„Cellular metabolism of different life stages of marine teleosts during Ocean Acidification and Warming “

“Zellulärer Metabolismus verschiedener Lebensstadien mariner Knochenfische unter Ozeanversauerung und -erwärmung.”

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Elettra Leo

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GUTACHTER

1. Gutachter: Prof. Dr. Hans O. Pörtner Universität Bremen
   Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung
   Integrative Ecophysiology
   Am Handelshafen 12 27570 Bremerhaven, Germany

2. Gutachterin: Prof. Dr. Inna Sokolova Universität Rostock
   MNF / Institut für Biowissenschaften, Meeresbiologie
   Albert-Einstein-Straße 3
   18059 Rostock, Germany

3. Prüfer: Prof. Dr. Wilhelm Hagen Universität Bremen
   FB2 Biologie/Chemie
   Postfach 330 440
   D-28334 Bremen

4. Prüfer: Dr. Felix C. Mark
   Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung
   Integrative Ecophysiology
   Am Handelshafen 12 27570 Bremerhaven, Germany

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ADP: Adenosin-5'- diphosphate

ANOVA: analysis of variance

ATP: Adenosin-5'-triphosphate

AWI: Alfred-Wegener-Institute Helmholtz Center for Polar and Marine Research

B. saida: *Boreogadus saida,* polar cod

B. medius: *Bellapiscis medius*

BIOPS: biopsy relaxing and preservation medium

BSA: Bovine Albumin Serum

C. harengus: *Clupea harengus*, Atlantic herring

cAMP: adenosin-5'- monophosphate cyclic

CA: carbonic anidrase

CH_4: methane

CI: Complex I of the electron transport system; NADH - quinone oxidoreductase

CII: Complex II of the electron transport system; Succinate dehydrogenase

CIII: Complex III of the electron transport system; Ubiquinol – cytochrome c reductase

Cyt c: cytochrome c

CIV: Complex IV of the electron transport system; Cytochrome c oxidase

CV: Complex V of the electron transport system; ATP synthase or F_0-F_1-ATPase

cO_2: Oxygen concentration

CO_2: carbon dioxide

CO_3^{2-}: carbonate ion

CCO: Cytochrome c oxidase; Complex IV

CS: Citrate synthase

DCPIP: 2,6-dichlorphenolindophenol

DIC: Dissolved Inorganic Carbon

dph: days post hatching

DTNB: 5,5'-dithio-bis-(2-nitro-benzoic acid)

EGTA: Ethylene glycol tetraacetic acid

ELSD: evaporative light scattering detector

ETS: Electron Transport System

F. malcolmi: *Forsterygion malcolmi*

F_0-F_1-ATPase: ATP synthase; Complex V

$FADH_2$: Flavin dinucleotide, reduced

FAO: food and agriculture organisation

FCCP: carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone

G. morhua: *Gadus morhua*, Atlantic cod

GAM: Generalized Additive Model

GHG: greenhouse gas

H^+: hydrogen ion, here proton

HCO_3^-: bicarbonate ion

H_2CO_3: carbonic acid

H_2O: water

Hb: haemoglobin

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HiT: Havbruksstasjonen i Tromsø

HPLC: high performance liquid chromatography

IPCC: Intergovernmental Panel on Climate Change

K_2SO_4: potassium sulfate

$KHSO_4$: potassium bisulfate

L-LDH: lactate dehydrogenase

LEAK: proton leak induced by oligomycin or atractyloside; mitochondrial respiratory State IV^+

MES: 2-(N-Morpholino)ethanesulfonic acid hydrate

MiR05: mitochondrial respiration medium

MO_2: respiration rates

MS-222: tricaine methane sulphonate

N. celidotus: *Notolobarus celidotus*

N. fucicola: *Notolabrus fucicola*

N_2O: nitrous dioxide

NADH: nicotin ammide adenin dinucleotide, reduced

NBS: national bureau of standards

NEAC: Northeast Arctic cod (*Gadus morhua*)

OA: ocean acidification

OAW: ocean acidification and warming

OW: ocean warming

OXPHOS: oxidative phosphorylation capacity; mitochondrial respiratory State III

PC: phosphatidylcholine

PCO_2: carbon dioxide partial pressure

PE: phosphatidylethanolamine

PK: piruvate kinase

POS: polarographic oxygen sensors

Q: quinone pool

Q_{10}: temperature coefficient

R: respiratory rate

RCP: representative concentration pathway

ROS: reactive oxygen species

ROX: residual oxygen consumption

rpm: round per minute

sAC: soluble adenylyl cyclase

SDH: Succinate dehydrogenase

SMR: standard metabolic rate

SST: sea surface temperature

SUIT: Substrates-Uncoupler-Inhibitors Titration

T: temperature

T. lunare: *Thalassoma lunare*

TCA: tricarboxylic acid

TMPD: N,N,N1,N1-tetramethyl-p-phenylenediamine

UV: ultraviolet light

VIS: visible light

Zusammenfassung

Der anthropogene Ausstoß von Treibhausgasen führt zu einem Anstieg der Temperaturen in der Atmosphäre und den Meeren. Der Anstieg der Wassertemperaturen geht mit einem Abfall des pH-Wertes einher, da das atmosphärische CO_2 mit dem Wasser reagiert. Dieses Phänomen ist als Ozeanversauerung und Erwärmung bekannt (OAW).
Steigende Temperaturen und ein verringerter pH im Wasser können zu Modifikationen der Energiebilanz von Fischen führen, wobei mehr Energie für den Proteinumsatz sowie für das Ionen- und Säure-Base-Gleichgewicht benötigt werden. Da die meisten dieser Prozesse von der Bereitstellung von ATP durch die Mitochondrien abhängig sind, wurde in dieser Doktorarbeit untersucht, ob steigende Temperaturen und PCO_2 die Funktion der Mitochondrien beeinflussen und Auswirkungen auf die Energiebilanz und somit auf das Akklimatisierungspotential der Tiere an Klimaveränderungen haben.
Mitochondrien sind spezialisierte zelluläre Organellen, deren Grad der Spezialisierung je nach Art und Lebensstadium variieren kann. Um ein breites Spektrum dieser Variabilität abzudecken, wurden in dieser Arbeit zunächst (i) die Mitochondrien juveniler Polardorsche (*Boreogadus saida*) als Beispiel polarer Fische und die juveniler Atlantischer Kabeljau (*Gadus morhua*) aus der nordöstlichen arktischen Population (NEAC) als Beispiel gemäßigter Fische analysiert. Beide Arten kommen momentan gemeinsam in den Gewässern um Spitzbergen vor. Weiterhin wurden (ii) die Mitochondrien juveniler Atlantischer Kabeljau mit den Mitochondrien von Embryonen derselben Art (Øresund Population) verglichen, um Unterschiede zwischen den Lebensstadien aufzuklären. Letztlich (iii) wurden Mitochondrien von Embryonen Atlantischer Kabeljau und von Embryonen Atlantischer Heringe (*Clupea harengus*) wegen des unterschiedlichen Laichverhaltens der Arten (Kabeljau laicht pelagisch, Hering benthisch) verglichen.

Polardorsch und NEAC wurden 4 Monate in je 8 Kombinationen von Temperatur (Polardorsch: 0, 3, 6, 8°C; NEAC: 3, 8, 12, 16°C) und PCO_2 (400 and 1170 µatm) akklimatisiert; im Anschluss daran wurde die mitochondriale Stoffwechselkapazität im Herzgewebe getestet. Weiterhin wurde die Zusammensetzung der Lipidklassen in Zellmembranen und die Kapazität einer Reihe mitochondrialer Enzyme analysiert.
Embryonen von Atlantischem Kabeljau und Hering wurden von der Befruchtung bis zum Schlüpfen unter gegenwärtigen und erwarteten Temperaturen (Atlantischer Kabeljau: 0, 3, 6, 9, 12°C; Hering: 6, 10, 14°C) und PCO_2 (400 and 1100 µatm) inkubiert. Sobald die Embryonen das Entwicklungsstadium “50% Augenpigmentierung” erreicht hatten, wurde die mitochondriale Funktion in kompletten Embryonen analysiert. Darüber hinaus wurde der Schlupferfolg, die Länge beim Schlüpfen und die

Missbildungsrate der Larven dokumentiert.
Die mitochondrialen Parameter, die in allen Arten gemessen wurden, sind OXPHOS, Protonenleck, Kapazität der Citrat-Synthase (CS) sowie die Kapazität der einzelnen Komponenten der Elektronentransportkette (ETC), d.h. Komplex I (CI), Komplex II (CII) und Komplex IV (CCO).

Juveniler Polardorsch zeigte einige stenotherme Merkmale wie zum Beispiel eine unveränderte Zusammensetzung der Membranlipide, gleichbleibende OXPHOS und ETS Werte trotz steigender Temperaturen sowie niedrige CCO/ETS Werte. Das Verhältnis zwischen OXPHOS und Protonenleck deutet darauf hin, dass die optimale Temperatur zur ATP-Produktion im Bereich 3-6°C liegt. Unter 8°C stieg das Protonenleck drastisch, was nicht in OXPHOS reflektiert wurde, so dass die Effizienz der ATP-Produktion und somit die verfügbare Energie in den Herzzellen sank. Da das Herz eine wesentliche Rolle bei der Akklimatisierung an Temperaturen spielt, könnte die geringere Energieleistung mit der erhöhten Mortalität bei diesen Temperaturen zusammenhängen. Der erhöhte PCO_2 hatte jedoch keinen Einfluss auf die Mitochondrien des Polardorschs, mit Ausnahme eines Anstiegs der CS Aktivität, welcher vermutlich eine kompensierende Reaktion auf die Hemmung der CS darstellte.
NEAC, andererseits, zeigte eher eurytherme Merkmale wie die Anpassung der Lipidkomponenten in den Zellmembranen, hohe CCO/ETS Werte sowie steigende OXPHOS und ETS bei ansteigenden Temperaturen. Obwohl auch das Protonenleck mit den Temperaturen anstieg, weist die konstante Effizienz der ATP-Produktion auf die Fähigkeit hin, das Protonenleck zu regulieren und die benötigte Energie für zelluläre Vorgänge in einem breiteren Temperaturbereich zu gewährleisten. Die Herzmitochondrien wurden jedoch negativ von erhöhtem PCO_2 beeinflusst, insbesondere in Kombination mit der höchsten getesteten Temperatur (16°C). Individuen dieser Gruppe zeigten niedrigere OXPHOS, ETS und Kapazität der ETS Enzyme CI und CCO, wohingegen die Enzyme des TCA-Kreislaufs CS und CII angeregt wurden. Möglicherweise inhibierte erhöhter PCO_2 die Enzyme CS und CII, welche daraufhin als Kompensation hochgeregelt wurden. Falls die Kompensation unvollständig war, könnte der Abfall in der Aktivität des TCA-Kreislaufs zur Abnahme der ETS Aktivität und damit der OXPHOS Kapazität geführt haben, was sich negativ auf die Energieleistung der Herzzellen auswirkt.
Im Gegensatz zu ihren juvenilen Artgenossen besaßen die Embryonen des Atlantischen Kabeljau Mitochondrien, die ein engeres Temperaturfenster aufwiesen und unempfindlich gegenüber erhöhtem PCO_2 waren. OXPHOS, ETS und CI stiegen mit zunehmenden Temperaturen bis bei 9°C ein Plateau erreicht wurde. CII wies die gleiche Kapazität unter Kontroll- und erhöhtem CO_2 auf. Die Kombination von hoher Temperatur (12°C) und erhöhtem PCO_2 wirkte sich jedoch negativ auf höherer Organisationsebene aus und reduzierte den Schlupferfolg und die Länge der geschlüpften Larven.

Weiterhin führte der erhöhte PCO_2 zu gesteigerten Missbildungsraten der Larven unter allen Inkubationstemperaturen.
Ähnliche Trends wurden in den embryonalen Mitochondrien des Atlantischen Hering gefunden. In dieser Art stiegen OXPHOS und CI mit zunehmenden Temperaturen bis sie bei 10°C ein Plateau erreichten, während erhöhter PCO_2 keinen Einfluss auf die Funktion der Mitochondrien ausübte. Obwohl erhöhter PCO_2 insbesondere unter hohen Temperaturen die Überlebenschancen der Embryonen des Atlantischen Kabeljaus verringerte, hing der Schlupferfolg sowie die Größe der geschlüpften Larven beim Atlantischen Hering nur von der Temperatur ab. Dies deutet auf eine höhere CO_2 Toleranz des Herings hin, welcher ein benthisches Laichverhalten aufweist.

Generell lässt sich auf der Basis mitochondrialer Daten sagen, dass NEAC eher eurytherm und plastisch in dem Temperaturbereich zu sein scheinen, der für Ende des Jahrhunderts in den Gewässern um Spitzbergen vorhergesagt wird. Trotz ihrer höheren CO_2-Empfindlichkeit könnten sie Polardorsch daher verdrängen oder in das Bodenwasser der Fjorde zwingen.
Diese Plastizität der juvenilen NEAC fand sich nicht in Embryonen der NEAC wieder, bei welchen das Temperaturfenster enger und empfindlicher gegenüber erhöhtem PCO_2 war. Dies weist darauf hin, dass Embryonen einen Engpass für den Akklimatisierungsprozess der Population darstellen könnte. Weiterhin könnte die Toleranz gegenüber hohen CO_2-Werten mit dem Laichverhalten zusammenhängen, wobei benthische Arten toleranter als pelagische sind.

Summary

The anthropogenic emissions of greenhouse gases are causing an increase in atmospheric and oceanic temperatures. In the oceans, seawater temperature rises in parallel to the decrease in pH caused by the reaction of rising atmospheric CO_2 with water. The combined phenomenon is known as Ocean Acidification and Warming (OAW).

Rising temperatures and decreased water pH may induce adjustments in the energy budget of fish, requiring more energy for protein turnover and for ion and acid-base balance. Since most of these processes depend on the mitochondrial provision of ATP, this PhD project investigated if rising temperature and PCO_2 affect the mitochondrial functioning with consequences for the energy budget and acclimation potential of the animals to climate changes.

Mitochondria are specialised cellular organelles and their degree of specialisation may vary according to species and life-stage. To cover a broad range of this variability, this thesis analysed firstly the mitochondria of juvenile polar cod (*Boreogadus saida*) as polar fish and of juvenile Atlantic cod (*Gadus morhua*) from the Northeast Arctic population (NEAC) as temperate fish, which presently co-occur in the waters around Svalbard. Secondly, the mitochondria of juvenile NEAC were compared with the mitochondria of embryos of the same species (Øresund population) to assess the differences between life-stages. Lastly, mitochondria of Atlantic cod embryos were analysed together with the ones of Atlantic herring embryos (*Clupea harengus*) because of the different spawning behaviour of the two species (pelagic for Atlantic cod, benthic for herring).

Polar cod and NEAC were acclimated for four months at combinations of temperature (polar cod: 0, 3, 6, 8°C; NEAC: 3, 8, 12, 16°C) and PCO_2 (400 and 1170 µatm) at the end of which their cardiac mitochondrial respiration was tested. In addition, the lipid class composition in pooled cellular membranes and the capacity of a number of mitochondrial enzymes were analysed.

Embryos of Atlantic cod and herring were incubated from fertilization to hatch at present and projected temperatures (Atlantic cod: 0, 3, 6, 9, 12°C; herring: 6, 10, 14°C) and PCO_2 (400 and 1100 µatm). When the embryos reached the "50% eye pigmentation" developmental stage, whole-body mitochondrial functioning was assessed. Moreover, the hatching success, length at hatch and larval malformation rates were recorded.

The mitochondrial parameters measured in all species were OXPHOS, proton leak, citrate synthase (CS) capacity and the capacity of the single components of the Electron Transport System (ETS) i.e., Complex I (CI), Complex II (CII) and Complex IV (CCO).

SUMMARY

Juvenile polar cod presented some stenothermal traits like the lack of adjustments of the membrane lipids, stable values of OXPHOS and ETS despite increasing temperatures and low values of CCO/ETS. The relation between OXPHOS and proton leak suggested an optimum temperature for ATP production in the 3-6°C range, while the proton leak increased dramatically at 8°C, which was not paired by OXPHOS, hence decreasing the ATP production and therefore the available energy in the cardiac cells. Since the heart plays a fundamental role in acclimation to temperature, the lower energy yield may be related to the higher mortality occurring at this temperature. Yet, polar cod mitochondria were not affected by elevated PCO_2 besides the increase in CS activity, probably as compensatory response to overcome its inhibition.

NEAC, on the other hand, displayed more eurythermal features like modifying the lipid components of the cellular membranes, high CCO/ETS and increasing OXPHOS and ETS with rising temperatures. Although proton leak also increased with temperature, the stable ATP production efficiency indicates the ability to control proton leak and ensure the required energy to the cellular processes in a broader range of temperatures. However, the cardiac mitochondria of NEAC were negatively impacted by incubation under elevated PCO_2, especially in combination with the highest tested temperature (16°C). Individuals from that group presented lower OXPHOS, lower ETS and lower capacity of the ETS enzymes CI and CCO whereas the TCA cycle-related enzymes CS and CII were stimulated. Possibly, elevated PCO_2 inhibited CS and CII which were up-regulated in order to compensate for the lower activity. If the compensation was just partial, the decrease in activity of the TCA cycle may have led to a decrease of the ETS activity and therefore of the OXPHOS capacity with negative consequences on the energy yield of the heart cells.

In contrast to their juvenile conspecifics, Atlantic cod embryos possessed mitochondria with a narrower thermal window which were not sensitive to elevated PCO_2. In fact, OXPHOS, ETS and CI increased with temperature until 9°C, where they reached a plateau, and CII presented the same capacity at control and high levels of CO_2. However, the combination of high temperature (12°C) and elevated PCO_2 exerted a negative effect at higher organizational levels, decreasing hatching success and hatchlings' length. Moreover, elevated PCO_2 increased the larval malformation rates at all incubation temperatures.

Similar trends were found in the embryonic mitochondria of Atlantic herring. In this species OXPHOS and CI increased with temperature until 10°C and then reached a plateau, while elevated PCO_2 did not affect the mitochondrial functioning. While elevated PCO_2, especially in combination with high temperatures, decreased the survival of Atlantic cod embryos, herring hatching success and hatchlings' size was only related to temperature, suggesting higher CO_2-tolerance in this benthic spawner.

In conclusion, with regard to the mitochondrial functioning, NEAC appeared more eurytherm and plastic in the range of temperatures projected for the waters around Svalbard at the end of the century. Despite their higher CO_2- sensitivity they may outperform polar cod, displacing them or forcing them to retreat in the fjord bottom waters.

The plasticity of juvenile NEAC was lowest at the embryonic level, where the thermal window was narrower and more susceptible to elevated PCO_2, suggesting that embryos may be a bottle-neck for the population acclimation process. Moreover, tolerance to high CO_2 may be related to the spawning behaviour, with benthic species being more tolerant than pelagic ones.

1. Introduction

Climate change represents a compelling issue for the whole planet, including the oceans. Increasing atmospheric concentrations of anthropogenic greenhouse gases are causing a decrease in water pH and a rise in oceanic water temperatures in a phenomenon called ocean acidification and warming (OAW) (Doney *et al.*, 2009; IPCC, 2019).

As climate change intensifies, ecosystems and organisms are facing changes beyond the natural variability, with negative impacts on their functioning (Henson *et al.*, 2017; Pörtner, 2014). Changes in the environment physical properties such as temperature, salinity and pH can affect the organisms' energy balance, requiring additional energy to recover and maintain homeostasis (Pörtner and Knust, 2007). This holds true especially for ectothermic animals such as fish because they are not able to control their internal temperature (Fry, 1971). For this reason, environmental temperature plays a key role in all aspects of their life such as metabolic rates, development, activity, growth and reproduction (Pörtner and Farrell, 2008; Sokolova, 2013). These processes have an optimum temperature and can be efficient only within a specific thermal range. Changes in the environmental temperature or the addition of further drivers such as ocean acidification can affect them irreparably with consequences at the individual, population and ecosystem levels (Pörtner *et al.*, 2017; Sokolova, 2013). Therefore, it is essential to understand the mechanisms underlying the organism's thermal tolerance and adaptation capacities to the new conditions.

Most of the processes ruling the organism's life are sustainable only under aerobic metabolism (Pörtner, 2002; Sokolova, 2013) and mitochondria are the primary site of aerobic energy production. At elevated but sublethal temperatures, mitochondria may become ineffective to supply ATP to power these processes (Clarke and Fraser, 2004; Hardewig *et al*., 1999; Iftikar *et al*., 2014) and little is known about the conjunction with ocean acidification (Strobel *et al.*, 2012; Strobel *et al.*, 2013a; Strobel *et al.*, 2013b; Kreiss *et al.*, 2015a) or different life stages. Therefore, this thesis aims to investigate the effects of OAW on the mitochondrial metabolism of juveniles and embryos in a selection of fish species and attempts to put it into relation with their acclimation capacity and fitness.

1.1 Climate changes

Human activities have been the main cause of climate change observed since the middle of the last century. Rising emissions in the atmosphere of greenhouse gases (GHG) such as CO_2, CH_4, N_2O and halocarbons have increased the global mean temperature of about 1°C since the 1850s, (IPCC, 2014; 2019) while the increase in global CO_2 concentration is up to 10 times faster than any rise that occurred

in the past 800,000 years (Lüthi *et al.*, 2008; Bereiter *et al.*, 2015).

In parallel with the global mean temperature, the mean sea surface temperature (SST) rises, a phenomenon called ocean warming (OW). Over the period 1950 –2016 the surfaces of the Indian, Atlantic and Pacific Oceans have warmed on average by 0.33°C, 0.21°C and 0.15°C respectively, with the greatest changes occurring in the highest latitudes (IPCC, 2019) There is also evidence of increased frequency and intensity of extreme warming events (Oliver *et al.*, 2018), which are expected to intensify further (Cai *et al.*, 2014; IPCC, 2019; Jacox, 2019).

Increased GHG concentration in the atmosphere causes a rise in concentration of the same gases in the ocean, since gas dissolution in the water depends on the gas partial pressure in the atmosphere. In particular, the ocean has absorbed approximately 30% of all anthropogenic CO_2 (Sabine *et al.*, 2004; Zeebe *et al.*, 2008) altering the ocean chemistry in an unprecedented way in at least 65 million years (Ridgwell and Schmidt, 2010 or even 300 million years as reported in Hönisch *et al.*, 2012). The absorbed CO_2 reacts with water to carbonic acid, which dissociates according to equation (1), lowering the ocean pH in a process known as ocean acidification (OA) (Doney *et al.*, 2009).

$$(1) \qquad CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons 2H^+ + CO_3^{2-}$$

1.1.1 The Representative Concentration Pathways

The temporal evolution of a specific scenario component (e.g., GHG emissions or socio – economic development) is termed pathway. The Representative Concentration Pathways (RCPs) describe a set of GHG concentration trajectories in the period from 1850 to 2100, with extensions up to the year 2300 (van Vuuren *et al.*, 2011; IPCC, 2019).

The RCPs are named and classified according to the radiative forcing levels reached by the end of the century (van Vuuren *et al.*, 2011). In details: RCP8.5 assumes constant emissions after 2100 until 2150 and constant concentrations afterwards, leading to an ocean warming of 3.7°C. RCP6 and RCP4.5 describe intermediate stabilization pathways, RCP2.6 describes a decline in emissions and constant concentrations after 2100 with an increase in global temperature of 2°C by the end of the century compared to the period 1986 – 2005 (IPCC, 2019). The lowest emissions scenario is RCP1.9 which limits the global temperature rise to 1.5°C as pledge by the Paris Agreement (UNFCCC, 2015).

In relation to ocean acidification, average surface ocean pH has decreased by 0.1 units from the pre-industrial value of 8.2 (Caldeira and Wickett, 2003; Orr *et al.*, 2005; IPCC, 2019) and is expected to

further decrease by up to 0.315 units by the year 2100 according to the RCP8.5 and up to 0.065 units according to RCP2.6 as shown in figure 1.h (IPCC, 2019). Moreover, ocean acidification will be even more pronounced in upwelling areas, where CO_2-rich deep water is brought to the ocean surface and in polar regions where low temperatures allow the water to hold more gas (Sabine *et al.*, 2004; Doney *et al.*, 2009; IPCC, 2019).

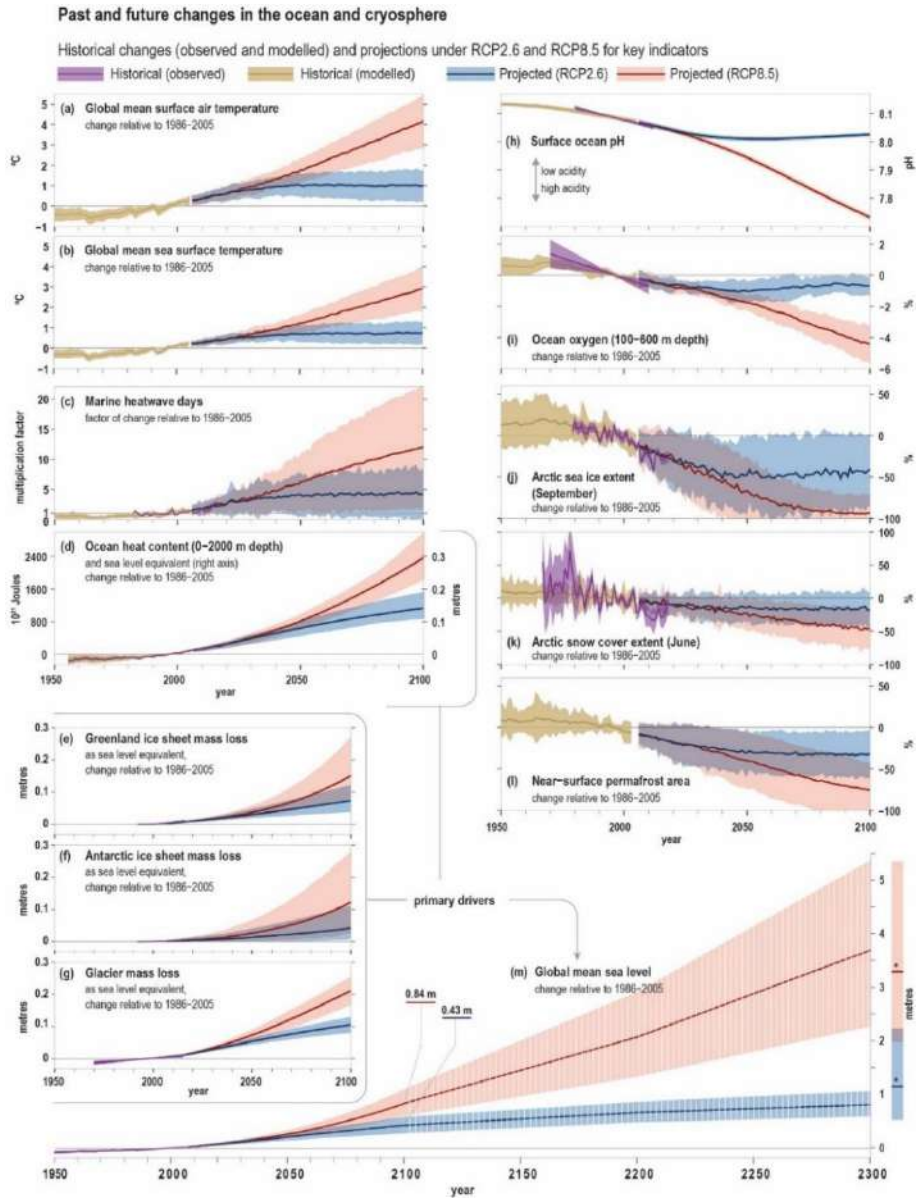


Figure 1: Changes in the ocean and cryosphere since 1950s (observed and modelled) and projected changes according to low (RCP2.6) and high (RCP8.5) emissions scenarios. Variations are illustrated for: (a) Global mean surface air temperature, (b) Global mean sea surface temperature, (c) Days of marine heatwaves, (d) Global ocean heat content (0–2000 m depth), (h) Global mean surface pH (on the total scale) (i) Global mean ocean oxygen change (100–600 m depth), (m) Global mean sea level change, (e) Greenland ice sheet mass loss, (f) Antarctic ice sheet mass loss, (g) Glacier mass loss, (j) Arctic sea ice extent for September; (k) Arctic snow cover for June (land areas north of 60º; and (l) near-surface (within 3–4 m) permafrost area in the Northern Hemisphere. Figure by SROCC (IPCC, 2019)

1.2 Effects of OAW on fish

A key factor in shaping the distribution of fish species is temperature.
Physiological processes and energy efficiency are confined to specific thermal windows (Guderley and St-Pierre, 2002). The baseline energy demand is mainly constituted by cellular maintenance (i.e. ion transport, protein turnover, anabolism) and respiratory and circulatory costs essential for the oxygen and nutrient supply of all body tissues (Jobling, 1994). At elevated but sub-lethal temperatures, the cellular energetic costs rise, increasing energy demand. Although most organisms have the capacity to compensate for increased metabolic baseline costs during long-term warm exposure, acclimatization capacities are species-specific and limited in time and specific to life stages. For example, while individuals may survive, they might experience a reduction in energy available for reproduction, or conditions at spawning grounds might not be suitable for embryonic development, resulting in changes in population distribution and size (Bozinovic and Pörtner, 2015; Pörtner, Bock and Mark, 2017).
Marine fish are generally considered to be less sensitive towards elevated CO_2 levels (and thus to OA) than other taxa because they are efficient acid-base regulators (Ishimatsu *et al.*, 2005; Melzner *et al.*, 2009; Portner, Walther and Wittmann, 2013; Heuer and Grosell, 2014). However, the processes responsible for acid-base and ion balance are energetically costly (up to 1.8% of standard metabolic rates in cutthroat trout *Oncorhynchus clarkii* at ambient PCO_2; Morgan and Iwama, 1999) and can add onto baseline metabolic costs (Pörtner, 2010). The altered tissue energy demand will have implications first on metabolic performance (Enzor *et al*., 2013; Rummer *et al.*, 2013; Strobel *et al.*, 2013a), and then on reproduction (Miller *et al.*, 2012), embryonic and larval development (Frommel *et al.*, 2012, 2016; Sswat *et al.*, 2018) and eventually on population well-being (Heuer and Grosell, 2014).

Despite regional differences, in the field increased CO_2 levels causing OA appear to be linked to increasing temperatures (OW), making it more difficult to disentangle and distinguish the biological effects of the individual drivers (Parmesan *et al.*, 2013). Several studies on various taxa indicate an increased sensitivity towards OA at temperatures close to the species' upper limit of the thermal window (Pörtner, 2012; Portner, Walther and Wittmann, 2013) and even a narrowing of the thermal window in presence of high CO_2 levels (Lannig *et al.*, 2010; Pörtner and Peck, 2010; Feidantsis *et al.*, 2015), most probably because of the elevated energetic requirements for homeostasis (Cattano *et al.*, 2018).
Therefore, to draw ecologically realistic conclusions, it is important to investigate the mechanisms underlying the acclimation capacities under multiple-driver scenarios (Anttila *et al.*, 2015).
Since alterations in the mitochondrial properties can contribute to trade-offs in the whole-animal energy budget (Pörtner, 2002), it is crucial to understand the effects of OAW on the mitochondrial physiology.

1.2.1 Mitochondria under OAW

Mitochondria are suggested to play a central role in defining the thermal responses of aerobic metabolism in ectothermic animals (Pörtner, 2002). Therefore, the effects of heat stress on the mitochondrial respiration and its potential acclimation capacity are widely studied.

High temperature decreases the coupling between oxygen consumption and phosphorylation by increasing the proton leak through the inner mitochondrial membrane (Chamberlin, 2004). Proton leak (LEAK) accounts for 20-25% of oxygen consumption in isolated cells (Rolfe and Brand, 1997; Chamberlin, 2004) and during thermal challenges, it rises with whole-animal metabolic rates (Hardewig, Peck and Pörtner, 1999) decreasing the mitochondrial capacity and ATP production efficiency (Hardewig, Peck and Pörtner, 1999; Hilton, Clements and Hickey, 2010; Iftikar and Hickey, 2013; Strobel *et al.*, 2013b).

Proton leak at high temperature could be also promoted by elevated mitochondrial membrane fluidity (Brand and Nicholls, 2011). If not counteracted, high temperature increases the membrane fluidity altering diffusion rates (e.g. proton leak) and barrier properties but also altering the functions of membrane associated proteins such as ion pumps and enzymes (e.g. Complex I; Complex IV and Complex V) (Hazel, 1972; Guderley and Johnston, 1996). Thermally induced adjustments to preserve the membranes' fluid liquid-crystalline phase are generally known as homeoviscous adaptation and include changes in the proportion of phospholipid classes (mainly phosphatidylcholine and phosphatidylethanolamine) and variation of the proportion on unsaturated fatty acids and the double bonds inside the unsaturated fatty acids (Hazel, 1995; Kraffe, Marty and Guderley, 2007; Lemieux, Blier and Tardif, 2008; Hofmann and Todgham, 2009). The time scale of this type of adjustments has not been described yet in fish but there are evidences of rapid changes in mollusks. For example, the cellular membranes of intertidal mussels can be rearranged within hours in response to temperature fluctuations (Williams and Somero, 1996).

The electron transport system (ETS) is a series of enzymes and molecules linked with the mitochondrial inner membrane that shuttles electrons from the initial electron donors (reduction equivalents NADH and $FADH_2$) to the final acceptor (oxygen) via redox reactions (Fig.2). For its key role in the mitochondrial respiration, the effects of temperature on the ETS and its single components are widely studied.

Complex I (NADH quinone oxidoreductase, CI in figure 2) is reported to be limiting at high temperature indicating a key role of this respiratory complex in setting the mitochondrial thermal constrains (Hilton, Clements and Hickey, 2010; Dhillon and Schulte, 2011). Complex I (CI) sensitivity to heat is suggested

to be linked to both its structure and function. CI is by far the largest respiratory complex, composed up to 40-47 subunits, 14 of which are conserved from prokaryotes to humans (Brandt, 2006; Papa *et al.*, 2008; Vinothkumar, Zhu and Hirst, 2014). It has a L-shape with the central body embedded in the inner mitochondrial membrane and the peripheral arm protruding in the mitochondrial matrix (see Hirst, 2013 for review). Mammalian CI is susceptible to ischemic damage resulting from reactive oxygen species (ROS) (Papa *et al.*, 2008; Murphy, 2009). Hence, also dysfunctions in ectotherms may be partially caused by ROS released once CI is damaged by high temperature. Moreover, the increased proton leak at high temperatures may decrease the membrane potential, inhibiting the electrogenic import of substrates and depressing the capacity of CI (Iftikar, MacDonald and Hickey, 2010; Vinogradov and Grivennikova, 2016).

Complex II (Succinate dehydrogenase, CII in figure 2) is part of both the tricarboxylic acid (TCA) cycle and the ETS. Eukaryotic Complex II (CII) is composed by four subunits and is the only respiratory complex to not to pump protons across the inner mitochondrial membrane (see Rutter, Winge and Schiffman, 2010 for review). Because of its smaller structure and because the import of succinate is not electrogenic, CII is generally considered to be more thermally stable than CI (Iftikar, MacDonald and Hickey, 2010). However, the New Zealand wrasses *Thalassoma lunare* and *Notolabrus celidotus* showed limited CII activity upon warming suggesting that CI exerted some sort of control over CII activity (Iftikar *et al.*, 2014, 2015).

Complex III (Ubiquinol– cytochrome c oxidoreductase, CIII in figure 2) consists of 11 subunits (in vertebrates) embedded in the inner mitochondrial membrane (Widger *et al.*, 1984). It is so far the least studied complex of the respiratory chain and the studies focused mainly on its role in hypoxia-induced ROS production in mammals (Guzy *et al.*, 2005). In fish, it is generally studied in association with CI (CI:CIII) (Mark *et al.*, 2012; Hunter-Manseau *et al.*, 2019). Mark *et al.* (2012) reported increased activity of CI:CIII upon warming in two Antarctic fish *Notothenia rossii* and *Notothenia coriiceps,* findings paired by the results from Hunter-Manseau *et al.* (2019) on eight fish species in a polar – equator latitudinal cline, while Lemieux *et al.* (2010b) reported decreased capacity of CIII and CI (individually measured) at temperatures approaching the upper thermal limit of the Atlantic wolffish (*Anarhichas lupus*). The parallel response of CI and CIII may be explained by stable supercomplexes consisting of CI and dimeric CIII (CI + $CIII_2$), as found in the mitochondria of yeast (Schägger and Pfeiffer, 2000), plants (Dudkina *et al.*, 2010) and mammals (Bianchi *et al.*, 2004).

Complex IV (Cytochrome c oxidase CCO, CIV in figure 2) consists of 7-12 subunits and is often assumed to be present in excess (Brunori *et al.*, 1987; Gnaiger, Lassnig, *et al.*, 1998; Rossignol *et al.*, 2000; Blier and Lemieux, 2001). However, in some ectotherms it was shown to have a thermal sensitivity similar to that of the oxidative phosphorylation capacity (OXPHOS) (Dahlhoff and Somero, 1993; Blier

and Lemieux, 2001; Lemieux *et al.*, 2010b) with increased activity at moderately high temperature (Cai and Adelman, 1990; Foster, Hall and Houlihan, 1993) until reaching a plateau (Lemieux *et al.*, 2010b) and decreased activity at temperatures close to the upper thermal limit of the animal (Iftikar *et al.*, 2014). Complex V (ATP synthase or F_0-F_1-ATPase, F_0-F_1-ATPase in figure 2) is composed of two functional, structurally well-defined parts: a proton channel F_0 embedded in the membrane containing eight subunits and a hydrophilic F_1, formed by five subunits of which the catalytic activity produces ATP (Senior, Nadanaciva and Weber, 2002). Low temperature is reported to slow Complex V (CV) catalytic speed (Kikuchi, Itoi and Watabe, 1999) with the consequence that some temperate eurytherm fish such as carp (*Cyprinus carpio*) increase the muscular content of CV to compensate for the decreased capacity upon cold acclimation (Kikuchi, Itoi and Watabe, 1999). On the other hand, little is known about the effects of warm temperature on CV. For example, Kreiss *et al.* (2015b) report an increase in activity and reduced thermal sensitivity after acclimation at high temperature and high levels of CO_2 (2500µatm) in the gills of Atlantic cod (*Gadus morhua*).

