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ORIGINAL PAPER

Impact of ocean acidification and warming on mitochondrial enzymes and membrane lipids in two Gadoid species

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Abstract

Mitochondrial respiration is a multi-step pathway that involves matrix and membrane-associated enzymes and plays a key role in acclimation to variable environmental conditions, but until now it has not been clear which of these steps would be most important in acclimation to changing temperatures and CO_2 levels. Considering scenarios of ocean warming and acidification we assessed the role and limitation to phenotypic plasticity in the hearts of two Gadoid species adapted to different thermal ranges: the polar cod (*Boreogadus saida*), an Arctic stenotherm, and the Northeast Arctic population of Atlantic cod (NEAC, *Gadus morhua*), a cold eurytherm. We analysed the capacity of single enzymes involved in mitochondrial respiration [citrate synthase (CS), succinate dehydrogenase (SDH), cytochrome *c* oxidase (CCO)], the capacity of the electron transport system and the lipid class composition of the cellular membranes. Juveniles of the two species were held for four months at four temperatures (0, 3, 6, 8 °C for polar cod and 3, 8, 12, 16 °C for NEAC), at both ambient and elevated P_{CO_2} (400 µatm and 1170 µatm, respectively). Polar cod showed no changes in mitochondrial enzyme capacities and in the relative lipid class composition in response to altered temperature or elevated P_{CO_2}. The lack of cardiac cellular plasticity together with evidence at the whole-animal level coming from other studies is indicative of little or no ability to overcome stenothermy, in particular during acclimation to 8 °C. In contrast, eurythermal NEAC exhibited modifications of membrane composition towards a more rigid structure and altered enzyme capacities to preserve functionality at higher temperatures. Furthermore, in NEAC, the capacities of SDH, CCO and CS were increased by high levels of CO_2 if combined with high temperatures (12 and 16 °C), suggesting the compensation of an inhibitory effect. These results indicate that the cold eurythermal species (NEAC) is able to alter its mitochondrial function to a far greater extent than the Arctic stenotherm (polar cod), indicating greater resilience to variable environmental conditions. This difference in plasticity may underpin differences in the resilience to climate change and affect future species distributions and, eventually, survival.

Keywords Atlantic cod · Climate change · Lipid class · Metabolism · Mitochondria · Polar cod · Mitochondrial enzyme

Abbreviations

NEAC	Northeast Arctic population of Atlantic cod
RCP	Representative concentration pathway
CS	Citrate synthase
CCO	Cytochrome *c* oxidase
SDH	Succinate dehydrogenase
CI:CIII	NADH:cytochrome *c* oxidoreductase
Chol	Cholesterol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
TCA cycle	Tricarboxylic acid cycle

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Introduction

Temperature has a defining role in the life of ectotherms, especially those living in an aquatic environment given their much-reduced ability to alter body temperatures through behavioural means. In some cases, species have developed mechanisms to compensate for temperature effects, notably visible in cold water fish which show partial compensation for the suppressing effect of cold temperatures on metabolic rates, for example through enhanced enzyme activities and/or adjustments to the density and capacity of mitochondria (Torres and Somero 1988; Crockett and Sidell 1990; Lannig et al. 2003). However, these adjustments are suggested to be different between cold stenothermal and cold eurythermal fish (Pörtner et al. 2000; Pörtner 2002). While cold stenotherms tend to have high mitochondrial densities but low-mitochondrial capacity in aerobic tissues (Pörtner et al. 2000, 2008), eurythermal cold adaptation rather involves maximized mitochondrial capacities (Pörtner et al. 2008; Blier et al. 2013). This reveals the presence of alternative strategies to cope with an extreme conditions depending on the level of environmental variability. The on-going increase in oceanic temperature, coupled with elevated P_{CO_2} (causing ocean acidification), raises the question of how cold stenothermal and cold eurythermal fish will respond to the future climate, given their different physiologies.

In a previous study, we analysed the potential for mitochondrial acclimation in permeabilized heart fibres of two Gadoid species living in the waters off the Svalbard archipelago (Leo et al. 2017). We chose polar cod (*Boreogadus saida*) as a cold (Arctic) stenotherm (thermal range around Svalbard − 2 to + 7 °C) (Laurel et al. 2016; Mark and Rohardt 2016) and the Northeast Arctic population of Atlantic cod (NEAC, *Gadus morhua*) as a cold eurytherm (experiencing temperatures ranging from − 1.5 °C in winter in the Barents Sea up to 10 °C when moving along the Norwegian coast at spawning time) (Righton et al. 2010; Michalsen et al. 2014). The fish heart is a key organ since it is highly responsive to temperature change (Farrell 2007; Ekström et al. 2016). Most of the energy demand of the fish heart is supplied by mitochondrial metabolism. The increased requirement for energy at higher temperatures is mirrored by shifts in the activity of individual enzymes or even between metabolic pathways (Lucassen et al. 2006; Melzner et al. 2009; Windisch et al. 2011; Strobel et al. 2013a, b). We also found that mitochondria of polar cod subjected to relatively high temperatures (8 °C) displayed increased proton leak, causing decreasing mitochondrial efficiency, and a decreased capacity of cytochrome *c* oxidase (CCO); they did not respond to changes in P_{CO_2}. In contrast, mitochondrial function and efficiency of NEAC was preserved at elevated temperatures (16 °C), but overall mitochondrial capacity was depressed by a combination of high temperature and high P_{CO_2} (Leo et al. 2017).

