an increased expression during warm acclimation, which perfectly mirrors the decrease in PPAR\(\alpha\). Thereby, the correspondence analyses indicate contrasting functions of both receptors (Fig. 4A). Fast responses of PPAR\(\alpha\) and -\(\beta\) 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 PPAR\(\beta\) also modulates glucose homeostasis (69). A significant positive correlation was found between expression levels of PPAR\(\beta\) and GYS; however, PPAR\(\gamma\) and the cofactor PGC1\(\alpha\) 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\(\alpha\) and PPAR\(\beta\), and furthermore, PPAR\(\gamma\). The small angle between these factors in Fig. 4A indicates strong correlations. During long-term exposure to 5°C PPAR\(\gamma\) seems to become more important (shift along the incubation time axis) in supporting the storage of glycogen (Table 5) in association with PGC1\(\alpha\).

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 PPAR\(\alpha\) and -\(\beta\) may reflect a well-controlled sensing of initial, fast changes and indicate a direct interdependency of both receptors. In mammals, the message levels of PPAR\(\beta\) and -\(\gamma\) were found to act as the dominant repressors of PPAR\(\alpha\)-mediated responses (30). Here, we observed inverse correlations (Table 5), i.e., suppression for nearly all genes that were positively correlated with the \(\alpha\)-receptor. Hence, it is possible that PPAR\(\alpha\) expression and PPAR\(\alpha\)-mediated responses are also controlled by PPAR\(\beta\) in fish.

PGC1\(\alpha\) 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\(\alpha\) for all transcription factors. In goldfish, temperature-dependent expression of PGC1\(\alpha\) were found in several tissues, albeit its expression increased both
upon cooling or warming in liver (35). NRF-1 was correlated with PGC1α (Table 5), as the expression of NRF-1 was significantly reduced. Besides, recent sequence analyses of PGC1α 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. brachyccephalum. 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 PPAR1α 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. brachyccephalum (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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