The mitochondrial effects of exposure to high CO_2 partial pressure (PCO_2) have received increased attention in the last years (see Heuer and Grosell, 2014 for review). In the Antarctic fish *Notothenia rossii*, mitochondrial pathways of several tissues were altered and mitochondrial metabolic capacity was decreased after long term acclimation at 2000 µatm CO_2 (Strobel et al., 2013a). Changes included decreased OXPHOS (mainly attributable to a decrease of CII activity) and decreased CCO activity, while signs of compensatory responses, such as increased P/O ratios were reported in liver mitochondria (Strobel *et al*., 2013b). This compensation was suggested to act through the stimulation of anaplerotic pathways (e.g., oxidative decarboxylation of dicarboxylic acids) which feed directly to CI, bypassing the CO_2 induced inhibition of CII (Strobel *et al*., 2013b). On the other hand, in gilthead seabream (*Sparus aurata*) exposed to 5000 µatm CO_2, increased glycolytic enzymes such as pyruvate kinase (PK) and lactate dehydrogenase (L-LDH), and decreased citrate synthase (CS) in red muscle, white muscle and heart indicate the onset of anaerobic metabolism under very high PCO_2 (Michaelidis, Spring and Pörtner, 2007; Feidantsis *et al.*, 2015).

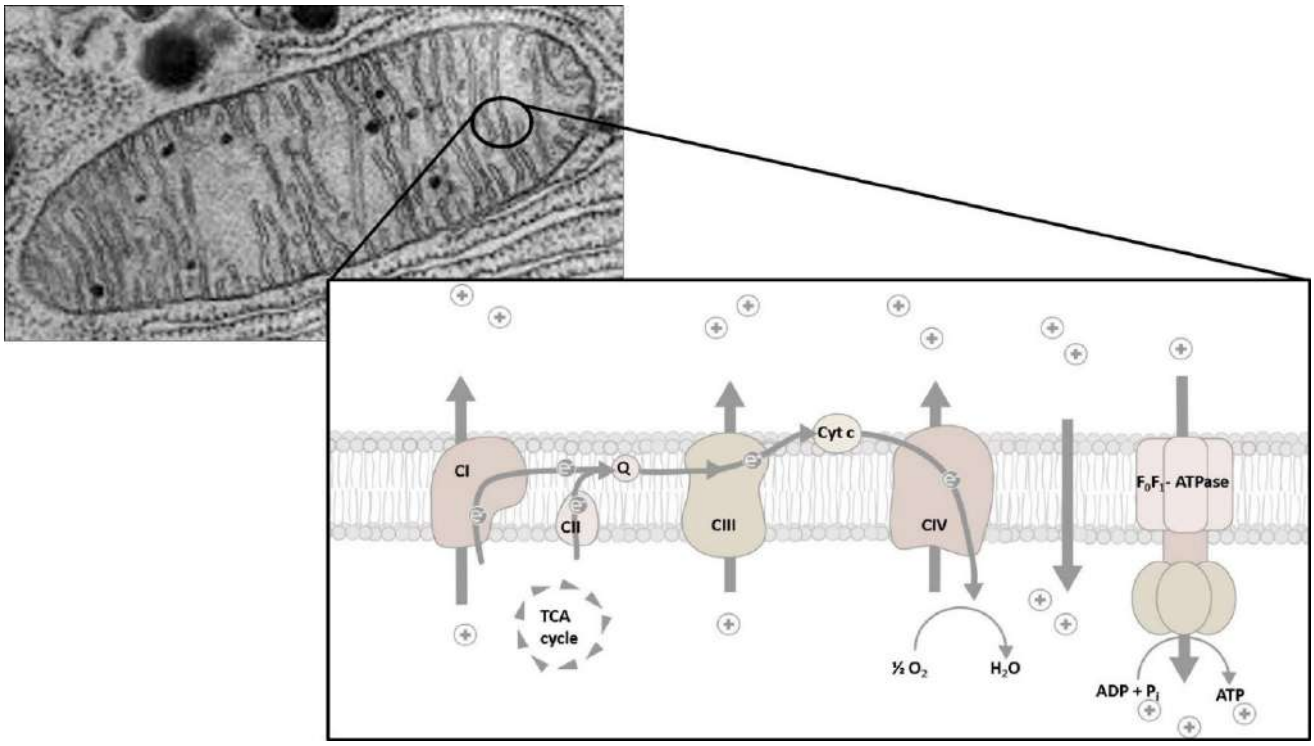


Figure 2: Mitochondrion and schematic of the mitochondrial Electron Transport System (ETS) situated in the cristae of the inner mitochondrial membrane. The complexes of the ETS linked with the inner mitochondrial membrane transport electrons produced by the reactions of the tricarboxylic acid cycle (TCA) located into the mitochondrial matrix on the one hand, and pump protons from the matrix into the intermembrane space on the other hand. The ETS has two entry points*:*
- Complex I (CI), which can oxidize NADH produced by the TCA-cycle, glycolysis and other mitochondrial pathways (e.g., fatty acid ß-oxidation and glutamate dehydrogenase).
- Complex II (CII), which is directly linked to the TCA cycle and through $FADH_2$ adds electrons into the quinone pool (Q). The electrons are then transferred from Q to Complex III (CIII) and Complex IV (CIV) to the final acceptor: O_2. The electron transfer is coupled to the pumping of protons into the intermembrane space creating a proton gradient across the inner mitochondrial membrane. This gradient is used by Complex V (CV) or F_0F_1-ATP synthase to synthetize ATP. Mitochondrion picture by Keith R. Porter/Science Source

1.2.2 Acclimation capacity

The energy requirements for stress response and homeostatic regulation can be modulated through acclimation and adaptation. Because of the broad thermal tolerance of eurytherm fish, the studies on their thermal acclimation potential and the associated physiological changes are crucial to shed light on the mechanistic background of eurythermy, in particular in comparison with stenothermy, and the related trade–offs in the light of the ongoing climate changes (Guderley and St-Pierre, 2002; Pörtner, 2006). Metabolic adaptation to temperature changes can involve adjustments in energy allocation and switch between metabolic pathways for energy provision and conversion. A typical example is the change of preferred fuel from lipids to carbohydrates and metabolic rearrangements towards protein catabolism and decreased lipid synthesis upon warming (Brodte, Knust and Pörtner, 2006; Windisch *et al.*, 2011).

At the mitochondrial level, metabolic adaptation to specific temperature ranges include mitochondrial density, enzyme kinetic parameters (such as substrate affinity and turnover number) and membrane composition (Hochachka and Somero, 2002). Hence, exposure to adverse temperatures will affect the kinetic properties of the enzymes in metabolic pathways, the leakiness of membranes and the stability of functional proteins with the consequential cost of protein turnover (Pörtner, 2006).

Extreme stenotherms such as high Antarctic fish (which have evolved in a cold but stable environment), have high mitochondrial densities but low mitochondrial capacities and low proton leak in aerobic tissues ensuring low costs of mitochondrial maintenance in a narrow thermal window (Hardewig, Peck and Pörtner, 1999; Pörtner, 2006). In contrast, cold-adapted eurytherms (such as sub – Antarctic and North Atlantic species) developed moderate mitochondrial densities with high individual capacities allowing more active lifestyles and broader thermal windows but carrying high maintenance costs related to higher proton leak (Egginton, Cordiner and Skilbeck, 2000; Lannig *et al.*, 2003; Pörtner, 2006).

Another element that could contribute to determine the mitochondrial thermal window is CI of the ETS. Studies on eurytherm and stenotherm fish from various habitat (sub-Antarctic vs. high-Antarctic Strobel *et al*., 2013b; temperate vs. tropical waters Iftikar *et al*., 2014; sub-tidal vs. intertidal Hilton, Clements and Hickey, 2010) showed common features. Species with Complex I activities that were stable over a broader thermal range were those species living in habitats with wider temperature variation (the sub-Antarctic *Lepidonotothen squamifrons;* the temperate wrasses *Notolabrus celidotus* and *Notolabrus fucicola* and the intertidal *Bellapiscis medius*) compared to the species with CI functional in a narrower thermal range (the high-Antarctic *Notothenia rossii;* the tropical wrasse *Thalassoma lunare* and the subtidal *Forsterygion varium* and *Forsterygion malcolmi*).

1.3 Sensitivity across life stages

The thermal tolerance of an individual might change during its life (Fig. 3). Embryos generally have limited thermal tolerance due to developmental constraints and incomplete development of the cardiovascular system and ion-regulatory organs (Pörtner, Bock and Mark, 2017; Dahlke *et al.*, 2018; Dahlke *et al.*, 2020). Juveniles and young adults have higher thermal tolerance due to high capacity linked with small body size. Larger individuals then become more thermally sensitive, due to limitations in the oxygen supply capacity and spawners have decreased thermal tolerance as consequence of the great energy investment in sperm and egg masses (Pörtner and Knust, 2007; Pörtner, Bock and Mark, 2017; Dahlke *et al.*, 2020).

Embryos are therefore considered one of the most susceptible life stages to climate change

(Pörtner and Peck, 2010; Dahlke *et al.*, 2018). For example, a recent study by Dahlke *et al.* (2018) found the hatching success of polar cod to decline above 2°C while studies on juveniles showed successful growth in a broader thermal range (0-6°C Kunz *et al.*, 2016; 0-9°C Laurel *et al.*, 2016).

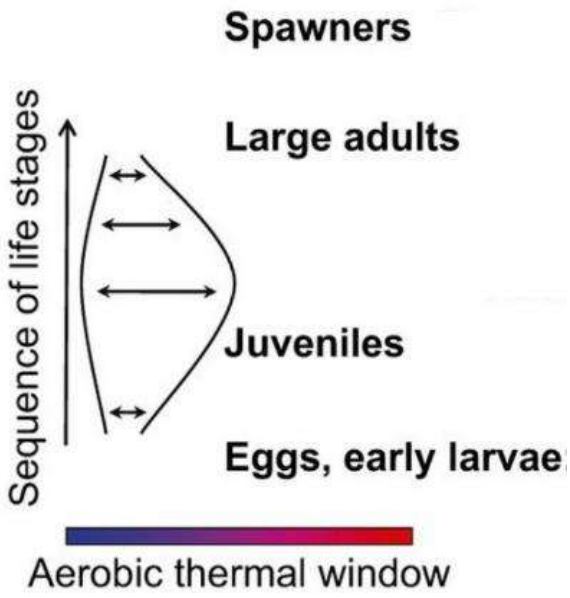


Figure 3: Modifications of the thermal window width according to life stage. Picture from Pörtner, Bock and Mark (2017).

Temperature influences the embryonic development acting on time of development, hatching success and energy allocation (Rombough, 2011). In the limits of the preferred temperature range, increasing temperature reduces developmental time and increases growth rates which can be beneficial for the animal because a rapid growth reduces the time spent in the small and more vulnerable size (Mueller *et al.*, 2011; Rombough, 2011). Beyond the upper limit of this range, temperature may cause the embryos to use more energy resources for maintenance (e.g., increased protein turnover) at the disadvantage of growth, decreasing hatching success and/or producing smaller and therefore more vulnerable hatchlings (Mueller *et al.*, 2011).

Similar to thermal sensitivity, increasing evidence suggests high sensitivity to high PCO_2 in early life stages while adult fish are considered relatively tolerant (Ishimatsu *et al.*, 2005; Melzner *et al.*, 2009). High PCO_2 is reported to cause behavioural changes potentially due to disrupted neurotransmitter function (Nilsson *et al.*, 2012), abnormal otolith growth, and increased metabolic costs due to impaired acid-base balance (Melzner *et al.*, 2009) resulting in impaired growth and decreased survival in embryos and larvae of many fish species such as *Menidia beryllina, Rachycentron canadum* and others (Baumann,

Talmage and Gobler, 2012; Kroeker *et al.*, 2013; Bignami, Sponaugle and Cowen, 2014; Di Santo, 2015). However, CO_2 sensitivity seems to be highly dependent on the ecology of the species investigated. Studies on Atlantic herring (*Clupea harengus*) found no significant effects of high PCO_2 (4600 µatm) on mortality, hatch rate, embryonic malformations and size at hatch (Franke and Clemmesen, 2011). Likewise, the coral reef fish *Amphiprion percula* showed no effects of PCO_2 up to 1030 µatm on mortality and hatch rates and larval length (Munday *et al.*, 2010). Both fish species are benthic spawners and their eggs usually experience a broad variation of PCO_2 during the embryonic development suggesting an increased tolerance towards habitat related high PCO_2.

In general, elevated PCO_2 may increase metabolic costs, especially at the limits of the thermal range, narrowing the thermal tolerance window (Pörtner, Bock and Mark, 2017; Melzner *et al.*, 2020). Embryos have limited aerobic scope (Killen *et al.*, 2007) and their resources depend only on the maternal provision of yolk (Rombough, 2011). Elevated temperature and PCO_2 may increase the energy requirements of developing embryos, making them unable to obtain the energy needed for all the processes due to metabolic limitations. In that case part of the energy from one process could be shifted to another one (Rombough, 2011). For example, to maintain the high protein turnover caused by elevated temperature or the high ion exchange stimulated by CO_2, they may shift energy from growth to protein syntheses and ion pumps. Therefore, since embryonic development and growth are almost entirely dependent on aerobic metabolism (Finn and Fyhn, 2010; Rombough, 2011), a detrimental effect of temperature and CO_2 on the mitochondrial physiology of embryos may even further diminish the energy available for developmental mechanisms and, as a consequence, decrease growth or hatching success.

1.4 Experimental background

Because of the adverse effects of high temperature on the energy balance (see section 1.2), fish tend to respond to increasing temperatures by moving poleward (Perry *et al.*, 2005) or deeper (Dulvy *et al.*, 2008) to occupy cooler environments closer to their optimum thermal range. However, fish may encounter physical limitations to their poleward migration such as the presence of land masses or constraints to their vertical migration, like bathymetric boundaries in shallow coastal areas, restrictions of habitat availability and the presence of oxygen minimum zones together with biological limitations such as unsuitable food webs (Garciá Molinos *et al.*, 2016). Moreover, ocean acidification and warming (OAW) influences the embryonic development acting on time of development, hatching success and energy allocation (Rombough, 2011; Bignami *et al*., 2014) displacing suitable spawning areas and eventually stopping the supply of new generations. Therefore, sensitive fish species with little acclimation capacity will experience a compression of the habitat, resulting in the shrinking of the

distribution areas and even to local extinctions at the southern limits (Pörtner, Bock and Mark, 2017). To study the relationship between mitochondrial sensitivity to OAW and acclimation capacity, this thesis compares the mitochondrial metabolism of the juveniles of two confamiliar fish species with different thermal preferences, the Arctic stenotherm polar cod (*Boreogadus saida*) and the boreal eurytherm Atlantic cod (*Gadus morhua*).

Since the same degree of stress can exert very different effects according to the life stage, this thesis analyses the effects of OWA also on developing embryos. For this purpose and to study the role of ecological differences in thermal tolerance, the selected species were Atlantic cod and Atlantic herring (*Clupea harengus*), two species with similar thermal preferences but different spawning behaviour.

1.4.1 Juveniles

Climate in the Arctic is changing more rapidly than in other areas, visible in a faster rise in temperatures and the effects of sea ice retreat as described in figure 1.j (IPCC, 2019). Moreover, PCO_2 levels in the Arctic Ocean are predicted to rise up to 1370µatm by the year 2100 (IPCC, 2014) due to increased gas solubility in cold waters.

Many fish species are shifting their distribution from boreal regions into the Arctic area as consequence of ocean warming (Fossheim *et al.*, 2015). One of these species is Atlantic cod, in particular its Northeast Arctic population (NEAC). NEAC is a boreal cold adapted eurytherm fish living mostly in the Barents Sea, experiencing temperatures reaching -1.5°C at the Atlantic – Arctic interface in winter and +11°C in summer when NEAC is moving to the Norwegian coast to spawn. Moreover, NEAC undergo a thermal variation of about 6°C during daily vertical migration (Righton *et al.*, 2010; Michalsen *et al.*, 2014). Despite distribution oscillations (Drinkwater, 2009), juvenile NEAC are observed in Svalbard waters year-round since 2008 (Renaud *et al.*, 2012), waters already inhabited by the native gadoid, polar cod (*Boreogadus saida*, Fig. 4).

Polar cod is a stenotherm species living in the Arctic waters with temperatures ranging between -2°C in winter and +8°C in summer (Drost, Carmack and Farrell, 2014). Because of the different widths of their thermal windows, it is important to understand the differences in the mitochondrial metabolism to shed light on the mechanism of thermal tolerance and thermal acclimation. This will help to understand the energetic constraints of acclimation to the new climate which may be reflected at the population level and eventually determine the success of one species and the displacement of the other.

1.4.2 Embryos

The second part of this thesis compares the mitochondrial metabolism during embryonic development of two co-occurring species (Atlantic cod and Atlantic herring). Atlantic cod is a pelagic spawner which lays buoyant eggs. The spawning events happen between March and May and spawning temperatures and sensitivity to elevated PCO_2 depend on population (Pörtner, 2008; Frommel *et al.*, 2012, 2013). For example, the spawning temperature ranges between 3°C for the Icelandic cod to 8°C for the North Sea cod. PCO_2 ranges from 410 µatm in the Lofoten spawning grounds to 2300 µatm in the Kiel Fjord (Frommel *et al.*, 2012, 2013).

Atlantic herring lays benthic eggs attached to the substrate. The spawning period and location is specific to season and population (Geffen, 2009). The sensitivity to elevated PCO_2 depends on population and spawning ground. For example, PCO_2 values up to 4600 µatm had no impact on hatch rate, development or otolith size in herring embryos from the Baltic sea (Franke and Clemmesen, 2011) while growth, development and tissue formation were already negatively affected by 1800 µatm CO_2 in larvae of the Norwegian population (Frommel *et al.*, 2014).

To understand how the ecology of the species could influence the mitochondrial metabolism during embryonic development and how this could impact the acclimation potential to the new climate conditions, the mitochondrial metabolism of embryos in the late developmental stages is studied and paralleled with indicators of whole animal fitness such as mortality, growth rates and hatching success obtained during the same experiment.

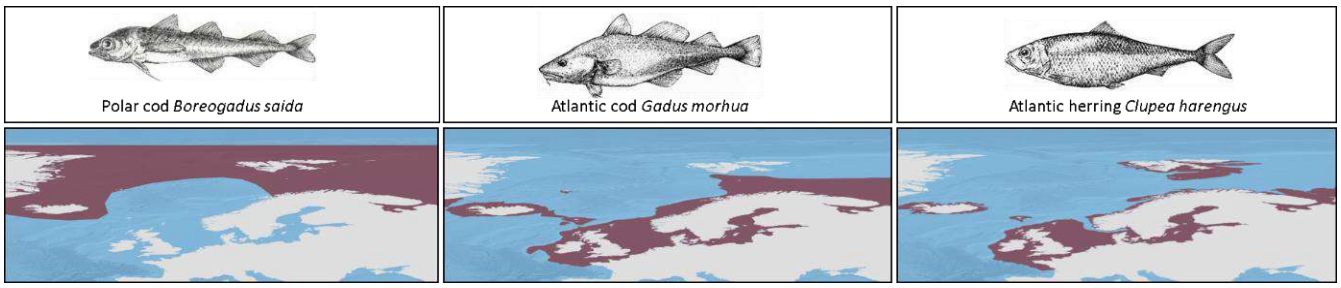


Figure 4:Species distribution of the three fish species considered in this thesis. From left to right: polar cod (*Boreogadus saida*), Atlantic cod (*Gadus morhua*), Atlantic herring (*Clupea harengus*). Maps from FAO, drawings by A. Bontempi.

1.5 Thesis objectives

This thesis aims to investigate the potential of metabolic adjustments of some marine fish species to ocean acidification and warming according to their latitude, life stage and spawning behaviour. In particular it focuses on the mitochondrial function, a key aspect in the aerobic metabolism.
This PhD project addresses the mechanistic differences and integrates the results up to the systemic level and intends to pursue its goal by asking the following three questions:

i. Does mitochondrial specialisation contribute to limit species ecology and response to climate change?

Thermal tolerance and acclimation capacity are highly dependent on the ecology and evolutionary history of a species. Stenotherm fish have very narrow thermal tolerance windows and lower acclimation capacity compared to eurytherm fish. Although many studies focused on the mitochondrial thermal tolerance of eurytherm fish (e.g. Fangue, Richards and Schulte, 2009; Iftikar and Hickey, 2013; Iftikar *et al.*, 2014) or highly stenotherm fish from Antarctica (Weinstein and Somero, 1998; Hardewig, Peck and Pörtner, 1999; Mark *et al.*, 2012; Martinez, Menze and Torres, 2013), only very few focused on the effects of temperature combined with high PCO_2, especially at ecologically relevant levels (Strobel et al., 2013a;b; Kreiss *et al.*, 2015a;b).
To target this question, publication I investigated the response of the mitochondrial phosphorylation system in the heart of a stenotherm fish (polar cod) and a cold eurytherm fish (NEAC) after 4 months incubation to temperatures and PCO_2 projected for the year 2100.

ii. Is the mitochondrial metabolism of developing embryos affected by ocean acidification and warming? How does the species' ecology relate to the response towards climate changes?

Early life stages are considered to be more sensitive towards temperature and CO_2 under many aspects (Chambers *et al.*, 2014; Di Santo, 2015; Pörtner, Bock and Mark, 2017; Dahlke *et al.*, 2018), however the role of mitochondria has never been taken into account in this context. This section investigates the mitochondrial thermal tolerance and the acclimation capacity toward OAW. To investigate if the ecology of the species could relate with the mitochondrial responses towards climate changes, Publication III describes the responses in the developing embryos of Atlantic herring, which attach their eggs on the sea

bed where they already experience broad PCO_2 and pH variations, while publication IV illustrates the responses of Atlantic cod, which produces buoyant eggs developing in a rather constant environment.

iii. Which are the mechanisms causing the different mitochondrial responses to ocean acidification and warming?

This section of the thesis aims to investigate the biochemical mechanisms responsible for the variation in sensitivity to increasing temperature and PCO_2 resulted in the previous sections.

Publication II analyses the cardiac tissue of polar cod and NEAC, and describes the capacity and thermal tolerance of key enzymes in the TCA cycle (CS, CII), in the Electron Transport System (CII, CI:CIII) and markers of the oxidative capacity (CCO) after long term incubation to the projected climate conditions. Moreover, the lipid class composition of the cellular membranes is analyzed to verify whether the acclimation process induces adjustments at the membrane level. Publication III and IV examine the modification of the contribution to ETS between CI and CII according to the incubation conditions in developing embryos of Atlantic herring and Atlantic cod.

2. Material and Methods

2.1 Experimental animals

The experiments conducted in this thesis focused on the tolerance and acclimation potential to ocean acidification and warming in fish living in the boreal and polar marine habitas.

The animals chosen for this thesis were polar cod (*Boreogadus saida*) to represent the Arctic area, Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*) to represent the boreal sector.

Polar cod is a member of the Gadidae family. It has a circumpolar distribution in the Arctic marked by the Polar Front in the Barents Sea (Hop and Gjøsæter, 2013), the fjords of Greenland (Christiansen *et al.*, 2016) and waters off Northern Labrador and Newfoundland (Markle, Scott and Scott, 1989) as southernmost limits. Water temperature preferences change according to life stage: during spawning the thermal range is narrow and above 0°C (1 – 2°C) (Hognestad, 1968). Pelagic juveniles avoid subzero habitat temperatures (4 – 7°C Olsen, 1962; 2 – 7°C Rass, 1968; 0.3 – 5.2°C Falk-Petersen et al., 1986). Adults commonly prefer subzero temperatures (>-1.5°C), but occasionally are found at up to 3.2°C (Falk-Petersen *et al.*, 1986).

Atlantic cod also belongs to the Gadidae family. It is a benthopelagic fish present in the North Atlantic and Arctic waters with temperatures ranging from -1 to 20°C (Drinkwater, 2005) Atlantic cod is sub-structured into several populations with different water temperature preferences (e.g. Mork *et al.*, 1985; Pogson, Mesa and Boutilier, 1995).

Atlantic herring is a fish from the Clupeidae family. It is benthopelagic with a distribution in the North Atlantic. It is widely distributed and divided into several populations according to their time and place of spawning (Geffen, 2009). Eggs and larvae are adapted to a broad range of temperature, light and hydrographic conditions.

The mitochondrial response to rising temperature and PCO_2 was measured in isolated cardiac tissue of juveniles via high-resolution respirometry measurements, enzyme activity and lipid composition, and in homogenates of developing embryos via respiration measurements to assess the sensitivity of fish towards these factors according to species and life stage.

2.1.2 Juveniles

On the 17th of January 2013, Polar cod juveniles were caught by bottom trawl in combination with a fish lift (Holst and McDonald, 2000) in the inner part of Kongsfjorden (Svalbard, 78° 97' N 12°51' E, Fig. 5) at 120 m depth and water temperatures of 2°C/3°C. They were transported to the facilities of the Tromsø Aquaculture Research Station, in Kårvik (Norway) and kept there at 3.3°C/3.8 °C until late April 2013. Then the specimens were transported to the aquarium facilities of the Alfred Wegener Institute (AWI) in Bremerhaven (Germany), where they were kept at 5°C, 32 PSU and ambient PCO_2 (400µatm) until the start of the incubation in June 2013.

In late August 2013, juvenile specimens of the Northeast Arctic population of Atlantic cod (NEAC) were collected in Rijpfjorden (80° 15.42' N 22° 12.89' E), Hinlopenstretet (79° 30.19' N 18° 57.51' E), and Forlandsundet (78° 54.60' N 11° 3.66' E) (Fig. 5) at 0/40 m depth and water temperatures of 3.5°C/5.5°C using a pelagic midwater trawl in combination with a fish lift (Holst and MacDonald, 2000) during RV Heincke cruise HE408. The fish were transported to the AWI facilities in Bremerhaven (Germany), where they were kept at 5°C, 32 PSU and ambient PCO_2 until the start of the incubation in May 2014.

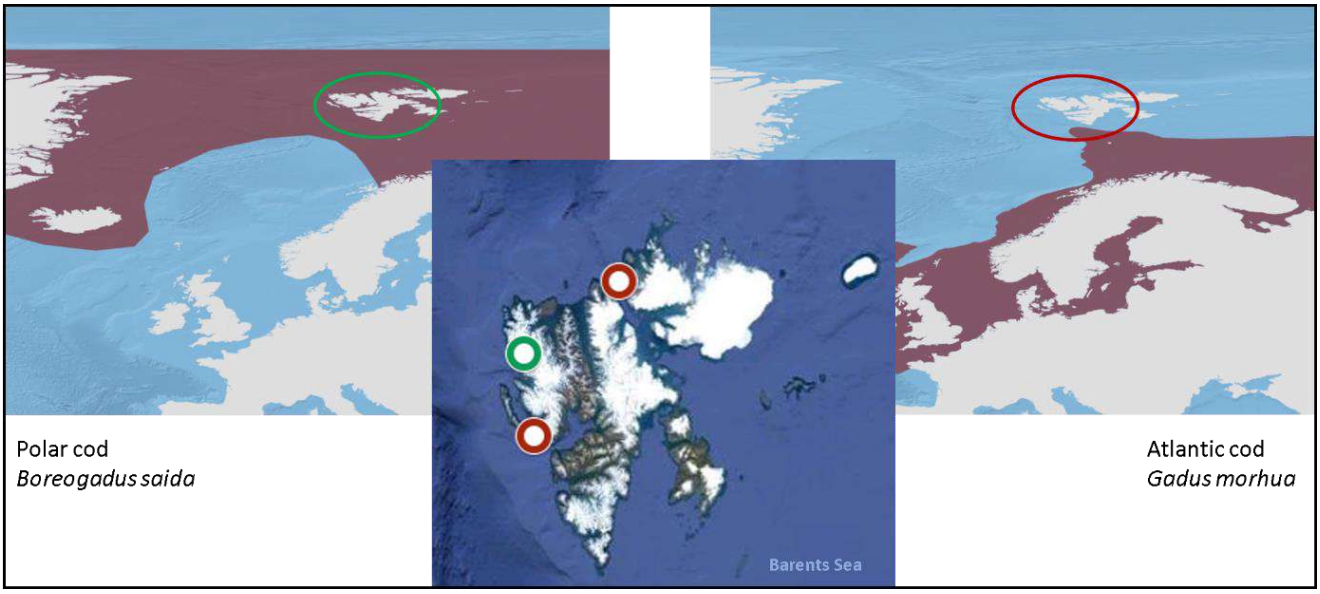


Figure 5: Distribution and sampling locations of polar and Atlantic cod. Map on the left: distribution of polar cod in the Eastern North Atlantic, Barents Sea and Arctic sea. Map on the right: distribution of Atlantic cod in the same areas.
Map in the center: Sampling points in the water around Svalbard for juvenile polar cod (green dot) and Atlantic cod, NEAC population (red dots).

2.1.2 Embryos

Adult Atlantic cod from the North Sea and Skagerrak population were collected in the Øresund Strait (55° 58' N 12° 38' E; Fig. 6) at 20/25m depth and water temperatures of 4.5-5°C by trawl and hand-gear during the spawning season in March 2013. Males (37– 68 cm; n = 12) and females (48–87 cm; n = 3) were strip-spawned to obtain gametes for *in vitro* fertilization. Fertilization was conducted at the Sven Lovén Centre of Kristineberg (Sweden) within 30 min after stripping in filtered (0.2 µm) and UV-sterilized seawater (salinity: 33 PSU) at ambient water temperature (4.5 ± 0.5°C) and two different seawater PCO_2 (present-day: 400 µatm, and future: 1100 µatm according to RCP8.5). The egg batch extracted from each female was split into two equal portions to be fertilized at the two different PCO_2 and was fertilized with milt aliquots from three to five males with a standardized milt–seawater dilution of 1:500 (Trippel, 2003). The fertilization process was stopped after 10 min by rinsing excess milt and ovarian fluid with filtered and sterilized seawater (Butts, Trippel and Litvak, 2009).

Adult Atlantic herring were caught in the inner Oslo Fjord close to the Biological Station Drøbak, University of Oslo (59˚40' N 10˚36’ E.; Fig.6) at 30 m depth during the spawning season in April 2013 with gill nets. Selected ripe fish were collected and killed with a blow on the head and were stored on ice to be transported to the Sven Lovén Centre of Kristineberg (Sweden). Upon arrival, males (n = 3) and females (n = 3) were stripped to obtain gametes for *in vitro* fertilization (approx. four hours after the fish had been caught). The adhesive eggs of each female were stripped onto plates of Polyethylene mesh (500 µm mesh size, 10 cm diameter) and posed in single layer to optimize fertilization success and oxygenation during development. The fertilization followed a wet-fertilization protocol (Geffen, 1999) in filtered (0.2 µm) and UV-sterilized seawater (salinity: 33 PSU) pre-adjusted at the desired combination of temperature and PCO_2. Individual egg-plates were placed in Petri dishes and fertilized with milt aliquots of three males with a 1:500 dilution with seawater (Trippel, 2003). The incubation was ended after 10 min by careful rinsing with seawater.

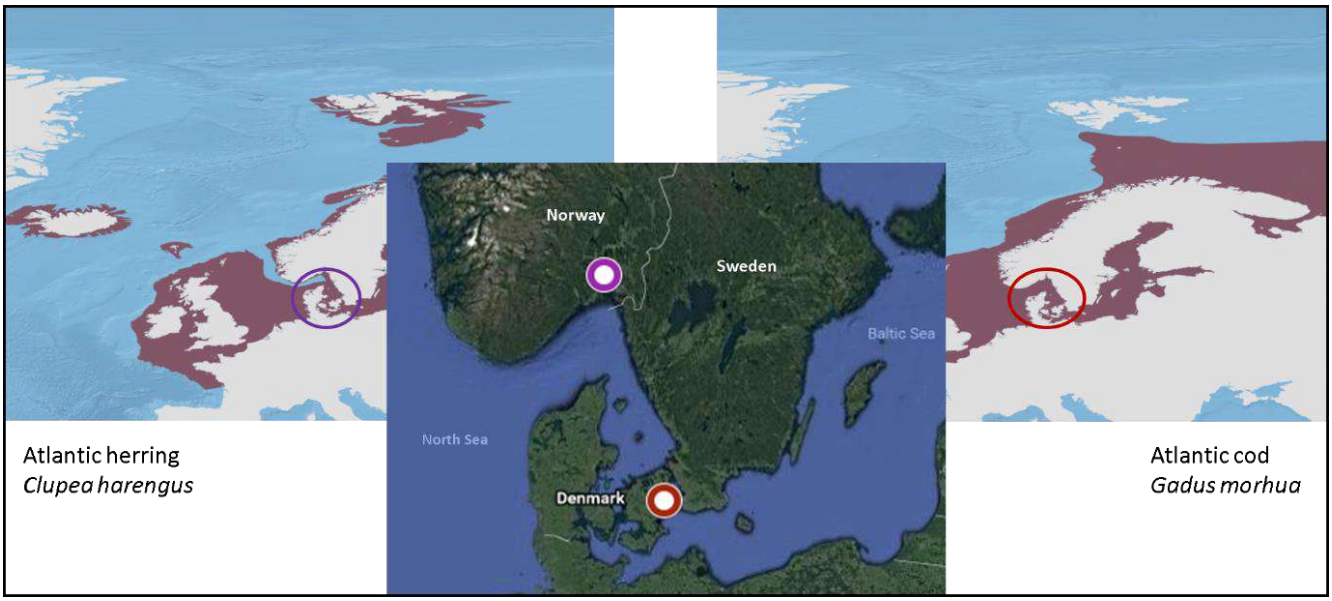


Figure 6: Distribution and spawning adults sampling location of Atlantic herring (purple dot) and Atlantic cod (red dot). The map on the left indicates the distribution area of Atlantic herring in the Eastern North Atlantic and Barents Sea, the map on the right describes the distribution of Atlantic cod in the same sector. The map in the center indicates the sampling points in the Oslo Fjord and Øresund for respectively spawning Atlantic herring (purple dot) and Atlantic cod (red dot).

2.2 Acclimation setup

2.2.1 Juveniles

All the experiments on juvenile fish were conducted in accordance with the ethical permission number AZ522-27-22/02-00 (113) released by the Senator for Healthcare, Bremen (Germany).

The incubation of polar cod and NEAC started in June 2013 and in May 2014, respectively. After at least four weeks of acclimation to laboratory conditions (seawater: 5°C, 32 PSU and ambient PCO_2), individuals from both species were randomly allocated to the single tanks assigned to each temperature and PCO_2 treatment (Fig.7) with a 12:12 day/night rhythm. Table 1 depicts the temperatures and PCO_2 used for this experiment.

PCO_2 conditions were pre-adjusted in a header tank containing ~200 L of seawater: virtually CO_2-free pressurized air and pure CO_2 were mixed using a mass flow controller (4 and 6 channel MFC system, HTK, Hamburg, Germany) to achieve the desired PCO_2.

Temperature was modified by 1 °C per day starting from 5 °C and, for the high PCO_2 groups, the desired PCO_2 was adjusted within one day after the incubation temperature was reached. The animals were kept under these conditions for four months and fed *ad libitum* with commercial pellet feed (Amber Neptun, 5 mm, Skretting AS, Norway) every fourth day (Kunz *et al.*, 2016, Publication AI).

In order to monitor the seawater chemistry over the entire incubation time, temperature,

salinity, DIC and pH (total scale) were measured once to twice a week in triplicate. Temperature and salinity were measured with a WTW LF 197 multimeter (WTW, Weilheim, Germany). pH was measured with a pH meter (pH 3310, WTW, Weilheim, Germany) calibrated with thermally equilibrated NBS-buffers (2-point-calibration). The pH-values were then corrected to pH total scale using thermally equilibrated pH-defined Tris-Buffer (Batch 4, Marine Physical Laboratory, University of California, San Diego, CA, USA). DIC was measured by a Seal QuAAtro SFA Analyzer (800 TM, Seal Analytical, Mequon, United States of America). Calculations of the carbonate system were conducted using CO2sys (Lewis and Wallace, 1998), applying the K_1, K_2 constants after Mehrbach *et al.* (1973), refitted after Dickson and Millero (1987) and using $KHSO_4$ dissociation constants after Dickson (1990) assuming a pressure of 10 dbar. A summary of the measured seawater parameters is presented in table A.1 of the Appendix while a full list of water chemistry raw data is given in PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.866369).

Sampling of polar cod and NEAC took place after 4 days of fasting. The specimens were anaesthetized with 0.2 g L^{-1} tricaine methane sulphonate (MS222) and killed by a spinal cut behind the head plate. Hearts of half of the fish were rapidly excised and washed with ice-cold modified relaxing buffer BIOPS (pH 7.4 at 4°C, 380 mOsmol L^{-1}, see Table A3 of the Appendix for the compounds list; modified after Gnaiger *et al.*, 2000) to be analysed with high resolution respirometry as permeabilized fibers. Hearts of the second half were quickly excised and frozen in liquid nitrogen and stored at -80°C until further usage for enzyme and lipid extraction. Because of a failure in the power supply part of the polar cod group incubated at 3°C and high PCO_2 died before the sampling for the high resolution respirometry could be performed. Table 2 summarizes the number of individuals per treatment and the mean length and weight at the time of sampling.