Here, we focus on the cellular and biochemical causes of these differences in mitochondrial response. Mitochondrial respiration is a complex multi-step process that involves several enzymes, some of them embedded in the mitochondrial membranes. Thus, the study of the capacity of specific enzymes involved in mitochondrial metabolism and the analysis of the composition of the membrane in which they are embedded can help us to understand and identify the causes of the difference in acclimation potential of the two species. Many studies have focused on the effect of high temperature on mitochondrial enzyme capacities. For example, warm-acclimated fish of a number of species displayed suppressed citrate synthase (CS) activity while CCO activity remained more or less unchanged (Hardewig et al. 1999; Lannig et al. 2003; Lucassen et al. 2006; Windisch et al. 2011). Few studies have addressed the effect of elevated P_{CO_2} on mitochondrial enzymes: Michaelidis et al. (2007) reported a decrease in CS activity in the heart of the temperate sea bream (*Sparus aurata*) incubated at high P_{CO_2} and unchanged temperature. Strobel et al. (2013a) found decreased activities of both CS and CCO in the heart of Antarctic *Notothenia rossii* acclimated to high temperature and elevated P_{CO_2}. It is unclear whether and how these changes in mitochondrial enzyme activity may be paralleled by a change in mitochondrial membrane structure. Long-term exposure to high temperatures alters the structure of cellular membranes, with more rigid membranes in warm-adapted or warm-acclimated individuals to counteract the destabilizing effects of elevated temperature (Dahlhoff and Somero 1993; Kraffe et al. 2007). The remodelling of membrane lipids owing to increasing temperature involves changes in the head group composition (e.g. choline lowers the membrane fluidity and ethanolamine increases it), length and saturation of the acyl chains and where present, changes in cholesterol (Chol) content (Hazel 1995; Kraffe et al. 2007; Hoffmann and Todgham 2010). However, little is known about the effects of elevated P_{CO_2} on the lipid composition of cellular membranes (Strobel et al. 2013b).

In this study, we analysed the combined effects of temperature and ocean acidification on the capacity of key enzymes involved in mitochondrial metabolism: CS (located in the matrix) and succinate dehydrogenase (SDH, membrane-bound complex II), being enzymes of the tricarboxylic acid cycle (TCA) cycle, and CCO and NADH:cytochrome *c* oxidoreductase (CI:CIII) as indicators of the capacity of the electron transport system which are all membrane-associated complexes. We also quantified the lipid composition of the pool of cellular membranes. These mitochondrial traits are measured in the hearts of polar cod and NEAC held

for 4 months at either their present ambient or at elevated temperatures (0, 3, 6 or 8 °C and 3, 8, 12 or 16 °C according to the species), and either at current P_{CO_2} levels (400 µatm) or at those projected for the year 2100 in the Arctic (1170 µatm; representative concentration pathway, RCP 8.5, Van Vuuren et al. 2011). These measurements allow us to evaluate how adjustments in mitochondrial enzyme capacities and in membrane lipids contribute to the acclimation potential of the two species and when put in relation with higher organismic levels they can help to understand the differences in thermal plasticity.

Materials and methods

Animal collection and incubation

Animal collection and incubation are described in detail in Kunz et al. (2016) and Leo et al. (2017). Briefly, juvenile polar cod were caught by the University of Tromsø (NO) in January 2013 from the inner part of Kongsfjorden (Svalbard, NO). Afterwards, they were kept at 3.3–3.8 °C and 32 PSU in the facilities of the Havbruksstasjonen i Tromsø AS (HiT) in Kårvik (NO) until late April 2013 when they were transported to the Alfred Wegener Institute's (AWI) facilities in Bremerhaven (Germany) and held at 5 °C and 32 PSU. Juvenile NEAC were caught during the RV Heincke cruise HE408 in August 2013 from various locations of the Svalbard Archipelago and transported to the AWI facilities in Bremerhaven, where they were kept at 5 °C and 32 PSU until the beginning of the incubation.

The incubations of polar cod and NEAC started in June 2013 and in May 2014, respectively. Fish from both species were kept for at least 4 weeks at 5 °C and 32 PSU to acclimate to laboratory conditions, then they were placed in single tanks and randomly allocated to temperature and P_{CO_2} treatment groups ($n = 12$ for each group). Individual polar cod were held at water temperatures of 0, 3, 6 or 8 °C, while NEAC were held at 3, 8, 12 or 16 °C, with each temperature treatment being combined with a P_{CO_2} of 400 µatm (control P_{CO_2}) or 1170 µatm (high P_{CO_2}) in a 4×2 cross design for both species. In all cases, fish were held in temperature-controlled rooms on a 12 h:12 h light/dark cycle. The seawater temperature, salinity, DIC and pH (total scale) were measured once to twice a week in triplicates throughout the incubation period; these parameters are reported in the Open Access Library PANGAEA (Schmidt et al. 2016). The animals were held under their treatment conditions for 4 months and were fed ad libitum with commercial pellet feed (Amber Neptun, 5 mm, Skretting AS, Norway) every fourth day.

Sampling

Sampling took place after four days of fasting at the end of the 4-month treatment period. Half of the fish present at the end of the incubation time were used in the study by Leo et al. (2017), the second half was used in this study. Fish were anaesthetized with 0.2 g L^{-1} tricaine methane sulphonate (MS222) and killed by a spinal cut behind the head plate. Hearts were rapidly excised and frozen in liquid nitrogen. Mean length, weight and number of the specimens used this study are given in Table 1.

Enzymatic activity

Frozen heart samples were homogenized in 9 volumes (w:v) of ice cold extraction buffer (Tris–HCl 20 mM, Na–EDTA 1 mM, Triton X-100 0.1%, pH 7.4 at 4 °C) with a tissue

Table 1 Total length, body weight and sample size (*n*) of polar cod (*Boreogadus saida*) and NEAC (*Gadus morhua*)

Treatment	Polar cod			NEAC		
	Total length (cm)	Body weight (g)	*n*	Total length (cm)	Body weight (g)	*n*
0 °C control	15.30 ± 0.96	26.25 ± 4.51	6	–	–	–
0 °C high	15.62 ± 0.48	24.28 ± 2.97	6	–	–	–
3 °C control	16.20 ± 0.29	28.33 ± 1.41	6	18.67 ± 2.02	52.55 ± 21.85	3
3 °C high	16.20 ± 0.29	29.08 ± 1.88	6	21.67 ± 0.95	79.49 ± 10.66	6
6 °C control	16.43 ± 0.20	26.97 ± 1.50	6	–	–	–
6 °C high	16.30 ± 0.65	27.24 ± 3.36	5	–	–	–
8 °C control	16.24 ± 0.38	25.60 ± 1.80	5	23.37 ± 2.50	114.68 ± 36.84	3
8 °C high	17.12 ± 1.52	29.08 ± 6.71	4	22.77 ± 1.31	98.15 ± 16.76	4
12 °C control	–	–	–	24.55 ± 0.55	128.23 ± 1.22	6
12 °C high	–	–	–	24.36 ± 1.36	113.89 ± 22.09	5
16 °C control	–	–	–	22.56 ± 0.54	95.67 ± 5.57	3
16 °C high	–	–	–	27.27 ± 1.98	180.35 ± 45.21	3

