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

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ABSTRACT

Mitochondrial plasticity plays a central role in setting the capacity for acclimation of aerobic metabolism in ectotherms in response to environmental changes. We still lack a clear picture if and to what extent the energy metabolism and mitochondrial enzymes of Antarctic fish can compensate for changing temperatures or PCO_2 and whether capacities for compensation differ between tissues. We therefore measured activities of key mitochondrial enzymes (citrate synthase (CS), cytochrome *c* oxidase (COX)) from heart, red muscle, white muscle and liver in the Antarctic fish *Notothenia rossii* after warm- (7 °C) and hypercapnia- (0.2 kPa CO_2) acclimation vs. control conditions (1 °C, 0.04 kPa CO_2). In heart, enzymes showed elevated activities after cold-hypercapnia acclimation, and a warm-acclimation-induced upward shift in thermal optima. The strongest increase in enzyme activities in response to hypercapnia occurred in red muscle. In white muscle, enzyme activities were temperature-compensated. CS activity in liver decreased after warm-normocapnia acclimation (temperature-compensation), while COX activities were lower after cold- and warm-hypercapnia exposure, but increased after warm-normocapnia acclimation. In conclusion, warm-acclimated *N. rossii* display low thermal compensation in response to rising energy demand in highly aerobic tissues, such as heart and red muscle. Chronic environmental hypercapnia elicits increased enzyme activities in these tissues, possibly to compensate for an elevated energy demand for acid–base regulation or a compromised mitochondrial metabolism, that is predicted to occur in response to hypercapnia exposure. This might be supported by enhanced metabolisation of liver energy stores. These patterns reflect a limited capacity of *N. rossii* to reorganise energy metabolism in response to rising temperature and PCO_2.

1. Introduction

In light of the ongoing ocean acidification of warming of the oceans (IPCC, 2007), the synergistic effects of both ocean warming and acidification have recently been found to reduce aerobic scope of marine ectotherms by further increasing their aerobic energy demand or by suppressing efficiency of oxygen supply (Pörtner, 2010, 2012). As a result, the capacity of an animal to increase its rate of aerobic energy turnover is likely to be reduced possibly even at temperatures within the optimal range of thermal tolerance (Pörtner and Farrell, 2008).

Mitochondria are the primary site of cellular oxygen consumption and aerobic energy production. Because oxygen is required for the aerobic production of ATP, mitochondrial function is closely connected to the ventilatory and circulatory capacities of the animal. Accordingly, limitations in mitochondrial energy metabolism caused by oxygen supply or substrate availability will contribute to a loss of whole animal aerobic scope (Pörtner, 2001, 2002). Due to this central role in aerobic energy metabolism, mitochondria are a key factor in defining metabolic capacities of ectothermal animals to respond to changes in abiotic, environmental conditions, such as increasing temperature and PCO_2.

Prolonged elevations in temperature usually cause an increase in physiological rates and associated energy demand. Functional responses to changes in tissue-specific aerobic energy demand include concomitant adjustments of the tissue's metabolic demand, such as shifts in substrate turnover (e.g., seasonal shifts in glycogen and lipid usage in *Arenicola marina*, (Sommer and Pörtner, 1999, 2002)), or changes in mitochondrial abundance and/or mitochondrial aerobic metabolism. For example, mitochondrial warm-compensation as seen in various temperate fish would involve reverse mitochondrial proliferation (e.g., Guderley, 1990; Lannig et al., 2003, 2005; Lucassen et al., 2006) or high mitochondrial activation energies (Hardewig et al., 1999b; Pörtner et al., 2000) in order to keep mitochondrial maintenance costs low. Furthermore, a high aerobic demand may cause shifts in the activities of individual enzymes or even between metabolic pathways, such as an increased net use of storage compounds such as carbohydrates and lipids or metabolic rearrangements towards enhanced protein catabolism and reduced lipid biosynthesis

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in many temperate fish species (Brodte et al., 2006; Lucassen et al., 2006; Michaelidis et al., 2007; Melzner et al., 2009; Windisch et al., 2011). Such shifts may support aerobic capacities under conditions of elevated energy demand, but also lead to a depletion of the animal's energy stores (Brodte et al., 2006; Windisch et al., 2011).

Changes in aerobic demand and mitochondrial adjustments may become visible in activities of the mitochondrial matrix enzyme citrate synthase (CS). This enzyme plays a central role in several metabolic pathways as it catalyses the first step of the tricarbonic acid cycle (TCA-cycle). The mitochondrial transmembrane protein cytochrome *c* oxidase (COX) is a substantial part (Complex IV) of the electron transport system. COX plays a crucial role in aerobic life because of its specific role as terminal electron acceptor of the electron transport system, where molecular oxygen is reduced to water (Gnaiger, 2009, 2012). Previous studies found COX to be the controlling site of mitochondrial respiration and ATP synthesis (Villani and Attardi, 2001; Kadenbach et al., 2010). Both enzymes, CS and COX, are frequently used as indicators of tissue specific aerobic capacity (Cai and Adelman, 1990). Ideally, CS capacities would reflect the entrance of acetyl-CoA into the TCA-cycle after final oxidation of fatty acids and carbohydrates. If acetyl-CoA is present in excess, it can be shuttled via citrate into the cytosol for fatty acid synthesis. Thus, the TCA-cycle in liver also supports biosynthetic processes, such as the lipid-biosynthesis or gluconeogenesis from malate (Owen et al., 2002; Windisch et al., 2011). Accordingly, the COX to CS ratio can be used to reflect preferred metabolic pathways and relative metabolic adjustments in response to warming and hypercapnia in a tissue. Furthermore, changes in COX activity may be related to alterations in mitochondrial membrane structure (Wodtke, 1981; O'Brien and Mueller, 2010), and in CS activity to changes in mitochondrial matrix volume (e.g., Hardewig et al., 1999b; Guderley and St-Pierre, 2002; Guderley, 2004).