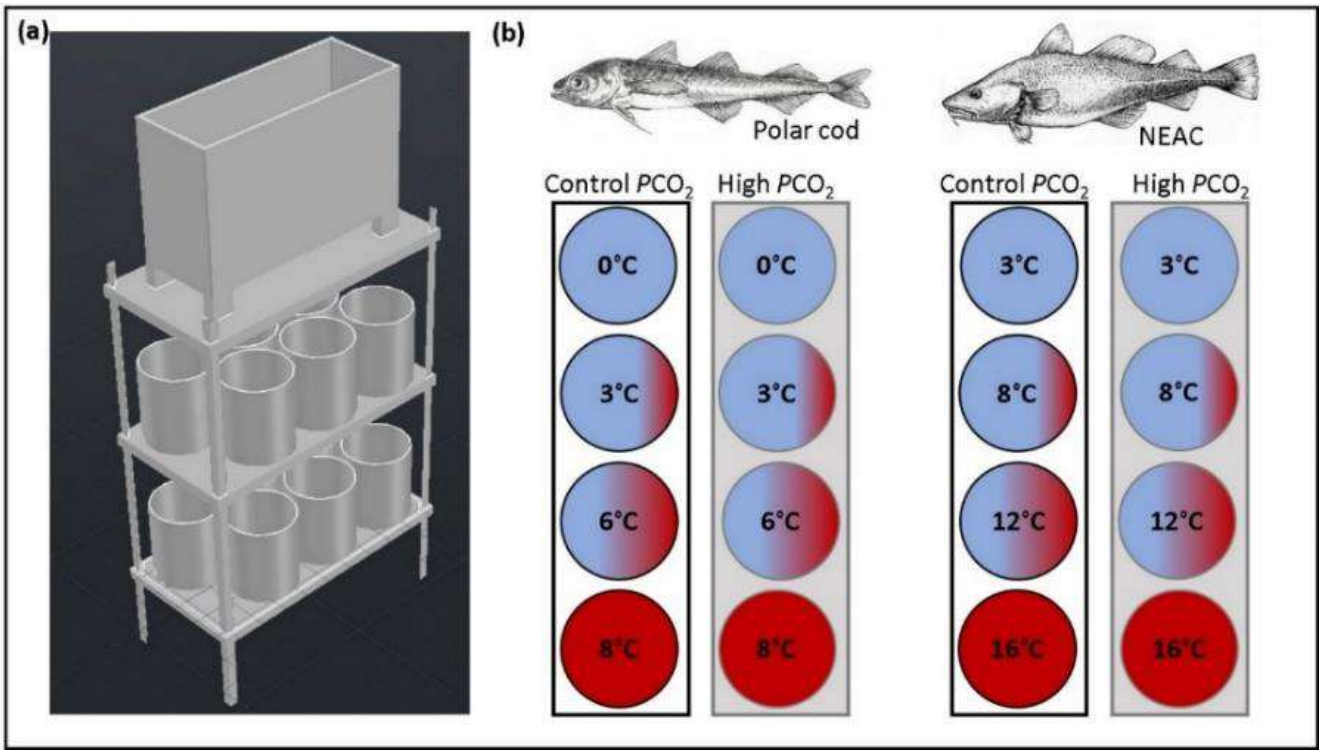


Figure 7: Acclimation set up for juvenile polar cod and NEAC. Panel a: rendering of an acclimation shelf. One shelf constitutes one acclimation unit in terms of temperature and PCO_2. Each tank on the shelf contains one single fish. Panel b: series of temperatures and PCO_2 used for the acclimation according to the species. Each circle represents one acclimation shelf. Control PCO_2:400 µatm, high PCO_2: 1100 µatm.

Table 1: Nominal temperature and PCO_2 treatments used for the incubation of polar cod and NEAC.

	Temperature	PCO_2	
Polar cod	0°C	400 µatm	1170 µatm
	3°C	400 µatm	1170 µatm
	6°C	400 µatm	1170 µatm
	8°C	400 µatm	1170 µatm
	Temperature	PCO_2	
NEAC	3°C	400 µatm	1170 µatm
	8°C	400 µatm	1170 µatm
	12°C	400 µatm	1170 µatm
	16°C	400 µatm	1170 µatm

Table 2: Total length (cm), body weight (g) and sample size (n) at the end of the acclimation period of the specimens used for assessing mitochondrial respiration in permeabilized cardiac fibers of polar cod and NEAC. "control" and "high" indicate control (400 µatm) and high (1170 µatm) CO_2 concentrations. Values are presented as mean ± S.E.M. Number of replicates differs due to occurred natural mortality while the 3°C/high PCO_2 group for polar cod died before sampling due to technical failure of the acclimation system.

	Acclimation	Total length (cm)	Body weight (g)	Sample size (*n*)
Polar cod	**0°C** control	15.28 ± 0.37	22.88 ± 2.05	5
	0°C high	14.30 ± 0.64	19.22 ± 2.61	6
	3°C control	15.62 ± 0.98	27.16 ± 6.25	3
	3°C high	-	-	-
	6°C control	15.73 ± 0.21	25.21 ± 1.14	6
	6°C high	17.52 ± 0.61	32.17 ± 2.90	5
	8°C control	15.18 ± 0.72	20.52 ± 2.56	6
	8°C high	15.07 ± 0.47	18.76 ± 1.11	4
	Acclimation	Total length (cm)	Body weight (g)	Sample size (*n*)
NEAC	**3°C** control	20.04 ± 0.92	60.84 ± 9.81	5
	3°C high	21.61 ± 0.46	78.19 ± 6.91	8
	8°C control	23.26 ± 1.75	99.04 ± 22.13	5
	8°C high	21.51 ± 0.82	80.51 ± 10.46	8
	12°C control	22.70± 0.80	98.70 ± 13.14	6
	12°C high	23.42 ± 0.72	100.75 ± 9.22	8
	16°C control	21.56 ± 0.69	81.48 ± 9.37	4
	16°C high	24.27 ± 1.91	133.13 ± 31.87	6

2.2.2 Embryos

All the experiments done on fish embryos were conducted in accordance with the ethical permission number 332-2012 released by the Swedish Board of Agriculture.

Fertilized Atlantic cod free floating eggs were transferred into airtight Polyethylene containers until the start of the incubation experiment. The containers were filled with seawater adjusted at the same PCO_2 and temperature conditions used during fertilization. 3 to 6 h after fertilization the eggs were split into five different incubators with different temperatures (0, 3, 6, 9 and 12°C) and incubated at present-day or future PCO_2 (according to the fertilization conditions, Tab. 3; Fig.8) with a 12:12 day/night rhythm until hatching as shown in figure 10. Atlantic herring eggs were treated in the same way but, due to their adhesive nature, were attached to mesh-plates prior fertilization. The plates were then placed into incubation cones and submerged in water baths thermostatted to 6, 10 and 14°C (Tab. 3, Fig. 9, 10).

In detail, the incubators (Imhoff sedimentation cones, 1000 ml volume) filled with 6°C filtered (0.2 µm) and UV-sterilized seawater (400/1100 µatm CO_2, 33 PSU) were stocked with eggs at a density of 0.35–0.5 eggs mL^{-1}. Afterwards, the incubators were submerged into 400L seawater baths thermostatted to different temperatures (0, 3, 6, 9, 12°C for Atlantic cod and 6, 10, 14°C for Atlantic herring) to ensure a gradual temperature change inside the incubator. Every 24 h 80-90% of the water volume of each incubator was replaced by filtered and UV-sterilized seawater to avoid oxygen depletion and bacterial or fungal infestation. In order to maintain constant water temperature in the 400-L baths, thermostats and recirculating pumps (40 L min^{-1}) were installed and the water temperature was recorded automatically every 15 min by a multichannel aquarium computer (IKS-Aquastar; IKS Systems, Karlsbad, Germany). Future PCO_2 conditions (1100 µatm) were obtained by injecting pure CO_2 gas into the submerged 60-L reservoir tanks at each temperature. A multichannel feedback system (IKS-Aquastar) connected to individual pH probes (IKS-Aquastar) was used to adjust water pH and PCO_2 values. A schematic of the incubation system is given in figure 10. The PCO_2 of the reservoir tanks was measured prior to every third water exchange with an infrared PCO_2 probe (Vaisala GM7; Vaisala, Vantaa, Finland). Before the daily water exchange, pH values of the reservoir tanks were measured with a pH electrode (Mettler Toledo InLab Routine Pt 1000 with temperature compensation, Mettler Toledo, Columbus, OH, USA), which was connected to a WTW 3310 pH-meter calibrated with NBS buffers (three-point calibration). To convert NBS to the free proton concentration scale for seawater pH (Waters and Millero, 2013), the electrode was recalibrated with Tris–HCl seawater buffers (Dickson et al., 2007), which were acclimated to the corresponding incubation temperature prior to each measurement. Values of the water chemistry for this experiment are presented in PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.884124) and summarized in Table A2 of the Appendix.

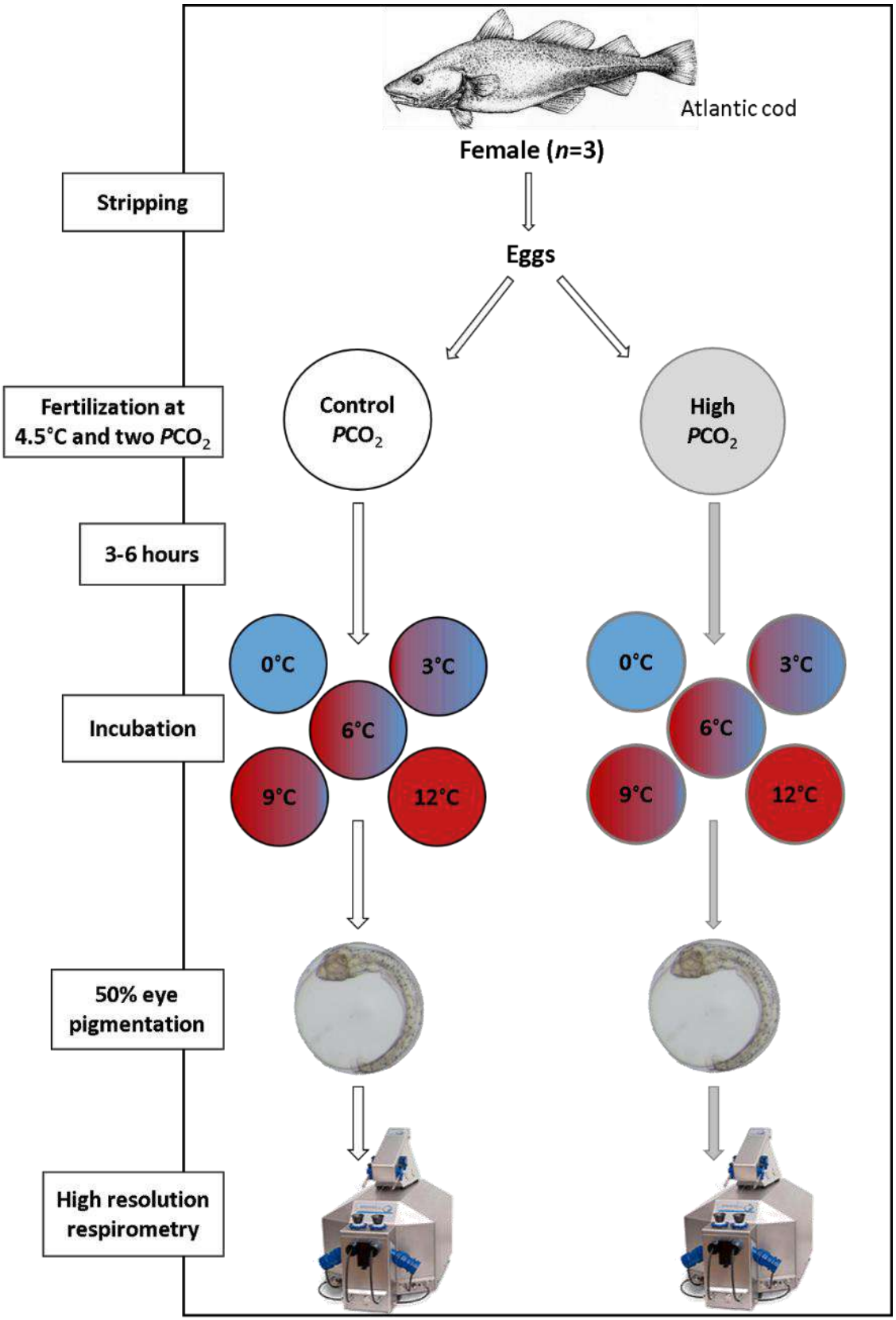


Figure 8: Acclimation design for Atlantic cod embryos. Adult Atlantic cod were stripped to obtain gametes for *in vitro* fertilization. The egg batch obtained from each of the three females was split in two to be fertilized at different conditions (4.5°C/400µatm PCO_2 or 4.5°C/1100µatm PCO_2). After 3-6h, fertilized eggs were transferred into five different incubators with different temperature (0, 3, 6, 9 and 12°C) and following the PCO_2 treatment at which were fertilized. At "50% eye pigmentation" developmental stage, about 100 eggs from each incubator were sampled, homogenized and the mitochondrial activity was measured by high resolution respirometry.

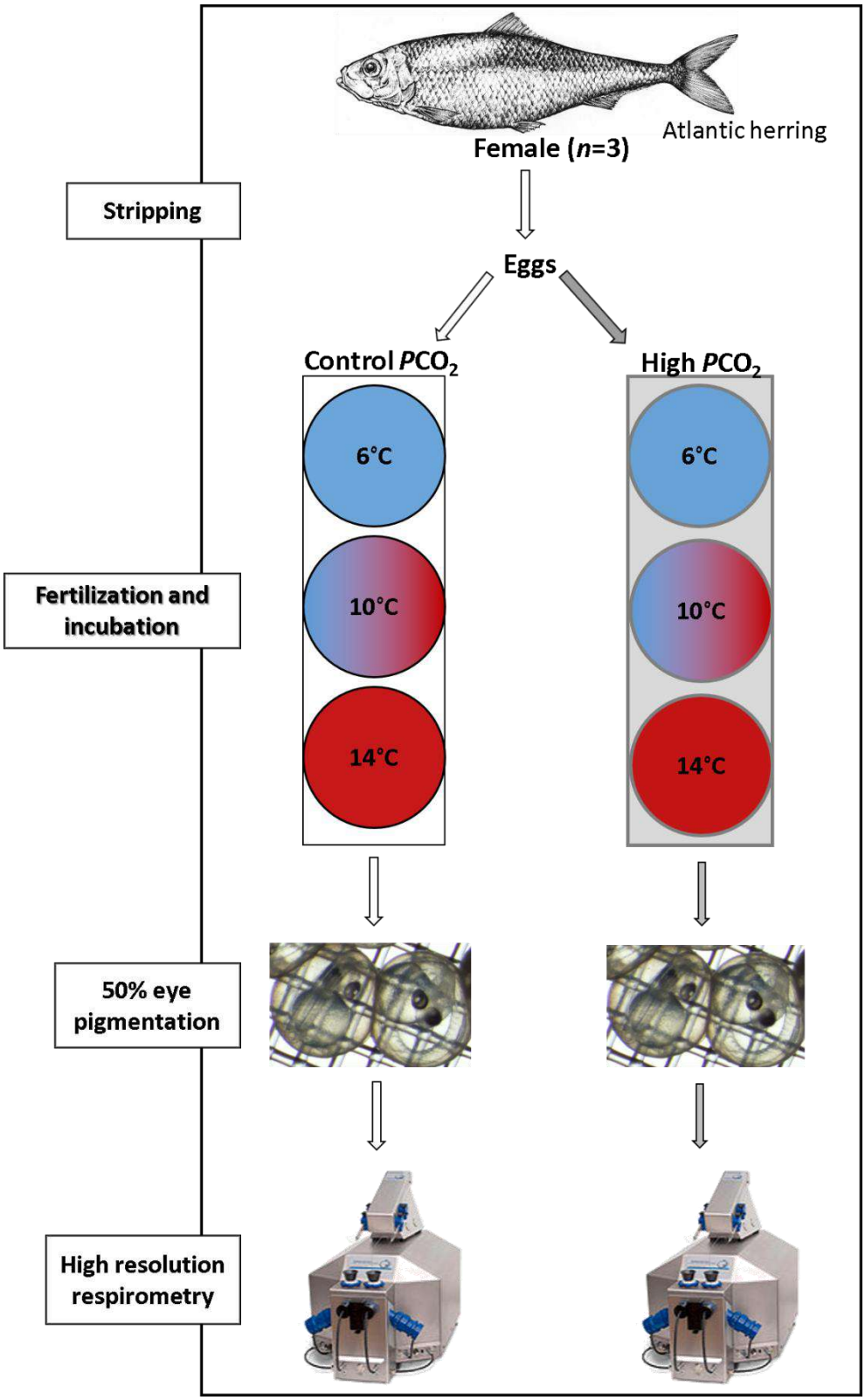


Figure 9: Acclimation design for developing embryos of Atlantic herring. Adult Atlantic herring were caught and stripped to obtain gametes for *in vitro* fertilization. The eggs of each of the three females were stripped onto mesh plates and fertilized in seawater pre-adjusted at the desired combination of temperature and PCO_2 (6-10-14° and 400-1100 µatm). 3-6 h after fertilization, the mesh plates were transferred into incubation cones with the same water parameters used during the fertilization. Once they reached the "50% eye pigmentation" developmental stage, about 100 eggs from each incubator were sampled, homogenized and the mitochondrial activity was measured by high resolution respirometry.

Table 3: Nominal temperature and PCO_2 chosen for the incubation design of developing embryos of Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*). The number of spawning females (n = 3) was used as replicate.

	Temperature	PCO_2	
Atlantic cod	0°C	400 µatm	1100 µatm
	3°C	400µ atm	1100 µatm
	6°C	400 µatm	1100 µatm
	9°C	400 µatm	1100 µatm
	12°C	400 µatm	1100 µatm
	Temperature	PCO_2	
Atlantic herring	6°C	400 µatm	1100 µatm
	10°C	400 µatm	1100 µatm
	14°C	400 µatm	1100 µatm

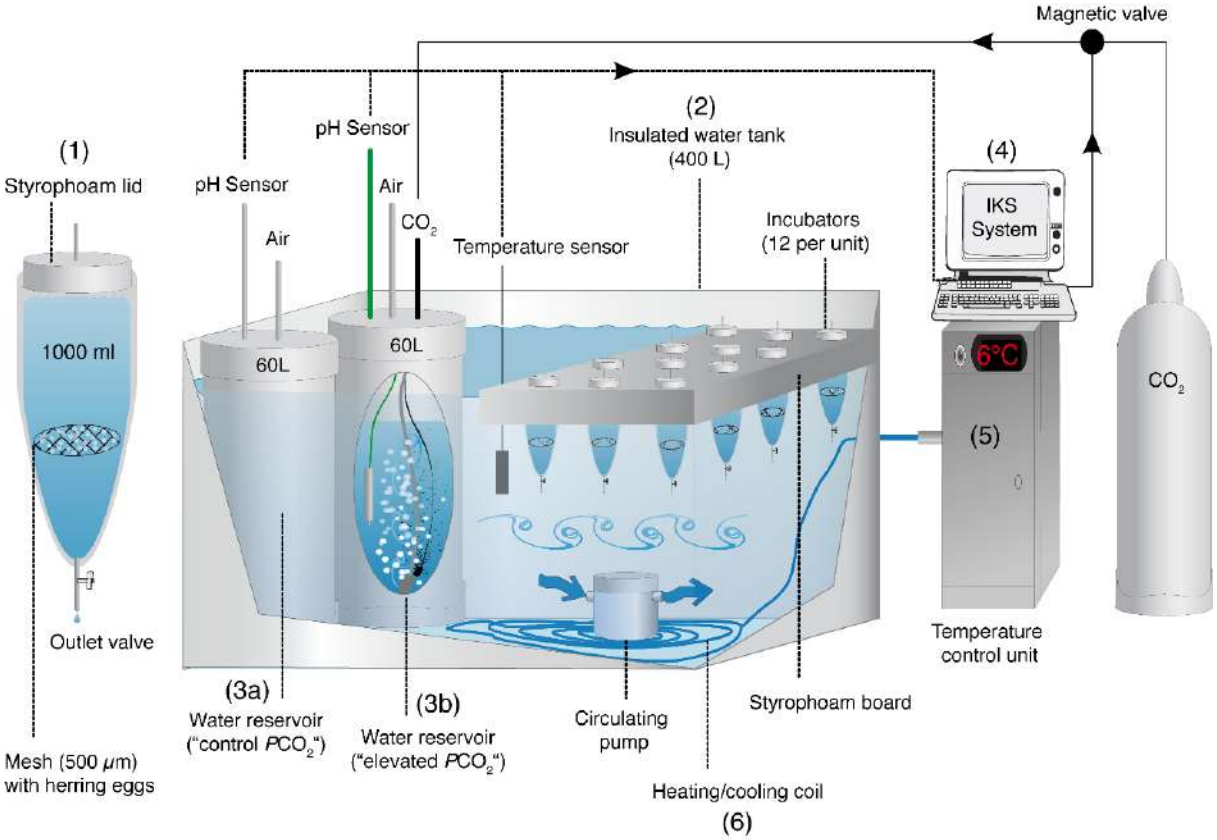


Figure 10: Incubation set up used for embryos of Atlantic cod and Atlantic herring. Fertilized eggs were placed into airtight Polyethylene cones **(1)**. The incubators were submerged into 400L seawater baths **(2)** thermostatted to different temperatures (0, 3, 6, 9, 12°C for Atlantic cod and 6, 10, 14°C for Atlantic herring). Thermostats **(5)** and recirculating pumps were installed to keep the water temperature constant. Future PCO_2 conditions (1100 µatm) were obtained by injecting pure CO_2 gas into the submerged 60-L reservoir tanks at each temperature **(3b)**. A multichannel feedback system (IKS-Aquastar) connected to individual pH probes (IKS-Aquastar) was used to adjust water pH and PCO_2 values **(4)**. Drawing by F. Dahlke

2.3 Sampling and Mitochondrial processing

2.3.1 High Resolution Respirometry

As a general definition, respirometry is based on the measurement of the change in oxygen concentration in a chamber using electrochemical sensors. The oxygen consumption of a biological sample measured as oxygen decline can then be plotted against time (Hütter *et al.*, 2006). High resolution respirometry provides a better resolution than conventional respirometry due to the development of more sensitive Clark-type polarographic oxygen sensors (POS) and electronics, minimization of oxygen diffusion using inert materials, special chamber design and online correction of background flux caused by the POS (Gnaiger, Kuznetsov, *et al.*, 1998; Hütter *et al.*, 2006).

In this thesis, mitochondrial respiration was measured by high resolution respirometry in juvenile permeabilized heart fibers and embryo homogenate using Oroboros Oxygraph-2 k™ respirometers (Oroboros Instruments, Innsbruck, Austria).

2.3.2 Permeabilized heart fibers from juveniles

Mitochondrial assays on cardiac tissue skinned heart fibers permeabilized with saponin have many advantages. Saponin is a detergent that binds selectively to membranes rich in cholesterol (e.g. plasma membrane) causing the formation of pores in the membranes while membranes with lower cholesterol content (e.g. mitochondrial) remain intact and functional. This form of permeabilization allows to equilibrate the cytosol of the cell with the surrounding respiration medium and thus enables instant experimental manipulation (Kuznetsov *et al.*, 2008). Moreover, the use of permeabilized fibers allows the mitochondria to stay in their cellular environment and interact with other cellular components, maintaining the functionality of all the respiratory complexes for several hours and requiring only small amounts of tissue (Sperl *et al.*, 1997; Saks *et al.*, 1998; Kuznetsov *et al.*, 2008).

After having been washed in the relaxing medium BIOPS (pH 7.4 at 4°C, 380 mOsmol L^{-1}, see table A3 of the Appendix for composition), hearts were mechanically divided in fibers and placed in 2 mL ice-cold BIOPS added with 50 µg mL^{-1} saponin and gently shaken on ice for 20 min. Fibers were then washed three times for 10 min in 2 mL ice-cold modified mitochondrial respiration medium MIR05 (pH 7.4 at 4°C, 380 mOsmol L^{-1}, see Table A3 of the Appendix for composition) (Gnaiger *et al.*, 2000; Iftikar and Hickey, 2013). Just before the start of the measurement, about 10 mg fibers were blotted dry, weighed and introduced into the Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria) sample chambers.

2.3.3 Embryo homogenate

Due to the limited amount of material and the difficulty to select a specific tissue in developing embryos, a homogenate of entire individuals was chosen to study mitochondrial function.

Embryos from Atlantic cod and Atlantic herring were homogenated once they reached the 50% eye pigmentation developmental stage (stage 22 according to Hall, Smith and Johnston, 2004). One hundred eggs were gently ground on ice in a glass potter filled with 2-mL ice-cold modified mitochondrial respiration medium MiR05 (pH 7.4 at 4°C, 380mOsmol L^{-1}, see Table A3 of the Appendix for composition) (Gnaiger *et al.*, 2000; Iftikar and Hickey, 2013). The resulting suspension was collected with care and filtered through a coarse mesh to prevent intake of eggshells. Subsequently, mitochondrial respiration was analysed using Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria).

2.3.4 Respirometry

Oxygen fluxes were calculated in real time using Oroboros DatLab Software 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria). Oxygen flux for permeabilized heart fibers was fiber weight specific (pmol O_2 (mg fresh weight * sec)$^{-1}$). For embryos the flux was corrected for the number of eggs included into the homogenate (nmol O_2 (egg * h)$^{-1}$). All measurements were run in MIR05 equilibrated at the respective incubation temperatures and PCO_2 at atmospheric levels (400µatm), with cO_2 ranging from ~370 nmol mL^{-1} (100% air saturation) to 100 nmol mL^{-1}.

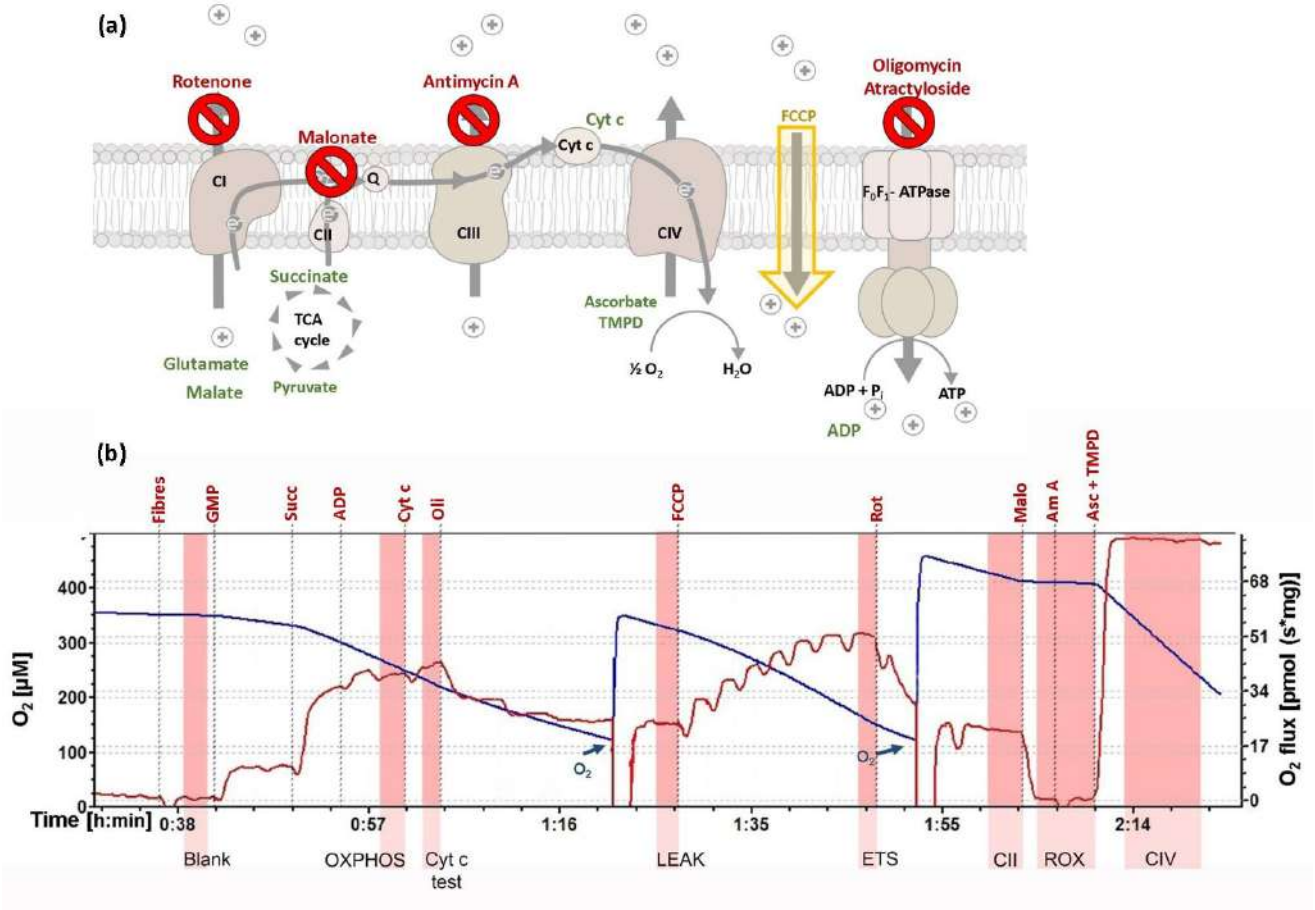
To assess the oxygen flux of specific part of the mitochondrial respiration and identify the contribution of the single components of the Electron Transport System (ETS), a substrate-uncoupler-inhibitor titration (SUIT, Fig. 11) protocol was applied (Gnaiger *et al.*, 2000).

In detail: first, a substrate combination that matches physiological intracellular conditions was applied in the absence of ADP. Glutamate and malate were added to stimulate Complex I (CI, NADH - Coenzyme Q oxidoreductase), then pyruvate and succinate were used to trigger Complex II (CII, Succinate dehydrogenase) and activate the convergent electron flow from Complex I and II into the Q-cycle.

Afterwards a series of manipulations started to reach the desired endpoints:

1) Saturating concentrations of ADP were added to stimulate oxidative phosphorylation (**OXPHOS**, State III) limited only by substrate supply and 2) the **Cytochrome c test** was performed to assess the integrity of the outer membrane. 3) **LEAK** (State IV^+) in the presence of high membrane potential was measured by addition of atractyloside or oligomycin (the former for polar cod and the latter for Atlantic

cod and herring) and 4) step-wise titration of carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) was used to uncouple mitochondria and assess the **maximum capacity of ETS**. 5) With the inhibition of Complex I by rotenone, the capacity of ETS is measured with the contribution of electrons from **Complex II** only into the Q-cycle. 6) The subsequent inhibition of Complex II and Complex III (CIII, Coenzyme Q – cytochrome c reductase) by the addition of malonate and antimycin a (respectively) was used to measure residual oxygen consumption (**ROX**) not coming from mitochondrial respiration. 7) At last, the **capacity of Complex IV** (CIV, Cytochrome c oxidase) was measured by the addition of the electron donor couple ascorbate and N,N,N1,N1-tetramethyl-p-phenylenediamine (TMPD). The



concentration of the chemicals used for the SUIT protocol is described in table A4 of the Appendix.

Figure 11: SUIT protocol. Substrates, inhibitors and uncoupler used to assess the mitochondrial functioning in permeabilized heart fibers of polar cod and NEAC and in embryo homogenate of Atlantic herring and Atlantic cod. Panel a depicts the targets of the chemicals used. Panel b exemplifies a typical DatLab output. The blue line represents the trend of the oxygen concentration, the red line illustrates the trend of the oxygen flux in permeabilized heart fibers. Dashed black lines indicate the addition points of the chemicals. Red squares highlight the sections used to calculate the mitochondrial respiration endpoints on the oxygen flux line.

2.4 Enzymatic assays

Capacities of enzymes were measured by spectrophotometry. Due to lack of material because of small heart size or high mortality in the acclimation group, the number of replicates was n = 4 to 6 for polar cod and n = 3 to 6 for NEAC

2.4.1 Tissue homogenate from juvenile heart

Portions of frozen heart samples (ca. 10 mg) were homogenized in 9 volumes (w:v) of ice-cold extraction buffer (20 mM Tris HCl, 1 mM Na-EDTA, Triton X-100 0.1%, pH 7.4 at 4°C) with a tissue homogenizer (Precellys 24, Bertin Technologies, France) at 5000 rpm and 4°C, 3 times for 15 sec. The obtained homogenate was then centrifuged at 1000 g and 4°C for 10 min. The supernatant was carefully collected and used to analyse the activities of four mitochondrial enzymes.
Enzyme activities of each sample were measured at 0-3-6-8°C (cuvette inner temperature) for polar cod and 3-8-12-16°C for NEAC using a UV/VIS spectrophotometer (Specord S600, Analytik Jena AG, Germany) equipped with a thermostatted cell holder.

Citrate synthase (CS) activity was measured as the increase in absorbance at λ =412 nm in a buffer containing 75 mM Tris-HCl pH 8.0, 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mM acetyl-CoA and 0.5 mM oxaloacetate according to the reaction:

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + \text{DTNB} + H_2O \rightarrow \text{Citrate} + \text{DTNB-S-CoA}$$

The increase in absorbance due to the formation of DTNB-S-CoA from DTNB and coenzyme A (CoA), was calculated using the extinction coefficient ε_{412}=13.61 $mol^{-1}*cm^{-1}$ (Sidell *et al.*, 1987).

Cytochrome c oxidase (CCO, CIV in ETS) activity was determined in a buffer containing 20 mM Tris-HCl pH 7.8, 0.057 mM cytochrome c_{red}, Tween 20 0.5%. The activity was calculated from the decrease in extinction at λ = 550 nm due to the oxidation of cytochrome c, using the extinction coefficient ε_{550} = 19.1 $mol^{-1}*cm^{-1}$ (modified after Moyes *et al.*, 1997) and following the reaction:

$$4\ \text{Cyt}\ c_{red} + O_2 + 4\ H^+ \rightarrow 4\ \text{Cyt}\ c_{ox} + 2\ H_2O$$

Succinate dehydrogenase (SDH, CII in ETS) buffer contained 100 mM Imidazole/HCl buffer pH 8.0, 5 mM $MgCl_2$, 20 mM succinate, 4 mM sodium azide, 0.04 mM antimycin A, 0.005 mM rotenone, 0.1 mM 2,6-dichlorphenolindophenol (DCPIP), 0.1 mM ubiquinone Q_1. The activity was determined following the reactions:

$$\text{Succinate} + Q \rightarrow \text{Fumarate} + QH_2 \qquad QH_2 + \text{DCPIP} \rightarrow Q + \text{DCPIPH}_2$$

and detecting 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.*, 2010a).

NADH:cytochrome c oxidoreductase (CI:CIII in ETS) was determined in a buffer containing 25 mM Imidazole /HCl buffer pH 7.4, 2 mM $MgCl_2$, 4 mM sodium azide, 0.08 mM cytochrome c_{ox}, 0.2 mM NADH following the reaction:

$$\text{NADH} + \text{Cyt } c_{ox} \rightarrow NAD^+ + \text{Cyt } c_{red}$$

The activity was calculated 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 Møller and Palmer, 1982).

Enzymatic activity was calculated using the Lambert-Beer law (2):

$$\text{Enzyme activity} = (A * V) / (\varepsilon * d * v * \Delta t) \qquad (2)$$

where A is the change in absorption over time (Δt), ε is the extinction coefficient, d is the cuvette path length, V is the total volume in the cuvette and v is the sample volume. The activity was expressed as U* mg fresh $weight^{-1}$.

Since CCO activity may indicate the amount of mitochondrial cristae and CS activity is supposed to be explicative of the mitochondrial matrix volume, the CCO/CS ratio helps to interpret the relationship between the mitochondrial inner membrane and the mitochondrial matrix space in the different acclimation groups. The CCO/CS ratio was calculated for each acclimation group only at the acclimation temperature.

The temperature coefficient (Q_{10}) of the enzyme activities was calculated according to equation (3) for the temperature range 0-6°C and 6-8°C for polar cod and 3-12°C and 12-16°C for NEAC.

(3) $$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$$

where R are the respiration rates (enzyme activities) and T the assay temperatures.

2.5 Lipid analysis from juvenile hearts

2.5.1 Lipid extraction

Portions of frozen hearts from the groups incubated at the lowest and highest temperatures (0°C and 8°C for polar cod n = 4 each group, 3°C and 16°C for NEAC n = 3 each group) and both PCO_2 treatments were used for this analysis. Membrane lipids were extracted using the method described by Folch, Lees and Stanley (1957). The samples were homogenized three times in a glass homogenizer with 6 ml dichloromethane:methanol (v 2:1) at 1200 U min^{-1} for two minutes. The homogenate was diluted with 2 ml of 0.88% KCl and centrifuged for five minutes at 1000 rpm to separate the apolar phase from the aqueous phase. The apolar phase containing the first batch of lipids was carefully removed and the aqueous phase was diluted again with 2 ml dichloromethane and centrifuged for five minutes at 1000 rpm for three times to fully extract the lipids from the aqueous phase. The lipid phase was then dried under nitrogen and the resulting debris was suspended in 50 µl chloroform.

2.5.2 Lipid class analysis

The lipid classes were separated and identified on a monolithic silica column (ChromolithPerformance-Si) using high-performance liquid chromatography (HPLC, LaChromElite HPLC system) with an evaporative light scattering detector (ELSD) according to Graeve and Janssen (2009). A gradient program with combination of three solvent mixtures was used:
eluent A: isooctane:ethylacetate (99.8:0.2, v/v),
eluent B: aceton:ethylacetate (2:1, v/v) with acetic acid (0.02% (v/v),
eluent C: 2-propanol:water (85:15, v/v) with acetic acid and ethanolamine, each 0.05% (v/v).
The analysis on membrane lipids revealed the presence of the following lipid classes: triacylglycerol, phosphatidylinositol, phosphatidylserine, cardiolipin, cholesterol, phosphatidylcholine and phosphatidylethanolamine.
The membrane composition was calculated as percentage of the single class over the total amount.

2.6 Data analysis

Normal distribution of the data was evaluated using Shapiro – Wilk test, homoscedasticity was evaluated by F-test (two groups) or Bartlett's test and Levene's test (more than two groups).