"Control" and "high" indicate control (400 µatm) and high (1170 µatm) P_{CO_2}. Values are given as means ± SEM

homogenizer (Precellys 24, Bertin Technologies, France) at 5000 rpm and 4 °C, 3 times for 15 s. The homogenate was then centrifuged at 1000 g and 4 °C for 10 min. The supernatant was used for analysis of enzymatic activities. Enzyme activities of each sample were measured at all four treatment temperatures for the species using a UV/Vis spectrophotometer (Specord S600, Analytik Jena AG, Germany) equipped with a thermostatted cell holder. The buffer pH was set to be constant at the acclimation temperatures to simulate the acclimation and to change according to temperature variation during the acute test. CS activity was detected in a buffer containing Tris–HCl 75 mM pH 8.0, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) 0.25 mM, acetyl-CoA 0.4 mM, oxaloacetate 0.5 mM. The activity was determined from the increase in absorbance at $\lambda = 412$ nm, due to the transfer of sulfhydryl groups from coenzyme A to DTNB, using the extinction coefficient $\varepsilon_{412} = 13.61$ mol^{-1} cm^{-1} (Sidell et al. 1987).

CCO activity was detected in a buffer containing Tris–HCl 20 mM pH 7.8, cytochrome c_{red} 0.057 mM, Tween 20 0.5%. The activity was determined from the decrease in extinction at $\lambda = 550$ nm due to the oxidation of cytochrome c, using the extinction coefficient $\varepsilon_{550} = 19.1$ mol^{-1} cm^{-1} (modified after Moyes et al. 1997).

SDH activity was detected in a buffer containing imidazole/HCl buffer 100 mM pH 8.0, $MgCl_2$ 5 mM, succinate 20 mM, sodium azide 4 mM, antimycin A 0.04 mM, rotenone 0.005 mM, 2,6-dichlorphenolindophenol (DCPIP) 0.1 mM, ubiquinone Q_1 0.1 mM. The activity was determined from the decrease in extinction at $\lambda = 600$ nm due to the reduction of DCPIP, using the extinction coefficient $\varepsilon_{600} = 19.2$ mol^{-1} cm^{-1} (modified after Lemieux et al. 2010).

CI:CIII was determined in a buffer containing imidazole/HCl buffer 25 mM pH 7.4, $MgCl_2$ 2 mM, sodium azide 4 mM, cytochrome c_{ox} 0.08 mM, NADH 0.2 mM. The activity was determined from the increase in extinction at $\lambda = 550$ nm due to the reduction of cytochrome c, using the extinction coefficient $\varepsilon_{550} = 19.1$ mol^{-1} cm^{-1} (modified after Möller and Palmer 1982). All chemicals were obtained by Sigma-Aldrich (Germany).

Lipid class determination

The lipid class composition of heart cellular membranes was determined for the polar cod and NEAC from the lowest and highest temperature treatments (0 vs. 8 °C and 3 vs. 16 °C, respectively) and both P_{CO_2} treatments. Membrane lipids were extracted after Folch et al. (1957). The frozen hearts were homogenized in a glass homogenizer containing 6 mL dichloromethane:methanol (v 2:1) for three times at 1200 U min^{-1} for 2 min. The extract was then diluted with 2 mL of 0.88% KCl and centrifuged for 5 min at 1000 rpm. The lipid phase was carefully separated and the aqueous phase was diluted again with 2 mL dichloromethane and centrifuged three times for five minutes at 1000 rpm to completely separate the lipid from the aqueous phase. After complete evaporation of the lipid phase with nitrogen, the raw extract was suspended in 50 µL chloroform. The lipid classes were separated and identified according to Graeve and Janssen (2009) on a monolithic silica column (ChromolithPerformance-Si) using high-performance liquid chromatography (HPLC, LaChromElite HPLC system) with an evaporative light-scattering detector (ELSD). A gradient program with combination of three solvent mixtures was used: eluent A consisted of isooctane:ethylacetate (99.8:0.2, v/v), eluent B was a mixture of acetone and ethyl acetate [2:1, v/v) containing acetic acid (0.02% (v/v)] and eluent C 2-propanol–water (85:15, v/v) [with acetic acid and ethanolamine, each 0.05% (v/v)].

Data analysis

Values are given as mean ± SEM if not stated otherwise. Data analysis was performed using R 3.2.0 (R Core Team 2015), with the level of statistical significance for all statistical tests set at $p < 0.05$.

Enzymatic activity

Enzyme activities were calculated per mg fresh-weight (U mg FW^{-1}) of heart tissue. The normality and the homoscedasticity of the data were tested by Shapiro–Wilk test and Bartlett's test, respectively. The differences in enzymatic activities were evaluated by two-way ANOVA followed by Tukey-HSD post hoc test. The temperature coefficients Q_{10} of the enzyme activities were calculated according to the equation:

$$Q_{10} = \left(R_2 \times R_1^{-1}\right)^{\left(10\times(T_2-T_1)^{-1}\right)}$$

where R is the enzyme capacity and T is the temperature in "°C" at which the enzyme capacities were measured. Q_{10} was calculated for the enzymes CS and CCO for the following temperature ranges: 0–6 °C and 6–8 °C for polar cod and 3–12 °C and 12–16 °C for NEAC.

The CCO:CS ratio was calculated for both species.