Most studies on mitochondrial aerobic enzyme capacities have been conducted on temperate zone fish (e.g., Dalziel et al., 2005; Hulbert et al., 2006; Grim et al., 2010; Martin-Perez et al., 2012). For example, COX activities were increased in heart and liver of carp (*Cyprinus carpio*) after warm-acclimation (Cai and Adelman, 1990). In contrast, a lack of change or even a loss of specific COX activities had been shown in liver of cold-acclimated cod *Gadus morhua* and eelpout *Zoarces viviparus*, while CS activity showed a strong thermal response, increasing in the cold and decreasing in the warmth (Lucassen et al., 2003; Lucassen et al., 2006). In cold-acclimated channel catfish, *Ictalurus punctatus*, a positive compensation by increased total liver CS activities was found, whereas total COX activities remained unchanged (Kent et al., 1988).

Owing to physiological adaptations for a life in permanently cold Antarctic waters, stenotherm fish may prove to respond differently to rising seawater temperature and PCO_2. For example, Antarctic fish possess extremely low metabolic rates, high enzyme quantities and high activation energies in their mitochondria. Thus, even small increases in temperature can cause a large increase in metabolic flux and thereby limit individual aerobic performance (Pörtner et al., 2000; Pörtner, 2006). Yet, hardly any study has analysed the role of mitochondrial aerobic enzymes in warm-acclimation of Antarctic fish. For example, the Antarctic eelpout *Pachycara brachycephalum* compensates for the warmth by reduced enzyme capacities (Lannig et al., 2005). In contrast, the notothenid *Pagothenia borchgrevinki* responds to warming by increasing muscle COX activities, while glycolytic and TCA enzyme levels remain unchanged. The authors suggest that high COX activities come along with increased oxidative capacities, which may support elevated metabolic costs at warmer temperatures in Antarctic fish (Seebacher et al., 2005).

Due to the enhanced CO_2 solubility in cold waters and body fluids, ocean acidification along with warming may become particularly threatening to polar ectotherms. Thereby, the combination of these two stressors may further reduce the very narrow thermal window of optimum performance in Antarctic species, with consequences for activity levels, growth rates and population survival (Pörtner, 2010; Munday et al., 2012). Similar to the changing metabolic demand of an organism at warmer temperatures, the maintenance of acid–base equilibria, i.e., elevated bicarbonate concentrations to compensate for increases in extra- and intracellular PCO_2 under chronic hypercapnia, may cause an increase in energy demand (Melzner et al., 2009). Accordingly, responses to changes in tissue-specific aerobic energy demand under elevated PCO_2 would involve shifts in mitochondrial abundance or aerobic metabolism. Up to now, only few studies have reported enzymatic responses to long-term elevated PCO_2 in fish, for example, a decrease in CS activity in heart, red muscle and white muscle of temperate sea bass *Sparus aurata* (0.5 kPa CO_2, Michaelidis et al., 2007). Although active organisms like fish are believed to possess adequate capacities to cope with hypercapnia-induced acid–base disturbances and shifts in energy demand, chronic hypercapnia exposure may exacerbate the effects of rising seawater temperature on cellular and whole animal metabolism (Munday et al., 2012; Pörtner, 2012). The response of fish to warming or hypercapnia has until now mainly been investigated for temperate species, and to our knowledge no study analysed the interaction of warming and hypercapnia on Antarctic fish at the mitochondrial enzyme level. In light of the physiological adaptations of Antarctic fish to their thermally stable environment, it is highly questionable if their energy metabolism displays similar acclimation capacities as temperate fish.

The present study was therefore designed to investigate enzymatic responses in tissues of different metabolic activity (liver, heart, red muscle and white muscle) of Antarctic notothenioid fish to ocean warming and acidification. We used the demersal notothenioid *Notothenia rossii*, which displays a circum-polar distribution at habitat temperatures between −1.9 and 2 °C (Everson, 1969; Gon and Heemstra, 1990; Schloss et al., 2008), as a representative for Antarctic stenotherms. We studied the acclimation capacities of two enzymes involved in aerobic mitochondrial metabolism, namely citrate synthase (CS) and cytochrome *c* oxidase (COX), in *N. rossii* acclimated for four to six weeks to the warmth (7 °C) and/or elevated PCO_2 of 0.2 kPa (2000 μatm). The characterisation of how exactly mitochondrial enzymes respond to long-term elevated CO_2 levels aims to increase the knowledge about the mitochondrial capacity of the unique group of Antarctic fish to respond or acclimate to rising temperature and PCO_2.