Mitochondrial respiration rates in permeabilized heart fibers were expressed as oxygen flux per mg fresh weight of cardiac fibers. For all the seven endpoints, differences across temperatures in the same PCO_2 treatment were evaluated with one-way ANOVA followed by Tukey's HSD test. Differences between PCO_2 treatments within the same temperature treatment were evaluated by Student's t-test (with Welch's correction in case of non-homoscedastic data).

Mitochondrial respiration in embryo homogenates is expressed as oxygen flux per number of eggs. Since preliminary two-way ANOVAs showed no significant effect of PCO_2 on mitochondrial functioning in Atlantic cod, data derived from the same female but different PCO_2 treatments were pooled together and the number of females was used as replicates. Subsequently, nonlinear regressions were used to describe the relationship between temperature and ETS (Complex I and Complex II) as well as temperature and OXPHOS coupling efficiency. In order to account for unequal variances, a generalized additive model (GAM) was applied to assess the effect of temperature on OXPHOS. For Atlantic herring, a two-way ANOVA with temperature and CO_2 as fixed factors. Female parent ID was used as a covariate. The effect between temperature levels was afterwards analyzed using Tukey's HSD test and between CO_2 levels using Student's t-test.

OXPHOS coupling efficiency was calculated according to equation (4) according to Gnaiger *et al.* (2015):

$$(\text{OXPHOS-LEAK}) * \text{OXPHOS}^{-1} \quad (4)$$

The enzyme activities were calculated as units per mg fresh-weight ($U*mg\ FW^{-1}$) of heart tissue. The differences in enzymatic activities were evaluated by two-way ANOVA followed by Tukey's HSD test and the temperature coefficient Q_{10} was calculated according to equation (3).

Preliminary Student's t-test (with Welch correction in case of non-homogeneous variances) highlighted equal lipid class compositions between PCO_2 treatments within the same temperature treatment. Therefore, data from different PCO_2 treatment were pooled according to temperature and only the differences between temperatures in the lipid class compositions were tested by Student t-test with Welch correction if needed.

All values shown in this thesis are provided as mean ± S.E.M. if not stated otherwise.

The level of statistical significance for all statistic tests was set at $p < 0.05$.

Data analysis was performed using R 3.2.0 (R Team, 2017).

2.7 Overview

An overview of the parameters measured in this thesis is given in table 4.

Table 4: Summary table of species, life stage, treatment and measured parameters used in this thesis and the respective publications.

Animal	Life stage	Treatment parameters	Measured parameters	Publication
Polar cod	Juvenile	Temp: 0 - 3 - 6 - 8°C PCO_2: 400 - 1100 µatm	OXPHOS, LEAK, ATP production efficiency	**I**
			Enzymatic capacity of CS, CI:CIII, SDH and CCO Membrane lipid composition	**II**
Atlantic cod (NEAC)	Juvenile	Temp: 0 - 3 - 6 - 8°C PCO_2: 400 - 1100 µatm	OXPHOS, LEAK, ATP production efficiency	**I**
			Enzymatic capacity of CS, CI:CIII, SDH and CCO Membrane lipid composition	**II**
Atlantic cod (Øresund)	Embryo	Temp: 0 - 3 - 6 - 9 - 12°C PCO_2: 400 - 1100 µatm	OXPHOS, ATP production efficiency, ETS, CI/ ETS, CII/ETS	**IV**
Atlantic herring	Embryo	Temp: 6 - 10 - 14°C PCO_2: 400 -1100 µatm	ETS, CI/ ETS, CII/ETS	**III**

3. Publications

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

Publication I

Elettra Leo, Kristina L. Kunz, Matthias Schmidt, Daniela Storch, Hans-O. Pörtner, Felix C. Mark (2017)

Mitochondrial acclimation potential to ocean acidification and warming of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*).

Frontiers in Zoology 14:21

The design of the experiments was developed by the candidate and the other co-authors. The candidate run the incubation together with the second and third author. The candidate performed the experiment and analysed the data. The candidate, together with the last author, wrote the manuscript which was revised by all the co-authors.

Publication II

Elettra Leo, Martin Graeve, Daniela Storch, Hans-O. Pörtner, Felix C. Mark (2020)

Impact of Ocean Acidification and Warming on mitochondrial enzymes and membrane lipids in two Gadoid species.

Polar Biology (doi: 10.1007/s00300-019-02600-6)

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

Publication III

Elettra Leo, Flemming T. Dahlke, Daniela Storch, Hans-O. Pörtner, Felix C. Mark (2018)

Impact of Ocean Acidification and Warming on the bioenergetics of developing eggs of Atlantic herring *Clupea harengus.*

Conservation Physiology (doi: 10.1093/conphys/coy050)

The study was planned by the candidate and the co-authors. The candidate performed the mitochondrial part of the experiment and the data analysis. The candidate wrote the manuscript together with the second author. The manuscript was revised my all the authors.

Publication IV

Flemming T. Dahlke, **Elettra Leo**, Felix C. Mark, Hans-O. Pörtner, Ulf Bickmeyer, Stephan Frickenhaus, Daniela Storch (2016)

Effects of ocean acidification increase embryonic sensitivity to thermal extremes in Atlantic cod, *Gadus morhua.*

Global Change Biology (doi: 10.1111/gcb.13527)

The candidate designed the experiment with the first, third, forth and last author. The candidate performed the mitochondrial part of the experiment and the data analysis of that part. The candidate wrote the manuscript in close cooperation with the first author. All the authors reviesed the final manuscript.

The candidate has also co-authored two more publications, included in the Appendix:

Publication AI

Kristina L. Kunz, Stephan Frickenhaus, Silvia Hardenberg, Torild Johansen, **Elettra Leo**, Hans-O. Pörtner, Matthias Schmidt, Heidrun S. WindischRainer Knust, Felix C. Mark (2016)

New encounters in Arctic waters: a comparison of metabolism and performance of polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*) under ocean acidification and warming.

Polar Biology (doi: 10.1007/s00300-016-1932-z)

Publication AII

Matthias Schmidt, Gabriele Gerlach, **Elettra Leo**, Kristina L. Kunz, Steffen Swoboda, Hans-O. Pörtner, Christian Bock, Daniela Storch (2017)

Impact of ocean warming and acidification on the behaviour of two co-occurring gadid species, *Boreogadus saida* and *Gadus morhua*, from Svalbard.

Marine Ecology Progress Series (571: 183–191)

PUBLICATION I

Mitochondrial acclimation potential to ocean acidification and warming of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*).

Elettra Leo, Kristina L. Kunz, Matthias Schmidt, Daniela Storch, Hans-O. Pörtner, Felix C. Mark

2017

Frontiers in Zoology 14:21

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Frontiers in Zoology

RESEARCH Open Access

Mitochondrial acclimation potential to ocean acidification and warming of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*)

Elettra Leo[1,2], Kristina L. Kunz[1,2,3], Matthias Schmidt[1,2], Daniela Storch[1], Hans-O. Pörtner[1,2] and Felix C. Mark[1*]

Abstract

Background: Ocean acidification and warming are happening fast in the Arctic but little is known about the effects of ocean acidification and warming on the physiological performance and survival of Arctic fish.

Results: In this study we investigated the metabolic background of performance through analyses of cardiac mitochondrial function in response to control and elevated water temperatures and PCO_2 of two gadoid fish species, Polar cod (*Boreogadus saida*), an endemic Arctic species, and Atlantic cod (*Gadus morhua*), which is a temperate to cold eurytherm and currently expanding into Arctic waters in the wake of ocean warming. We studied their responses to the above-mentioned drivers and their acclimation potential through analysing the cardiac mitochondrial function in permeabilised cardiac muscle fibres after 4 months of incubation at different temperatures (Polar cod: 0, 3, 6, 8 °C and Atlantic cod: 3, 8, 12, 16 °C), combined with exposure to present (400μatm) and year 2100 (1170μatm) levels of CO_2.
OXPHOS, proton leak and ATP production efficiency in Polar cod were similar in the groups acclimated at 400μatm and 1170μatm of CO_2, while incubation at 8 °C evoked increased proton leak resulting in decreased ATP production efficiency and decreased Complex IV capacity. In contrast, OXPHOS of Atlantic cod increased with temperature without compromising the ATP production efficiency, whereas the combination of high temperature and high PCO_2 depressed OXPHOS and ATP production efficiency.

Conclusions: Polar cod mitochondrial efficiency decreased at 8 °C while Atlantic cod mitochondria were more resilient to elevated temperature; however, this resilience was constrained by high PCO_2. In line with its lower habitat temperature and higher degree of stenothermy, Polar cod has a lower acclimation potential to warming than Atlantic cod.

Keywords: Arctic fish, RCP 8.5, Heart mitochondria, Mitochondrial capacity, Proton leak

Background

Ocean warming driven by anthropogenic CO_2 emissions influences the distribution of marine animals causing significant impacts on biodiversity and ecosystem structure [1, 2], such as local extinctions [3] and poleward migrations [4–6]. Fish (and other ectotherms) are particularly sensitive to fluctuations in temperature since their body temperature is in equilibrium with their environmental temperature [7]. Fish species distribution, in fact, is confined to a specific temperature window, due to the temperature dependency of physiological processes and to sustain maximal energy efficiency ([8] for review).

The increased CO_2 concentration in the atmosphere is one of the major causes for the global greenhouse effect and also causes a decrease in ocean pH, a phenomenon commonly known as ocean acidification [9]. High CO_2 partial pressure (PCO_2) is known to affect biological and physiological processes of marine organisms (e.g. [10–14]) and tolerances towards other stressors [15–17]. Moreover, high PCO_2 could provoke a narrowing of the thermal

* Correspondence: Felix.Christopher.Mark@awi.de
[1]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, Am Handelshafen 12, D-27570 Bremerhaven, Germany
Full list of author information is available at the end of the article

tolerance window of ectotherms, so that limits of its thermal acclimation capacity are met earlier [2, 18–21].

At the cellular level, exposure to high temperature can cause changes in the three dimensional structures of proteins, including the assembly states of multiprotein complexes and eventually protein denaturation and loss of activity [7]. Moreover, increasing temperatures can alter the cellular membranes packing order, which can cause changes in membrane-associated processes until a potential complete loss of function [22]. Furthermore, since cellular oxygen demand increases with increasing temperature, the production of mitochondrial reactive oxygen species (ROS) is likely to increase which can damage biological molecules, including lipids, proteins and DNA [23, 24]. Therefore, towards the upper limit of the thermal window, the cellular energetic costs for maintenance rise, increasing baseline energy turnover and allowing only for time-limited periods of passive tolerance. If high temperature persists over this period of passive tolerance, the costs of maintenance can only be covered at the expense of other functions such as growth and reproduction, decreasing the overall animal fitness [17]. Therefore, in light of ongoing ocean acidification and warming it is important to understand how fish respond to increasing habitat temperatures, their ability to adjust their thermal sensitivity and the role that high PCO_2 plays in thermal acclimation [2, 25].

The fish heart is highly aerobic and sensitive to temperature [26, 27]. Its capacity limits have been hypothesized to shape the warming-induced onset of sublethal thermal constraints in fishes [2, 28–31]. Recent studies have shown that high temperature leads to heart failure in various fish species like New Zealand triplefins and temperate and tropical wrasses [28, 29, 32, 33]. It was suggested that progressive impairment of several components of the mitochondrial function measured in permeabilised heart muscle fibres, such as oxidative phosphorylation (OXPHOS, respiratory state III), ATP production efficiency and the capacity of single complexes of the Electron Transport System (ETS) shape the temperature of heart failure (T_{HF}). High temperature changes the fluidity of mitochondrial membranes, which can entail increased proton leak through the inner membrane ([19] for review), resulting in decreased coupling ratios and causing decreased membrane potential [34, 35] and, as a consequence, inhibit the electrogenic transport of substrates, i. e. the transport of charged substrates like glutamate and malate that leads to the translocation of net charge across the membrane [36]. This indicates that mitochondrial metabolism is involved in functional constraints and thermal limitation of this tissue [28, 29, 32, 33]. Therefore, alterations in cardiac mitochondrial metabolism might lead to impaired cardiac energy turnover and, as a consequence, constraints in cardiac performance and ultimately affect the fishes' thermal sensitivity.

Although an extensive literature has been produced on the effects of temperature on fish cellular metabolism and mitochondrial function (e.g. [8, 33, 37] and the literature therein), only few studies have addressed the effects of moderately elevated PCO_2 on them [30, 38–41]. Moreover, as ocean warming and ocean acidification caused by high PCO_2 are two sides of the same coin, they must be considered in combination in order to draw ecologically realistic conclusions [17, 42, 43].

Ocean acidification and warming trends are projected to exert particularly strong effects in the Arctic. As one of the consequences, temperate species may become established in Arctic habitats (by poleward migration), potentially displacing resident taxa [1, 4, 6]. For example, in the past decade the Northeast Arctic population of Atlantic cod (*Gadus morhua*, NEAC) has expanded its range into the Barents Sea [44, 45], on the North-east Greenland shelf [46] and in the coastal waters around Svalbard, which are inhabited by native Polar cod (*Boreogadus saida*), a key species in this region [1, 47].

Polar cod is a permanently cold adapted Arctic fish (thermal habitat around Svalbard ranging from −2 to +7 °C [48, 49]) while NEAC is a cold acclimated sub-Arctic population of temperate Atlantic cod expanding into the Arctic (habitat thermal range around Svalbard: 0–8 °C [1, 50]). Cold-acclimated and -adapted fish are known to have elevated mitochondrial densities. Among cold adapted species, extreme stenotherms such as high Antarctic fish, have high densities but low mitochondrial capacities and low proton leak in aerobic tissues [37, 51–53]. This may result in the low maintenance costs derived by proton leak and narrow thermal windows of these species and, as a consequence, cause high sensitivity to ocean warming [53, 54]. On the other hand, eurythermal cold adaptation ensures mitochondrial function over a wider range of temperatures at lower mitochondrial densities and maximized capacities [53, 55]. As a permanently cold adapted fish, Polar cod may therefore not be able to adjust mitochondrial capacities during warming to a similar extent as NEAC, which apparently has a higher capacity to adjust to higher temperatures by decreasing mitochondrial densities and capacities and thereby developing the metabolic plasticity necessary to acclimate to new conditions [56]. The differences in thermal response and, in particular, the ability to acclimate to higher temperatures will play a central role for their interaction in a changing ecosystem.

Hence, the aim of this study was to investigate the acclimation potential of Polar cod *Boreogadus saida* and Northeast Arctic cod (NEAC) *Gadus morhua* exposed to water temperatures and PCO_2 projected for

the year 2100 in the Arctic i.e. 8 °C and 1170μatm PCO_2 (RCP 8.5 [57]). For a deeper understanding of the impact of ocean acidification and warming on the bioenergetics of the two species in relation to thermal tolerance, we further investigated mitochondrial function in the cardiac muscle of animals incubated for 4 months at four different temperatures (Polar cod: 0, 3, 6, 8 °C and Atlantic cod: 3, 8, 12, 16 °C), and two PCO_2 (400μatm and 1170μatm) in a cross factorial design. We used permeabilised cardiac muscle fibres to investigate a system resembling the living state as closely as possible [58–60], facilitating the extrapolation from measurements of cardiac mitochondrial capacities to their potential effects on the heart and eventually drawing conclusions on the effects of high temperature and high PCO_2 on the whole organism. Moreover, by analysing the mitochondrial function at the respective incubation temperature we could investigate the acclimation potential of the two species. We hypothesized that NEAC had higher thermal limits and a larger acclimation capacity than Polar cod and found accordingly that mitochondrial functions are constrained at lower temperatures in Polar cod than in NEAC. We discuss our results in light of the findings reported by Kunz et al. [61], who showed wider thermal windows for growth and standard metabolic rate (SMR) in NEAC than in Polar cod from the same acclimation experiment.

Methods

Animal collection

Juvenile Polar cod were collected by bottom trawl in combination with a fish lift [62] on January 17th, 2013 from the inner part of Kongsfjorden (Svalbard, 78° 97' N 12°51' E) at 120 m depth and a water temperature between 2 and 3 °C. They were kept at 3.3–3.8 °C in the facilities of the Tromsø Aquaculture Research Station, in Kårvik (Norway) until late April 2013 when they were transported to the aquarium facilities of the Alfred Wegener Institute (AWI) in Bremerhaven (Germany), where they were kept at 5 °C, 32 PSU and ambient PCO_2 until the start of the incubation.

Juvenile Northeast Arctic cod (NEAC) were caught in late August 2013 in several locations off Western Svalbard during RV Heincke cruise HE408 in Rijpfjorden (80° 15.42' N 22° 12.89' E), Hinlopenstretet (79° 30.19' N 18° 57.51' E), and Forlandsundet (78° 54.60' N 11° 3.66' E) at 0–40 m depth and water temperatures between 3.5 and 5.5 °C using a pelagic midwater trawl in combination with a fish lift [62]. The specimens were transported to the AWI facilities in Bremerhaven (Germany), where they were kept at 5 °C, 32 PSU and ambient PCO_2 until the start of the incubation.

Incubation

Polar cod incubation started in June 2013 and of NEAC in May 2014. After at least 4 weeks of acclimation to laboratory conditions (5 °C, 32 PSU and ambient PCO_2), individuals from both species were housed in single tanks and randomly allocated to the temperature and PCO_2 incubation set-up with a 12 h day/night rhythm. The respective PCO_2 conditions were pre-adjusted in a header tank containing ~200 l of seawater. Virtually CO_2-free pressurized air and pure CO_2 were mixed by means of mass flow controllers (4 and 6 channel MFC system, HTK, Hamburg, Germany) to achieve the desired PCO_2. Temperature was adjusted by 1 °C per day for each group starting from 5 °C. PCO_2 in the high PCO_2 group was adjusted within 1 day after the incubation temperature was reached. The animals were kept under incubation conditions for 4 months and fed *ad libitum* with commercial pellet feed (Amber Neptun, 5 mm, Skretting AS, Norway) every fourth day [61]. The sampling of Polar cod and NEAC took place after 4 days of fasting, due to sampling and experimental logistics three to six individuals of Polar cod and four to eight individuals of NEAC were sampled in one batch. Because of a failure in the power supply the group incubated at 3 °C and high PCO_2 died before the mitochondrial capacity could be investigated.

Average length and weight, as well as the number of the specimens per treatment at the time of sampling are given in Table 1.

CO_2 and carbonate chemistry

Temperature, salinity, DIC and pH (total scale) were measured once to twice a week in triplicates in order to monitor the seawater chemistry of the incubation. Temperature and salinity were measured with a WTW LF 197 multimeter (WTW, Weilheim, Germany). pH was measured with a pH meter (pH 3310, WTW, Weilheim, Germany) calibrated with thermally equilibrated NBS-buffers (2-point-calibration). The pH-values were then corrected to pH Total scale using pH-defined Tris-Buffer (Batch 4, Marine Physical Laboratory, University of California, San Diego, CA, USA).

DIC was measured by a Seal QuAAtro SFA Analyzer (800 TM, Seal Analytical, Mequon, United States of America). Calculations of the carbonate system were conducted using CO2sys [63], applying the K1, K2 constants after Mehrbach et al. [64], refitted after Dickson and Millero [65] and using KHSO4 dissociation constants after Dickson [66] assuming a pressure of 10 dbar.

Complete summaries of the seawater parameters and raw data for both species are available from the Open Access library PANGAEA [67, 68].

Table 1 Total length, body weight and number of fish (*n*) used for testing cardiac mitochondrial respiration in Polar cod (*B. saida*) and NEAC (*G. morhua*)

Acclimation	Species					
	B. saida			*G. morhua*		
	Total length (cm)	Body weight (g)	*n*	Total length (cm)	Body weight (g)	*n*
0 °C control	15.28 ± 0.37	22.88 ± 2.05	5	-	-	-
0 °C high	14.30 ± 0.64	19.22 ± 2.61	6	-	-	-
3 °C control	15.62 ± 0.98	27.16 ± 6.25	3	20.04 ± 0.92	60.84 ± 9.81	5
3 °C high	-	-	-	21.61 ± 0.46	78.19 ± 6.91	8
6 °C control	15.73 ± 0.21	25.21 ± 1.14	6	-	-	-
6 °C high	17.52 ± 0.61	32.17 ± 2.90	5	-	-	-
8 °C control	15.18 ± 0.72	20.52 ± 2.56	6	23.26 ± 1.75	99.04 ± 22.13	5
8 °C high	15.07 ± 0.47	18.76 ± 1.11	4	21.51 ± 0.82	80.51 ± 10.46	8
12 °C control	-	-	-	22.70 ± 0.80	98.70 ± 13.14	6
12 °C high	-	-	-	23.42 ± 0.72	100.75 ± 9.22	8
16 °C control	-	-	-	21.56 ± 0.69	81.48 ± 9.37	4
16 °C high	-	-	-	24.27 ± 1.91	133.13 ± 31.87	6

"control" and "high" indicate control (400μatm) and high (1170μatm) CO_2 concentrations. Values are given as means ± S.E.M

Preparation of permeabilised cardiac fibres

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 washed with ice-cold modified relaxing buffer BIOPS (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$, 20 mM taurine, 15 mM Na_2-phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES, 220 mM sucrose, pH 7.4, 380 mOsmol l^{-1}; modified after [69]). Hearts were then separated in fibres and placed in 2 ml ice-cold BIOPS containing 50 μg ml^{-1} saponin and gently shaken on ice for 20 min. Fibres were then washed three times for 10 min in 2 ml ice-cold modified mitochondrial respiration medium MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 160 mM sucrose, 1 g l^{-1} bovine albumine serum, pH 7.4, 380 mOsmol l^{-1}) [29, 69].

Directly before experimentation, a subsample of about 10 mg fibres was blotted dry, weighed and introduced into the oxygraph sample chambers.

Mitochondrial respiration

Mitochondrial respiration was recorded using Oroboros Oxygraph-2 k™ respirometers (Oroboros Instruments, Innsbruck, Austria) and measured as weight-specific oxygen flux [pmol O_2 (mg fresh weight sec)$^{-1}$] calculated in real time using Oroboros DatLab Software 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria).

All analyses were performed at the respective incubation temperatures, with cO_2 in a range from ~370 nmol ml^{-1} (100% air saturation) to 100 nmol ml^{-1} and PCO_2 at atmospheric levels.

A substrate-uncoupler-inhibitor titration (SUIT) protocol was used on the permeabilised cardiac fibres to investigate the partial contributions of the single components of the phosphorylation system [69]). NADH - Coenzyme Q oxidoreductase (Complex I, CI) and Succinate dehydrogenase (Complex II, CII) substrates (10 mM glutamate, 2 mM malate, 10 mM pyruvate and 10 mM succinate) were added. Saturating ADP (3 mM) was added to stimulate oxidative phosphorylation (OXPHOS). Cytochrome c (10 μM) was added to test the integrity of the outer membrane. Respiration state IV^+ was measured by addition of atractyloside (0.75 mM) or oligomycin (6 μM) (for Polar cod and NEAC respectively) and step-wise (1 μM each) titration of carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) was used to uncouple mitochondria (ETS). Complex I, Complex II and Coenzyme Q – cytochrome c reductase (Complex III, CIII) were inhibited by the addition of rotenone (0.5 μM), malonate (5 mM) and antimycin a (2.5 μM), respectively. Lastly the activity of the Cytochrome c oxidase (Complex IV, CIV) was measured by the addition of the electron donor couple ascorbate (2 mM) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mM).

All chemicals were obtained from Sigma-Aldrich (Germany).

Data analysis

Mitochondrial respiration rates are expressed per mg fresh weight of cardiac fibres and the values are given as means ± S.E.M. OXPHOS coupling efficiency was calculated as [(OXPHOS-State IV^+) $OXPHOS^{-1}$] after Gnaiger [70].

Normal distribution of the data was assessed by Shapiro-Wilk test and homoscedasticity was evaluated by F-test or Bartlett test in case of two or more groups, respectively. Differences between PCO_2 treatments within the same temperature treatment were evaluated by Student's *t*-test (with Welch's correction in case of non-homoscedastic data). Differences across temperatures in the same PCO_2 treatment were evaluated with one-way ANOVA followed by Tukey's test for the comparison of means.

The level of statistical significance was set at $p < 0.05$ for all the statistical tests.

All statistical tests were performed using R 3.2.0 and the "stats" package [71].

Results

The maximal oxidative phosphorylation capacity (OXPHOS) of permeabilised heart fibres of both species is shown in Fig. 1. In Polar cod, the groups incubated under control PCO_2 showed significantly lower OXPHOS flux in the 0 °C acclimated fish than in all further incubation groups (3 °C, $p = 0.007$; 6 °C, $p = 0.007$; 8 °C, $p = 0.001$). Mitochondrial respiration was at a similar level in the groups incubated at 3, 6 and 8 °C ($p > 0.05$). High PCO_2 levels did not affect OXPHOS, with no differences between the OXPHOS of the groups incubated at the two PCO_2 levels within a temperature treatment ($p > 0.05$). The groups incubated under high PCO_2 displayed fluxes that were similar at 6 and 8 °C ($p > 0.05$) but significantly higher than in the 0 °C incubated group ($p = 0.04$, Fig. 1a).

Temperature had a significant effect on the OXPHOS of NEAC, with fluxes increasing with incubation temperature (control PCO_2: $F = 4.74$, $p = 0.02$; high PCO_2: $F = 3.78$; $p = 0.02$, Fig. 1b). Moreover, the 16 °C/high PCO_2 incubated group showed a lower OXPHOS compared to the 16 °C/control PCO_2 group ($p = 0.03$). This resulted in a more evident plateauing of OXPHOS between 12 and 16 °C in the group incubated under high PCO_2. Comparing the two species, Polar cod had significantly higher OXPHOS capacities than NEAC at both 3 °C ($p = 0.01$, Fig. 1 blue box) and 8 °C (control PCO_2: $p = 0.04$; high PCO_2: $p = 0.04$, Fig. 1 red box).

In both species, state IV^+ was sensitive to temperature (Fig. 2): in Polar cod it remained unchanged in the groups incubated at 0, 3 and 6 °C ($p > 0.05$) but was significantly higher in animals incubated at 8 °C compared to the other incubation groups (6 to 8 °C/control PCO_2: $p = 0.01$; 6 to 8 °C/high PCO_2: $p = 0.04$) as shown in Fig. 2a. Quantifying State IV^+ as a percent fraction of OXPHOS, it was close to 20% and thus lowest in the 3 °C and 6 °C groups of *B. saida*, while at 0 and 8 °C the fraction of State IV^+ exceeded these values about two-fold as shown in Fig. 3.

In NEAC, State IV^+ increased along with incubation temperature (control PCO_2: $F = 5.96$; $p = 0.02$, high PCO_2: $F = 12.43$; $p < 0.001$) as depicted in Fig. 2b, however, State IV^+ increased under high PCO_2 at 8 °C compared to the group incubated under control PCO_2 at the same temperature ($p = 0.02$). Fractional values of State IV^+ in OXPHOS (Fig. 3) for the groups incubated under present levels of CO_2 revealed values close to 20% in the groups incubated to 3 and 8 °C and two-fold higher values after incubation to 12 and 16 °C. In the groups incubated under high PCO_2, State IV^+ of the group incubated at 8 °C showed values similar to the groups incubated to 12 and 16 °C (Fig. 3). In consequence, sensitivity to CO_2 varied with incubation temperature and

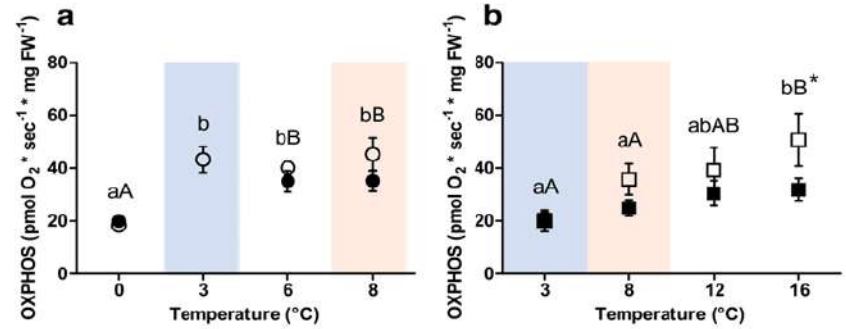

Fig. 1 Maximal oxidative phosphorylation capacity (OXPHOS) of permeabilised heart muscle fibres of (**a**) Polar cod (*B. saida*) and (**b**) NEAC (*G. morhua*). Different letters within panels indicate significant differences ($p < 0.05$) between temperature treatments; *lower case letters*: control PCO_2 (400μatm), *upper case letters*: high PCO_2 (1170μatm), * indicates significant differences ($p < 0.05$) between CO_2 groups at the same temperature. All values are reported as means ± S.E.M. (for *n* refer to Table 1). *Open symbols*: control PCO_2 (400μatm), *filled symbols*: high PCO_2 (1170μatm). *Circles*: Polar cod, *Squares*: NEAC. *Blue box*: cold shared incubation temperature (3 °C), *Red box*: warm shared incubation temperature (8 °C) between the two species

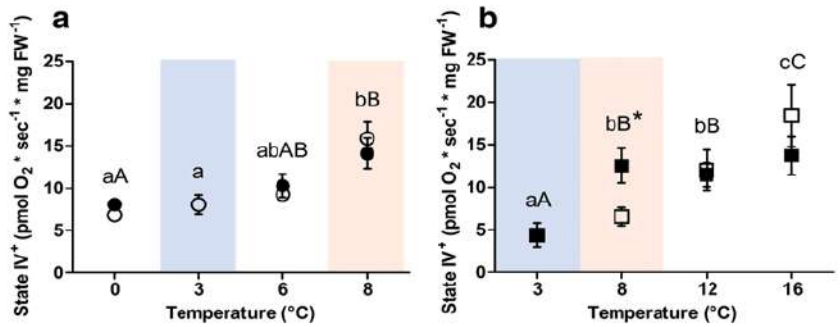

Fig. 2 State IV* of permeabilised heart muscle fibres of (**a**) Polar cod (*B. saida*) and (**b**) NEAC (*G. morhua*). Different letters within panels indicate significant differences (p <0.05) between temperature treatments; *lower case letters*: control PCO_2 (400μatm), *upper case letters*: high PCO_2 (1170μatm), * indicates significant differences (p <0.05) between CO_2 groups at the same temperature. All values are reported as means ± S.E.M. (for n refer to Table 1). *Open symbols*: control PCO_2 (400μatm), *filled symbols*: high PCO_2 (1170μatm). *Circles*: Polar cod, *Squares*: NEAC. *Blue box*: cold shared incubation temperature (3 °C), *Red box*: warm shared incubation temperature (8 °C) between the two species

was maximal but with opposite effects at 8 °C (stimulation of state IV$^+$ above controls) and 16 °C (depression of OXPHOS below controls at 16 °C).

OXPHOS coupling efficiency in Polar cod under control PCO_2 was maximal in the group incubated to 3 °C (0.82 ± 0.02), and decreased at 8 °C to values comparable to the 0 °C group (control PCO_2: 0.61 ± 0.03, high PCO_2: 0.58 ± 0.05), mainly because of increased State IV$^+$ at 8 °C (Fig. 2, 3 and 4). In NEAC (Fig. 4b), the OXPHOS coupling efficiency was maximal at 8 °C and control PCO_2 (0.81 ± 0.02) and minimal at 16 °C (0.64 ± 0.06). In the groups incubated under high PCO_2, the maximum of OXPHOS coupling efficiency fell to 3 °C (0.77 ± 0.03) and reached its minimum at 8 °C (0.46 ± 0.08) to rise again at 12 °C and 16 °C (0.58 ± 0.05 and 0.56 ± 0.05, respectively). However, these changes in OXPHOS coupling efficiency were not significant (control PCO_2: $F = 5.27$; $p = 0.82$, high PCO_2: $F = 9.7886$, $p = 0.072$). At 8 °C, the OXPHOS coupling efficiency was significantly lower under high PCO_2 than in the control PCO_2 group ($p = 0.003$). Comparing the OXPHOS coupling efficiency between the two species, NEAC and Polar cod showed similar values in the 3 °C/control PCO_2 group (Fig. 4 blue box) and at 8 °C/high PCO_2 (p >0.05, Fig. 4 red box), while the coupling efficiency was higher in NEAC incubated at 8 °C/control PCO_2 than in Polar cod incubated under the same conditions (p <0.001, Fig. 4 red box).

The thermal sensitivity of Complex IV also differed between the two species (Fig. 5). In Polar cod, Complex IV capacity rose from 0 to 6 °C (control PCO_2: $F = 67.29$, p <0.001) and decreased between 6 °C and 8 °C (control PCO_2: p <0.001). This trajectory was only present as a non-significant trend in the groups incubated under high PCO_2 ($F = 3.88$, $p = 0.10$) because of the non-significant decrease of the mean

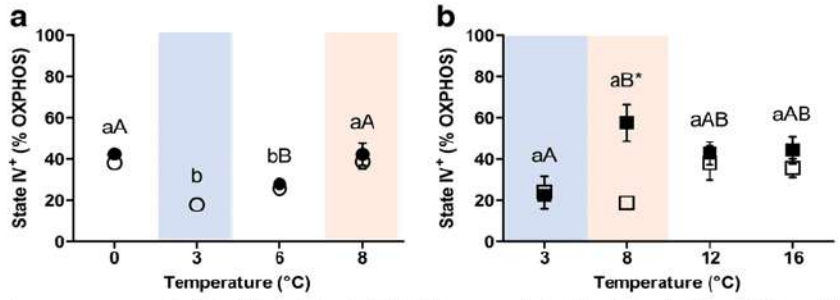

Fig. 3 Percentage of oxygen consumed by State IV$^+$ in relation to OXPHOS in permeabilised heart muscle fibres of Polar cod (*B. saida*, panel **a**) and NEAC (*G. morhua*, panel **b**). Different letters within the panels indicate significant differences (p <0.05) between temperature treatments; *lower case letters*: control PCO_2 (400μatm), *upper case letters*: high PCO_2 (1170μatm), * indicates significant differences (p <0.05) between CO_2 groups at the same temperature. All values are reported as means ± S.E.M. (for n refer to Table 1). *Open symbols*: control PCO_2 (400μatm), *filled symbols*: high PCO_2 (1170μatm). *Circles*: Polar cod, *Squares*: NEAC. *Blue box*: cold shared incubation temperature (3 °C), *Red box*: warm shared incubation temperature (8 °C) between the two species

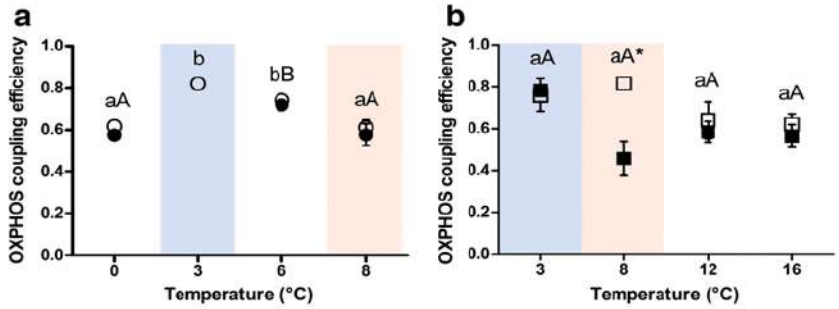

Fig. 4 OXPHOS coupling efficiency in permeabilised heart muscle fibres of (**a**) Polar cod (*B. saida*) and (**b**) NEAC (*G. morhua*). Different letters within panels indicate significant differences (p <0.05) between temperature treatments; *lower case letters*: control PCO_2 (400μatm), *upper case letters*: high PCO_2 (1170μatm), * indicates significant differences (p <0.05) between CO_2 groups at the same temperature. All values are reported as means ± S.E.M. (for n refer to Table 1). *Open symbols*: control PCO_2 (400μatm), *filled symbols*: high PCO_2 (1170μatm). *Circles*: Polar cod, *Squares*: NEAC. *Blue box*: cold shared incubation temperature (3 °C), *Red box*: warm shared incubation temperature (8 °C) between the two species

capacity of Complex IV at 6 °C/high PCO_2 compared to control PCO_2 at the same temperature ($p = 0.09$). In NEAC, Complex IV capacity increased with increasing temperatures in the groups incubated under control PCO_2 ($F = 3.25$, $p = 0.05$), but not in the groups incubated under high PCO_2 ($F = 2.18$, $p = 0.12$). At 16 °C, the capacity of NEAC Complex IV was lower under high PCO_2 ($p = 0.099$) than under control PCO_2. Comparing the two species, the capacity of Complex IV was similar (non-significant differences) in all shared treatments (3 °C/control CO_2, 8 °C/control CO_2 and 8 °C/high CO_2: p >0.05, Fig. 5 blue and red boxes).

Discussion

Our study shows differences in mitochondrial metabolism between a cold-adapted Arctic and a cold-acclimated sub-Arctic fish from the same area, potentially leading to differences in acclimation capacities to ocean acidification and warming.