Lipid class composition

After the assessment of normality (Shapiro–Wilk test) and homoscedasticity (F test), the differences in lipid class compositions between CO_2 treatments were tested by Student's t test with Welch correction in the case of non-homogeneous variances. Since there was no significant effect of CO_2 treatment, the data from the same temperature groups were pooled to examine effects of temperature

alone. The Chol, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) contents were calculated as percentages of total lipid content (polar and neutral lipids). In addition, the PE:PC ratio was calculated. The differences between temperatures in the lipid class compositions were tested using Student's *t* test with the Welch correction if needed.

Results

Enzymatic capacity

The capacity of the four enzymes analysed in this study (CS, CCO, SDH, CI:CIII) is illustrated in Fig. 1 for polar cod and Fig. 2 for NEAC. Q_{10} values are presented in Table S1 of the Supplementary Material.

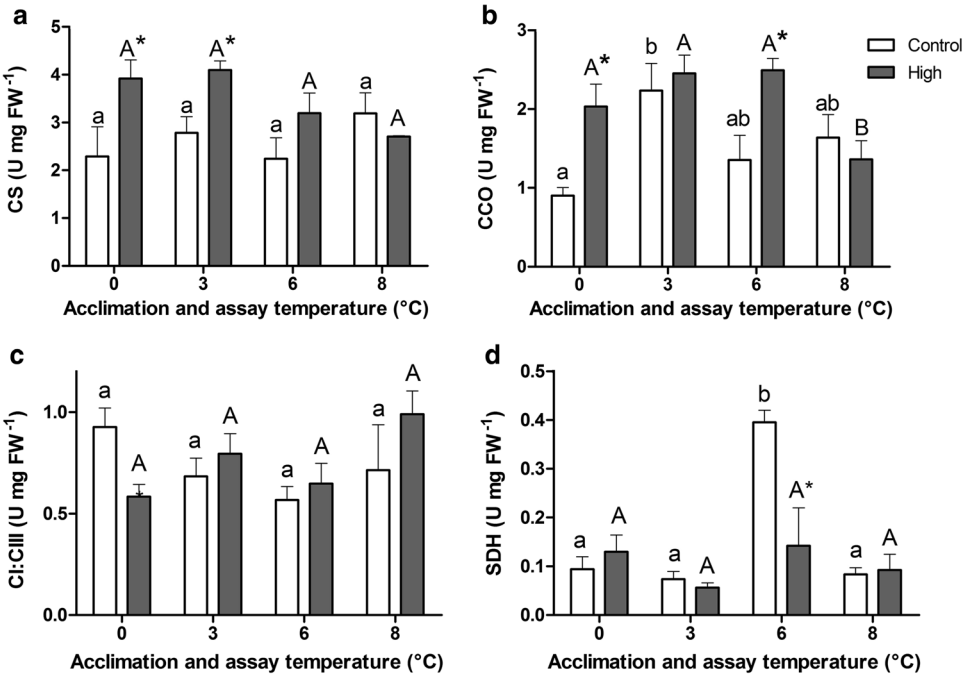

Fig. 1 Enzymatic capacities of heart tissue of polar cod (*Boreogadus saida*) acclimated and measured at the respective acclimation temperature. **a** Citrate synthase (CS), **b** cytochrome *c* oxidase (CCO), **c** NADH:cytochrome *c* oxidoreductase (CI:CIII), and **d** succinate dehydrogenase (SDH). Within panels: *indicates the difference between CO_2 treatments at the same incubation temperature. Lower case letters indicate significant differences across temperature at control P_{CO_2}, upper case letters indicate the difference across temperature treatments at high P_{CO_2}. White bars: control P_{CO_2} (400 µatm), grey bars: high P_{CO_2} (1170 µatm). Values are given as means ± SEM

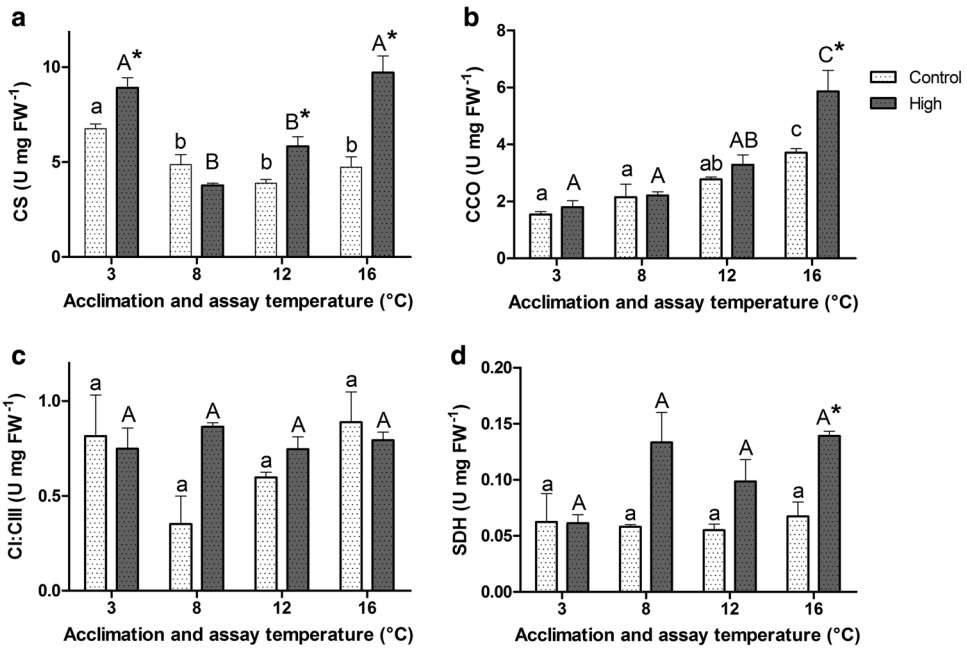

Fig. 2 Capacities of mitochondrial enzymes from heart tissue of NEAC (*Gadus morhua*) measured at the respective acclimation temperatures. **a** Citrate synthase (CS), **b** cytochrome *c* oxidase (CCO), **c** NADH:cytochrome *c* oxidoreductase (CI:CIII), and **d** succinate dehydrogenase (SDH). Values are presented as means ± SEM. In the panel: *indicates the difference between CO_2 treatments at the same incubation temperature. Lower case letters indicate differences between temperature treatments at control P_{CO_2}, upper case letters indicate differences between temperature treatments at high P_{CO_2}. White bars: control P_{CO_2} (400 µatm), grey bars: high P_{CO_2} (1170 µatm)