2. Material and methods

2.1. Animal capture and acclimation

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

The fish were reared and acclimated in the aquaria facilities at Dallmann Laboratory, Carlini Station (formerly Jubany Station, King George Island) under natural light conditions. Animals were fed to satiation every other day with chopped fish muscle and snails. For acclimation, animals were randomly selected and acclimated to 1 °C, 0.04 kPa CO_2 (control group, $n = 9$, mass 155–804 g; total length 25–39.4 cm), 1 °C, 0.2 kPa CO_2 (cold hypercapnic group, $n = 10$, mass 144–510 g; total length 23.8–32.8 cm), 7 °C, 0.04 kPa CO_2 (warm normocapnic group, $n = 5$, mass 151–412 g; total length 23.6–33.9 cm) and 7 °C, 0.2 kPa CO_2 (warm hypercapnic group, $n = 10$, mass 137–504 g; total length 21.4–31.3 cm). For detailed acclimation conditions, specific seawater conditions and seawater carbonate chemistry see (Strobel et al., 2012). Previous studies on the warm-acclimation capacities of *N. rossii* showed that they can survive temperatures of 5 °C for several weeks (Heise and Abele, 2007).

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Therefore, we chose the acclimation temperature of 7 °C to acclimate *N. rossii* at the maximum possible temperatures in order to gain results on their acclimation capacities. After five weeks acclimation to 7 °C, *N. rossii* was still healthy and in an acceptable condition (see Strobel et al., 2012). Following the Intergovernmental Panel on Climate Change's 'business-as-usual' scenario, atmospheric CO_2-concentrations may exceed 0.2 kPa by the year 2200 (IPCC, 2007). Therefore, we chose 0.2 kPa CO_2 for our hypercapnia acclimation of *N. rossii*.

2.2. Sampling

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

2.3. Enzyme assays

Frozen liver tissue was ground into powder by mortar and pestle under liquid nitrogen and homogenised in a glass homogeniser in 9 vol.% buffer containing 20 mmol L^{-1} Tris–HCl, 1 mmol L^{-1} EDTA, 0.1% Triton X-100 (pH 7.4) and afterwards with an Ultra Turrax (Silent Crusher M, Heidolph Instruments, Schwabach, Germany), followed by 10 min centrifugation at 1000 ×*g* at 4 °C. Enzyme activities of each sample extract were measured in the supernatant at 0, 6, 9 and 12 °C in a UV/VIS spectrophotometer (Beckman, Fullerton, CA, USA) equipped with a thermostatted cell holder. The assay temperatures 0, 6, 9 and 12 °C allow the comparison of mitochondrial capacities towards acute temperatures in control vs. acclimated *N. rossii*. The efficiency of the extraction procedure was optimised until no further enzymatic activity could be detected in re-extracted pellets.

Citrate synthase (CS; EC 2.3.3.1) activity was detected according to Sidell et al. (1987) in a buffer containing 75 mmol L^{-1} Tris–HCl, 0.25 mmol L^{-1} DTNB, 0.4 mmol L^{-1} acetyl-CoA, 0.5 mmol L^{-1} oxaloacetate. The activity was determined from the increase in absorbance at $\lambda = 412$ nm, caused by the transfer of sulfhydryl groups from coenzyme A to 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), quantified by use of the extinction coefficient (ε_{412}) of DTNB of 13.61 $mmol^{-1}$ cm^{-1}.

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

Protein concentration of the tissue extract was determined according to Bradford (Bradford, 1976) by measuring the absorbance at $\lambda = 595$ nm and 20 °C in a spectrophotometer (Pharmacia LKB Biochrom 4060, Pharmacia, UK). The enzyme activity (CS and COX) was calculated per mg tissue fresh-mass (nmol min^{-1} mg FW^{-1}) as well as per mg cellular protein (nmol min^{-1} mg $protein^{-1}$) to account for changes in mitochondrial density and composition on the one hand (normalised to FW), and on the other hand to standardise the enzyme activities to the amount of protein in each tissue extract. As different acclimation groups, particularly temperature-acclimated fish, may contain different amounts of lipids and mitochondria per gram fresh mass, the lipids may compromise the relation between enzyme activity and fresh weight. Via the standardisation per mg protein, the effect of changing amounts of tissue lipids in differently acclimated fish was compensated.

2.4. Calculations and statistics

The COX/CS ratio (mean across all assay temperatures as a measure for the enzyme–activity ratio between individual tissues) is given for the four tissue types of every acclimation group as a general indicator for the preferred metabolic pathways in each tissue type investigated. The temperature coefficient (Q_{10}) was calculated for the temperature ranges 0–6 °C, 6–12 °C, 0–9 °C and 0–12 °C. All data were tested for normality (Kolmogorov–Smirnov) and homogeneity of variance. We compared the enzyme activities of the temperature and CO_2-acclimated animals to their controls for each tissue-type using two-way analysis of variance (ANOVA) followed by a Tukey-test. A three-way ANOVA was conducted to test interactions between acclimation temperature, acclimation PCO_2 and assay temperature for each tissue using the R-software. All data are presented as means ±

Table 1
Mean citrate synthase (CS) and cytochrome *c* oxidase (COX) activities in nmol per minute and mg protein of *Notothenia rossii* in all tissues investigated and all acclimation conditions.