Mitochondria from permeabilised heart fibres appeared to be affected mainly by the incubation temperature while high levels of CO_2 significantly affected mitochondrial respiration only in NEAC (*Gadus morhua*) and mainly at the highest investigated temperature (16 °C). NEAC OXPHOS and Complex IV capacities decreased under elevated CO_2 at high temperature, although the latter only as non-significant trend. This suggests that the noxious effects of high PCO_2 are stronger at the upper end of the thermal window and might affect the heat tolerance of NEAC [2, 17]. Furthermore, proton leak at 8 °C was higher in the group incubated under high PCO_2 than in the control PCO_2 group, indicating that overall mitochondrial efficiency might be affected through alterations of membrane characteristics. Elevated PCO_2 is reported to inhibit Citrate Synthase and

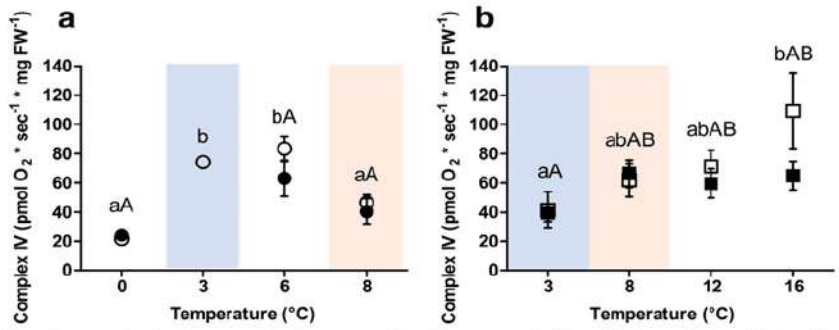

Fig. 5 Complex IV (Cytochrome c Oxidase) capacity. Panel **a**: permeabilised heart muscle fibres of Polar cod (*B. saida*). Panel **b**: permeabilised heart muscle fibres of NEAC (*G. morhua*). Different letters within the panels indicate significant differences (p <0.05) between temperature treatments; *lower case letters*: control PCO_2 (400μatm), *upper case letters*: high PCO_2 (1170μatm). All values are reported as means ± S.E.M. (for n refer to Table 1). *Open symbols*: control PCO_2 (400μatm), *filled symbols*: high PCO_2 (1170μatm). *Circles*: Polar cod, *Squares*: NEAC. *Blue box*: cold shared incubation temperature (3 °C), *Red box*: warm shared incubation temperature (8 °C) between the two species

Complex II in mammals and fish [40, 72, 73] with subsequent stimulation of the mitochondrial anaplerotic pathways to overcome this inhibition [40, 74]. The difference in sensitivity of the two species to elevated levels of CO_2 could be related to differences in preferential metabolic pathways, with Polar cod (*Boreogadus saida*) relying more than NEAC on anaplerotic pathways that feed directly into Complex I such as the oxidation of glutamate, pyruvate or palmitoyl carnitine [40, 73]. Further investigation, especially at the genetic level is needed. Furthermore, it is still unknown whether and to what extent elevated PCO_2 might alter the membrane characteristics and contribute to proton leak.

In Polar cod, OXPHOS of the groups incubated at 3-6-8 °C was higher at the respective incubation temperature than OXPHOS of the 0 °C treatments while the OXPHOS coupling efficiency was highest in the 3 °C group and lowest in the 0 and 8 °C groups. This indicates an optimum temperature for ATP production efficiency between 3 and 6 °C. At lower and higher temperatures, the increased proton leak in relation to OXPHOS created a less favourable ratio between ATP produced and oxygen consumed. These findings match those by Drost et al. [75], where heart rate of acutely warmed Polar cod increased until a first Arrhenius breakpoint at 3 °C. Heart rate still increased further but at a lower rate until 8 °C, passing a second break temperature. In our study, 8 °C corresponds to the highest rate of proton leak, and lowest Complex IV capacity, implying a direct participation of mitochondria in the thermal responses of the heart. The close similarity between the data from the acute study of Drost et al. [75], our 4-months incubation study and a study on behavioural thermal preference from Schurmann and Christiansen [76] indicates preferred temperatures of 3–6 °C within a thermal gradient from 0 to 8 °C for Polar cod, suggesting that Polar cod have only limited abilities to acclimate to higher temperatures.

In contrast, NEAC OXPHOS continued to increase with long-term incubation temperatures to even above those experienced within the natural habitat. This appears to occur without compromising OXPHOS coupling efficiency and reveals a higher acclimation potential than Polar cod, in line with the overall distribution area of Atlantic cod from temperate to (sub-) Arctic waters. This apparent plasticity is in line with the findings by Zittier et al. [77] in which NEAC specimens acclimated to 15 °C displayed critical temperatures (Tc, defined as the onset of the anaerobic metabolism, cf. Frederich & Pörtner [78]) about 10 °C higher than specimens kept at ambient temperature (4 °C). In Polar cod, the high proton leak at 8 °C is the main cause of reduced mitochondrial efficiency (OXPHOS coupling efficiency). This increase in proton leak can be caused by loss of membrane integrity in response to changes in membrane fluidity [7, 79]. In a previous study, Martinez et al. [80] found increased proton permeability of the inner mitochondrial membrane of the Antarctic silverfish *Pleuragramma antarcticum* after warming. In addition, Strobel et al. [40] found that this may be due to an unchanged saturation index of the mitochondrial membrane, observed in liver of the Antarctic *Notothenia rossii* after warm acclimation. These findings suggest a limited ability of Antarctic stenothermal fish to acclimate to temperature changes. Similar patterns may constrain acclimation of cold-adapted Arctic fish. The decreased capacity of Complex IV at 8 °C in Polar cod implies that the interactions between the inner membrane and embedded enzymes may also be affected by high temperatures [80, 81]. In NEAC, proton leak was lower than in Polar cod and reached 40% of OXPHOS at 12 °C, while in Polar cod the same relative values were found at 8 °C under control PCO_2. A strong thermal response of proton leak may reflect high temperature sensitivity of the organism [52, 82–84], and thus a higher baseline proton leak combined with its steeper increase upon warming may point towards a stronger degree of cold adaptation in Polar cod.

The findings in this study contrast earlier results obtained in isolated mitochondrial suspensions where mitochondria remained fully functional beyond whole organism heat limits [82, 85]. The present findings suggest that mitochondria may display wider thermal limits in suspensions than when embedded in permeabilised fibres. Mitochondria in permeabilised fibres may still interact with other cellular organelles and are thus integrated into a more complex system than are isolated mitochondria. These considerations suggest that thermal tolerance is more constrained in permeabilized fibres than in isolated mitochondria. Such findings may thus be in line with the assumed narrowing of thermal windows once molecular and mitochondrial functions are integrated into larger units up to whole organism [86]. While the experiment was carried out at non-limiting PO_2 in the media (>100 nmol ml^{-1}) [87], diffusion gradients of oxygen and/or other substances within the permeabilised cardiac fibres may cause this hierarchy in thermal constraints. In a study on growth, mortality and standard metabolic rates (SMR) of the same Polar cod and NEAC as examined in this study, Kunz et al. [61] found higher SMR in Polar cod than in NEAC at the same incubation temperatures. This is mirrored in the mitochondrial respiration presented in this study, where OXPHOS capacity in Polar cod was larger than in NEAC at both 3 and 8 °C. In Polar cod, the SMR of the 3 and 6 °C groups were lower than in the groups incubated at 8 °C, which is mirrored in the pattern of cardiac State IV^+ respiration. At 8 °C the OXPHOS coupling

efficiency (i.e. ATP production efficiency) decreased as State IV^{+} increased and the capacity of Complex IV decreased. Maybe these findings indicate decreased cardiac mitochondrial efficiency that may limit cardiac function and promote heart failure, which is consistent with a drop in cardiac function [75] and the onset of heart failure in Polar cod at 8 °C. At this temperature, oxygen demand and mortality increased, and growth decreased in this species [61]. In fact, the estimated decrease in ATP production efficiency at 8 °C was paralleled by a reduced feed conversion efficiency and concomitant increase in SMR. This likely indicates a shift in energy allocation due to an impaired balance between energy production and demand, e.g. due to increased mitochondrial proton leak (see [88] for review). According to these findings, 8 °C is close to the long-term upper thermal tolerance limit for the Svalbard population of Polar cod, which is again in line with the observed increased mortality [61].

In NEAC, the parallel rise of whole organism SMR and cardiac fibre OXPHOS and the parallel decrease of OXPHOS and SMR at high PCO_2 compared to controls at 16 °C indicates that cardiac mitochondrial function is adjusted to the level of whole animal energy demand at different incubation temperatures and that the effects of high PCO_2 are greatest close to the upper thermal limit. Thermal constraints setting in at whole animal level may again relate to the thermal sensitivity of cardiac mitochondrial function [28, 29, 32, 33]. The fact that first performance limitations are observed in the 16 °C/high PCO_2 incubation may not be of direct relevance for the Svalbard stock of NEAC over the next century, but marks a potential southern distribution limit for the Barents Sea and Norwegian Sea.

Polar cod is a cold adapted species and the constraint on cardiac mitochondrial metabolism at 8 °C, concomitant with increased mortality indicates that the animal's thermal window matches its current habitat temperature range. In contrast, adult NEAC show the ability to broaden their thermal window beyond the present sub-Arctic habitat temperatures (see above). Because of the habitat temperature range of the two species is similarly wide but shifted to lower temperatures in Polar cod, combined with the high metabolic baseline cost (SMR) of Polar cod the two species may be classified as cold-adapted (Polar cod) or cold-acclimated (NEAC) eurytherms. NEAC appear to be much more plastic than Polar cod, thus, Polar cod may be more vulnerable to future ocean conditions than NEAC.

Conclusions

Future ocean acidification and warming may impair cardiac mitochondrial function of Polar cod (*Boreogadus saida*) and Northeast Arctic cod (NEAC, *Gadus morhua*) in somewhat different ways. In Polar cod, high temperature (8 °C) increases proton leak and thereby decreases ATP production efficiency, while high CO_2 levels did not have a significant effect. In NEAC, mitochondrial respiration remained functional at higher temperatures, but capacity was depressed by the combination of high temperature and high PCO_2. Furthermore, in NEAC, incubation temperature leads to variable mitochondrial response patterns under elevated PCO_2. The causes of the different responses to elevated PCO_2 in the heart of these two species remain to be identified, for example, the role of anaplerotic pathways and their regulation should be further investigated.

As a result of the degree of cold adaptation, Polar cod display high metabolic maintenance costs (indicating that it is cold-eurythermal) and low acclimation capacity, while NEAC is cold acclimated and benefits from a lower rate of metabolism and a higher plasticity to acclimate to increasing temperature. As a consequence, mitochondrial function of NEAC hearts may be less constrained by rising temperatures than Polar cod, indicating that NEAC could outperform and possibly replace Polar cod in the waters around Svalbard if ocean warming and acidification further increase towards the conditions predicted for the end of the century (8 °C and 1170μatm PCO_2). Since Polar cod has a key role in Arctic ecosystems [48], temperature driven changes in the distribution of this species can be an important component in the impacts of climate change on Arctic ocean ecosystems.

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Availability of data and materials
The datasets analysed during the current study and the data regarding the incubation set-up are available from the Open Access library PANGAEA (www.pangaea.de; https://doi.pangaea.de/10.1594/PANGAEA.866369; https://doi.pangaea.de/10.1594/PANGAEA.873536).

Authors' contributions
EL, KK, MS, DS, HOP and FCM designed the study. EL, KK, MS carried out the animal incubations. EL performed the experiment on cardiac mitochondria and all data analyses and interpreted the results together with FCM. EL and FCM drafted the manuscript, KK MS DS and HOP contributed to writing the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
Experiments were conducted in accordance with the ethical permission number AZ522-27-22/02-00 (113) released by the Senator for Healthcare, Bahnhofsplatz 29, 28195 Bremen on February 21st, 2013 and valid until February 21st, 2017.

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Author details
[1]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, Am Handelshafen 12, D-27570 Bremerhaven, Germany. [2]University of Bremen, Fachbereich 2, NW 2/Leobener Strasse, D-28359 Bremen, Germany. [3]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Bentho-Pelagic Processes, Am Alten Hafen 26, D-27568 Bremerhaven, Germany.

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PUBLICATION II

Impact of Ocean Acidification and Warming on mitochondrial enzymes in two gadoid species.

Elettra Leo, Martin Graeve, Daniela Storch, Hans-O. Pörtner, Felix C. Mark

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ORIGINAL PAPER

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

Elettra Leo[1,2] · Martin Graeve[3] · Daniela Storch[1] · Hans-O. Pörtner[1,2] · Felix C. Mark[1]

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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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✉ Felix C. Mark
Felix.Christopher.Mark@awi.de

[1] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, Am Handelshafen 12, 27570 Bremerhaven, Germany

[2] University of Bremen, Fachbereich 2, NW 2 / Leobener Strasse, 28359 Bremen, Germany

[3] Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Ecological Chemistry, Am Handelshafen 12, 27570 Bremerhaven, Germany

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.

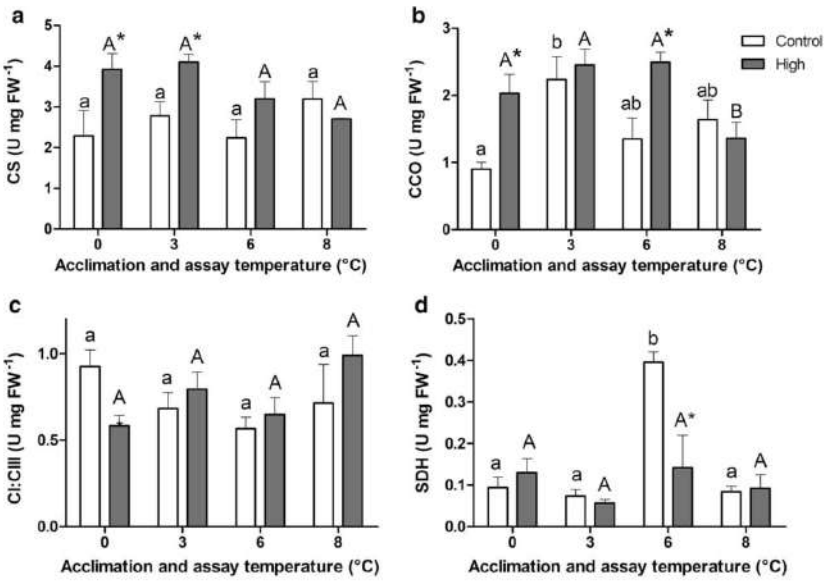

Fig. 1 Enzymatic capacities of heart tissue of polar cod (*Boreogadus saida*) acclimated and measured at the respective acclimation temperature. **a** Citrate synthase (CS), **b** cytochrome *c* oxidase (CCO), **c** NADH:cytochrome *c* oxidoreductase (CI:CIII), and **d** succinate dehydrogenase (SDH). 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

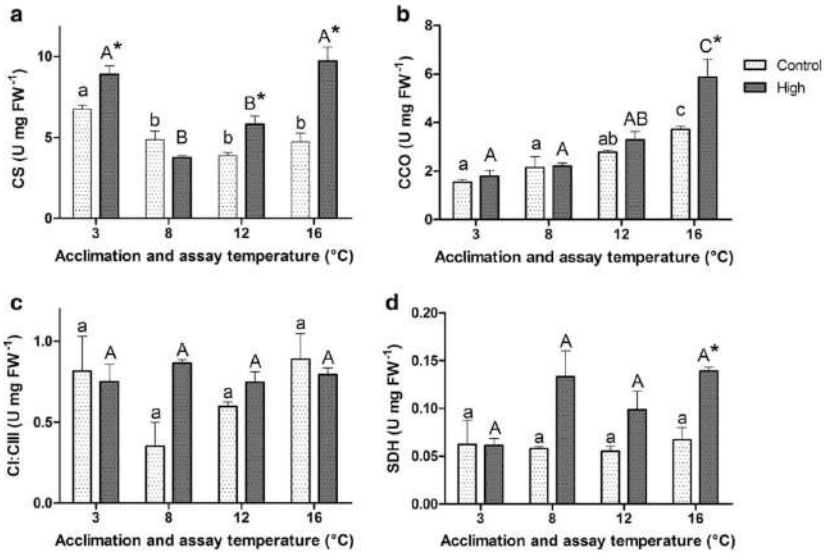

Fig. 2 Capacities of mitochondrial enzymes from heart tissue of NEAC (*Gadus morhua*) measured at the respective acclimation temperatures. **a** Citrate synthase (CS), **b** cytochrome *c* oxidase (CCO), **c** NADH:cytochrome *c* oxidoreductase (CI:CIII), and **d** succinate dehydrogenase (SDH). Values 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).

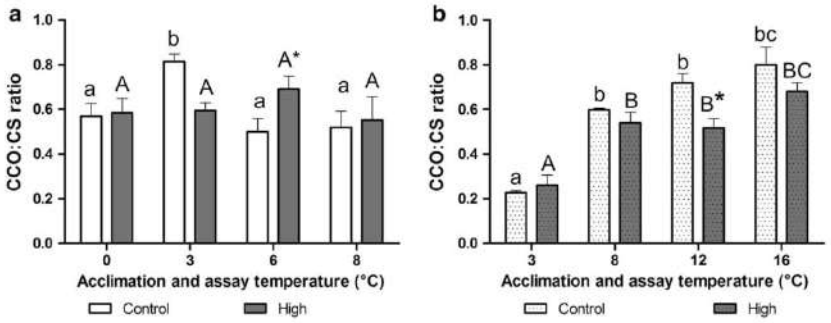

Fig. 3 CCO:CS ratios in polar cod (*Boreogadus saida*, panel **a**) and NEAC (*Gadus morhua*, panel **b**) heart tissue measured at acclimation temperature. Within panels: lower case letters indicate differences between temperatures at control P_{CO_2}, upper case letters indicate differences between 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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PUBLICATION III

Impact of Ocean Acidification and Warming on the bioenergetics of developing eggs of Atlantic herring *Clupea harengus.*

Elettra Leo, Flemming T. Dahlke, Daniela Storch, Hans-O. Pörtner, Felix C. Mark

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Research article

Impact of Ocean Acidification and Warming on the bioenergetics of developing eggs of Atlantic herring *Clupea harengus*

Elettra Leo[1,2], Flemming T. Dahlke[1,2], Daniela Storch[1], Hans-O. Pörtner[1,2] and Felix C. Mark[1,*]

[1]Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, Am Handelshafen 12, D-27570 Bremerhaven, Germany
[2]University of Bremen, Fachbereich 2 Biologie/Chemie, NW 2/Leobener Strasse, D-28359 Bremen, Germany

***Corresponding author:** Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Integrative Ecophysiology, Am Handelshafen 12, D-27570 Bremerhaven, Germany. Email: Felix.Christopher.Mark@awi.de

Atlantic herring (*Clupea harengus*) is a benthic spawner, therefore its eggs are prone to encounter different water conditions during embryonic development, with bottom waters often depleted of oxygen and enriched in CO_2. Some Atlantic herring spawning grounds are predicted to be highly affected by ongoing Ocean Acidification and Warming with water temperature increasing by up to +3°C and CO_2 levels reaching ca. 1000 µatm (RCP 8.5). Although many studies investigated the effects of high levels of CO_2 on the embryonic development of Atlantic herring, little is known about the combination of temperature and ecologically relevant levels of CO_2. In this study, we investigated the effects of Ocean Acidification and Warming on embryonic metabolic and developmental performance such as mitochondrial function, respiration, hatching success (HS) and growth in Atlantic herring from the Oslo Fjord, one of the spawning grounds predicted to be greatly affected by climate change. Fertilized eggs were incubated under combinations of two PCO_2 conditions (400 µatm and 1100 µatm) and three temperatures (6, 10 and 14°C), which correspond to current and end-of-the-century conditions. We analysed HS, oxygen consumption (MO_2) and mitochondrial function of embryos as well as larval length at hatch. The capacity of the electron transport system (ETS) increased with temperature, reaching a plateau at 14°C, where the contribution of Complex I to the ETS declined in favour of Complex II. This relative shift was coupled with a dramatic increase in MO_2 at 14°C. HS was high under ambient spawning conditions (6–10°C), but decreased at 14°C and hatched larvae at this temperature were smaller. Elevated PCO_2 increased larval malformations, indicating sub-lethal effects. These results indicate that energetic limitations due to thermally affected mitochondria and higher energy demand for maintenance occur at the expense of embryonic development and growth.

Key words: Atlantic herring, embryonic development, mitochondrial capacity, Ocean Acidification, Ocean Warming, respiration

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Introduction

The atmospheric CO_2 concentration has increased dramatically since the preindustrial era, from ca. 280 µatm to ca. 410 µatm nowadays causing an increase in average ocean surface temperatures of about 0.83°C and a decrease in surface water pH of 0.1 units (Bopp *et al.*, 2013). If the rate of emissions does not change, the level of atmospheric CO_2 is expected to rise to ca. 851–1370 µatm by the year 2100 causing an average warming of 3.15°C and a decrease of 0.41 pH units in the ocean surface waters (Henson *et al.*, 2017).

While juvenile and adult fish appear to tolerate CO_2 levels far beyond average climate change predictions (>2000 µatm, Ishimatsu *et al.*, 2008), early life stages, such as developing embryos and lecithotrophic larvae, appear to be more vulnerable to both, Ocean Acidification and Warming (Baumann *et al.*, 2011; Chambers *et al.*, 2014; Pimentel *et al.*, 2014; Frommel *et al.*, 2016; Stiasny *et al.*, 2016; Dahlke *et al.*, 2017; Sswat *et al.*, 2018). This is probably due to simple modes of respiration (dermal vs. gills) during development and insufficient acid–base regulation before the formation of gills, a situation possibly exacerbated by the higher surface to volume ratio of the early stages compared to adults (Kikkawa *et al.*, 2003; Ishimatsu *et al.*, 2008). Moreover, developing embryos and lecithotrophic larvae are entirely dependent on parental provisioning of resources (yolk) and molecular defence mechanisms (Kamler, 2008) which may become limiting in a changing environment. Exposure to elevated PCO_2 (CO_2 partial pressure) has been found to adversely affect embryonic development (Tseng *et al.*, 2013; Dahlke *et al.*, 2017), larval growth and survival (Baumann *et al.*, 2011) and tissue/organ health (Frommel *et al.*, 2016) in some fish species. However, in other species, studies have not detected any effect on embryogenesis (Franke and Clemmesen, 2011; Maneja *et al.*, 2015), hatching (Frommel *et al.*, 2012) or growth and development (Munday *et al.*, 2011; Frommel *et al.*, 2012; Hurst *et al.*, 2012, 2013; Bignami *et al.*, 2014). Thus, it is important to understand which are the mechanisms underlying the sensitivity towards Ocean Acidification and Warming in the early life stages to assess which fish species will be affected more by the ongoing climatic changes.

Thermal acclimation and acid–base regulation may increase the metabolic costs of development, causing the reallocation of the yolk-limited resources from development and growth to maintenance (Rombough, 2011; Dahlke *et al.*, 2017). Studies of energy metabolism in developing fish eggs have been concerned mainly with measuring levels of potential energy reserves, metabolites and relevant metabolic enzyme systems (Tocher *et al.*, 1985; Finn *et al.*, 1996; Finn and Fyhn, 2010), and only recently, studies have begun to address how Ocean Acidification and Warming affect the metabolism of fish embryos (Flynn *et al.*, 2015; Pimentel *et al.*, 2016; Dahlke *et al.*, 2017).

In an early study, Boulekbache (1981) described the energetic charge of ATP in the developing embryos of rainbow trout (*Oncorhynchus mykiss*). He found a decrease of ATP/ADP during cleavage (morula–blastula), then a slight increase during gastrulation followed by a plateau. This profile could represent a heavy utilization of pre-existing ATP during the early stage followed by a neosynthesis in parallel to increased embryo cell movement and diversification (Boulekbache, 1981; Tocher *et al.*, 1985). Since the ATP consumption profile seems to be correlated with specific moments of embryonic development and since at these stages ATP production may be limited by the endogenous resources (yolk), ATP production pathways, mitochondrial metabolism in particular, could play a key role in the embryonic sensitivity to ocean acidification and warming.

Atlantic herring (*Clupea harengus*) is a benthic spawner and schooling pelagic fish. It is widely distributed throughout the North Atlantic shelf regions from the East coast of North America to the West coast of Europe and the Baltic Sea Herring populations represent a major resource not only for other fish species, birds and whales (Lynam *et al.* 2015), but also for commercial fisheries, with annual catches of more than 2 million tones (FAO, 2018). Herring population dynamics are known to be sensitive to changes in water temperature: in the last centuries cool periods promoted the increase of herring biomass and the southward expansion of the distribution areas, while warming events exerted the opposite effect (Alheit and Hagen, 1997). Moreover, herring spawn over extended periods with a wide range of spawning locations specific to seasons and populations (Geffen, 2009). Therefore, herring populations spawning in areas predicted to be severely affected by climate changes may be more vulnerable to Ocean Acidification and Warming, especially those spawning during the summer/autumn season. For example, PCO_2 values above 4000 µatm could be reached in the future at important herring spawning grounds in the Baltic Sea such as the Kiel Fjord (Melzner *et al.*, 2013; Frommel *et al.*, 2014) and at higher latitudes including the Skagerrak and North Sea. PCO_2 is predicted to double with an increase in temperature of more than 3°C (Henson *et al.*, 2017; van Vuuren *et al.*, 2011: RPC 8.5). Studies of developing herring embryos from the Baltic Sea found no significant effect of high PCO_2 (4600 µatm) in hatch rate, development, or otolith size (Franke and Clemmesen, 2011). However, no studies have so far addressed the combined effects of increasing temperature and PCO_2 on the embryonic development of this fish.

It is thus important to understand the effects of ocean acidification and warming on the development of herring embryos under the conditions predicted to occur at the spawning grounds by the end of the century. To do so, we incubated developing embryos of Atlantic herring from the Scandinavian coast within a cross-factorial combination of three temperatures (6–10–14°C) and two PCO_2 levels

(400–1100 µatm), to mirror present conditions and the conditions projected for the end of the century (RCP. 8.5; van Vuuren *et al.*, 2011; Henson *et al.*, 2017) for the entire developmental period, from fertilization to hatch. We analysed mitochondrial respiration and the oxygen demand of late-stage embryos (50% eye pigmentation) to investigate mitochondrial function in relation to embryonic energy demand, respectively. Furthermore, we observed hatching success (HS), larval deformities and larval length at hatch to identify constraints on performance at the whole-organism level.

Methodology

This study was conducted at the Sven Lovén Centre for Marine Science, Kristineberg Biological Station (University of Gothenburg, Sweden) between April and May 2013 in accordance with the legislation of the Swedish Board of Agriculture (Permit: 332–2012).

Experimental animals

Ripe Atlantic herring, *C. harengus*, were caught with gill nets during the spawning season in April 2013 in the inner Oslo Fjord (Norway). Selected fish were caught and killed with a blow on the head and were stored on ice and transported to the Sven Lovén Centre. Gametes of males ($n = 3$) and females ($n = 3$) used for *in vitro* fertilizations were obtained by strip spawning approximately four hours after the fish had been caught.

Experimental design

A full-factorial design with three temperatures (6, 10 and 14°C) and two PCO_2 (400 µatm and 1100 µatm) was used for fertilization and incubation of herring eggs. Treatment conditions were selected to encompass ambient spawning temperatures (6–10°C and the PCO_2 recorded in the year 2013) as well as water warming and acidification projected for the end of this century according to IPCC's business-as-usual scenario (RCP 8.5, van Vuuren *et al.*, 2011). Eggs produced by different females were incubated separately and 'females' were treated as biological replicates ($n = 3$). Each female was represented by two incubators at each treatment combination (2 × 3 females × 6 treatments = 36 incubators in total). In order to avoid biased survival estimates, only one of both incubators was used to collect samples for measurements of oxygen consumption rates and mitochondrial capacities. The second incubator was used to evaluate HS and larval morphology at hatch. Incubator classification and arrangement within experimental units was done randomly.

Fertilization protocol

The eggs of each female were stripped onto 12 plates of Polyethylene mesh (500 µm mesh size, 10 cm diameter). To optimize fertilization success and oxygenation during development, care was taken to arrange the eggs in single layer. Two out of 12 egg-plates were fertilized at each of six different temperature × PCO_2 treatment combinations (Table 1) following a wet-fertilization protocol (Geffen, 1999). Individual egg-plates were placed in Petri dishes and incubated for 10 min with a milt-seawater dilution of 1:500 (10 ml, produced with milt aliquots from $n = 3$ males). After being carefully rinsed, the egg-plates were transferred into hatching jars filled with 1 l of filtered (0.2 µm) and UV sterilized seawater (adjusted to the respective treatment combination). The percentage of fertilized eggs on each plate (i.e. fertilization success) was determined by visual inspection under a stereomicroscope after 12 h of incubation. Mean values are shown in Table 1.

Incubation

The incubation set-up is shown in Supplementary Figure S1. Herring eggs adhered to mesh-plates were incubated within transparent, bottom tapered hatching jars (Imhoff sedimentation cones, 1000 ml volume, Supplementary Fig. S1A), which were submerged into 400 l seawater baths thermostatted to different temperatures (6, 10 and 14°C, Supplementary Fig. S1B). Each incubator was sealed with a Styrofoam lid to prevent outgassing of CO_2. Eggs received dim light with a daily rhythm of 12 h/12 h light/darkness. Every 24 h, 80% of the water volume of each incubator was replaced by filtered (0.2 µm) and UV-sterilized seawater (33 PSU) to avoid oxygen depletion and bacterial or fungal infestation. Herring eggs were not exposed to air during water exchange. Each water bath contained two 60-l reservoir tanks, which were used to pre-adjust exchange-seawater to the corresponding temperature and PCO_2. Water temperatures of the different water baths were recorded automatically every 15 min (±0.1°C, Table 2) by a multi-channel aquarium computer (IKS-Aquastar, IKS Systems, Germany).

Elevated PCO_2 conditions were administered by injection of pure CO_2 gas into the submerged 60 l reservoir tanks by bubbling through large aeration stones (20 cm length). A multi-channel feedback system (IKS-Aquastar), connected to individual pH-probes (IKS-Aquastar) and solenoid valves were used to adjust PCO_2 values. Pure CO_2 was infused via perforated silicone tubes until the desired pH/PCO_2 was reached. The PCO_2 of the reservoir tanks was measured *in situ* prior to every second water exchange with an infrared PCO_2 probe (Vaisala GM70, manual temperature compensation, ±10 µatm accuracy; Vaisala, Finland). The probe was equipped with an aspiration pump and sealed with a gas-permeable membrane to measure PCO_2 in air equilibrated with dissolved CO_2 in the water, as described by Munday *et al.* (2013) and Jutfelt and Hedgarde (2013). Factory calibration was confirmed by measurements of seawater previously bubbled with a technical gas mixture (1010 µatm CO_2 in air; AGA Sweden). Prior to the daily water exchange, pH-values of the reservoir tanks were measured with a lab-grade pH-electrode to three decimal places (Mettler Toledo InLab Routine Pt 1000 with temperature compensation, Mettler Toledo, Switzerland), which was connected to a WTW 3310

pH-meter. A two-point calibration with NBS-buffers was performed on a daily basis. To convert NBS to the free proton concentration scale for seawater pH (Waters and Millero, 2013), the electrode was recalibrated with Tris-HCl seawater buffers (Dickson *et al.*, 2007), which were acclimated to the corresponding incubation temperature prior to each measurement. Seawater pH-values refer to the free proton concentration scale throughout this manuscript (for summary see Table 2), Individual values for each measured parameter are available in the Open Access library PANGAEA (see 'Data availability' section).

Data collection

Whole-embryo oxygen consumption

Oxygen consumption rates (MO_2) of late-stage embryos (at 50% eye pigmentation) were measured in closed, temperature-controlled respiration chambers (OXY0 41 A, Collotec Meßtechnik GmbH, Germany, Supplementary Fig. S2) following methodologies described by Schiffer *et al.* (2014). All measurements were performed in duplicates (with two respiration chambers) at the same developmental stage (~50% eye pigmentation) and treatment as during incubation. Staging was done by visual inspection during the daily water exchange. Development times until 50% eye pigmentation (and hatching) did not differ between PCO_2 treatments (Supplementary Fig. S3 and Table S1), as was demonstrated for Baltic herring under more extreme PCO_2 conditions (4 600 µatm, Franke and Clemmesen, 2011). The stage at 50% eye pigmentation was selected because it represents a clearly discernible developmental landmark (Hill and Johnston, 1997) at which the embryonic cardiocirculatory system, and thus metabolic capacity, is already well-developed (Hill and Johnston, 1997). For each run, ~20 (±3) eggs were loaded into each of the two respiration chambers. The chambers were previously filled with a volume of ~2 ml sterilized seawater, whereby each chamber was alternately used for different PCO_2 treatments. The eggs were placed on a polyethylene mesh (500 µm mesh size) with a magnetic micro-stirrer (3 mm) underneath to avoid oxygen stratification within the respiration chamber (see Supplementary Fig. S2). The change in oxygen saturation was detected by micro-optodes (fiber-optic microsensor, flat broken tip, diameter: 140 µm, PreSens GmbH, Germany) connected to a Microx TX3 (PreSens GmbH, Germany). Recordings were stopped after 60 min (at 6°C) or as soon as the oxygen saturation declined below 80% air saturation (20–40 min at 10 and 14°C). After each run, the wet mass per egg and the exact water volume of the respiration chamber was determined by weighing on a precision balance (±0.01 mg). Bacterial oxygen consumption (always below 5%) and optode drift (always below 1%) was determined by blank measurements before and after three successive runs with eggs. Given that egg masses did not differ between temperature and PCO_2 treatments (Supplementary Fig. S3), MO_2 was expressed as (nmol O_2 egg^{-1} h^{-1}) according to the following formula: $MO_2 = DO_2 * Vol/N_{Eggs}$, where DO_2 is the decline in oxygen saturation (nmol l^{-1} h^{-1}), Vol is the water volume of the respiration (ml) chamber and N_{Eggs} is the number of eggs.

Mitochondrial function

Mitochondrial function was measured in a cellular suspension of late-stage eggs (at 50% eye pigmentation) as described in Dahlke *et al.* (2017). Briefly, one hundred eggs from $n = 3$ females were gently ground on ice in a glass potter filled with 2-ml ice-cold modified mitochondrial respiration medium MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 160 mM sucrose, 1 g l^{-1} bovine serum albumin, pH 7.4, 380 mOsmol l^{-1}) (Iftikar and Hickey, 2013; Gnaiger *et al.*, 2015). The resulting suspension was collected avoiding the collection of the eggshells and mitochondrial respiration was analysed using Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria). The

Table 1: Mean ± SEM fertilization success of Atlantic herring (*Clupea harengus*) eggs fertilized at different levels of temperature and PCO_2. Differences between temperature and PCO_2 treatments were statistically not significant ($F = 1.9$, $P = 0.192$ and $F = 0.97$, $P = 0.344$, respectively)

Temperature (°C)	Fertilization success (%)	
	Control PCO_2	High PCO_2
6	88.8 ± 2.8	79.4 ± 4.3
10	76.0 ± 9.0	72.2 ± 8.6
14	88.9 ± 8.5	85.3 ± 8.0

Table 2: Summary table of the water parameters measured during the incubation of Atlantic herring (*Clupea harengus*) eggs until hatch. Data are presented as mean ± SD

Duration (days)	Nominal T (°C)	Measured T (°C)	Oxygen (%)		PCO_2 (µatm)		pH_F	
			Control PCO_2	High PCO_2	Control PCO_2	High PCO_2	Control PCO_2	High PCO_2
27	6	6.15 ± 0.06	94.40 ± 0.71	94.40 ± 0.61	415 ± 10	1101 ± 47	8.15 ± 0.02	7.77 ± 0.02
16	10	10.04 ± 0.06	94.40 ± 0.63	94.40 ± 0.49	408 ± 10	1050 ± 46	8.17 ± 0.02	7.79 ± 0.03
11	14	14.07 ± 0.20	95.00 ± 0.00	95.00 ± 0.00	403 ± 12	1050 ± 29	8.18 ± 0.02	7.78 ± 0.02

oxygen flux (nmol O_2 (egg* h)$^{-1}$) was recorded and calculated in real-time using Oroboros DatLab 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria). Measurements were conducted in MiR05 buffer equilibrated to atmospheric PCO_2 and acclimation temperature of the eggs. The cO_2 ranged from atmospheric saturation (ca. 370 nmol ml^{-1}) to 150 nmol ml^{-1}. A substrate–uncoupler–inhibitor titration (SUIT) protocol was used to investigate the capacities of the single components of the electron transport system (ETS) measured as oxygen consumption attributable to each component (nmol O_2 (egg* h)$^{-1}$). In detail: ETS capacity was measured by step-wise (1 µM each) titration of carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) in the presence of Complex I (CI) and Complex II (CII) substrates (10 mM glutamate, 2 mM malate, 10 mM pyruvate and 10 mM succinate). CI, CII and Complex III (CIII) were inhibited by the addition of 0.5 µM rotenone, 5 mM malonate and 2.5 µM antimycin a, respectively. All chemicals were obtained from Sigma-Aldrich (Germany).

Hatching success

Once hatching started, free-swimming larvae were collected in the morning, euthanized with an overdose of tricaine methane sulphonate (MS-222) and counted after visual examination for morphological deformities under a stereomicroscope. The incidence of larval deformities was quantified as the percentage of hatchlings that exhibited severe deformations of the yolk sac, cranium or vertebral column. HS, defined as the percentage of non-malformed larvae that hatched from fertilized eggs, was calculated as:

$$HS = (L_i - L_d)/E_f * 100$$

where L_i represents the number of hatched larvae, L_d is the number of deformed larvae and E_f is the number of fertilized eggs (shown in Table 1).

Larval size at hatch

Subsamples of 10–30 non-malformed larvae of three females at each treatment combination were photographed for subsequent measurements of larval standard length (SL) using Olympus image analysis software (Stream Essentials©, ± 1 µm). Only samples obtained from the same daily cohort (during peak-hatch at each temperature treatment) were used for statistical comparison between PCO_2 treatments.

Data analysis

All data are presented as mean ± SEM.

Statistical analyses were conducted using R 3.2.0 (R Core Team, 2015) and the level of statistical significance was set at $P < 0.05$ for all the statistical tests.

Normal distribution and homoscedasticy of the data were assessed by Shapiro–Wilk and Bartlett's tests, respectively.

Multi factorial analyses of variance (two-way ANOVA) including the female parent ID as covariance were used to evaluate whether temperature and PCO_2 and the combination of both factors had an effect on the parameters object of this study. The two-way ANOVA was followed by Tukey's HSD test for temperature and Student's *t*-test for CO_2.

In addition, MO_2 data were analyzed with the female-specific egg mass included in the model as covariate.

The temperature coefficient Q_{10} was calculated according to the equation:

$$Q_{10} = (R_1/R_2)^{10/(T2-T1)}$$

R: respiration rate
T: temperature at which the respiration rate was measured.