Polar cod (Fig. 1)

In polar cod CS capacity was unaffected by temperature ($F=1.24$, $p>0.05$), but in the cold temperature treatments (0 and 3 °C) CS capacity was higher in the groups incubated at high P_{CO_2} compared to the control groups (Fig. 1a: 0 °C: $p=0.0471$; 3 °C: $p=0.0064$). The Q_{10} values for CS (Table S1) ranged from 0.65 ± 0.25 to 1.91 ± 0.40 for the 0–6 °C range and from 0.25 ± 0.06 to 1.08 ± 0.42 for the 6–8 °C range, and so were significantly higher when measured over the lower temperature range ($p=0.0009$). The temperature treatment also had an effect on Q_{10} ($p=0.0018$), with lowered values with increasing acclimation temperature.

CCO capacity was sensitive to both temperature and CO_2 treatments (temperature: $F_{3,32}=4.38$, $p=0.0108$; CO_2: $F_{1,32}=8.82$, $p=0.0056$), with thermal sensitivity being influenced by the CO_2 treatment (temperature × CO_2 interaction: $F_{3,32}=3.25$, $p=0.0345$; Fig. 1b). In fact, while CCO capacity followed a bell-shaped thermal reaction norm in the control P_{CO_2} groups with a maximum at 3 °C, CCO capacity in the high P_{CO_2} groups was high and similar across all three lower test temperatures but decreased at 8 °C. CCO Q_{10} values did not vary between the tested temperature ranges ($p>0.05$), being between 0.27 ± 0.03 and 2.03 ± 0.72 in the 0–6 °C range and between 0.16 ± 0.04 and 1.90 ± 0.54 in the 6–8 °C range. Both treatment temperature and CO_2 had an effect on Q_{10} (temperature: $p=0.0144$; CO_2: $p=0.0049$), with Q_{10} values generally lower in the high P_{CO_2} groups. The CCO:CS ratio was not affected by CO_2 treatment ($F_{1,33}=0.158$, $p>0.05$) but showed temperature sensitivity ($F_{3,33}=3.487$, $p=0.0265$), with higher values at intermediate temperatures (Fig. 3a).

CI:CIII capacity exhibited no differences between treatments but an interaction between the two drivers (temperature: $F_{3,33}=2.24$, $p>0.05$; CO_2: $F_{1,33}=0.24$, $p>0.05$; temperature × CO_2 interaction: $F_{1,33}=3.98$, $p=0.0158$; Fig. 1c). SDH capacity was not significantly affected by CO_2 treatment ($F_{1,33}=3.29$; $p>0.05$) but was influenced by temperature, being highest at the intermediate (6 °C) treatment (Fig. 1d).

NEAC (Fig. 2)

In NEAC, acclimation to high P_{CO_2} increased CS capacity ($F_{1,16}=30.66$, $p<0.0001$) compared to the control P_{CO_2} at all temperatures except at 8 °C ($p>0.05$). Acclimation temperature also affected CS capacity ($F_{3,16}=23.68$, $p<0.0001$), with the lowest capacities at intermediate temperatures (Fig. 2a). Q_{10} values for CS activity ranged between 0.47 ± 0.04 and 1.32 ± 0.17 for the 3–12 °C temperature range and between 0.06 ± 0.02 and 4.42 ± 0.14 for the 12–16 °C temperature range (Table S1). The values varied according to temperature range ($p=0.0409$) and to acclimation temperature ($p<0.0001$), but with an interaction between these two factors ($p<0.0001$). Q_{10} values were thus highest at temperatures farthest away from the acclimation temperature. For instance, for the groups acclimated at 3 °C the Q_{10} values were higher in the 12–16 °C range (control CO_2: $p<0.0001$; high CO_2: $p=0.0408$), while in the groups acclimated at 12 and 16 °C the Q_{10} values were higher in the 3–12 °C range (12 °C/control CO_2: $p<0.0001$; 12 °C/high CO_2: $p=0.0230$; 16 °C/control CO_2: $p=0.0434$; 16 °C/high CO_2: $p>0.05$).

CCO capacity increased with rising acclimation temperatures ($F_{3,20}=31.64$, $p<0.0001$; Fig. 2b). CO_2 acclimation decreased the activity of CCO ($F_{1,20}=8.402$, $p=0.0089$), but this effect was primarily driven by differences at the highest temperatures (temperature × CO_2 interaction: $F_{3,20}=3.62$, $p=0.0310$). Q_{10} values were statistically similar between temperature ranges ($p>0.05$), temperature treatments ($p>0.05$) and CO_2 treatment ($p>0.05$; Table S1).

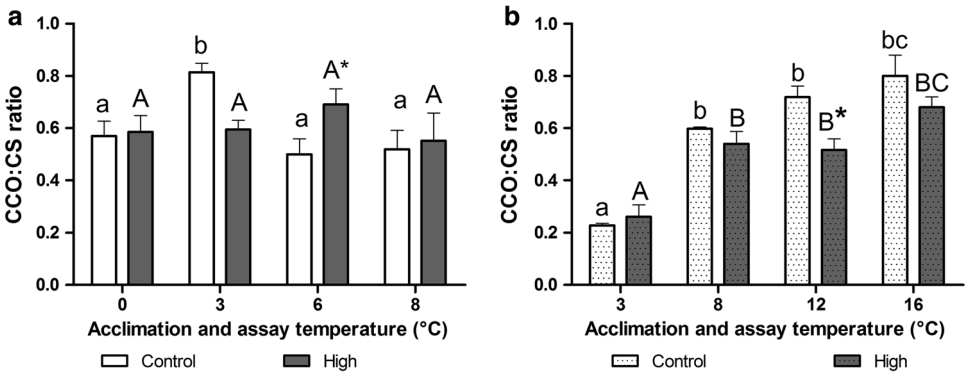

Fig. 3 CCO:CS ratios in polar cod (*Boreogadus saida*, panel **a**) and NEAC (*Gadus morhua*, panel **b**) heart tissue measured at acclimation temperature. Within panels: lower case letters indicate differences between temperatures at control P_{CO_2}, upper case letters indicate differences between temperatures at high P_{CO_2}, *indicates differences between CO_2 treatments at the same temperature. Values are reported as means ± SEM. White bars: control P_{CO_2} (400 µatm), grey bars: high P_{CO_2} (1170 µatm)