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

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

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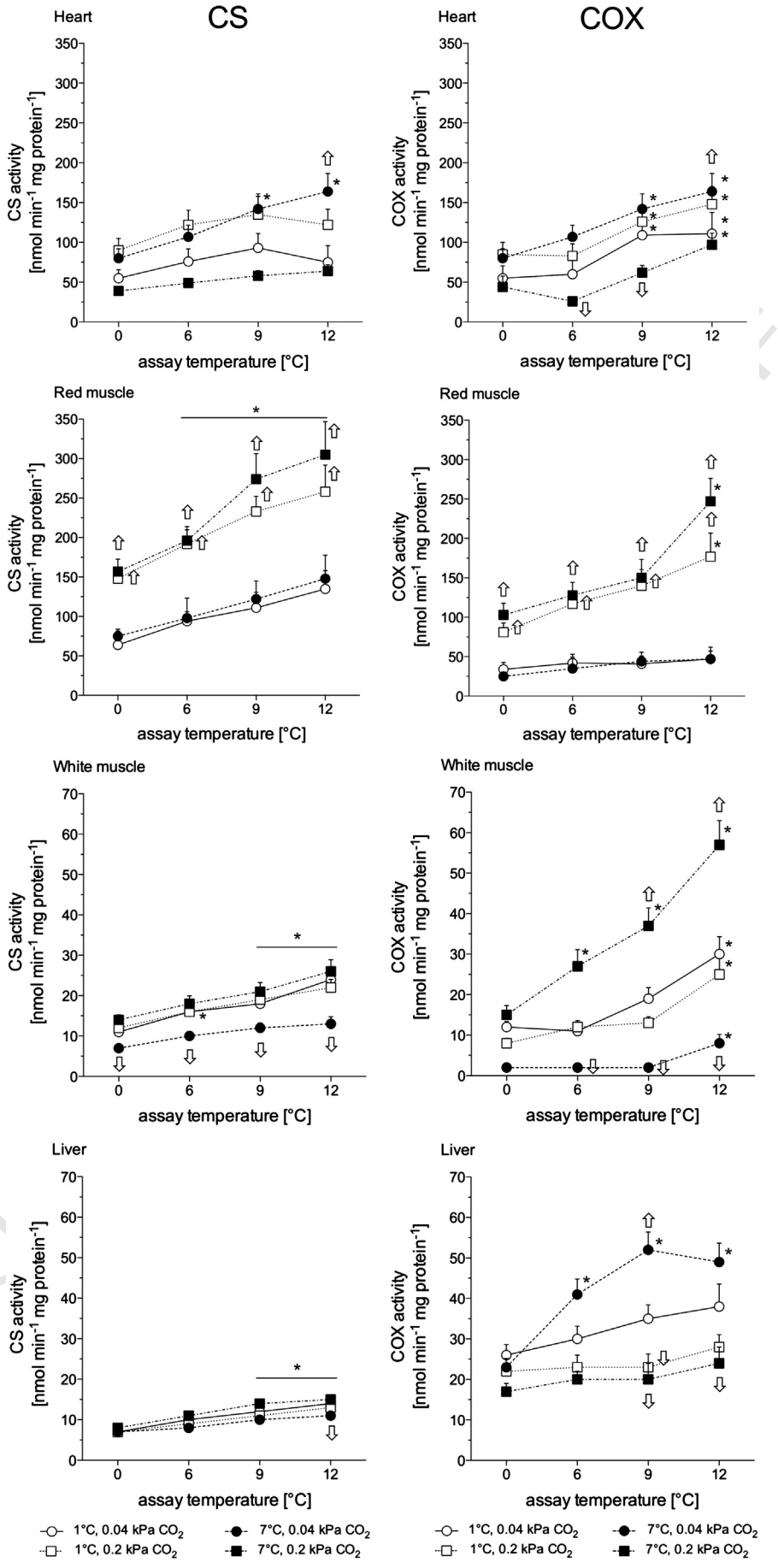

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standard error of the mean (SEM) per mg cellular protein or mg tissue fresh mass. Differences were considered significant if $p \leq 0.05$.

3. Results

3.1. Effects of warm-acclimation on enzyme activity

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

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

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

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

3.2. Effect of hypercapnia acclimation on enzyme activity

Cold-hypercapnia acclimated fish had a higher CS activity across all assay temperatures in heart (Table 1) and a significantly higher activity at 12 °C when expressed in nmol min^{-1} mg FW^{-1} compared to the control group (cold normocapnic). In red and white muscle, cold-hypercapnia acclimation had no effect on the enzyme activities. The liver of the cold-hypercapnia acclimated group had lower COX activities per mg protein compared to the control group at the 9 °C assay (Fig. 1).

3.3. Effects of warm and hypercapnia acclimation on enzyme activity

In the warm-hypercapnia acclimated fish, CS and COX activities in red muscle (per mg protein and FW) were significantly higher compared to the enzyme activities of the control group at all assay temperatures. In liver, COX activities at 9 °C and the mean across all temperatures were reduced after warm-hypercapnia acclimation compared to the control (Table 1, Figs. 1 and 2).