Results

Mitochondrial function and whole-embryo respiration

The *in vivo* oxygen consumption rates (MO_2) of Atlantic herring embryos were affected only by temperature ($F = 173.87$, $P < 0.001$, Fig. 1b). In general, MO_2 increased with temperature in a non-linear fashion: expressed as Q_{10}, the increase in MO_2 between 6 and 10°C (Control PCO_2: 2.30 ± 0.23; High PCO_2: 2.00 ± 0.20) was lower than between 10 and 14°C (Control PCO_2: 2.27 ± 0.14; High PCO_2: 3.17 ± 0.27).

In vitro, the mitochondrial oxygen flux corresponding to the maximum capacity of the ETS was affected only by temperature ($F = 5.61$, $P = 0.019$, Fig. 1a) and increased between 6 and 10°C ($P = 0.04$), but, unlike whole-embryo MO_2 (Fig. 1), reached a plateau between 10 and 14°C ($P > 0.05$). CI and CII contributed differently to the ETS according to temperature ($F = 17.28$, $P < 0.001$, Fig. 2). CI contribution declined with increasing temperature while CII contribution increased (Fig. 2). At 14°C, only 37% of the ETS capacity was contributed from CI, compared with 62% at 6°C (Fig. 2).

Viable hatch and length at hatch

HS (Fig. 3) was significantly affected by temperature ($F = 14.07$, $P = 0.001$) with a reduction of hatched larvae at 14°C compared with the other acclimation groups (6–14°C: $P = 0.005$; 10–14°C: $P = 0.001$; Fig. 3a). Elevated PCO_2 had no significant effects on HS but caused a significant reduction ($P = 0.02681$) of the HS in the group incubated at 6°C (64.26 ± 1.72%) compared to the group incubated at the same temperature but under control PCO_2 (75.43 ± 2.85%).

The proportion of larvae hatching with severe morphological malformations was higher in the groups incubated under high PCO_2 ($F = 13.03$, $P = 0.004$, Fig. 3b) with percentages almost doubled compared with the groups incubated under control PCO_2 (tab. 1). Larval malformations

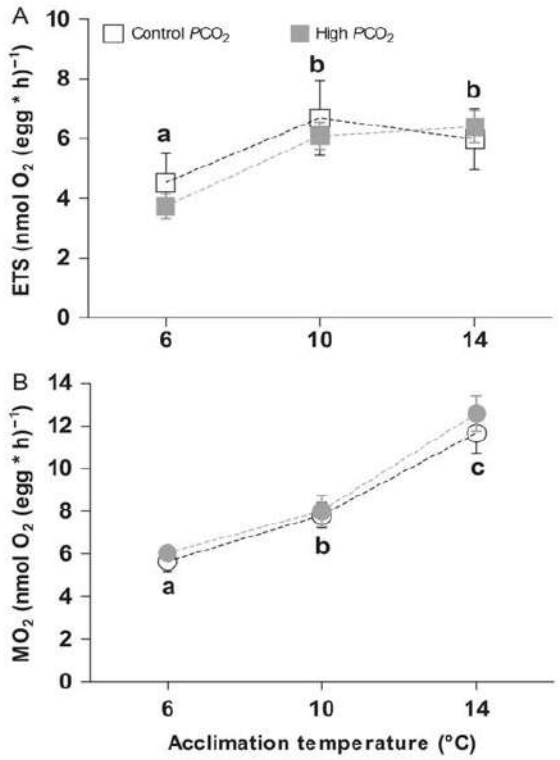

Figure 1: Respiration performance and mitochondrial capacity of Atlantic herring (*Clupea harengus*) embryos at 50% eye pigmentation stage. Values are reported as mean ± SEM. Panel **A**: Electron Transport System (ETS) capacity. Open squares: control PCO_2 (400 µatm), solid squares: high PCO_2 (1100 µatm). Panel **B**: Whole-embryo respiration. Open circles: control PCO_2 (400 µatm), solid circles: high PCO_2 (1100 µatm). Different letters within panels indicate significant differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

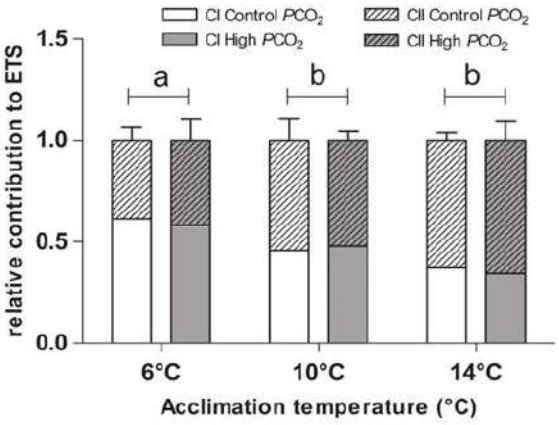

Figure 2: Contribution (%) of Complex I and Complex II to the electron transport system (ETS) in the embryos of Atlantic herring (*Clupea harengus*). Embryonic stage: 50% eye pigmentation. Values are reported as mean ± SEM. Open bars: Complex I, dashed bars: Complex II. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate statistical differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

were not significantly correlated with increasing temperatures ($F = 1.67$, $P > 0.05$) and there was no interactive effect between temperature and CO_2.

SL at hatch (Fig. 4) was significantly affected by temperature ($F = 43.12$, $P < 0.001$) with a trend toward reduction with warming. SL was not affected by elevated PCO_2 ($F = 9.11$, $P > 0.05$).

Discussion

In this study, we analysed the development and mitochondrial function of Atlantic herring (*C. harengus*) embryos that were incubated to either current water conditions, or to conditions projected for the end of this century in waters surrounding the Scandinavian coast; one of the main spawning grounds of this species in the North Atlantic.

In general, we found that elevated temperature reduced HS and high PCO_2 caused larval malformation. Mitochondrial function was not affected by elevated PCO_2; however, temperature played a major role in shaping mitochondrial respiration, with subsequent effects on embryonic respiration and body length at hatching; which is in line with other studies on Atlantic herring (Geffen, 2002; Peck *et al.*, 2012).

The capacity of the ETS increased with temperature between 6°C and 10°C without a further increase at 14°C; however, the relative contribution of CI and CII to the ETS changed with temperature (for the entire range 6–14°C), with the contribution of CI being negatively correlated to temperature. In developing teleost fish, embryos mainly rely on carbohydrates during the initial phase of development, until blastula (Kamler, 2008), then catabolize amino acids from protein (benthophils) or free amino acids (FAA, pelagophils), together with lipids (Finn and Fyhn, 2010). In a study on Atlantic cod (*Gadus morhua*) eggs, Fyhn and Serigstad (1987) showed that the FAA content of the yolk was depleted by ~90% during spawning to hatching, but without a corresponding increase in the protein content of the developing embryo. Moreover, they found that alanine, serine, leucine, isoleucine, lysine, and valine (in that order) quantitatively dominated the amino acids pool, and accounted for ~75% of the decrease. Alanine, serine, leucine, isoleucine and lysine enter the TCA cycle at the citrate synthase step, via pyruvate (alanine and serine) or via acetyl-coA and acetoacetyl-coA (leucine, isoleucine and lysine); both of which are fed into CI and CII. Only valine and isoleucine enter the cycle via succinyl-coA and feed directly into CII. Taking this into account, the reduction of CI contribution to

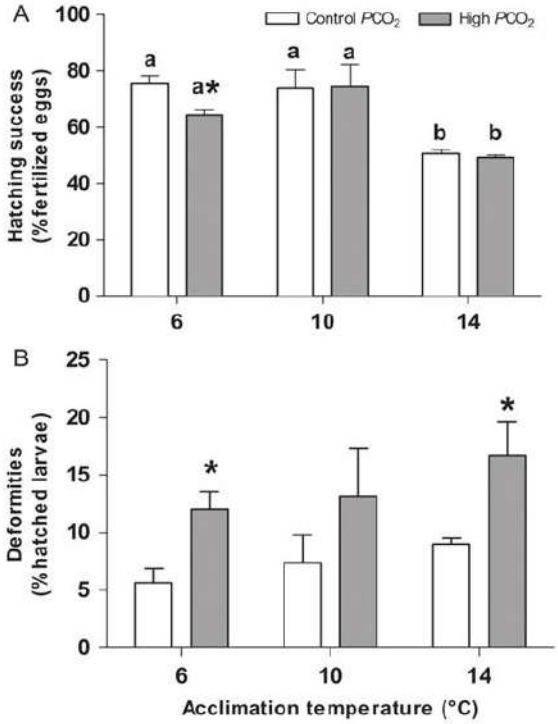

Figure 3: Viable hatch of Atlantic herring (*Clupea harengus*). Values are reported as mean ± SEM. Panel **A**: hatching success as percentage of fertilized eggs that hatch. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Panel **B**: Larval malformations as percentage of hatched larvae. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate statistical differences ($P < 0.05$) between temperature treatments, * indicates significant differences ($P < 0.05$) between CO_2 groups at the same temperature.

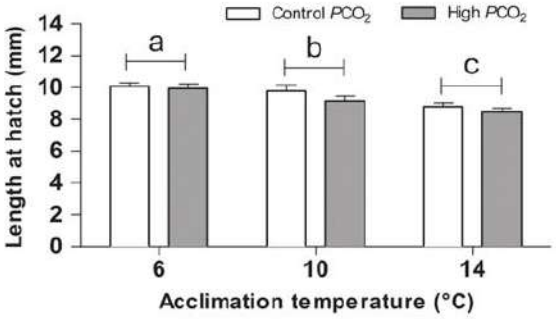

Figure 4: Length at hatch (mm) of Atlantic herring larvae (*Clupea harengus*). Values are expressed as mean ± SEM. Open bars: control PCO_2 (400 µatm), solid bars: high PCO_2 (1100 µatm). Different letters indicate significant differences ($P < 0.05$) between temperature treatments independent of the CO_2 treatment.

the ETS, in favour of CII, could indicate a shift in metabolic pathways from the preferred CI feeding amino acids (alanine, serine, leucine, isoleucine and lysine) to CII feeding amino acids (valine and isoleucine), as a result of increasing temperature. However, several studies have reported a reduced contribution of CI to the ETS with decreasing temperature in adult fish and embryos, with suggested causes being either a lack of substrates or a change in membrane fluidity (Hilton *et al.*, 2010; Iftikar *et al.*, 2015; Dahlke *et al.*, 2017). These two hypotheses are not contradictory, but complement (and even cause) each other.

A shift in ETS contribution from CI to CII results in a less efficient ATP production pathway, since each cycle of the TCA cycle theoretically produces ~7.5 ATP from CI, but only ~1.5 ATP from CII. The decreased ATP provision at higher temperatures would need compensation by an increase in embryonic respiration (MO_2) as seen at 14°C in this study. In addition to the shift in the contribution of the individual complexes to ETS, increasing temperature may also cause a rise in mitochondrial uncoupling, increasing oxygen demand to compensate for the increased proton leak (Weinstein and Somero, 1998; Hardewig *et al.*, 1999). Therefore, an animal's respiratory rate (MO_2) may increase in order to partially compensate for these constraints. However, a limit may be reached where the animal is no longer able to provide oxygen to mitochondria or aerobically produce enough ATP; which may lead to constraints on performance, the onset of anaerobic metabolism and eventually death (Hardewig *et al.*, 1999; Pörtner, 2002).

In this study, we identified several negative effects of decreased ATP production efficiency at a higher incubation temperature (14°C). There was a decreased HS at this elevated temperature and the larvae that hatched at 14°C were smaller than the larvae from other incubation temperatures, indicating that less energy was available for development. Therefore, high temperature (14°) may have limited mitochondrial function, which is mirrored at the whole-organism level, by the decreased length and HS. This provides a link between thermal sensitivity of energy metabolism and the effects of warming at the whole-organism level.

Elevated PCO_2 caused a significant increase in larval deformities. This is similar to the findings of Frommel *et al.* (2014), which showed that elevated PCO_2 caused significant organ damage and reduced growth in the larvae of Atlantic herring. However, in another study on Atlantic herring embryos, (Franke and Clemmesen, 2011) found no significant effect of elevated PCO_2 (levels up to 4635 µatm) on egg mortality or the occurrence of embryonic malformations. These contrasting findings could be partially explained by the different origins of the herring populations. The herring used in this study and the study by Frommel *et al.* (2014) came from the Scandinavian coast, while the herring used in the study by Franke and Clemmesen came from the Kiel Fjord in the Baltic Sea, where PCO_2 levels are above

2300 µatm due to upwelling events (Thomsen *et al.*, 2010). Atlantic herring display high plasticity in physiological tolerance (Geffen, 2009; Peck *et al.*, 2012), allowing different populations to spawn in different seasons and live in a broad range of temperatures and salinities (Geffen, 2009). Herring lay adhesive benthic eggs (Nash *et al.*, 2009; Schmidt *et al.*, 2009) and therefore encounter potentially challenging hydrographic conditions during egg development, since bottom waters are often depleted of oxygen and enriched in CO_2, relative to surface waters. These results contribute to the growing evidence of differences in the sensitivity towards Ocean Acidification and Warming between herring populations (Franke and Clemmesen, 2011; Sswat *et al.*, 2018) and compared to pelagic spawners such as Atlantic cod, flounder and many tropical reef species (Chambers *et al.*, 2014; Munday *et al.*, 2016; Dahlke *et al.*, 2017).

Conclusions

Our study assessed the effects of combined Ocean Acidification and Warming on developing eggs of Atlantic herring. By studying such effects at both the cellular level (e.g. mitochondrial functioning) and the organism level (e.g. body size at hatching), this study provides a link between the thermal sensitivity of an individual's energetic metabolism with the fitness of the individual as a whole.

Elevated temperature significantly affected mitochondrial function by shifting the relative ETS contribution from CI to CII. This may decrease ATP production, which could lead to a mismatch between the energy produced by the mitochondria and the energy requested by the organism for maintaining metabolism; which in turn could reduce the energy allocation to development indicated by reduced length at hatch.

Elevated PCO_2 did not affect HS; however, it did increase the occurrence of malformed larvae. This suggests that exposure to near future acidification levels may cause sub-lethal cellular damage that may not be reflected in vitality and survival rates. These sub-lethal effects of ocean acidification may present the largest risk to individuals and populations (Briffa *et al.*, 2012). For example, smaller larval size at hatch may increase the risk of predation and reduce foraging ability (Miller *et al.*, 1988).

Furthermore, herring populations experience high fishing mortality in addition to other environmental stressors such as pollution and hypoxia. Therefore, potential effects of ocean acidification and warming must be added to the list of anthropogenic perturbations leading to increased mortality in fish early life stages.

Data availability

The datasets containing the physiological and morphological parameters measured in this study and the data regarding the incubation physico-chemical parameters are available from the Open Access library PANGAEA (www.pangaea.de; https://doi.pangaea.de/10.1594/PANGAEA.884123 and https://doi.pangaea.de/10.1594/PANGAEA.884124).

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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Author contributions

E.L., F.T.D., F.C.M., D.S. and H.-O.P. designed the experiment; E.L., F.T.D. and F.C.M. collected, analysed and interpreted the data; E.L., F.T.D., F.C.M., D.S. and H.-O.P wrote the article.

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PUBLICATION IV

Effects of ocean acidification increase embryonic sensitivity to thermal extremes in Atlantic cod, *Gadus morhua.*

Flemming T. Dahlke, Elettra Leo, Felix C. Mark, Hans-O. Pörtner, Ulf Bickmeyer, Stephan Frickenhaus, Daniela Storch

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Effects of ocean acidification increase embryonic sensitivity to thermal extremes in Atlantic cod, *Gadus morhua*

FLEMMING T. DAHLKE[1,2], ELETTRA LEO[1,2], FELIX C. MARK[1], HANS-OTTO PÖRTNER[1,2], ULF BICKMEYER[1], STEPHAN FRICKENHAUS[1,3] and DANIELA STORCH[1]

[1]*Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany,* [2]*University of Bremen, NW 2 Loebener Str., 28359 Bremen, Germany,* [3]*Hochschule Bremerhaven, An der Karlstadt 8, 27568 Bremerhaven, Germany*

Abstract

Thermal tolerance windows serve as a powerful tool for estimating the vulnerability of marine species and their life stages to increasing temperature means and extremes. However, it remains uncertain to which extent additional drivers, such as ocean acidification, modify organismal responses to temperature. This study investigated the effects of CO_2-driven ocean acidification on embryonic thermal sensitivity and performance in Atlantic cod, *Gadus morhua*, from the Kattegat. Fertilized eggs were exposed to factorial combinations of two PCO_2 conditions (400 μatm vs. 1100 μatm) and five temperature treatments (0, 3, 6, 9 and 12 °C), which allow identifying both lower and upper thermal tolerance thresholds. We quantified hatching success, oxygen consumption (MO_2) and mitochondrial functioning of embryos as well as larval morphometrics at hatch and the abundance of acid–base-relevant ionocytes on the yolk sac epithelium of newly hatched larvae. Hatching success was high under ambient spawning conditions (3–6 °C), but decreased towards both cold and warm temperature extremes. Elevated PCO_2 caused a significant decrease in hatching success, particularly at cold (3 and 0 °C) and warm (12 °C) temperatures. Warming imposed limitations to MO_2 and mitochondrial capacities. Elevated PCO_2 stimulated MO_2 at cold and intermediate temperatures, but exacerbated warming-induced constraints on MO_2, indicating a synergistic interaction with temperature. Mitochondrial functioning was not affected by PCO_2. Increased MO_2 in response to elevated PCO_2 was paralleled by reduced larval size at hatch. Finally, ionocyte abundance decreased with increasing temperature, but did not differ between PCO_2 treatments. Our results demonstrate increased thermal sensitivity of cod embryos under future PCO_2 conditions and suggest that acclimation to elevated PCO_2 requires reallocation of limited resources at the expense of embryonic growth. We conclude that ocean acidification constrains the thermal performance window of embryos, which has important implication for the susceptibility of cod to projected climate change.

Keywords: aerobic metabolism, Atlantic cod, embryonic development, hatching success, mitochondrial respiration, ocean acidification, ocean warming, thermal tolerance window

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Introduction

Anthropogenic climate change will impact marine fish stocks in various ways (Gattuso *et al.*, 2015). Global ocean warming is currently the main environmental driver of changes, which scale from individual physiological responses (Pörtner & Knust, 2007) to shifting population dynamics (Petitgas *et al.*, 2013) and altered ecosystem productivity (Britten *et al.*, 2016). By the year 2100, continued warming of 3–4 °C is expected to occur in concert with an increase in the frequency of thermal extreme events and ocean acidification (Bopp *et al.*, 2013; IPCC, 2014). Ocean acidification results from the absorption of atmospheric CO_2 by the surface ocean (increase in PCO_2) and has been demonstrated to affect the performance and survival of various marine organisms, including many fish species (see reviews by Heuer & Grosell, 2014; Wittmann & Pörtner, 2013). Estimating species vulnerability to future ocean warming therefore requires knowledge of how additional drivers, such as ocean acidification, affect the susceptibility of organisms to increasing temperature means and extremes.

Ectothermic species can only tolerate specific conditions. Their ability to carry out vital functions over a range of temperatures is defined as their thermal performance window (Pörtner, 2002). Performance limits at extreme temperatures are species specific and may emerge from a mismatch between oxygen demand and supply to vital cellular functions, such as mitochondrial

Correspondence: Flemming T. Dahlke, tel. +49 471 4831 2197, fax +49 471 4831 1149, e-mail: flemming.dahlke@awi.de

ATP production, ion regulation and protein synthesis (Pörtner, 2002, 2010; Schulte, 2015). The resulting deficiency in oxidative energy production first compromises whole-animal performances like development, growth and reproduction (Pörtner & Knust, 2007). Beyond high critical temperatures, severe hypoxaemia sets in and organisms need to exploit protective mechanism (e.g. heat shock proteins and antioxidative defence) to avoid thermal death (Pörtner, 2010). In the long run, such performance limitations can translate into population decline, shifts in migration patterns or the abandonment of traditional habitats (Pörtner & Knust, 2007; Farrell *et al.*, 2008). Ocean acidification may act as an additional stressor because the effects of elevated PCO_2 can affect vital physiological processes, including those involved in oxidative energy production (Wittmann & Pörtner, 2013; Heuer & Grosell, 2014). Consequently, a narrowing of the thermal tolerance window occurs through exacerbated constraints on aerobic metabolism and functional capacities, as previously demonstrated for marine invertebrates (Walther *et al.*, 2009; Schiffer *et al.*, 2014).

Species vulnerability to direct effects of climate change may depend on tolerance thresholds of sensitive early life stages (Pörtner & Peck, 2010). Fish embryos are considered to possess particularly narrow tolerance windows because central organ systems and regulation pathways are not yet fully developed (Pankhurst & Munday, 2011). Moreover, developing embryos are passive recipients of their environment and entirely dependent on parental provisioning of resources (yolk) and molecular defence mechanisms (Kamler, 2008). Increased metabolic costs associated with thermal acclimation and acid–base regulation could therefore invoke trade-offs between the allocation of limited resources to vital maintenance functions and other energy-demanding processes like developmental growth (Rombough, 2011).

The present study investigated the effects of future ocean acidification (PCO_2 = 1100 μatm; pH = 7.77) on embryonic thermal sensitivity in Atlantic cod, *Gadus morhua*, from the Kattegat. We used a wide range of temperatures (0, 3, 6, 9, 12 °C), which allows identifying both lower and upper thermal tolerance thresholds of cod embryos (Geffen *et al.*, 2006). The Kattegat population was chosen as it is one of the southernmost populations of cod in the Northeast Atlantic and thus particularly at risk from ocean warming (Drinkwater, 2005). By integrating the effects of temperature and elevated PCO_2 on embryonic development, aerobic metabolism and resource utilization, we sought to answer the following questions: (1) Do temperature and elevated PCO_2 interact to affect embryo survival and hatching success? (2) Does exposure to elevated PCO_2 aggravate thermal constraints on whole-embryo oxygen uptake and mitochondrial energy production? (3) Are there energetic costs and developmental trade-offs associated with thermal acclimation and compensation of acid–base disturbance? To address the latter point, we evaluated larval morphometrics at hatch and the abundance of Na^+/K^+-ATPase-rich ionocytes on the yolk sac epithelium as morphological indicators for developmental growth and acid–base regulation effort, respectively. Our results provide novel mechanistic insight into the physiological consequences of coincident changes in temperature and PCO_2, relevant for the understanding of climate change impacts on cod and other marine ectotherms.

Materials and methods

Broodstock and gamete collection

The study was conducted at the Sven Lovén Centre for Marine Science, Kristineberg, Sweden, between March and May 2013, in accordance with the legislation of the Swedish Board of Agriculture (Permit: 332-2012). Adult Atlantic cod were caught by trawl and hand-gear in the Øresund Strait (55°58′N; 12°38′E) during the spawning season in March 2013. Water temperature and salinity at the spawning ground (20–25 m depth) was 4.5 ± 0.5 °C and 32 PSU, respectively. Gametes of males (37–68 cm; n = 12) and females (48–87 cm; n = 5; Table S1) for *in vitro* fertilizations were obtained by strip spawning.

Fertilization protocol

Fertilization was conducted within 30 min after stripping at ambient water temperature (4.5 ± 0.5 °C) and two different seawater PCO_2 treatments (present-day PCO_2: 400 μatm, pH_F: 8.15 and future PCO_2: 1100 μatm, pH_F: 7.77; Table S2). Seawater (salinity: 33 PSU) used for fertilization of cod eggs was filtered (0.2 μm) and UV-sterilized. Egg batches obtained from individual females were split into two separate portions for the use at the two different PCO_2 treatments and fertilized with milt aliquots from three to five males. A standardized milt–seawater dilution of 1:500 was used to maximize fertilization success (Trippel, 2003). The fertilization process was stopped after 10-min gamete contact time by rinsing excess milt and ovarian fluid (Butts *et al.*, 2009). Rinsed eggs were immediately transferred into airtight polyethylene containers previously filled with seawater adjusted to respective PCO_2 treatments and maintained at the same temperature conditions (4.5 ± 0.5 °C) as during fertilization until the start of the incubation experiment (3–6 h postfertilization). During storage, water pH did not change by more than 0.01 units and oxygen concentration remained above 95% (OXI 340i; WTW, Weilheim, Germany). Prior to the start of the incubation experiment, subsamples of 30 eggs per batch were collected and photographed with a digital imaging system (Stereomicroscope: Olympus SZX7; Camera: Olympus DP 26; Olympus, Shinjuku, Japan) to determine differences in mean egg

diameter between females (Table 2). Fertilization success was determined in subsamples of 100 eggs (n = 3 subsamples per batch and PCO_2 treatment). Triplicates were incubated within sealed petri dishes (20 mL) at 5 °C and the corresponding PCO_2 treatment for 12 h. Subsequently, the eggs were photographed to evaluate fertilization success at the 8- or 16-cell stage (Table 2).

Experimental design

Cod eggs previously fertilized at either present-day or elevated PCO_2 were maintained at respective PCO_2 treatments and incubated at five different temperatures (0, 3, 6, 9 and 12 °C) until hatch. Temperatures were selected to cover the entire range of thermal conditions during embryonic development of cod, including average spawning conditions (4–8 °C) in the Kattegat/Øresund area (Righton *et al.*, 2010; Børjesson *et al.*, 2013) and projected warming scenarios (+3–4 °C, IPCC, 2014, RCP8.5). Individual egg batches of five different females were used and females were treated as biological replicates. Each PCO_2 and temperature treatment group of an egg batch was subdivided into two stagnant incubators. In order to avoid biased survival estimates, only one of both incubators was used to evaluate embryo survival and larval morphology at hatch, while oxygen consumption rates, mitochondrial capacities and continuous monitoring of developmental stage were measured in subsamples taken from the second incubator.

Incubation protocol

Initially, all incubators (Imhoff sedimentation cones, 1000 mL volume) were filled with 6 °C filtered (0.2 μm) and UV-sterilized seawater (400/1100 μatm CO_2, 33 PSU) and stocked with eggs at a density of 0.35–0.5 eggs mL^{-1}. Subsequently, incubators with eggs were submerged into 400-L seawater baths thermostatted to different temperatures (0, 3, 6, 9 and 12 °C) to ensure a gradual temperature change inside the incubator. The transparent, bottom-tapered incubators were sealed with a custom-made Styrofoam cover to prevent outgassing of CO_2 and temperature fluctuations at the water surface. Eggs received dim light with a daily rhythm of 12-h/12-h light/darkness. Every 24 h 90% of the water volume of each incubator was replaced by filtered (0.2 μm) and UV-sterilized seawater (33 PSU) to avoid oxygen depletion and bacterial or fungal infestation. An outlet valve was mounted at the bottom of the incubators to drain the seawater with dead eggs, which lose buoyancy and descend to the bottom. The incubators were slowly refilled from underneath the positively buoyant, viable eggs. Oxygen saturation of drained seawater was never below 90% and water pH did not change by more than 0.01 units within 24 h. Seawater parameters are summarized in Table 1 and Fig. S1.

Each seawater bath contained two 60-L reservoir tanks, which were used to adjust seawater to the corresponding temperature and PCO_2 conditions for daily water exchange. Thermostats were used to maintain water temperatures; recirculating pumps (40 L min^{-1}) were installed to ensure homogeneous temperature conditions in the 400-L seawater baths. Water temperatures of the different water baths were recorded automatically every 15 min (± 0.1 °C) by a multichannel aquarium computer (IKS-Aquastar; IKS Systems, Karlsbad, Germany). Temperatures within randomly selected incubators (three per treatment) and reservoir tanks were measured manually on a daily basis. Temperature differences between the 400-L seawater bath and submerged egg incubators and 60-L reservoir tanks did not exceed 0.2 °C.

Future PCO_2 conditions were established by injection of pure CO_2 gas into the submerged 60-L reservoir tanks at each temperature. A multichannel feedback system (IKS-Aquastar) connected to individual pH probes (IKS-Aquastar) and solenoid valves was used to adjust water pH and PCO_2 values. Pure CO_2 was infused via perforated silicone tubes until the desired value was reached. The PCO_2 of the reservoir tanks was measured *in situ* prior to every third water exchange with an infrared PCO_2 probe (Vaisala GM70, manual temperature compensation, ±10 μatm accuracy; Vaisala, Vantaa, Finland). The probe was equipped with an aspiration pump and sealed with a gas-permeable membrane to measure PCO_2 in air equilibrated to dissolved water gases as described by Jutfelt & Hedgarde (2013). Factory calibration was confirmed by measurements of seawater previously bubbled with a technical gas mixture (1010 μatm CO_2 in air; AGA Sweden). Prior to the daily water exchange, pH values of the reservoir tanks were measured with a laboratory-grade pH electrode to three

Table 1 Summary table for mean seawater quality parameters measured during cod egg incubation until complete hatch. Partial pressures of dissolved carbon dioxide (PCO_2) were measured *in situ* in air equilibrated with dissolved water gases. Measurements of seawater pH_{NBS} were calibrated against tempered Tris–HCl seawater buffers and adjusted according to the free proton concentration scale (pH_F). Individual values of incubation temperature O_2, PCO_2 and pH_F are displayed in Fig. S1

Nominal T (°C)	Measured T (°C)	Duration (days)	Oxygen (%)		PCO_2 (μatm)		pH_F	
			Control	High-PCO_2	Control	High-PCO_2	Control	High-PCO_2
0	0.04	45	95.6	95.6	409	1114	8.15	7.77
3	3.05	30	95.4	95.5	409	1057	8.15	7.77
6	6.15	18	94.9	94.7	415	1122	8.14	7.76
9	9.14	13	94.3	94.6	422	1091	8.14	7.77
12	12.11	9	94.1	94.3	417	1097	8.14	7.77

decimal places (Mettler Toledo InLab Routine Pt 1000 with temperature compensation, Mettler Toledo, Columbus, OH, USA), which was connected to a WTW 3310 pH-meter. A three-point calibration with NBS buffers was performed on a daily basis. To convert NBS to the free proton concentration scale for seawater pH (Waters & Millero, 2013), the electrode was recalibrated with Tris–HCl seawater buffers (Dickson *et al.*, 2007), which were acclimated to the corresponding incubation temperature prior to each measurement. Seawater pH values refer to the free proton concentration scale throughout this manuscript.

Data collection

Cumulative survival, development and hatching success. Embryo mortality was recorded on a 24-h basis until all individuals within an incubator had either died or hatched. Cumulative embryo survival until complete hatch was expressed as the percentage of living individuals at day-x postfertilization relative to the initial number of fertilized eggs. Once hatching commenced, free-swimming larvae were collected in the morning, euthanized with an overdose of tricaine methanesulphonate (MS-222) and counted after visual examination for morphological deformities under a stereomicroscope. The incidence of larval deformities was quantified as the percentage of hatchlings exhibiting severe deformations of the yolk sac, cranium or vertebral column (Fig. S2).

Subsamples of 5 to 10 eggs of each female and treatment combination were obtained every 24 h (every 48 h at 0 °C and 3 °C) for the determination of developmental stage after Hall *et al.* (2004). The rate of development was evaluated as the time (days) from fertilization until stage 17 (end of gastrulation), stage 22 (50% eye pigmentation) and peak hatch, which was defined as the time point when more than 50% of the remaining embryos of an incubator had hatched (Peck *et al.*, 2012).

Hatching success as a synonym for overall embryo viability was defined as the percentage of nonmalformed larvae that hatched from the initial number of fertilized eggs. The proportion of fertilized eggs within an incubator was estimated from the mean fertilization success of the respective egg batch (Table 2). Statistical analyses for larval deformities and hatching success are based on $n = 4$ to 5 females at each treatment combination ($n = 5$ females at 0 °C, 6 °C, 12 °C; $n = 4$ females at 3 °C, 9 °C).

Table 2 Mean egg diameter and fertilization success of cod eggs fertilized at ambient temperature and different PCO_2 conditions

Female	Mean egg diameter (mm, ±SD)	Fertilization success (%)			
		400 μatm	SD	1100 μatm	SD
1	1.48 (0.03)	75.0	0.2	83.4	6.7
2	1.54 (0.02)	96.7	2.2	95.9	2.8
3	1.48 (0.03)	97.2	2.2	98.1	1.3
4	1.28 (0.02)	92.9	2.4	88.7	3.4
5	1.36 (0.03)	96.9	1.3	95.3	1.7

Whole-embryo oxygen consumption. Oxygen consumption rates (MO_2) of eyed-stage embryos (50% eye pigmentation, stage 22 according to Hall *et al.*, 2004; Fig. 1iii) were measured in closed, temperature-controlled respiration chambers (OXY0 41 A, Collotec Meßtechnik GmbH, Niddatal, Germany). The double-walled chambers were connected to a flow-through thermostat to adjust the temperature of the respiration chamber to the corresponding incubation temperature of the eggs. Measurements were conducted in triplicate with eggs of each female using $n = 3$ different females in total. For each run, a mean number (± SD) of 20 ± 5 eggs were placed in the chamber with a volume of roughly 1 mL sterilized seawater adjusted to the PCO_2 values of the corresponding treatment. A magnetic microstirrer (3 mm) was placed underneath the floating eggs to avoid oxygen stratification in the respiration chamber. The change in oxygen saturation was detected by micro-optodes (fibre-optic microsensor, flat broken tip, diameter: 140 μm; PreSens GmbH, Regensburg, Germany) connected to a Microx TX3 (PreSens GmbH). Recordings were stopped as soon as the oxygen saturation declined below 80% air saturation. Subsequently, the exact water volume of the respiration chamber and wet weight of the measured eggs were determined by weighing (±0.01 mg) on a precision balance. Oxygen consumption was expressed as [nmol O_2 (egg * h)$^{-1}$]. Bacterial oxygen consumption and optode drift were determined by measurements of the blank respiration chamber before and after three successive egg respiration measurements.

Mitochondrial respiration. Mitochondrial respiration was measured in a homogenate of eyed-stage embryos (50% eye pigmentation, stage 22 according to Hall *et al.*, 2004; Fig. 1iii). One hundred eggs from $n = 3$ females were gently ground on ice in a glass potter filled with 2 mL ice-cold modified mitochondrial respiration medium MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 160 mM sucrose, 1 g L^{-1} bovine serum albumin, pH 7.4, 380 mOsmol L^{-1}) (Iftikar & Hickey, 2013; Gnaiger *et al.*, 2015). The resulting homogenate was collected and mitochondrial respiration was estimated using Oroboros Oxygraph-2k™ respirometers (Oroboros Instruments, Innsbruck, Austria). The oxygen flux [nmol O_2 (egg * h)$^{-1}$] was recorded and calculated in real time using Oroboros DatLab 5.2.1.51 (Oroboros Instruments, Innsbruck, Austria). Measurements were conducted in MIR05 buffer equilibrated to atmospheric PCO_2, at the acclimation temperature of the eggs. The PO_2 ranged from atmospheric saturation (ca. 370 nmol mL^{-1}) to 150 nmol mL^{-1}. A substrate-uncoupler-inhibitor titration (SUIT) protocol was used to investigate the capacities of the single components of the electron transport system (ETS); 10 mM glutamate, 2 mM malate, 5 mM pyruvate, 10 mM succinate and 3 mM ADP were added to stimulate oxidative phosphorylation (OXPHOS), and 3 μM oligomycin was used to inhibit F_0-F_1 ATP synthase and assess LEAK respiration. Repeated titration of carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone (FCCP, 0.5 μM each step) was used to uncouple mitochondria (ETS) and complex I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3) and complex II (succinate dehydrogenase, EC 1.3.5.1) were inhibited by the addition of

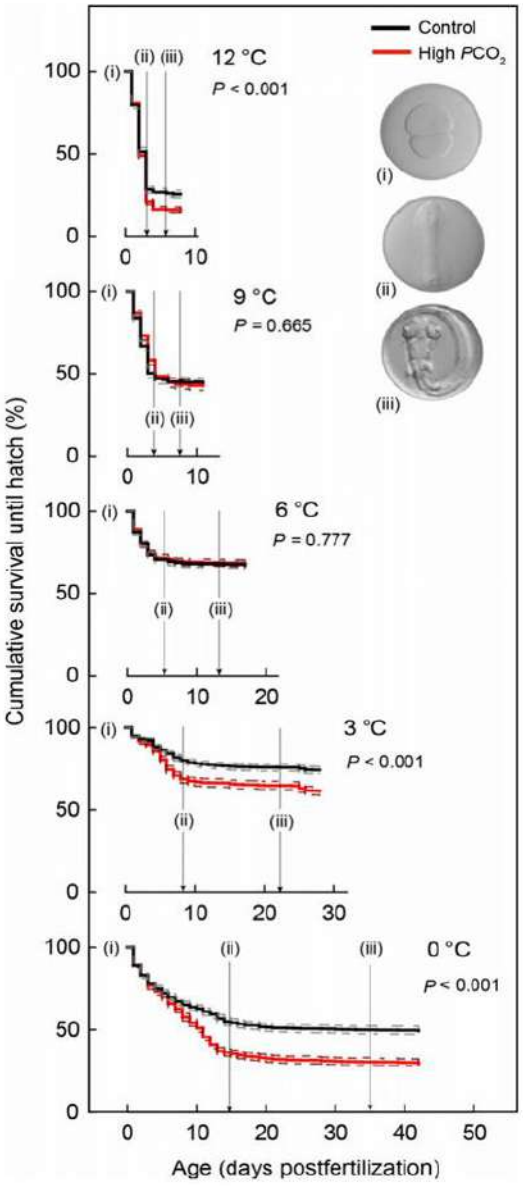

Fig. 1 Cumulative survival until hatch of Atlantic cod embryos reared at different temperatures (from bottom to top: 0, 3, 6, 9 and 12 °C) and PCO_2 conditions. Survival probability functions and *P*-values are based on Kaplan–Meier survival analysis. Dotted lines indicate 95% confidence intervals. Grey vertical lines labelled with (i), (ii) and (iii) denote successive stages during embryonic development also demonstrated in the pictures: (i) initial cleavage (start of the incubation experiment), (ii) closure of blastopore (end of gastrulation) and (iii) 50% eye pigmentation (stage sampled for respiration measurements). [Colour figure can be viewed at wileyonlinelibrary.com]

rotenone (0.5 μM) and malonate (5 mM), respectively (c.f. Shama *et al.*, 2014). OXPHOS coupling efficiency was calculated as [(OXPHOS-LEAK)/OXPHOS] according to Gnaiger *et al.* (2015). All chemicals were obtained from Sigma Aldrich, Germany.