They ranged between 1.01 ± 0.22 and 1.96 ± 0.17 for the 3–12 °C range and between 0.51 ± 0.14 and 1.92 ± 0.84 for the 12–16 °C range. The CCO:CS ratio was sensitive to both temperature and CO_2 (temperature: $F_{3,16} = 35.10$, $p < 0.0001$; CO_2: $F_{1,16} = 4.518$, $p = 0.0456$), increasing with temperature but at each temperature tending to be lower for the high P_{CO_2} groups (Fig. 3b).

As with polar cod, CI:CIII in NEAC did not vary with treatment ($p > 0.05$; Fig. 2c). SDH capacity was unaffected by temperature treatment ($F_{3,21} = 2.22$, $p > 0.05$) but was higher in the fish held under high P_{CO_2} (3 °C: $p > 0.05$, 8 °C: $p = 0.0501$, 12 °C: $p = 0.0629$, 16 °C: $p = 0.0064$; Fig. 2d).

Comparison between species

NEAC had greater CS capacities than did polar cod at 3 °C (control CO_2: $p < 0.0001$; high CO_2: $p = 0.0355$), but this difference became smaller at 8 °C (control CO_2: $p = 0.0500$; high CO_2: $p < 0.0001$) due to a reduction in the CS capacity of NEAC. However, the two species did not differ in activities for the other mitochondrial enzymes (CCO, CI:CIII and SDH) at either of the two common test temperatures ($p > 0.05$).

Lipid classes

The Chol, PE and PC composition and the PE:PC ratio are shown in Table 2 for both polar cod and NEAC. In polar cod, none of the lipid classes changed in relative abundance with rising incubation temperatures (Chol: $p > 0.05$, PE: $p > 0.05$, PC: $p > 0.05$, PE:PC: $p > 0.05$). In NEAC, the percentage of Chol and PE did not change with temperature (Chol: $p > 0.05$, PE: $p > 0.05$), however, the PC content tended to be higher in the 16 °C group compared to the 3 °C group, although not significantly ($p = 0.0626$).

Discussion

This study highlights the effects of current levels and future scenarios of ocean acidification and warming on the activity levels and capacity of key mitochondrial enzymes, and on the lipid composition of cellular membranes. We studied an Arctic stenotherm (polar cod) and a cold eurytherm (the NEAC) currently living in the same region (the water off the Svalbard Archipelago) but both being at the limit of their thermal range (upper limit for polar cod, lower limit for NEAC). Unlike earlier studies on the effects of temperature on mitochondrial enzymes of cold-adapted fish (Lucassen et al. 2003, Lucassen et al. 2006; Strobel et al. 2013a, b), which analysed only CS and CCO capacity, we chose four enzymes (CS, CCO, SDH and CI:CIII) to broaden our view on the mitochondrial respiration processes. Moreover, while many previous studies used limited acclimation time or unrealistically high P_{CO_2} levels (e.g. Lucassen et al. 2003, Lucassen et al. 2006; Michaelidis et al. 2007), our study includes long-term acclimation (4 months) as well as temperature and P_{CO_2} values projected for the year 2100 (RCP 8.5, Van Vuuren et al. 2011).

In general, the data reflect the importance of looking at the capacity of a more complete set of enzymes involved in mitochondrial metabolism, as they varied in their responses to temperature and P_{CO_2}. Among those measured, CS and SDH were the most sensitive to CO_2, while CCO was mainly affected by temperature (as well as the lipid layer in which it is embedded) and CI:CIII showed little acclimation to either temperature or P_{CO_2}. Furthermore, data will be discussed in the light of the previous results obtained from the same acclimation project by Kunz et al. (2016) and Leo et al. (2017). The enzymes analysed in this study were selected to be the same or to be directly linked with the features examined in the above-mentioned studies.

Table 2 Cholesterol (Chol), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) content of the cellular membranes in the heart from polar cod (*Boreogadus saida*) and NEAC (*Gadus morhua*)

Incubation temperature (°C)	Polar cod				NEAC			
	Lipid class				Lipid class			
	Chol	PE	PC	PE:PC	Chol	PE	PC	PE:PC
0	14.21 ± 1.24	25.02 ± 0.85	58.00 ± 0.65	0.47 ± 0.03	–	–	–	–
3	–	–	–	–	13.48 ± 0.69	22.87 ± 2.19	46.81 ± 1.38	0.50 ± 0.05
8	14.10 ± 0.76	23.02 ± 1.14	56.42 ± 2.15	0.41 ± 0.03	–	–	–	–
16	–	–	–	–	13.83 ± 0.40	18.83 ± 1.74	52.85 ± 2.72*	0.36 ± 0.04*

Each lipid class content is reported as percentage of the total amount of lipids. Values are given as means ± SEM (polar cod $n = 8$, NEAC $n = 6$, CO_2 treatments of the same temperature were pooled because there was no CO_2 effect)

*Trends ($0.1 > p > 0.05$) between incubation temperatures for the same species

Enzymatic capacity

Polar cod exhibited very little modification of enzyme capacities after being held for several months under elevated temperatures and high P_{CO_2}. CS capacity did not change between polar cod held at different temperatures, although the Q_{10} decreased upon warming, indicating a slight warm acclimation. CS capacity was higher in fish held under elevated P_{CO_2} but the Q_{10} values were similar to the control P_{CO_2} groups suggesting that incubation under high levels of CO_2 does not affect the acute thermal tolerance of this enzyme. CCO showed different thermal trends in response to the P_{CO_2} incubation levels (Fig. 1b; Table 3), indicating an interaction between these two drivers. Under control levels of P_{CO_2} the thermal reaction norm highlights 3 °C as the optimum temperature for this enzyme, whereas in fish held at high P_{CO_2} levels the marked decrease at 8 °C paralleled by a very low Q_{10} for this group may suggest the onset of thermal constraints.