In the heart of the warm-hypercapnia acclimated fish, COX activity (per mg protein and per mg FW) was significantly lower than in control animals at the 6 and 9 °C assays, and also at 12 °C when depicted per mg tissue mass. The CS activity in the heart of the warm-hypercapnic group was significantly reduced compared to the control when plotted per mg FW (Fig. 2). Both COX and CS enzyme activities (expressed per mg protein and per mg FW) were elevated in red muscle after warm-hypercapnia acclimation.

In white muscle, COX activities showed a significant interaction/elevation in activity per mg cellular protein (three-way ANOVA, $F_{1,82} = 4.59$, $P < 0.035$) and per FW (three-way ANOVA, $F_{1,90} = 4.119$, $P < 0.045$) with respect to warm and hypercapnia acclimation (Fig. 1).

In liver of the warm-hypercapnic group, COX activity per mg protein was significantly decreased below control values (Fig. 1), and both CS and COX activity showed a significant interaction between temperature and PCO_2 (three-way ANOVA; CS: $F_{1,79} = 4.02$, $P < 0.048$; COX $F_{1,71} = 5.87$, $P < 0.018$).

For the sake of clarity, Table 2 provides a simplified overview on the trends of enzyme activities in response to warm and/or hypercapnia acclimation in all four tissues of *N. rossii* investigated in the present study. A significant interaction between acclimation temperature, acclimation PCO_2 and assay temperature only occurred in the COX activities per mg protein in white muscle (three-way ANOVA, $F_{3,70} = 3.02$, $P < 0.0352$).

3.4. COX to CS ratio, tissue protein content

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

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

4. Discussion

Measurements of maximal CS and COX activities are frequently used to determine changes in aerobic metabolic capacity in response to changes in environmental abiotic conditions (O'Brien and Mueller, 2010). In this study, we assessed the different responses in CS and COX activity to long-term elevated warming and/or hypercapnia in tissues characterised by high rates of aerobic energy generation (heart, red muscle), as well as to the aerobically active liver, which represents the hub of intermediary metabolism, compared to the metabolically less active white muscle. Heart and red muscle had up to six-fold higher enzymatic activities than white muscle and liver, indicating a higher mitochondrial density and metabolic capacity in the former tissues, which is often found in fish (Dalziel et al., 2005). The enzyme activities we measured in heart, red muscle, white muscle and liver fibres were consistent with values reported previously for cold-adapted fish (Crockett and Sidell, 1990; Hardewig et al., 1999b; Lucassen et al., 2006; Mark et al., 2012). Our findings thus Q4

Fig. 1. Maximum activities of citrate synthase (CS) and cytochrome *c* oxidase (COX) per mg protein in heart, liver, white muscle and red muscle of *Notothenia rossii*. White circles represent the control group (cold normocapnia: 1 °C, 0.04 kPa CO_2, $n = 6$), black circles the warm normocapnia (7 °C, 0.04 kPa CO_2, $n = 3$–4), white squares the cold hypercapnia (1 °C, 0.2 kPa CO_2, $n = 7$–8) and black squares the warm hypercapnia (7 °C, 0.2 kPa CO_2, $n = 6$–8) acclimated *N. rossii*. Values are given as means ± SEM. Activities were assayed at 0, 6, 9 and 12 °C. Arrows depict a significant ($p \leq 0.05$) increase/decrease in enzyme activity of a given treatment compared to the control group at the respective assay temperature. * denotes a significant ($p \leq 0.05$) increase in enzyme activity within an acclimation group compared to the 0 °C assay.