Larval morphometrics at hatch. Subsamples of 10–30 non-malformed larvae of four to five females at each treatment combination ($n = 5$ females at 6 °C; $n = 4$; females at 0 °C, 3 °C, 9 °C, 12 °C) were photographed for subsequent measurements of larval standard length somatic body area and yolk sac area using Olympus image analysis software (Stream Essentials©, ± 1 μm). Only samples obtained from the same daily cohort (during peak hatch at each temperature treatment) were used for statistical comparison of larval morphometrics between PCO_2 treatments (Politis *et al.*, 2014).

Immunohistochemistry and confocal microscopy. Subsamples of 5–10 larvae were obtained from $n = 3$ females and each treatment combination within 24 h posthatch. Larvae were fixed in 4% buffered formaldehyde (PBS, pH 7.4) for 12 h at 15 °C and stored in 70% PBS-buffered isopropanol (pH 7.4). For immunohistochemical staining of Na^+/K^+-ATPase (NAK), fixed larvae were rehydrated in 0.1 M PBS (pH 7.4) and incubated in 3% bovine serum albumin (BSA) for 30 min to block nonspecific binding. Subsequently, larvae were incubated with the primary antibody (monoclonal mouse antiserum against the α-5 subunit of chicken NKA, D.M. Fambrough, Johns Hopkins University, Baltimore, USA, diluted by 1: 100 in PBS) for 12 h at 2 °C on a rotator. Afterwards, larvae were rinsed in PBS and incubated with a secondary antibody (DyLight© 488-conjugated goat anti-mouse; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. Finally, larvae were rinsed once more with PBS and placed on a fluorescence slide prior to image acquisition with a confocal laser scanning microscope (Leica TCS SP5 II). Negative controls were performed without application of the primary antibody. The surface area of individual immunopositive cells (IPCs) on the yolk sac epithelium was measured (± 1 μm) with the image processing freeware ImageJ©. The total number of IPCs on the yolk sac was evaluated using a cell counter plugin for ImageJ© (ATCN 1.6). Reliability of the software was verified by manual counts (<1% variation). IPC density was calculated as the number of cells divided by the yolk sac area (cells per mm^2). The total surface area of IPCs on the yolk sac ('IPC-cover') was estimated as a function of cell area (CA) and cell density (CD). In order to express IPC-cover as the percentage of the total yolk sac area for each individual, we used the following formula: IPC-cover = CA × CD × 100.

Data analysis

If not stated otherwise, statistical analyses were conducted using the program SigmaPlot version 12.5 (Systat Software, San Jose, CA, USA). Cumulative survival functions (with 95% confidence intervals) of embryos exposed to different water temperatures and PCO_2 conditions are based on

Kaplan–Meier survival analysis. Embryos that did not die but emerged as larvae towards the end of the experiment were treated as right-censored observations (Kaplan & Meier, 1958). Nonparametric log-rank tests were applied to compare survival curves among different PCO_2 conditions (fixed factor) at each of five incubation temperatures separately.

Multifactorial analyses of variance (two-way ANOVA) was used to evaluate whether temperature and PCO_2 (treated as fixed factors) and the combination of both factors had an effect on the following dependent variables: developmental time until embryos reached morphological landmarks (end of gastrulation, 50% eye pigmentation and peak hatch), larval malformations, hatching success, whole-embryo MO_2 and ionocyte morphology. Levene's and Shapiro–Wilk methods confirmed normality and homoscedasticity, respectively.

Data on mitochondrial functioning (OXPHOS, contribution of complex I and complex II to ETS and OXPHOS coupling efficiency) were checked for outliers by Nalimov's test at the level of $P < 0.01$. Preliminary two-way ANOVAs showed no significant effect of PCO_2 on mitochondrial functioning (all $P > 0.5$); as a result, data derived from different PCO_2 treatments were pooled within groups of $n = 3$ females in order to increase precision and statistical power for the detection of temperature effects (Quinn & Keough, 2002). Subsequently, nonlinear regressions were used to describe the relationship between temperature and ETS (complex I and complex II) and OXPHOS coupling efficiency. To account for unequal variances, a generalized additive model (GAM) was applied to assess the effect of temperature on OXPHOS. This spline-based, semiparametric method has been implemented in several statistical tools of the open source software R (R Developmental Core Team, 2013), such as the package *mgcv* (Wood, 2006). The *gam()* function in *mgcv* has the benefit of avoiding prior assumptions about the shape of the regression function, while it provides an estimate of the predictor effect (i.e. temperature), including regression coefficients and *P*-values (Wood, 2006). Data on whole-embryo MO_2 and OXPHOS were displayed as Arrhenius reaction norms, in order to linearize exponential data and to resolve changes in metabolic states (Kumamoto *et al.*, 1971).

Differences in larval morphometrics (standard length, somatic body area and yolk sac area) were determined by multifactorial analysis of covariance (ANCOVA) using general linear models in R (R Developmental Core Team, 2013). The models were run with temperature and PCO_2 as fixed factors, while the female-specific egg diameter was treated as a continuous covariate. Homogeneity of regression slopes was confirmed graphically prior to ANCOVA procedures. All data are presented as means (± SEM), and statistical tests with $P < 0.05$ were considered significant.

Results

Fertilization success

Fertilization success did not differ among PCO_2 conditions (paired t-test: $n = 5$, $P = 0.803$) and ranged between 75% and 98% (Table 2).

Cumulative survival and developmental time

Cumulative survival until hatch followed a general pattern characterized by two distinct periods at either treatment combination (Fig. 1). The major portion of mortality occurred during an early developmental period, which lasted from the cleavage stage (Fig. 1i) until the end of gastrulation (closure of blastopore, Fig. 1ii). Only few losses were recorded during subsequent development and hatching. Under present-day PCO_2 conditions, cumulative embryo survival increased from 52% at 0 °C to 73% °C at 3 °C, but decreased progressively at warmer temperatures reaching 63% at 6 °C, 42% at 9 °C and 25% at 12 °C. Exposure to elevated PCO_2 caused a significant decrease in embryo survival at 0 °C, 3 °C and 12 °C (log-rank test: all $P < 0.001$), but not at 6 and 9 °C (Fig. 1).

As expected, the rate of embryonic development was highly temperature dependent such that the time from fertilization until peak hatch increased from 9 days at 12 °C to 42 days at 0 °C (two-way ANOVA, $F_{4,45} = 723.5$, $P < 0.001$, Fig. 1). The influence of elevated PCO_2 on the development time until embryos reached stage 17 (Fig. 1ii), stage 22 (Fig. 1iii: 50% eye pigmentation) and peak hatch was not significant (two-way ANOVA, all $P > 0.5$, Fig. S3).

Hatching success

Hatching success decreased from the thermal optimum (3 °C to 6 °C) towards colder and warmer temperatures, yielding a dome-shaped response pattern across the applied temperature range (two-way ANOVA: $F_{4,45} = 15.9$, $P < 0.001$, Fig. 2). Elevated PCO_2 caused a significant reduction in hatching success (two-way ANOVA: $F_{1,45} = 11.1$, $P = 0.002$), especially at extremely cold and warm temperatures. The relative difference between PCO_2 treatments increased from Δ11% at 9 °C to Δ47% at 0 °C and Δ42% at 12 °C.

The proportion of unviable larvae hatching with severe morphological malformations increased with temperature and ranged from 6% at 0 °C to 22% at 12 °C (two-way ANOVA: $F_{4,45} = 9.1$, $P < 0.001$). Exposure of embryos to elevated PCO_2 had an additive effect, such that the incidence of deformities was consistently increased by an average of Δ37% (two-way ANOVA: $F_{1,45} = 10.0$, $P = 0.003$, Fig. 1, inlet).

Respiratory performance and capacity of eyed-stage embryos

Temperature and PCO_2 interactively affected *in vivo* oxygen consumption rates (MO_2) of eyed-stage embryos (two-way ANOVA: $F_{4,29} = 4.4$, $P = 0.011$). In

general, MO_2 increased with temperature, whereas extremely cold (0 °C) and warm temperatures (12 °C) caused changes in metabolic states, indicated by discontinuous Arrhenius reaction norms (Fig. 3a). In the range from 0 to 9 °C, MO_2 of embryos exposed to elevated PCO_2 was increased by an average of Δ11% relative to control conditions. By contrast, simultaneous exposure to elevated PCO_2 and warmer temperatures (12 °C) led to a significant reduction in MO_2 when compared with present-day PCO_2 (paired t-test: $P < 0.01$, $n = 3$). The increase in MO_2 in response to elevated PCO_2 between 0 and 9 °C was statistically significant if

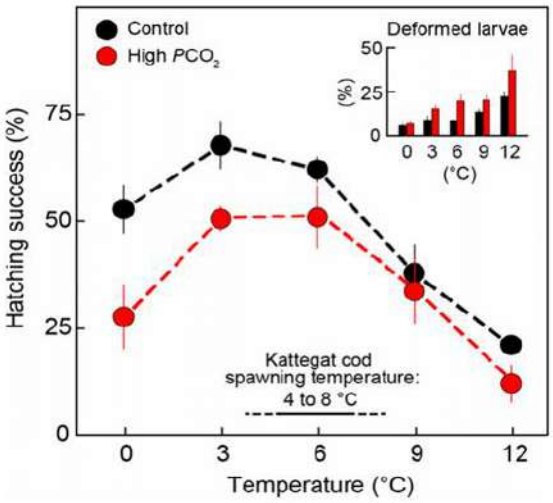

Fig. 2 Hatching success of Atlantic cod embryos as a function of temperature and different PCO_2 conditions. Where not visible, error bars fall within data symbols. (Inset) Bar graph shows the proportion of malformed larvae hatching at different temperature and PCO_2 conditions. Data are means (±SEM). [Colour figure can be viewed at wileyonlinelibrary.com]

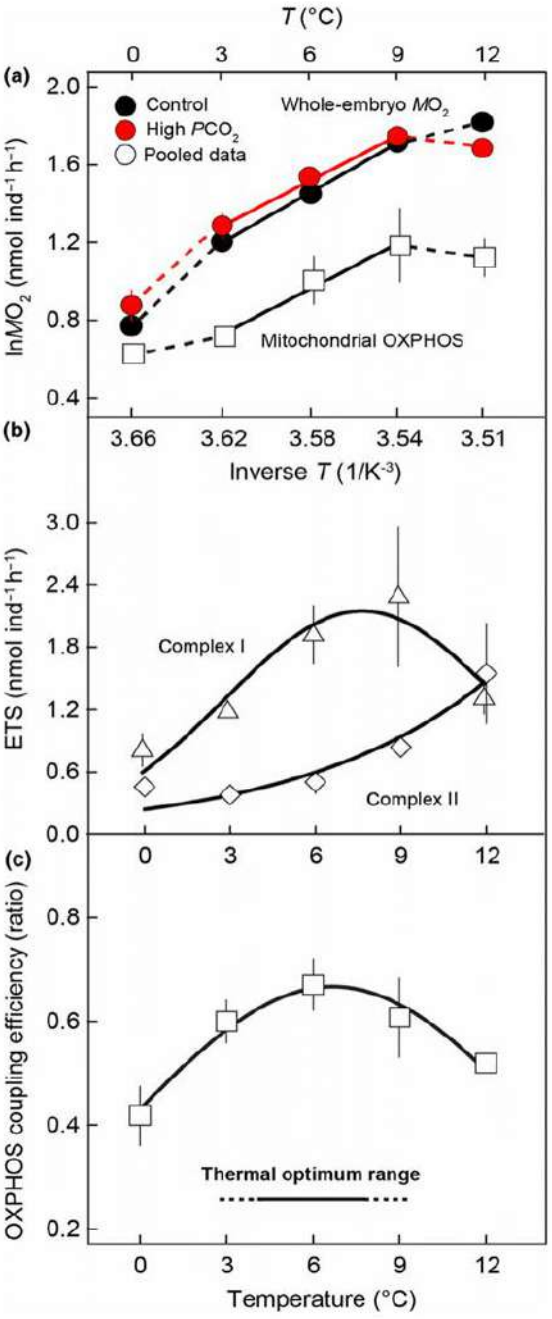

Fig. 3 Respiratory performance and mitochondrial capacities of Atlantic cod embryos as a function of temperature at different PCO_2 conditions (filled symbols). Open symbols refer to pooled data from both CO_2 treatments. (a) Arrhenius plots of *in vivo* whole-embryo oxygen consumption rates (MO_2) and *in vitro* mitochondrial phosphorylation capacity (OXPHOS). Solid lines are linear regressions indicating constant thermal sensitivity of MO_2 and OXPHOS in the range from 3 to 9 °C. (b) Changes in the contribution of complex I (open triangles) and complex II (open diamonds) to the electron transport system (ETS). Thermal performance curves (solid lines) were described by fitting a three-parameter Gaussian function for complex I ($2.148*exp(-5*(T-7.714)/4.823)^2$, $r^2 = 0.40$) and a two-parameter exponential growth function for complex II ($0.242*exp0.151*T$, $r^2 = 0.40$). (c) OXPHOS coupling efficiency expressed as the ratio of consumed oxygen to produced ATP (open squares). Thermal performance curve of OXPHOS coupling efficiency was described by a three-parameter Gaussian function ($0.667*exp(-5*((T-6.68)/7.152)2$, $r^2 = 0.40$). [Colour figure can be viewed at wileyonlinelibrary.com]

values measured at 12 °C were removed from the model (two-way ANOVA: $F_{1,23} = 6.16$, $P = 0.025$).

In vitro mitochondrial oxidative phosphorylation capacity (OXPHOS) was significantly influenced by temperature (GAM, $F_{4,14} = 4.27$, $P = 0.011$) such that OXPHOS increased until 9 °C, but levelled off at 12 °C. Thermal sensitivity and limitations upon warming of OXPHOS were consistent with those observed for whole-embryo MO_2, as was illustrated by parallel Arrhenius reaction norms (Fig. 3a).

The relative contribution of complex I (CI) and complex II (CII) to the electron transport system (ETS) was positively correlated with temperature. However, the response patterns differed markedly between the two complexes (Fig. 3b). Oxygen consumption rates of CI displayed an increase from 0 to 9 °C and a subsequent decrease towards 12 °C (three-parameter Gaussian function, $F_{4,14} = 4.03$, $P = 0.046$). Conversely, oxygen consumption of CII increased exponentially from 0 to 12 °C (two-parameter exponential growth function, $F_{4,14} = 13.62$, $P = 0.003$), indicating dissimilar thermal performance thresholds among respiratory complexes.

OXPHOS coupling efficiency, expressed as the ratio of consumed oxygen to produced ATP, was significantly correlated with temperature (three-parameter Gaussian function: $F_{4,14} = 5.36$, $P = 0.022$). Coupling efficiency was stable in the range from 3 to 9 °C, but decreased towards both extremely cold (0 °C) and warm temperatures (12 °C), yielding a dome-shaped response across the applied temperature range (Fig. 3c).

Larval morphometrics at hatch

Both standard length (SL, Fig. S4a) and somatic body area (SBA, Fig. 4a) of newly hatched larvae were significantly affected by temperature (ANCOVA, SL: $F_{4,45} = 10.7$, $P < 0.001$; SBA: $F_{4,45} = 2.8$, $P = 0.040$) and tended to decrease in response to warming. SL of larvae reared under elevated PCO_2 was reduced by an average of Δ5% relative to present-day PCO_2 (ANCOVA: $F_{1,45} = 6.6$, $P = 0.015$). The reduction in developmental growth in response to elevated PCO_2 became more apparent through the measure of SBA (Δ11%, ANCOVA: $F_{1,45} = 15.4$, $P < 0.001$), indicating that larvae were leaner rather than shorter. Yolk sac area was also influenced by temperature (ANCOVA: $F_{4,45} = 4.2$, $P = 0.007$), but did not differ among PCO_2 treatments (ANCOVA: $F_{1,45} = 0.25$, $P = 0.628$; Fig. S4b).

Abundance of NKA-rich ionocytes on the yolk sac of newly hatched larvae

Na^+/K^+-ATPase-immunopositive cells (IPCs) were most abundant on the yolk sac epithelium of newly hatched cod larvae. Highest densities were observed on the walls of the primordial gill cavity (Fig. 4b ii). The percentage of the yolk sac area covered by IPCs (IPC-cover) decreased linearly with increasing temperature (two-way ANOVA: $F_{4,29} = 35.2$, $P < 0.001$) from 25% at 0 °C to 17% at 12 °C under present-day PCO_2

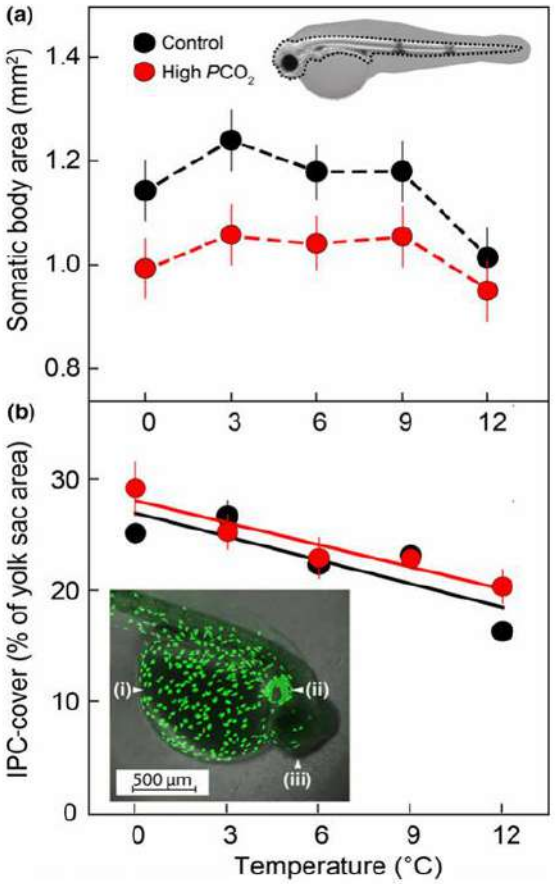

Fig. 4 Effects of temperature and PCO_2 on larval morphology at hatch. (a) To estimate developmental growth, we measured larval somatic body area as illustrated by the inserted image. Data are means (±SEM) derived from ANCOVA. Results for larval standard length and yolk sac area are displayed in Fig. S4. (b) The abundance of Na^+/K^+-ATPase-immunopositive cells (IPCs) on the yolk sac epithelium of newly hatched larvae was used as a morphological indicator of embryonic acid–base regulation effort. Linear regressions indicate the correlation between IPC-cover and temperature. Where not visible, error bars fall within data symbols. The inserted confocal image shows a representative cod larva with IPCs stained in green. Arrowheads indicate (i) IPCs on the yolk sac, (ii) densely aggregated IPCs on the walls of primordial gill cavity and (iii) the head of the larva. Data are means (±SEM).

conditions. An apparent difference in IPC-cover between PCO_2 treatments was not significant (two-way ANOVA: $F_{1,29} = 1.9$, $P = 0.172$).

Discussion

This study aimed to assess the effects of ocean acidification on embryonic thermal sensitivity in a key marine fish species, *Gadus morhua*. Our results demonstrate that constraints imposed by elevated PCO_2 impair the capacity of embryos to survive under low and high temperature extremes, which has important implications for the susceptibility of cod to future climate change.

Latitudinal ranges of marine ectotherms are fundamentally determined by their ranges of thermal tolerance (Sunday *et al.*, 2012). Understanding species- and life stage-specific thermal windows, and secondly the effects of ocean acidification on these windows, is thus crucial to anticipate future changes in species abundance and distribution patterns (Pörtner & Farrell, 2008; Pörtner & Peck, 2010). The present study shows that exposure to future PCO_2 levels can increase the sensitivity of cod embryos to thermal extremes and *vice versa*. Hatching success of embryos acclimated to intermediate temperatures (6–9 °C) was only marginally reduced in response to elevated PCO_2, whereas exposure at 0 °C and 12 °C caused a reduction in hatching success by more than 40% relative to ambient CO_2 conditions (Fig. 2). This effect corresponds to a narrowing of the thermal tolerance window of cod embryos and implies that ocean acidification constrains the range of suitable spawning conditions for this economically important species. Future levels of PCO_2 are thus likely to exacerbate the impacts of warming and/or thermal extreme events, such as cold snaps or heat waves, which are expected to become more frequent and more intense with climate change (Stocker *et al.*, 2013). Reduced heat tolerance in response to experimental ocean acidification has been reported for early life stages of marine fish species (Pimentel *et al.*, 2014, 2016; Flynn *et al.*, 2015) and various marine invertebrates (reviewed by Przeslawski *et al.*, 2015). In line with these previous studies, our results strengthen the hypothesis that physiological constraints imposed by elevated PCO_2 enhance the susceptibility of organisms to thermal extremes (Pörtner, 2012), in this case possibly due to an increase in oxygen demand (Fig. 3).

Lethal effects of elevated PCO_2 and critical temperatures occurred primarily during early embryogenesis (Fig. 1), likely reflecting ineffective homeostatic control mechanisms until the end of gastrulation (Hamdoun & Epel, 2007). Thermal tolerance thresholds of hatching success presented here (≤0 and ≥9 °C) agree well with those reported in other studies (Nissling, 2004; Geffen *et al.*, 2006; Dahlke *et al.*, 2016). These values also match the thermal niche (3–7 °C) occupied by most cod populations during spawning (Righton *et al.*, 2010), which is narrow compared with temperature preferences of non-breeding adult conspecifics (−1.5 to 20 °C). Ontogenetic differences in thermal tolerance can be linked to a shift in the capacity for oxygen supply from simple diffusion across the integument of embryos to fully developed ventilation and cardiovascular systems in postlarval stages (Pörtner & Farrell, 2008). Physiological constraints associated with thermal specialization of embryos may therefore constitute one important reason for the climate sensitivity of cod and other marine ectotherms. Our results imply that future warming (+3 to 4 °C) will exceed embryonic tolerance thresholds of southern cod populations, for example in the Celtic Sea, North Sea and adjacent Kattegat, especially if combined with ocean acidification.

Changes in cod abundance and distribution patterns further depend on factors other than temperature, such as foodweb dynamics, habitat availability and demography, all of which are influenced by climate and human exploitation of marine resources (Petitgas *et al.*, 2013; Kjesbu *et al.*, 2014). These complexities can at least in part be captured through their energy demand and associated routine metabolic scope shaping distribution limits (Deutsch *et al.*, 2015). Efforts to estimate the susceptibility of marine ectotherms to climate change must therefore build on a mechanistic understanding of individual physiological responses to temperature (Pörtner & Farrell, 2008). Mechanisms that have been suggested to play an important role in shaping thermal tolerance windows of water-breathing animals include protein thermal stability (Somero, 2010), the regulation of ventilation and cardiac performance (Pörtner & Farrell, 2008; Eliason *et al.*, 2011), in relation to aerobic energy supply and demand (Pörtner, 2010; Sokolova *et al.*, 2012; Schulte, 2015). Limits found at different levels of biological organization are suggested to follow a systemic to molecular hierarchy of thermal tolerance (Pörtner, 2002).

Our measurements of whole-embryo oxygen consumption rates ($\dot{M}O_2$) and mitochondrial functioning (Fig. 3) indicate that heat tolerance of cod embryos is shaped by the organismal capacity to cover oxygen and energy (ATP) demand at thermal extremes. Limitations to $\dot{M}O_2$ upon warming were paralleled by decrements in mitochondrial phosphorylation capacity (OXPHOS) and coupling efficiency resulting from functional constraints to the electron transport system (ETS). Defective performance of ETS was primarily related to a decrease in the activity of complex I (Fig. 3c), which is the main entrance of electrons into the ETS and

contributes significantly to ATP synthesis (Hochachka & Somero, 2016). This lack of capacity to further increase aerobic energy production matches organismal heat tolerance limits beyond which vital functions cease (Pörtner & Knust, 2007), as was evident from reduced hatching success under these conditions (≥9 °C, Fig. 2). Similarly, progressive insufficiency of mitochondrial ATP production was seen at low temperatures, possibly related to the onset of hatching failure. Compromised cellular energy balance and thereby energy-demanding processes such as acid–base regulation and protein synthesis (Pörtner, 2012; Sokolova *et al.*, 2012) may ultimately lead to reduced CO_2 resilience and survival in the cold (≤3 °C, Fig. 2).

Increased energy demand of embryos acclimated to elevated PCO_2 was reflected by a rise in MO_2 (Δ11%) within the range from 0 to 9 °C. By contrast, simultaneous exposure to elevated PCO_2 and warmer temperatures (12 °C) caused a significant decrease in MO_2, suggesting that additional energetic loads associated with CO_2 compensation caused an earlier onset of thermal constraints on MO_2. This synergistic response pattern links impaired aerobic capacity with reduced embryo survival under exposure to warming and elevated PCO_2. Previous studies investigating CO_2 effects on the thermal sensitivity of aerobic metabolism in marine fish embryos reported similar results (Rosa *et al.*, 2014, 2016; Di Santo, 2015), while other species or life stages may display higher acclimation capacities (Gräns *et al.*, 2014; Flynn *et al.*, 2015).

Increased energy requirements for maintenance imposed by elevated PCO_2 and warming coupled with reduced mitochondrial energy provision translated into diminished developmental growth, evidenced by significantly reduced larval standard length and somatic body area at hatch (Fig. 4a). Interestingly, exposure of embryos to elevated PCO_2 caused a significant reduction in larval body size, while yolk reserves remaining at hatch did not differ between PCO_2 treatments. Hence, our results imply that energy provision was already at its maximum and increased energetic costs associated with acclimation to elevated PCO_2 induced reallocation of limited resources at the expense of embryonic growth (Rombough, 2011). This observation corresponds with the idea that resource allocation follows a hierarchical order where vital homeostasis-related functions take priority over other energy-demanding processes such as developmental growth (Wieser & Krumschnabel, 2001).

The abundance of extrabranchial ionocytes in newly hatched larvae serves as a morphological indicator for embryonic acid–base regulation effort (Hiroi & Mccormick, 2012). These cells contain large amounts of ion pumps such as Na^+/K^+-ATPase (NKA), which is considered the major energy sink for homeostasis-related processes (Melzner *et al.*, 2009). The observed increase in ionocyte surface area towards low temperatures hence indicates additional efforts associated with cold acclimation (Pörtner *et al.*, 1998). Exposure to elevated PCO_2 did not induce significant changes in ionocyte morphology (Fig. 4b). It is therefore plausible that not only acid secretion, but also further regulation and defence mechanisms (e.g. protein turnover, DNA repair) contributed to increased energy demands under high-PCO_2 conditions as previously demonstrated in sea urchin larvae (Stumpp *et al.*, 2012; Pan *et al.*, 2015).

The regulation of energy allocation to maximize growth during embryogenesis is important for the fitness and survival of hatching larvae because predation pressure during fish early life history is strongly size dependent and generally corresponds with the principle of 'bigger is better' (Houde, 1997; Pepin *et al.*, 2015). Furthermore, reduced body size at hatch affects larval foraging ability and starvation resistance (Miller *et al.*, 1988), thus leading to increased vulnerability throughout the critical first-feeding period, which can be decisive in terms of year-class strength (Chambers & Trippel, 2012). When effects of elevated PCO_2 were investigated in isolation, increased larval deformities and mortality were demonstrated for Atlantic silverside, *Menidia menidia* (Baumann *et al.*, 2012), Atlantic herring, *Clupea harengus* (Frommel *et al.*, 2014) as well as for cod from the Barents Sea, Norwegian Sea and Øresund (Frommel *et al.*, 2012; Stiasny *et al.*, 2016). These results corroborate our findings and emphasize the susceptibility of cod to future ocean acidification. However, populations may differ in their sensitivity to direct effects of temperature and PCO_2 (Frommel *et al.*, 2013; Oomen & Hutchings, 2016). Further research is therefore needed to account for existing differences in life history characteristics and acclimatization capacity among more than 20 cod populations located across the North Atlantic.

In summary, warming imposed limitations to oxygen uptake and mitochondrial capacities indicate a mechanistic link between aerobic energy homeostasis and heat tolerance. Exposure to elevated PCO_2 exacerbated negative effects of both warm and cold temperatures on survival and aerobic performance at the whole-embryo level, reflecting a narrowing of the aerobic thermal tolerance window. Increased embryonic energy demand under elevated PCO_2 was evidenced by higher metabolic rates and reduced larval size at hatch, while the consumption of yolk reserves remained unaffected. We conclude that cod embryos lack sufficient physiological capacities to convert and allocate energy to systemic functions that facilitate homeostasis and, at the same time, maximize organismal performance under stressful

environmental conditions. Projected ocean acidification and warming are thus likely to exert negative effects by narrowing this developmental bottleneck, possibly leading to the abandonment of traditional spawning habitats in the Kattegat and other regions along the southern boundary of the species' distribution.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Measured standard length and body weight of female and male Atlantic cod used in the present study.
Table S2 Summary table of seawater quality parameters measured prior to the fertilisation procedure.
Figure S1. Seawater parameters measured during cod egg incubation.
Figure S2. Representative examples for typical larval deformities.
Figure S3. Temperature-dependent development rates of cod embryos exposed to different PCO_2 conditions.
Figure S4. Larval standard length (a) and yolk sac area (b) of newly hatched cod larvae as a function of temperature and different PCO_2 conditions.

4. Discussion

This thesis aims to investigate how mitochondrial plasticity influences the acclimation capacity to ocean acidification and warming (OAW) of Arctic and boreal fish species. The role of mitochondrial specialization to define the sensitivity or tolerance to elevated temperature and PCO_2 is addressed in the present thesis by comparing the cardiac mitochondria of juvenile polar cod (*Boreogadus saida*) and Northeast Arctic cod (NEAC, *Gadus morhua*), respectively a polar and a boreal species co-occurring in the waters off the Svalbard archipelago; and the mitochondria in embryos of Atlantic cod (*G. morhua*) and Atlantic herring (*Clupea harengus*), two boreal species with distinct spawning behaviour.

The obtained results will be discussed in the light of data from a parallel study that investigated the very same specimens of polar cod and NEAC (Kunz *et al.,* 2016; Publication AI) and Atlantic cod and herring embryos (Dahlke in the co-authored publications) at higher organizational level to provide a wider point of view and to link their mitochondrial mechanism to the effects at the whole-animal level. In addition, the mitochondrial functioning of juvenile and embryonic Atlantic cod will be compared to assess the role of mitochondria in defining the width of the thermal window across life-stages.

The discussion will follow the objectives of the thesis as presented in section 1.5. The first section (4.1) analyses the influence of mitochondrial specialisation on the acclimation potential of the cardiac cells of polar cod and NEAC and the effects at higher organizational level (1.5 question *i*.). The second section (4.2) compares the mitochondrial metabolism of developing embryos of Atlantic cod and Atlantic herring to assess differences in their sensitivity to OAW and the effects at the whole-animal level (section 1.5 question *ii*.). The third section investigates in details the mitochondrial mechanisms linked to the sensitivity or tolerance towards OAW (section 1.5 question *iii*.). Lastly, section 4.4 provides a comprehensive summary and a general overview.

4.1 *How does mitochondrial specialisation contribute to species ecology and response to climate change?*

Metabolic adaptations to environmental stress such as variable temperature include flexible allocation of energy and switch between different metabolic processes responsible for energy production and conversion (Sokolova *et al.*, 2012). Increasing temperature speeds the cellular energy consuming processes up with the subsequent acceleration of the energy producing pathways to ensure metabolic coupling between energy demand and production (Hochachka and Somero, 2002). This coupling is the

result of the balance between substrates acquisition/conversion (Beukema and Cadée, 2001; Sokolova *et al.*, 2012) and mitochondrial efficiency (Guderley and Pörtner, 2010; Sokolova *et al.*, 2012).
Stenotherm and eurytherm fish are suggested to have different mitochondrial features, influencing their efficiency at elevated temperatures: stenotherms have high mitochondrial density with low individual capacity whereas eurytherms have fewer mitochondria with higher capacity (Pörtner, 2006). Moreover, the capacities of mitochondrial components (e.g. Complex I; Complex IV, Citrate Synthase, membrane leakiness) are reported to vary between stenotherm and eurytherm fish influencing the animal's habitat thermal range and thermal tolerance (Lemieux *et al.*, 2010; Strobel *et al.*, 2012; Iftikar *et al.*, 2014; Hunter-Manseau *et al.*, 2019).
In fish, the heart plays a key role in temperature adaptation. In some species cardiac mitochondrial efficiency is likely to decrease at temperatures close to the animal upper thermal limit (Iftikar *et al.* 2010; Iftikar *et al.*, 2014), less efficient cardiac mitochondria may lead to less efficient cardiac output which in consequence may lead to cardiocirculatory fail during heat stress contributing significantly to define the species thermal habitat range (Farrell, 2002; Christen *et al.*, 2018).

Publication I presents heart mitochondrial functioning and efficiency in polar cod. The bell-shaped reaction norm of OXPHOS coupling efficiency indicates an optimum temperature for ATP production efficiency between 3 and 6 °C. Moreover, OXPHOS, although not affected by elevated PCO_2, was around 98% of ETS at 0°C and 95% at 8°C but only 80% of ETS at 3 and 6°C (Tab. 5). The OXPHOS/ETS ratio is considered a marker of the reserve capacity of the oxidative pathway and species with narrow thermal window presented decreasing reserve capacity (OXPHOS/ETS) with rising temperature indicating a loss of capacity to generate membrane potential at elevated temperatures (Iftikar *et al.,* 2014). These results are in line with the behavioural thermal preference found by Schurmann and Christiansen (1994), suggesting that polar cod prefer to linger in the temperatures range (3-6°C) where the mitochondrial efficiency is at its maximum and there is still some ETS reserve capacity to draw from if needed.

Table 5: OXPHOS as percentage of ETS (OXPHOS/ETS) in the cardiac permeabilized fibres of polar cod (B. saida). Control PCO_2 is 400 µatm, High PCO_2 is 1170µatm. Values are given as mean ± S.E.M. (for *n* see table 2).

Acclimation Temperature	Control PCO_2	High PCO_2
0°C	98.06 ± 6.92	95.42 ± 8.05
3°C	77.74 ± 4.88	-
6°C	79.00 ± 4.19	79.00 ± 3.99
8°C	95.00 ± 3.79	94.00 ± 3.77

At 8°C, the lower efficiency in ATP synthesis is mainly to ascribe to increased proton leak indicating that membrane stability could be a key factor in temperature acclimation. Proton leak decreases membrane potential, which, in case of intense metabolic activity may lead to membrane dielectric breakdown (Rolfe and Brand, 1997; Brand and Nicholls, 2011), but causes intensified oxygen demand to maintain the level of ATP production (Pörtner, 2006; Žūkienė *et al.*, 2007; Iftikar *et al.*, 2014). This undesirable increase in mitochondrial oxygen demand, together with increasing mitochondrial metabolic rates entail the increase in standard metabolic rates (SMR) at elevated temperatures. SMR represents the energy costs of basal maintenance (such as acid-base regulation, protein turnover), is the major component of the energy budget (Guderley and Pörtner, 2010; Sokolova *et al.*, 2012) and cannot be reduced below a certain limit (Sokolova *et al.*, 2012).

Polar cod showed increased standard metabolic rates (SMR) and reduced growth at 8°C (Kunz *et al.*, 2016, Publication AI). At this temperature the increased energy requirement and mitochondrial oxygen demand suggested by higher oxygen flux for SMR is paralleled with a decrease in mitochondrial efficiency due to increased proton leak. Since the allocation of energy to maintenance has priority over growth and storage, a mismatch between energy demand and energy provision could provoke the stop of these processes if the available energy is only sufficient to support the basal maintenance costs (Sokolova *et al.*, 2012) explaining the reported decrease in growth. Moreover, the heart is a highly aerobic organ with 90% of the produced ATP conveyed to contractile machinery and ion pumps responsible for the heart beat (Farrell, 2002). Therefore, in this organ, a mismatch between mitochondrial efficiency and energy requirement could lead to negative consequences as indicated by the dramatic decrease in heart rates at 8°C found by Drost, Carmack and Farrell (2014) with severe outcomes for the animal, as indicated by the increased mortality of polar cod at 8°C (Kunz *et al*., 2016, Publication AI).

While for polar cod the optimum temperature ranged between 3 and 6°C, at 8°C (just two degrees warmer) SMR and proton leak increased exponentially followed by decreased growth and increased mortality. Hence, it appears worthwhile to investigate the cellular and mitochondrial functioning of polar cod in the temperature range 6-8°C in more detail to cast light on the transition from optimum temperature to upper thermal limit, with special attention to the heart.

On the opposite, OXPHOS in the cardiac mitochondria of NEAC rose with increasing temperature, suggesting higher plasticity of the mitochondrial metabolism to meet the cellular energetic requirements (Publication I). Moreover, despite the increase of proton leak with temperature, the OXPHOS coupling efficiency remained constant, indicating the ability to control the effects of temperature and accelerated metabolism on the inner mitochondrial membrane. Increased oxidative phosphorylation capacity (OXPHOS) and stable efficiency may contribute to keep the metabolic coupling between energy demand and production, allowing the animals to grow even at high temperature

as indicated by Kunz *et al.* (2016, Publication AI).

Despite the higher plasticity related to temperature, NEAC mitochondria appeared more sensitive to elevated PCO_2 than polar cod. At 16°C, OXPHOS, CI and CCO decreased in the group incubated under high PCO_2 (Publication I). Decreased OXPHOS, even with stable mitochondrial efficiency, means decreased ATP production which translates into less energy available for the cellular processes. In response to elevated PCO_2, the ATP demand increases due to increased activities of ion pumps such as Na^+/K^+-ATPase and H^+-ATPase (Cornelia M. Kreiss *et al.*, 2015; Cattano *et al.*, 2018). Increased ATP demand, paralleled with decreased ATP production, may lead to a mismatch in the energetic budget and a shift in the energy allocation towards basal maintenance processes (such as acid-base and ion balance). Despite this, the growth of NEAC was not affected by elevated PCO_2 (Kunz et al., 2016, Publication AI). However, the limited tank space and the individual feeding might have removed the energy necessary for locomotion and for food competition out of the budget, allowing positive growth even with increased maintenance costs and detrimental effects of CO_2 on the energy provision processes (see section 4.3 for details).