In NEAC, the CS capacity of the control P_{CO_2} groups follows the thermal trajectory well described for cold eurythermal fish, including other studies on NEAC (Lannig et al. 2003; Lucassen et al. 2003, 2006; Khan et al. 2014), with decreased capacity at warmer temperatures; the Q_{10} values indicate warm acclimation through downregulation in the 12 and 16 °C groups. However, the increased capacity upon CO_2 acclimation suggests compensatory upregulation.

Unlike in polar cod, the CCO capacity of NEAC increased with increasing acclimation temperature, with each acclimation group showing similar Q_{10} values, indicating that NEAC is able to modulate the capacity of this enzyme, following an increased respiratory demand. An increased CCO capacity and decreased CS capacity during warm acclimation in cold-adapted fish has previously been reported (Windisch et al. 2011). Windisch et al. (2011) suggested that the alteration of the CCO:CS ratio was due to changes in respiratory capacity (through CCO) as well as a change in the multiple functions of CS, especially a reduction in the provision of intermediates from the TCA cycle, e.g. to fatty acids and amino acid anabolism. In our study, the CCO:CS ratio showed little modification in polar cod but increased with temperature in NEAC, highlighting the greater potential for warm acclimation in NEAC compared to polar cod.

The differences in enzymatic capacities of polar cod and NEAC could be explained by different fuel preferences in their heart tissues. Cold-adapted fish show a preference towards fatty acids metabolism rather than carbohydrates (Guderley and Gawlicka 1992; Rodnick and Sidell 1994; Driedzic et al. 1996). The β-oxidation of fatty acids produces acetyl-CoA for the TCA cycle as well as $FADH_2$ and $NADH + H^+$, which feed directly into the Electron Transport System. This could explain why polar cod showed lower capacity of CS (a key enzyme of the TCA cycle) at 3 and 8 °C compared to NEAC despite similar capacities of CI:CIII and CCO (directly fed by $FADH_2$ and $NADH + H^+$).

The suggested preference of polar cod heart for catabolizing fatty acids and the subsequent excess of CCO and CI:CIII enzymes compared to CS could explain the relative thermostability in mitochondrial function for this species. However, combining this study with earlier studies examining other aspects of the same acclimation experiment (Kunz et al. 2016; Leo et al. 2017; summarized in Table 3) suggests an inability to increase enzymatic capacity and/or switching to other metabolic pathways at 8 °C. At this temperature the mitochondrial proton leak (LEAK) increased exponentially but this was not paralleled by an increase in oxidative phosphorylation capacity (OXPHOS, Table 3). At the whole-animal level, the 8 °C groups were characterized by increased SMR (Table 3) and increased mortality (Kunz et al. 2016), indicating that the metabolic restrictions evident at the mitochondrial level at 8 °C could be translated into long-term constraints on whole-animal performance. A caveat might still be that the common source of data may lead to interpretations that are limited by the lack of independence between the features analysed in this study.

The performance of NEAC was affected more by the combination of temperature and high P_{CO_2}. In the groups incubated at 16 °C, CS, CCO and SDH had a higher capacity in the fish incubated under high P_{CO_2}. However, we previously showed that the OXPHOS of permeabilised heart tissue from the animals incubated under these conditions was lower than in the control P_{CO_2} group (Leo et al. 2017; Table 3). Exposure to elevated levels of CO_2 in the water can cause an increase in intracellular bicarbonate in fish (Brauner et al. 2004; Michaelidis et al. 2007; Strobel et al. 2012, 2013b). High bicarbonate levels can competitively inhibit citrate oxidation in mammalian kidney mitochondria, with a resulting increase in intracellular citrate concentration (Simpson 1967) that can inhibit CS by feedback mechanisms, thus reducing the activity of the whole TCA cycle. An increase in the capacity of CS, like the one seen in this study on NEAC, may compensate for the initial inhibition by bicarbonate. In a previous study, Strobel et al. (2013b) found a decreased flux through SDH under high P_{CO_2} levels in the Antarctic fish *N. rossii*, suggesting an inhibition by elevated CO_2 (Wanders et al. 1983). To overcome this inhibition (seen in respiring mitochondria), the enzymatic capacity of SDH may be enhanced, e.g. by increasing the enzyme abundance, and this enhancement will result in an increased capacity of the enzyme in vitro as seen in the present study.

Lipid classes

The relative composition of lipid classes in cellular membranes differed only in relation to species and incubation temperature but not in relation to P_{CO_2}. While in polar cod there were no

Table 3 Summary table of the acclimation features of polar cod (*Boreogadus saida*) and Northeast Arctic population of Atlantic cod (NEAC, *Gadus morhua*)