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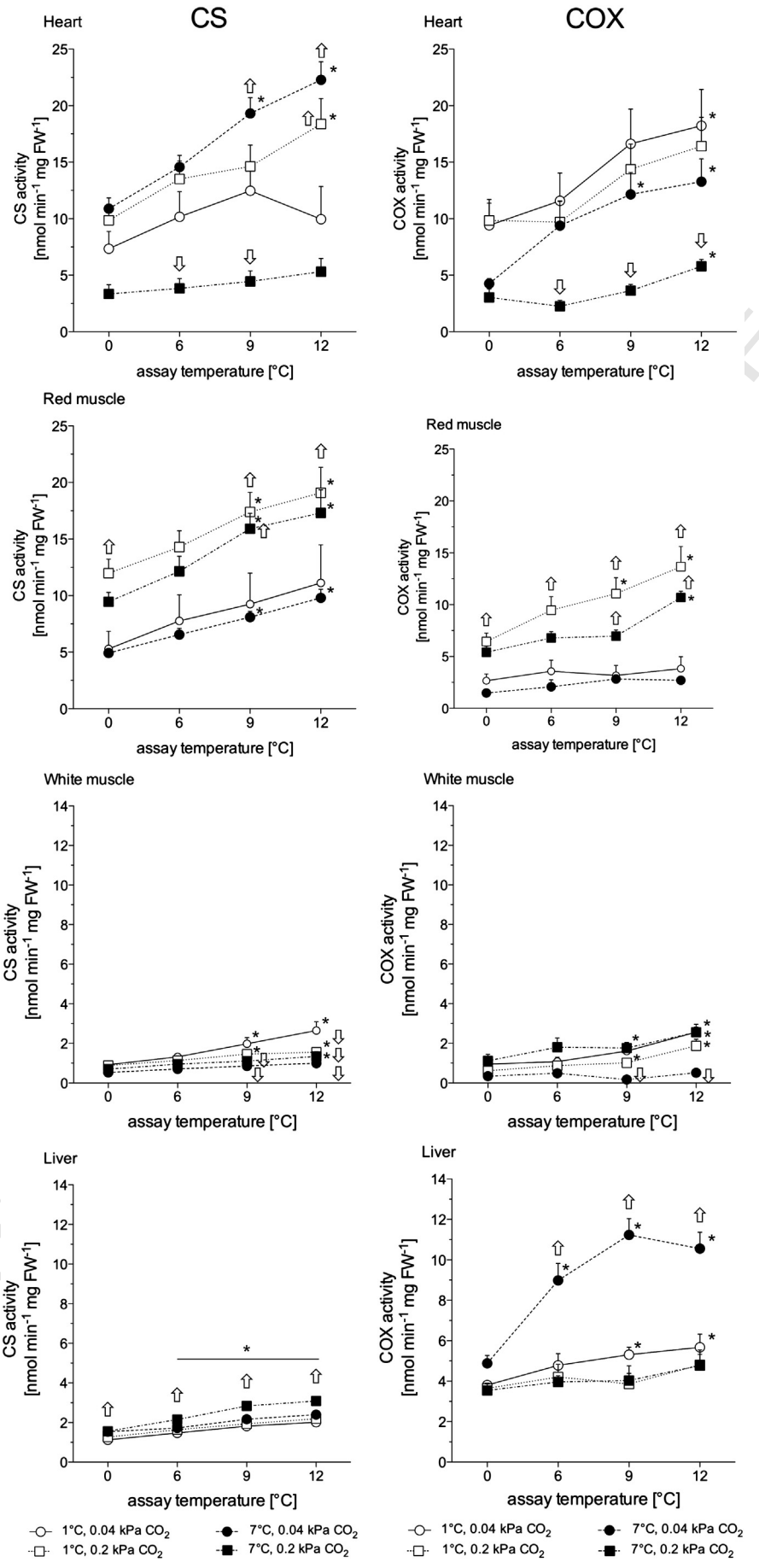

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Table 2
Simplified trends of citrate synthase (CS) and cytochrome *c* oxidase (COX) Q_{10} values of *Notothenia rossii* in all tissues investigated and all acclimation conditions compared to control.

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

→ indicates similar enzyme activities compared to control. ↑/↓ displays elevated/decreased enzyme activities compared to control, respectively. ↗/↘ depicts a slight trend of increasing/decreasing enzyme activities towards warmer assay temperatures. Trends of enzyme activities are given per mg protein (Prot) and per mg fresh mass (FW). Differences in enzyme activity trends per mg protein and per g fresh mass are highlighted in grey.

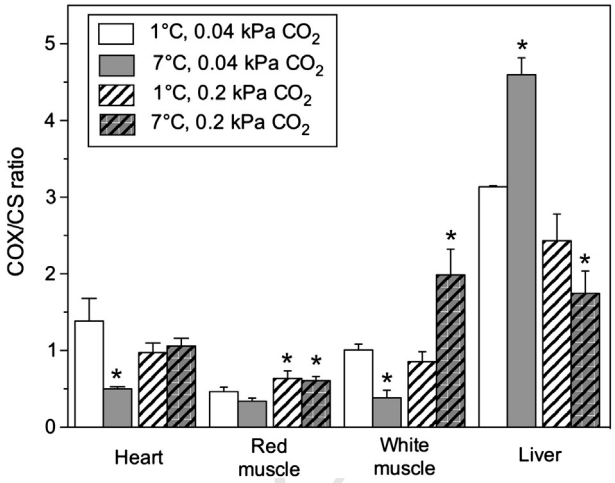

Fig. 3. Effect of warm and hypercapnia acclimation on the COX/CS ratio in *Notothenia rossii*. COX/CS ratio in heart, liver, white muscle and red muscle of control (white bars; 1 °C, 0.04 kPa CO_2, $n = 6$), warm normocapnia (grey bars; 7 °C, 0.04 kPa CO_2, $n = 3$–4), cold hypercapnia (white hatched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

confirm the higher oxidative capacity (and consequently oxygen demand) of the heart compared to red muscle (Walesby and Johnston, 1980) and the higher oxidative capacity of red muscle compared to white muscle in *N. rossii*.

4.1. Effect of warm-acclimation on enzyme activities

In many temperate fish species, a positive cold compensation involves mitochondrial proliferation, including tissue enzyme contents and activities, or enhanced aerobic capacities of individual mitochondria (e.g. in cod *G.* morhua, stickleback *Gasterosteus aculeatus* and trout *Oncorhynchus mykiss*; Egginton et al., 2000; Guderley et al., 2001; Lannig et al., 2003). Conversely, one would expect a reduction of mitochondrial content and enzyme activities in order to reduce mitochondrial maintenance costs towards the warmth (Pörtner, 2002). Our data on the enzyme activities in warm normocapnia acclimated *N. rossii* suggest that heart tissue does not compensate for warming to 7 °C in the conventional sense, as this would imply a down-regulation of mitochondrial capacities (e.g., Lannig et al., 2005; Lucassen et al., 2006). Instead, COX activities per mg tissue measured in *N. rossii* were hardly reduced compared to the control, and CS activities were even increased towards the warmth (Fig. 2, Table 2). Although higher oxidative capacities may be needed to meet the energy demand of the heart at warmer ambient temperature, uncompensated mitochondrial capacities or densities also imply elevated maintenance costs of heart mitochondria that may need support by the energy stores of other tissues or shifts in metabolic pathways. Similarly, COX activity was stimulated in liver of warm-normocapnia acclimation of *N. rossii* (Fig. 1, Table 2). This picture also contradicts the general pattern of reversed mitochondrial proliferation in the warmth (Johnston et al., 1998; Guderley and St-Pierre, 2002) and indicates metabolic reorganisation or shunting of metabolic pathways in the liver.