Level	Function	Trend		Data
		Polar cod	NEAC	
Whole-animal	Growth	Stable across temperatures and P_{CO_2}	Increases with temperature. No CO_2 effects	Kunz et al. (2016)
	SMR	Stable until 6 °C with a dramatic increase between 6 and 8 °C. No CO_2 effects	Increases with temperature. No CO_2 effects	Kunz et al. (2016)
Permeabilized heart fibres	LEAK	Stable until 6 °C with a dramatic increase between 6 and 8 °C. No CO_2 effects	Increases between 3 and 8 °C, stabilises until 12 °C and increases at 16 °C. No CO_2 effects	Leo et al. (2017)
	OXPHOS	Increases between 0 and 3 °C, than stable. No CO_2 effects	*Increases with increasing temperature. At 16 °C the control P_{CO_2} group reaches the plateau while the high P_{CO_2} group increases further*	Leo et al. (2017)
	CCO	Increases between 0 and 3 °C, than stable. No CO_2 effects	*Increases between 3 and 8 °C, at 8 °C the control P_{CO_2} group reaches the plateau while the high P_{CO_2} group further increases at 16 °C*	Leo et al. (2017)
Heart mitochondrial enzymes	CCO	*The capacity in the control P_{CO_2} groups increases from 0 to 3 °C and decreases afterwards. In the high P_{CO_2} groups the capacity increases between 0 and 3 °C, stays high at 6 °C and decreases at 8 °C*	*Increases with increasing temperatures. At 16 °C the capacity of the high P_{CO_2} group is higher than in the control P_{CO_2} group*	This study
	CS	Stable across temperatures and P_{CO_2}	Capacity higher at the temperature extremes and higher in the high P_{CO_2} groups	This study
	SDH	Stable across temperatures and CO_2. Only the group at 6 °C and control P_{CO_2} has higher capacity than the other groups	Stable across temperatures but higher in the high P_{CO_2} groups	This study
	CI:CIII	Stable across temperatures and P_{CO_2}	Stable across temperatures and P_{CO_2}	This study

Data on temperature and P_{CO_2} acclimation presented here are combined with datasets from the same study on the whole-animal level (Kunz et al. 2016) and from permeabilized heart fibres (Leo et al. 2017). Acclimation temperatures for polar cod were 0–3–6–8 °C, for NEAC 3–8–12–16 °C. Control P_{CO_2} was set at 400 µatm and high P_{CO_2} was set at 1170 µatm for both species. Features for which the two P_{CO_2} groups show different trends are highlighted in italics

changes in the relative composition of the lipid classes, in NEAC there were signs of modulation of the membrane composition that may maintain membrane fluidity and properties at high temperatures. This modulation, termed homeoviscous adaptation (Hazel 1995), is mainly present in eurythermal fish (Cossins and Bowler 1987; Hazel 1995; Kraffe and et al. 2007; Grim et al. 2010; Hoffmann and Todgham 2010). Such modifications of membrane composition in warm-adapted or warm-acclimated fish imply an increased proportion of saturated fatty acids, increased Chol content and changes in the PE:PC ratio to counteract the destabilizing effects of elevated temperature (Dahlhoff and Somero 1993; Kraffe et al. 2007). In our study, the NEAC groups incubated at 16 °C had a similar Chol content but a higher PC content and a lower PE:PC ratio compared to the cod kept at 3 °C. This is in line with the study of Kraffe et al. (2007) on rainbow trout (*Oncorhynchus mykiss*), in which the PE:PC ratio was the most responsive marker to increases in acclimation temperature. As PC is considered a bilayer stabilizer, a ratio in favour of PC would lower membrane fluidity (Hazel 1995; Kraffe et al. 2007; Hoffmann and Todgham 2010), causing the membrane to be more rigid, and therefore to maintain fluidity at high temperatures (Dahlhoff and Somero 1993; Kraffe et al. 2007). Although the determination of the relative lipid class composition was conducted on the entire pool of cellular membranes, we assume that the same pattern of adjustments is reflected in the mitochondrial membranes alone. Mitochondrial membranes have a lower Chol content and PC and PE are their main constituents, accounting together for about 80% of the total phospholipid composition (Daum 1985). During thermal adaptation and acclimation, the PE:PC ratio changes in the mitochondrial membranes in a similar way as in other cellular membranes (Wodtke 1978, 1981) and as presented in this study. Since mitochondrial membrane lipids play a role in the modulation of mitochondrial processes (Schlame et al. 2000; Kraffe et al. 2007), the inability of polar cod to adjust the properties of the membranes may contribute to the decrease in capacity of the membrane-associated enzymes SDH and CCO in the groups incubated at 8 °C and the abrupt increase in proton leak in the heart mitochondria from the same acclimation group compared to the colder groups (Table 3). On the other hand, the trend in NEAC towards membrane modification in response to temperature could in part explain the lack of thermal sensitivity of the membrane-bound enzymes SDH and CI:CIII and the increase in CCO capacity at high temperature (Table 3).

Conclusions

In this study, we analysed how temperature and P_{CO_2} levels alter the enzymatic capacities of four enzymes involved in mitochondrial respiration and the lipid composition of the cellular membranes, comparing an Arctic stenotherm and a cold eurytherm fish. Furthermore, we discussed how the differences in plasticity between the two species could affect their relative response to future ocean acidification and warming.

The Arctic stenotherm polar cod showed little acclimation potential at the level of mitochondrial enzymes as well as in terms of membrane composition. The inability of polar cod to modify the capacity of their mitochondrial enzymes upon warm acclimation may have little impact at temperatures below 6 °C, but could set long-term limitations to performance at 8 °C since a less efficient mitochondrial respiration is paired with increased SMR (Table 3) and mortality (Kunz et al. 2016). Moreover, the lack of modification of membrane composition could become detrimental through compromising the functionality of membrane-associated enzymes such as CCO and SDH at higher temperatures. In contrast, NEAC displays all the features of a cold eurytherm, showing thermal adjustments in CCO and CS capacities, and in membrane composition. Even if mitochondrial respiration in the heart of NEAC is negatively affected by the combination of high temperature and CO_2 (Table 3), this happens only at temperatures far above the projections for the end of the century for the Arctic region. Therefore, cardiac mitochondrial metabolism of polar cod appears to be more sensitive to future Arctic Ocean conditions than NEAC and only a profound change in the cardiac mitochondrial function could generate the plasticity needed by polar cod to cope with future temperatures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

Ethical approval The handling of the specimens of polar cod and NEAC was carried out according to the Ethical Permission Number AZ522-27–22/02–00 (113) released by the Senator for Healthcare, Bahnhofsplatz 29, 28195 Bremen on February 21st, 2013 (permit valid until February 21st, 2018).

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