The COX to CS ratio can be used to reflect preferred metabolic pathways and relative metabolic adjustments in response to environmental challenges in a tissue. In fact, a strong thermal stimulation of CS indicates an enhanced TCA-activity from anaplerotic pathways (Lucassen et al., 2003) in heart of acclimated *N. rossii* which was also reflected in a significantly lower COX/CS ratio in the warm-acclimated compared to the control group (Fig. 3).

In liver of warm acclimated of *N. rossii*, we observed different COX/CS ratios compared to the other tissues in this study, which are mainly due to low CS activities and may reflect the different metabolic duties of this tissue. The TCA-cycle in liver is a metabolic sink for succinyl-CoA from the oxidation of odd chain fatty acids and supports biosynthetic processes, e.g. lipid-biosynthesis from excess citrate or gluconeogenesis from malate (Owen et al., 2002; Windisch et al., 2011). The elevated COX/CS ratio in liver after warm-acclimation (Fig. 3) is a result of increased COX activities that can serve to enhance oxygen affinity (Gnaiger et al., 1998). This also may entail a shift from high-energy substrates (fatty acids) to carbohydrate fuels (pyruvate entry from carbohydrate oxidation) and glycogen catabolism, which are energy generating pathways that consume less oxygen (Sidell et al., 1987; Windisch et al., 2011). As a result, the higher aerobic capacities might be accompanied by a concomitant degradation of the liver energy stores at warmer temperatures, in line with observations of a lower hepatosomatic index of warm-acclimated *P. brachycephalum* (Lannig et al., 2005). Furthermore, the tissue protein content in liver was slightly higher in the warm-acclimated *N. rossii* compared to control (Fig. 4), which mirrors a reduced lipid or glycogen content and concomitantly higher cellular protein fraction per mg liver tissue following warm-exposure. The energy reserves in the liver may be used to support enhanced aerobic capacities in the heart of warm-acclimated *N. rossii* (see above), but in the long run reduced ATP supply by the liver will contribute to limit the performance of highly aerobic tissues like heart or red muscle.

Indeed, the CS (mean Q_{10} 1.7 ± 0.1) or COX activities in red muscle of warm-normocapnia acclimated *N. rossii* remained similar to the enzyme activities in the control group (Table 2), indicating no enzymatic temperature compensation or inverse mitochondrial proliferation in red muscle. The present findings are similar to those in the high-Antarctic notothenioid *P. borchgrevinki*, which even increases oxidative phosphorylation capacities in the red pectoral muscle to cover elevated metabolic costs for labriform locomotion in the warmth (Seebacher et al., 2005). In line with findings of high capacities to increase critical swimming speed in *P. borchgrevinki* following

Fig. 2. Effects of warm and hypercapnia acclimation on activities of citrate synthase (CS) and cytochrome *c* oxidase (COX) per mg tissue fresh mass (FW) in heart, liver, white muscle and red muscle of *Notothenia rossii*. White circles represent the control group (cold normocapnia: 1 °C, 0.04 kPa CO_2, $n = 6$), black circles the warm normocapnia (7 °C, 0.04 kPa CO_2, $n = 3$–4), white squares the cold hypercapnia (1 °C, 0.2 kPa CO_2, $n = 7$–8) and black squares the warm hypercapnia (7 °C, 0.2 kPa CO_2, $n = 6$–8) acclimated *N. rossii*. Enzyme activities are given as means ± SEM at 0, 6, 9 and 12 °C. Arrows depict a significant ($p \leq 0.05$) increase/decrease in enzyme activity of a given treatment compared to the control group at the respective assay temperature. * depicts a significant ($p \leq 0.05$) increase in enzyme activity within an acclimation group compared to the 0 °C assay.

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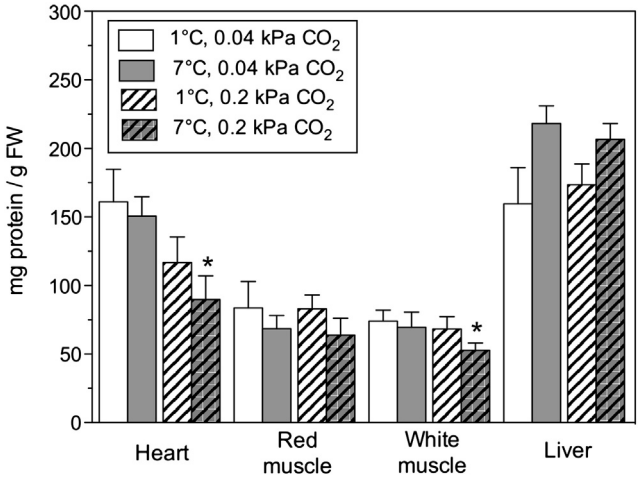

Fig. 4. Total protein content in heart, red muscle, white muscle and liver of control, warm and hypercapnia acclimated *Notothenia rossii*. White bars: control — 1 °C, 0.04 kPa CO_2, $n = 6$; grey bars: warm normocapnic — 7 °C, 0.04 kPa CO_2, $n = 3$–4; white hatched bars: cold hypercapnic — 1 °C, 0.2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

warm-acclimation (Seebacher et al., 2005), the uncompensated, high enzymatic activities observed in red muscle of *N. rossii* imply elevated metabolic rates but also costs in the warmth, which may challenge heart performance and liver metabolism.

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

4.2. Synergistic effects of warming and hypercapnia on enzyme activities

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

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

A high aerobic demand of red muscle was actually mirrored by enhanced enzyme capacities in warm and hypercapnia acclimated *N. rossii*: In both cold- and warm-hypercapnic groups, enzyme activities and COX/CS ratios were significantly higher compared to control- and warm-normocapnic *N. rossii* (Table 2 and Fig. 3). Thus, the findings in cold- and warm-hypercapnic fish were clearly an acclimation response triggered by 0.2 kPa CO_2, which caused a 2- to 2.5-fold increase in enzyme activity, independent of temperature. Furthermore, tissue protein content was not different between the control and both hypercapnia acclimated groups (Fig. 4). A change in mitochondrial structure or abundance (as shown for muscle of several temperate species, Egginton and Sidell, 1989; Johnston et al., 1998) seems therefore unlikely in red muscle of warm-normocapnia or -hypercapnia acclimated *N. rossii*. Instead, *N. rossii* appears to increase mitochondrial aerobic capacities in red muscle in response to chronic hypercapnia. This enhancement may be the result of a compensation for inhibitory effects by elevated bicarbonate (Simpson, 1967; Strobel et al., in press) or elevated costs for acid–base balance under elevated environmental CO_2 levels (Deigweiher et al., 2008), which consequently involves mobilisation of energy stores, such as liver fat or protein.

Indeed, the results of enzyme activities in liver tissue of cold- and warm-hypercapnic fish show decreased COX activities at unchanged CS activities. Such a picture indicates shifts in metabolic pathways in liver independent of temperature, e.g. shunting TCA-cycle intermediates away from the electron transport system towards gluconeogenesis to support other tissues, such as red muscle. Nevertheless, hypercapnia compensation in liver may be time-limited, as indicated by significantly reduced hepatosomatic indices in cold and warm-hypercapnia acclimated *N. rossii* already after five weeks acclimation time (Strobel et al., 2012). Thus it remains unclear whether a shift in metabolic pathways in response to warm- and hypercapnia acclimation is sufficient to support an elevated energy demand of other, highly oxidative tissues in the long run.

In white muscle, hypercapnia acclimation (cold/warm) had no effect on CS activity per mg protein, but led to reduced total thermal capacities in white muscle tissue, which corresponds to reduced CS activity in white muscle in the warm temperate seabream *S. aurata* exposed to 0.5 kPa CO_2 (Michaelidis et al., 2007). The authors postulate a shift from aerobic to anaerobic metabolism in hypercapnia acclimated *S. aurata*, which is unlikely for *N. rossii* due to a different preference of metabolic pathways in white muscle of these two species: in warm temperate *S. aurata*, glycolytic pathways dominate (Bone et al., 1978), while in Antarctic *N. rossii* ATP synthesis of white muscle strictly depends on aerobic mitochondrial energy metabolism (Walesby and Johnston, 1979) and thus possesses generally low anaerobic capacities. Furthermore, *S. aurata* is using the white trunk musculature for (anaerobic) locomotion, while notothenid fish mainly use labriform swimming with the red musculature of the pectoral fins (Walesby and Johnston, 1979). Thus, their white muscle is not used intensively and is therefore energetically not very demanding. In white muscle of *N. rossii*, COX activities per mg protein and consequently COX/CS ratio, were elevated in the warm-hypercapnic group, an effect that was not visible when enzyme activities were related to tissue weight. This difference occurred due to a significantly reduced amount of protein per g tissue after warm-hypercapnia acclimation (Fig. 4). Although white muscle plays a minor role in whole animal energy metabolism, the reduced CS activities and lower protein content after hypercapnia acclimation indicate a slight compensation or even catabolism of white muscle protein, possibly in response to an elevated energy demand under elevated seawater PCO_2.

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

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5. Conclusions

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

Red muscle, the most important muscle for locomotion in *N. rossii*, showed no temperature compensation but increased capacities following Q_{10}, which may be necessary to sustain swimming performance at warmer temperatures. The elevated energy demand resulting from the high enzymatic activities in heart and red muscle may be supported by using fatty acids or glucose from liver energy stores.

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

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

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

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

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

These compensating mechanisms involved may not be complete and entail a net depletion of energy stores, which in the long run may reduce the capacities of other energy-demanding processes such as reproduction or growth, as observed in reduced hepatosomatic indices in cold- and warm-hypercapnia acclimated *N. rossii*. This species will therefore only have a limited ability to compensate the effects of ocean acidification and warming.

6. Uncited references

Q8

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