4.2 *Is the mitochondrial metabolism of developing embryos affected by ocean acidification and warming? How does the species ecology relate to the response towards climate changes?*

During embryonic development, significant changes in the structure and physiology occur with life-long impacts on the animal's performances (Jonsson and Jonsson, 2019; Dahlke *et al.*, 2020).

In fish, embryonic growth and development are sustained almost entirely by aerobic metabolism fuelled by amino acids (Finn and Fyhn, 2010).

While the effects of high temperature on embryonic development are largely studied, the knowledge about influence of elevated PCO_2 is relatively small (Di Santo, 2015; Dahlke *et al.*, 2020). However, Baumann *et al.* (2012) indicate the egg stage as the most vulnerable to elevated PCO_2 suggesting a strong selection towards tolerant individuals when addressing the question of CO_2 tolerance in fish larvae. Among the egg stages, Dahlke *et al.* (2020) reported the gastrulation stage as most sensitive to OAW in Atlantic cod. Studies on different taxa suggest that early embryonic stages are protected by maternal factors such as metallothionines and chaperon proteins (Meistertzheim *et al.*, 2009; Lister, Lamare and Burritt, 2015; Byrne *et al.*, 2020) or epigenetic effects (Eirin-Lopez and Putnam, 2019) while by the time of hatch, larvae have developed proper ion and acid-base balance systems to counteract the elevated PCO_2. The embryos of Atlantic cod and Atlantic herring presented in this thesis reached the developmental stage of 50% eye pigmentation, not protected any more by the

maternal provisions but less efficiently equipped than larvae at hatch.

Embryonic mitochondrial respiration was not affected by elevated PCO_2 in both species but was sensitive to temperature and showed similar reaction norms (Publication III and IV). In fact, mitochondrial respiratory capacity (expressed either as OXPHOS or ETS) increased with temperature up to 9°C for Atlantic cod and 10°C for Atlantic herring, and then plateaued.

In both species, the main cause of this plateau seemed to be a decrease of Complex I (CI) activity at warm temperatures. While the mitochondrial oxygen consumption depending on the electron flux through Complex II (CII) continued to rise with increasing temperature in both species, in Atlantic cod the oxygen consumption depending on the electron flux through CI increased until 9 °C and then decrease at 12°C (less than 50% compared to 9°C). In Atlantic herring the decline rates were consistent (6°C: 62% contribution of CI to ETS; 14°C: 37% contribution) but more progressive suggesting a different control of the electron flux through the two complexes. The loss of CI function, not only decreases the overall mitochondrial capacity (represented by plateaus in the ETS and OXPHOS capacities) but may lead to an even lower net ATP production, since the H^+ pumped following the electron flux through CI generate more ATP than through CII (for details see section 4.3).

Less ATP available at high temperature could explain the decreased hatching success in both species (Publication III and IV). At elevated temperature the costs for maintenance increase, leaving less energy available for growth and development, if the ATP production is impaired. Standard length at hatch also decreased with temperature suggesting less energy available for growth. The diminution of hatchling size occurred at colder temperatures than the decrease in hatching success, indicating a possible partitioning of the available energy in favour of development over growth at intermediate temperatures.

While elevated PCO_2 did not influence the mitochondrial functioning in both species, the two fish species presented different whole-animal tolerance. Atlantic herring embryos were tolerant to high PCO_2 with no effects on hatching success and length at hatch indicating the presence of compensatory mechanisms. Atlantic cod, instead, showed lower respiration rates (MO_2) and lower hatching success, especially at the highest temperature analysed (12°C). At this temperature OXPHOS plateaued, the flux through CI decreased sharply (providing less ATP/cycle) and the ATP production efficiency decreases possibly causing a diminution of the general cellular ATP load. If extra costs were added under high PCO_2 incubation, for example for ion and acid-base balance, the energy available for development and growth may have decreased with consequences on hatching success and hatchling size (Sokolova, 2013; Heuer and Grosell, 2014; Cattano *et al.*, 2018).

The different sensitivity of the two species to OA could relate to diverse ecologies. Studies have illustrated that tolerant species generally occur in habitats with daily and seasonal CO_2 fluctuations (Munday *et al.*, 2011; Bignami *et al.*, 2013; Chevin and Hoffmann, 2017; Gaitán-Espitia *et al.*, 2017).

Moreover, a recent study showed greater mortality under elevated PCO_2 in pelagic fish compared to benthic species (Cattano *et al.*, 2018). Atlantic herring are benthic spawners, therefore the high tolerance to OA of their embryos could be explained by the higher and more variable CO_2 levels experienced in their natural environment.

Despite the different impact of OA on the metabolism, exposure to high levels of CO_2 increased the malformations rates in both species (Publication III and IV). A possible explanation is the upregulation of the collagen α-1 gene col27a1b under elevated PCO_2, linked with the ossification of cartilage, in particular the notochord (Mittermayer *et al.*, 2019; Stiasny *et al.*, 2019).

Elevated temperature decreased standard length at hatch in both species (Publication III and IV), making the hatchlings more vulnerable to predation (Rombough, 2011). However, fish larvae are one of the fastest growing vertebrates (Finn *et al.*, 2002) and, in an appropriate nutrition regime, could rapidly exit this unfavourable phase. Yet, elevated temperature reduces the time available for development, as illustrated in Publication IV for Atlantic cod. Earlier hatch due to elevated temperature could not match phyto- and zooplankton blooms (regulated also by light). This match (or mismatch) is particularly important at the time of switching from yolk reserves to exogenous food, with consequences on larval survival and growth (Neuheimer, MacKenzie and Payne, 2018). Moreover, in the field, rising temperatures may be associated with high PCO_2 (OAW) which increases the malformation rates in embryos, further decreasing the survival expectancy of the new generations.

4.3 *Which are the mechanisms causing the different mitochondrial responses to ocean acidification and warming?*

The mechanisms underlying the different sensitivity to elevated temperature and PCO_2 can be divided into membrane-related and enzyme-related processes, which are tightly interlinked.

4.3.1 Membrane properties

It is fundamental for cellular homeostasis to keep cellular membranes fluid. Membrane fluidity increases with temperature, changing the functional properties (Hazel, 1972; Guderley and Johnston, 1996; Brand and Nicholls, 2011). To keep a 'cold' membrane functioning at warm temperature it is necessary to modify its composition towards more stable lipids. This form of adjustment is defined homeoviscous adaptation (Hazel, 1995) and it has been observed upon warming in eurytherm fish such as rainbow trout, *Oncorhynchus mykiss* (Kraffe, Marty and Guderley, 2007), and killifish, *Fundulus heteroclitus* (Grim, Miles and Crockett, 2010), but not in the stenotherm high-Antarctic *Notothenia rossii* (Strobel *et*

al., 2013b). This observation suggests that the ability to alter membrane fluidity plays a role in cellular acclimation potential. Moreover, since mitochondria are surrounded by two membranes (and the inner membrane being highly specialised), this form of adaptation could influence mitochondrial sensitivity to temperature and therefore contribute to set the limits of the aerobic metabolism.

Publication II reports the lipid classes composition of the cellular membranes (pooled) in the heart of polar cod and NEAC at the lowest and highest incubation temperatures.

Both fish species showed no effects of elevated PCO_2 on membrane composition but a different response to temperature (see Publication II: Table 2). Polar cod did not change the relative abundance of lipid classes between 0 and 8°C while in NEAC phosphatidylcholine (PC) content relative to the total lipid content was slightly higher at 16°C compared to 3°C, altering its ratio with phosphatidylethanolamine (PE).

Despite the higher temperature for gel/fluid transition of PE lipids (Brown *et al.*, 1986), a high content of PE at elevated temperature could result in a loss of membrane integrity due to the change of the molecular geometry from cylindrical to conical and therefore unsuitable for the bilayer phase (Israelachvili, Marceljia and Horn, 1980; Seddon, 1990). Hence, at high temperature, an increase of PC (which maintains its structure even at high temperature) over PE is beneficial to ensure membrane integrity and function, as seen in NEAC.

Thermal perturbation of the lipid phase state increases the permeability to cations and water, presumably because of packing defects (Singer, 1981; Carruthers and Melchior, 1986).

Although this thesis presents data relative to the entire pool of cellular membranes, some general conclusions could be drawn about mitochondrial membranes. In fact, 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).

The inner mitochondrial membrane has a higher content of PE (40%) than other organelles (about 20%) (Hazel, 1995). Thus, elevated temperature could alter the bilayer integrity increasing the leak of protons from the intermembrane space into the mitochondrial matrix (Brand *et al.*, 1994).

The proton circuit is central to the ATP production mitochondrial function. Protons pumped during substrate oxidation into the intermembrane space re-enter the matrix through ATP synthase to produce ATP from ADP (Fig. 2). Under physiological conditions, all mitochondria possess an endogenous proton leak (Brand and Nicholls, 2011), which may serve to prevent dielectric breakdown of the membrane and limit the leakage of single electrons from the electron transport system to form ROS (Rolfe and Brand, 1997; Brand and Nicholls, 2011). However, an increase in proton leak may decrease the oxidative phosphorylation efficiency by uncoupling substrate oxidation from ATP production (Hardewig, Peck and Pörtner, 1999; Hilton, Clements and Hickey, 2010; Mueller *et al.*, 2011; Iftikar and Hickey, 2013; Strobel

et al., 2013b).

Publication I shows the effects of temperature on proton leak (named State IV^{+}) in heart permeabilized fibres. Proton leak was not affected by elevated PCO_2, neither in polar cod nor in NEAC. These results, together with the absence of CO_2–induced adjustments in the lipid classes (Publication II), suggest that elevated PCO_2 (and therefore ocean acidification) does not impact the mitochondrial function through enhancing proton leak. Conversely, increasing temperature influenced proton leak in both species, although in different ways.

Polar cod had unchanged proton leak between 0 and 6°C, with values reaching 20% OXPHOS at 3 and 6°C but was significantly higher in fish incubated at 8°C, where the fraction of proton leak doubled. The absence of modifications in the lipid classes, in particular in the PE/PC ratio, suggests that the dramatic increase of proton leak at 8°C could be caused by loss of integrity of the inner mitochondrial membrane.

In NEAC, proton leak increased along with incubation temperature without exceeding the 40% of concomitantly rising OXPHOS at the highest temperatures. The gradual increase in proton leak was paralleled by lower PE/PC (Publication II) indicating a certain degree of adjustment and control over the negative effects of temperature.

Lipids determine the physical properties of the membranes and therefore may play a role in the regulation of the proteins embedded in them (Hazel, 1972; Guderley and Johnston, 1996).

In polar cod, the lack of lipid class modifications in the cellular membranes and therefore assumed in the mitochondrial membranes, could contribute to the decrease in capacity of the membrane-associated enzyme CCO (Publication II) due to the loss of membrane integrity. In NEAC on the other hand, the stability of CI:CIII and the increase in CCO capacity upon warming, could be partially explained by the trend towards membrane adjustment (Publication II).

These findings, all together, suggest that membranes could represent a constraint for the acclimation capacity of polar cod compared to NEAC during ocean warming.

4.3.2 Mitochondrial enzymes

As temperature rises, the cellular energy demand increases. To adjust the energy production to the new requirements, metabolic adaptation may set in, according to species and life stage.

Metabolic adaptation typically implies changes in the preferred fuel and shifts from macromolecules (e.g. lipids and proteins) synthesis to catabolism (Brodte, Knust and Pörtner, 2006; Windisch *et al.*, 2011).

These changes can involve directly the mitochondria as intended place of the shifted metabolic pathways

(e.g. the fatty acids β-oxidation takes place into the mitochondrial matrix), or indirectly by removing or adding intermediates of the TCA cycle (Hochachka and Somero, 2002; Windisch *et al.*, 2011; Strobel *et al.*, 2013b; Iftikar *et al.*, 2015). Therefore, mitochondrial enzymes may change their activity in a regulated manner, according to substrate availability and changes in the physical environment such as medium pH, membrane potential and membrane fluidity. These adjustments may include substrate affinity, turnover number and enzyme quantity (Hochachka and Somero, 2002). However, the new conditions, although functional, may not be as efficient as the previous ones. If temperature rises above a critical values, enzymes lose catalytic capacity and eventually denaturate adding consequential cost for protein turnover (Pörtner, 2006).

Elevated PCO_2 may affect the mitochondrial enzymes by changing the matrix pH, inhibiting specific enzymes (Strobel, *et al.*, 2013b), or interfering with the substrates transport (Robinson *et al.*, 1977).

Because of the important role of the aerobic metabolism in the energy provision for acclimation and adaptation to OAW, this thesis aimed to study the effects of elevated temperature and PCO_2 on the mitochondrial enzymes involved in the cellular respiration to enlighten the biochemical mechanisms underlying the mitochondrial functioning and therefore the match/mismatch between energy demand and production and the effects seen at the whole-animal level.

Citrate Synthase

Citrate Synthase (CS) represents the link between TCA cycle and pyruvate/acetyl-CoA producing pathways (e.g., glycolysis, fatty acids oxidation) and it is situated in the mitochondrial matrix. Publication II shows that the CS capacities of polar cod and NEAC have species-specific responses to elevated temperature but common responses to elevated PCO_2. Polar cod exhibited very little modification of enzyme capacities after being held for several months under elevated temperatures whereas in NEAC the enzyme capacity decreased with increasing temperature. Since CS is the link between TCA cycle and the fatty acids metabolism (catabolism and synthesis), variations of the CS capacity with temperature may indicate the ability to shift from fatty acids catabolism to synthesis (Windisch *et al.*, 2011) suggesting a higher degree of metabolic plasticity.

Both species presented higher CS capacity in fish held under elevated PCO_2. In rat kidneys (Robinson *et al.*, 1977), increasing levels of HCO_3^- (from 0.1 mM to 0.3 mM) stimulate the export of citrate from the intramitochondrial compartment involving three different carriers. First HCO_3^- enters the mitochondria exchanged with phosphate by a phosphate carrier, then, phosphate is exchanged with malate by a dicarboxylate carrier, lastly malate is exchanged with citrate by a tricarboxylate carrier in a net exchange of HCO_3^- for citrate. Hence, the loss of citrate from the mitochondrial matrix may require higher CS capacity to replenish the citrate load necessary for the TCA cycle under high PCO_2 conditions.

However, if the export of citrate is not fully compensated for, it could cause the slowdown of the TCA cycle and consequently the depression of the mitochondria respiration.

Complex I

Complex I (CI) activity variation is relevant to understand the mitochondrial acclimation potential in all the investigated animals.

Embryonic CI was not affected by elevated PCO_2 but its activity decreased at high temperature in both species (Fig.12a). This type of thermally induced decline has already been observed in aquatic ectotherms, from portunid crabs (Iftikar, MacDonald and Hickey, 2010) to triplefin fish (Hilton, Clements and Hickey, 2010). CI thermal instability may be linked to its size and shape and its sensitivity to ROS and/or to impaired substrate supply resulting from lower membrane potential (see section 1.2.1). Moreover, the proton channel is located into the membrane arm of the enzyme (Efremov, Baradaran and Sazanov, 2010), thus thermally induced instability of the membrane could lower the enzyme efficiency. The activity of CI from polar cod was not influenced by high PCO_2 but increased from 0 to 3°C and then stabilized (Fig.12b), indicating little thermal sensitivity. Publication II investigated the capacity of CI:CIII, which appeared thermally stable and in excess with respect to Citrate Synthase (CS). Cold-adapted fish are thought to use lipids as preferred fuel (Guderley and Gawlicka, 1992; Rodnick and Sidell, 1994; Driedzic, Bailey and Sephton, 1996). The β-oxidation of fatty acids produces acetyl-CoA for the TCA cycle and $FADH_2$ and $NADH + H^+$, which feed directly into the ETS explaining the apparent excess of CI:CIII in relation to CS and its thermal stability, partly overcoming the electrogenic supply of substrates. Yet, CI maximum capacity was measured as CI:CIII, thus CI apparent thermal stability might be the result of interactions between CI and CIII, for example the formation of supercomplexes $CI+CIII_2$ (Bianchi *et al.*, 2004)

In NEAC, CI activity was slightly lower than in polar cod and was not affected by elevated PCO_2 at 3 and 8°C but at 8°C the reaction norms of the two PCO_2 groups split (Fig. 12b). In the control group, CI activity increased up to 12°C and decreased at 16°C, possibly for the reasons listed above, whereas in the high PCO_2 group CI activity began to decrease already after 8°C. Up to now, little is known on the effects of CO_2 and HCO_3^- on CI and the results are often contradictory (Melzner *et al.*, 2020). While CI activity was enhanced by HCO_3^- either via anaplerotic pathways (Strobel *et al*., 2013b) or via phosphorylation mediated by cAMP/sAC (Acin-Perez *et al.*, 2009; Tresguerres *et al.*, 2014), a range of studies found elevated oxidative stress under OA conditions (see Melzner *et al.*, 2020 for review) suggesting higher ROS production, of which CI is one of the main targets. Increased capacities of CS and CII after incubation under elevated PCO_2 suggest an attempt to compensate for *in vivo* inhibition of the TCA cycle (Publication II). If the compensation is not complete the entire mitochondrial respiration

mechanism slows down, decreasing OXPHOS (Publication I) and CI activity. Moreover, decreased activity of CI (especially at high temperature) increases ROS production, which could damage CI and further decrease its activity. In conclusion, the negative effects of elevated PCO_2 on CI activity may not arise from the direct action of CO_2 on the enzyme but from the inhibition of the TCA cycle. These assumptions are supported by the absence of effects of elevated temperature or PCO_2 on the capacity of CI:CIII in the photometric analysis, untied from the TCA cycle by the experimental protocol (Publication II).

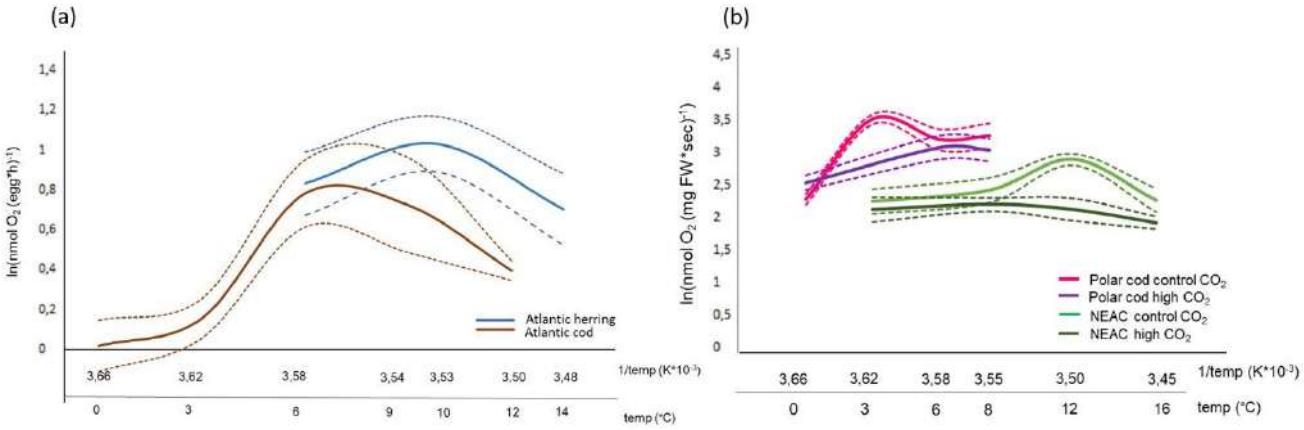


Figure 12: Arrhenius plot of the O_2 flux through Complex I (CI) in **(a)** the whole-body homogenate of "50% eye pigmentation" embryos of Atlantic cod (G. morhua) and Atlantic herring (C. harengus) and **(b)** the permeabilised cardiac fibers of juvenile polar cod (B. saida) and NEAC (G. morhua) after acclimation treatments (from fertilization on for embryos, four months for juveniles). Solid lines indicate the mean and dashed lines represent the S.E.M. range. In panel (a) the control and high PCO_2 groups were pooled because not statistically significant.

Another important aspect to consider is the contribution of CI to the ETS and its relation to CII. CI and II contribute differently to the generation of membrane potential. CI pumps 4 H^+ in the intermembrane space for each couple of electrons translocated. These 4 H^+ are followed by other 4 H^+ pumped by Complex III and 2 by Complex IV for a total of 10 H^+ pumped for couple of electrons. Due to the stoichiometry of the ATP-synthase, these 10 H^+ contribute to the formation of 2.5 ATP molecules whereas CII, incapable of pumping protons, provides electrons which flow only through Complex III and IV (6 H^+) generating only 1.5 ATP molecules. Hence, for every pyruvate entering the TCA cycle, 10 ATP derive from CI (4 NADH oxidised) and 1.5 ATP from CII (1 $FADH_2$ oxidised). Therefore, it is essential to investigate the contribution of CI to the ETS, since a shift in it may lead to less efficient ATP production pathways.

The embryos presented in this thesis (Publication III and IV) have an excess of CI (60-80% of ETS, Fig.13). This result is in line with their metabolic fuel preferences during development. From blastula

until hatch the main fuel is amino acids, stored as proteins (herring) or free amino acids (cod) (Fyhn and Serigstad, 1987). The most abundant and consumed amino acids are, in quantitative order: alanine, serine, leucine, isoleucine, lysine and valine. The first five enter the TCA cycle as CI substrates while only isoleucine and valine enter the cycle as CII substrates (Fig.14), supporting the observed higher contribution of CI to the ETS.

The decreased activity of CI at high temperature (see above) was followed by a decrease in its contribution to the ETS in both species (Fig.13), paralleled by an increase of the activity of Complex II as compensatory mechanism resulting in a shifted contribution of the two complexes to the ETS. Yet, shifted contribution in favour of Complex II may result in a decrease of ATP production (10 ATP vs. 1.5 ATP) which could lead to a mismatch between energy requested and energy provided by mitochondria.

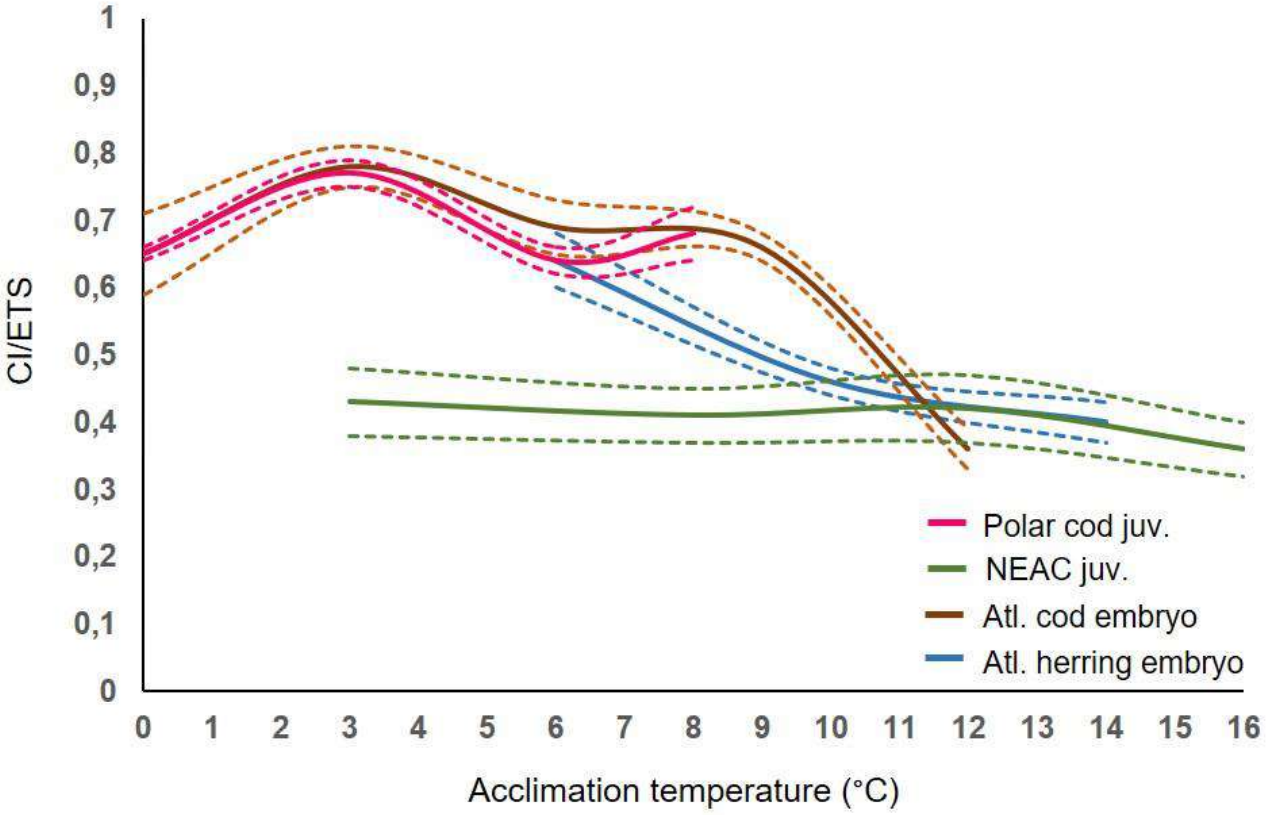


Figure 13: Contribution of Complex I (CI) to the electron transport system (ETS) according to acclimation temperature in all the fish presented in this thesis. Continuous lines represent the means and dashed lines represent the S.E.M. interval. Control and high PCO_2 groups were pooled as they were not statistically different.

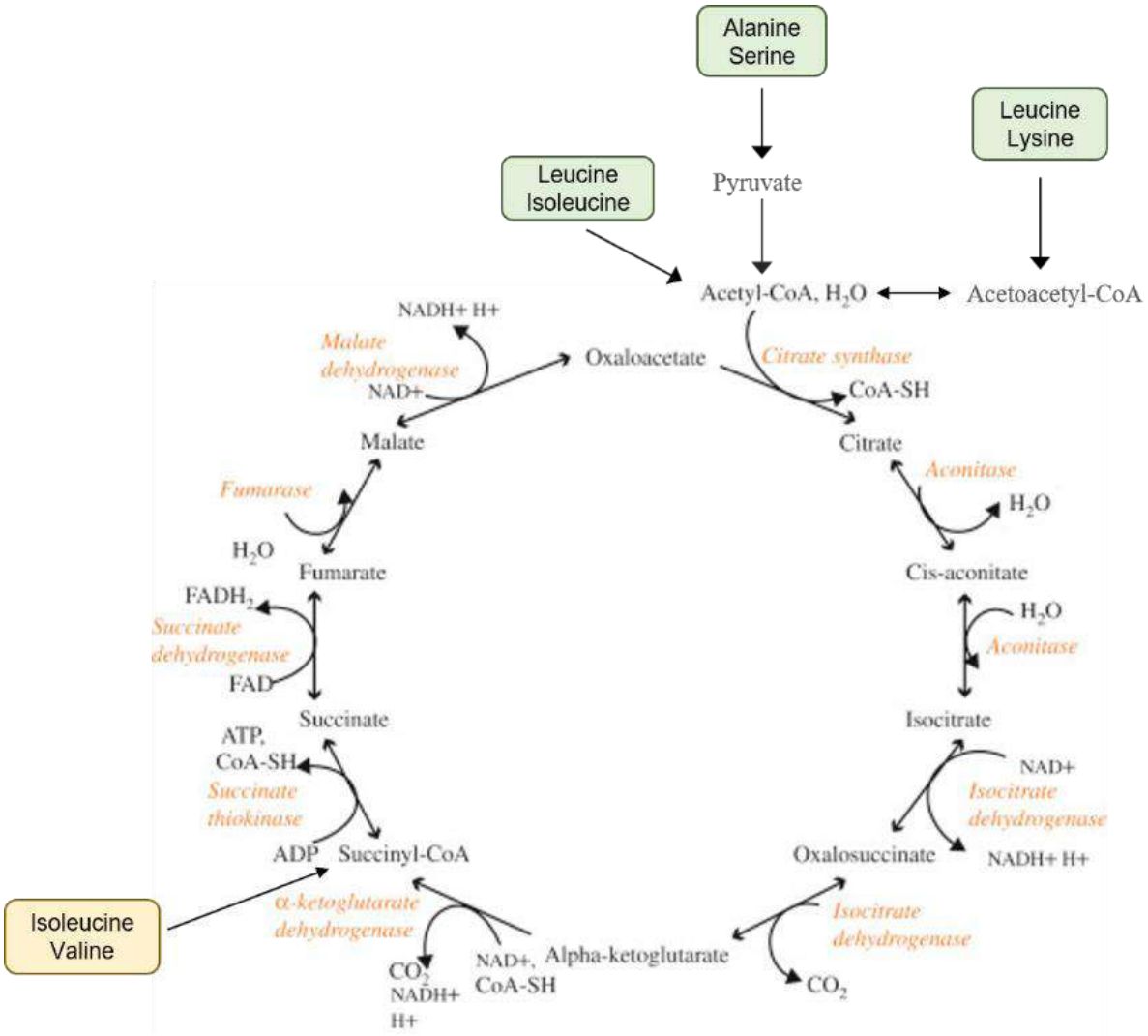


Figure 14: Tricarboxylic acids cycle (TCA) entry point of the six amino acids used as major metabolic fuel by fish embryos. TCA drawing by ScienceDirect.

In contrast with their conspecific embryos, juvenile NEAC showed a lower contribution of CI to the ETS (40%, Fig.13). This value is in line with other studies on temperate fish such as triplefin fish (Hilton, Clements and Hickey, 2010) and sea bass *Dicentrarchus labrax* (Howald *et al.*, 2019), but also Antarctic fish such as *Notothenia rossii* and *Notothenia coriiceps* (Mark *et al.*, 2012), suggesting different metabolic pathways between embryos and adult fish. At high temperature, the overall ETS capacity of NEAC decreased with CI activity maintaining the relative contribution stable.

In juvenile polar cod, CI contributed for 60-80% to ETS (Fig.13). Elevated contribution of CI to the ETS allows to obtain more ATP molecules per couple of electrons translocated and therefore for O_2 consumed (high ATP/O ratio), resulting in a lower need of O_2 to maintain the ATP load, hence it is associated with hypoxia tolerance in adult fish (Hilton, Clements and Hickey, 2010). For example, a recent study from Christen *et al.*, (2018) found CI contribution around 60-80% of the ETS (similar to what found in polar

cod) in the heart of the Arctic char (*Salvelinus alpinus*), a cold-adapted, hypoxia tolerant salmonid (Anttila *et al.*, 2015).

The high CI contribution to the ETS supports previous studies on hypoxia tolerance in polar cod (Kempf, 2020). Usually, polar fish are highly aerobic because they evolved in O_2-rich waters, however polar cod was reported to have higher hypoxia tolerance compared to Antarctic fish (Whiteley, Christiansen and Egginton, 2006), possibly due to the shorter period of stable low habitat temperatures in the Arctic compared to the Antarctic (Eastman, 1997; Whiteley, Christiansen and Egginton, 2006). Hence, although lipids may remain the preferred fuel in normoxic conditions (see above), polar cod may be able to shift to metabolic pathways more favourable to CI (e.g., carbohydrates and some amino acids) when retreating to the cold and hypoxic fjord bottom waters for avoiding high temperatures and predators.

In a general view, CI seems to play a key role in the mitochondrial plasticity but the mechanisms remain unclear and require further investigation.

For example, a recent study (Wilson *et al.*, 2020) found a mutation of the second codon of the ND1 gene in polar cod. This mutation substitutes a threonine residue with a leucine residue and was not found in related species such as Arctic cod (*Arctogadus glacialis*), walleye pollock (*Gadus chalcogrammus*) and saffron cod (*Eleginus gracilis*).

Threonine is a polar amino acid whereas leucine is an apolar amino acid therefore this mutation could alter dramatically the protein structure. The ND1 gene codes for the subunit core 1, one of the 14 highly conserved subunits of CI. In particular, subunit core 1 is part of the transmembrane domain responsible for proton translocation and mutations of this subunit cause, in humans, mito-myopathy, Optic Nerve Disease and encephalopathy (Wilson *et al.*, 2020). At the moment, there are no evidences of the effects of this mutation on the physiology of CI in polar cod (Wilson *et al.*, 2020), nonetheless this residue change could have fundamental consequences on the protein structure and on the interaction between the protein and the surrounding environment, thus it could be the key to understand some of the characteristics of the mitochondrial metabolism of this species such as the little mitochondrial plasticity or the high hypoxia tolerance. However, the study was conducted on specimens of the Pacific population of polar cod (Wilson *et al.*, 2020), and it is not clear, at the moment, if the mutation is present also in the Svalbard population.

CI activity could decrease due to loss of membrane potential caused by excessive proton leak. Proton leak increased with increasing temperature (Publication I) but the measurements of membrane potential are missing to support this hypothesis. Moreover, CI activity in NEAC decreased at high temperature, despite the ability of this fish species to adjust the membrane composition to counteract the increase in fluidity (promoting proton leak, Publication II). Possibly, ROS released at high temperature by CI could react with the membrane inducing lipids peroxidation chain reactions. Hence, lipid peroxides

may still destabilize the inner mitochondrial membrane affecting the capacity of CI without being detected by the lipid classes measurements.

For these reasons, CI features should be further investigated and paralleled with the analysis of membrane potential and ROS production.

Complex II

Complex II (CII) is a small complex embedded in the inner mitochondrial membrane. Despite the destabilizing effects of elevated temperature on the lipid bilayers, CII appeared to be more stable upon warming compared to other membrane-linked enzymes such as CI and CIV in the animals investigated in this thesis. Possibly, the key of its stability resides in the incapacity of pumping protons and in the small number of subunits (Rutter, Winge and Schiffman, 2010). Proton translocation, in fact, requires conformational changes which could cause higher instability, while a high number of subunits increases the conformational flexibility of the enzyme (Daniel, 1996). Since conformational flexibility and thermal instability are interrelated (Daniel, 1996), the small and stiff CII may be less prone to thermal instability. Moreover, the small size and the conformational stability may avoid CII to be negatively affected by alterations of the membrane fluidity and to be an easy target for ROS.

However, CII seems to be sensitive to elevated PCO_2, at least in NEAC (Publication II). Although a specific inhibition by bicarbonate was suggested by Strobel et al. (2013b), the mechanism behind is still unclear. In polar cod and in the embryos of Atlantic cod and Atlantic herring the contribution of this complex to the ETS increased while the contribution of CI decreased upon warming, suggesting a compensatory function of CII at high temperature in some species. Therefore, it would be worth to study the mechanisms underlying its sensitivity to temperature and CO_2 and to investigate whether the shift in relative contribution to the ETS depends on a shift in substrates preferences or also on the direct modulation of CII properties.

Complex III.

Complex III (CIII) is the least investigated of all the ETS complexes. However, it is the first complex situated after the Q-junction, thus receiving electrons from both CI and CII and it is embedded into the inner mitochondrial membrane, therefore sensitive to its changes. In this thesis CIII was investigated only in association with the other complexes, mainly CI. Therefore, some of the observed features of CI might be ascribable to CIII characteristics or to the interaction of the two complexes (e.g., supercomplex CI + $CIII_2$ Bianchi *et al*., 2004). Furthermore, CIII is thought to be involved in hypoxia tolerance (Guzy *et al.*, 2005). Since polar cod showed some traits related to hypoxia tolerance, it would be worth to

investigate the role of this complex in setting the mitochondrial plasticity, especially in polar cod.

Complex IV

Complex IV (CCO) activity in fish generally increases with warm acclimation (Cai and Adelman, 1990; Foster, Hall and Houlihan, 1993; Lemieux *et al.*, 2010b; Strobel *et al.*, 2013a; Iftikar *et al.*, 2014). However, while in some fish species CCO thermal window is greater than the individuals' thermal window, in other cases CCO capacity decreases while approaching the individuals' upper thermal limit. In particular, CCO seems to cover a broader thermal range in temperate fish (such as *Notolabrus fucicola* and *Notolabrus celidotus*, Iftikar *et al*., 2014 or common carp *Cyprinus carpio*, Hunter-Manseau *et al*., 2019) than in warm and cold – adapted fish (e.g., *Thalassoma lunare*, Iftikar *et al*., 2014; *Anarhichas lupus*, Lemieux *et al*., 2010b; *Gobionotothen gibberifrons* Hunter -Manseau *et al.*, 2019).

In this thesis, polar cod CCO capacity increased up to 6°C and decreased between 6 and 8°C (Publications I and II) and at the latter temperature its flux values approached the overall ETS flux suggesting a direct control of CCO over the ETS. Since the heart relies mainly on aerobic metabolism, a decrease in CCO capacity relative to ETS at 8°C could cause an impairment in energy supply leading to heart failure and eventually to increased mortality, as actually reported for this temperature by Kunz *et al.* (2016, publication AI). However, literature points to Complex I and V to be the most sensitive complexes to temperature variations (Kikuchi, Itoi and Watabe, 1999; Lemieux *et al.*, 2010a; Hunter-Manseau *et al.*, 2019), therefore the decrease in CCO activity could be ascribed, at least partially, to a general loss of functions of the mitochondrial membrane in which it is embedded (see proton leak, section 4.3.1) rather than a direct sensitivity to temperature and the drop in mitochondrial efficiency leading to increased mortality may be due to a group of factors (inability to modulate Complex I activity, decreased CCO activity, increased proton leak) rather than a direct consequence of the control exerted by CCO on ETS and on the phosphorylation capacity.

The CCO capacity of NEAC increased with increasing acclimation temperature (Publication I and II), and each acclimation group showed similar Q_{10} values (Publication II), indicating that NEAC is able to modulate the capacity of this enzyme, following the increased energy demand with temperature. This difference between polar cod and NEAC is in line with the idea that temperate fish have a CCO thermal window broader than the whole animal's, while in more stenotherm fish the upper thermal limit for this enzyme is close to the animal's limit (Iftikar *et al*., 2014; Hunter-Manseau *et al*., 2019).

In NEAC, CCO capacity decreased under elevated PCO_2 at high temperature, paralleled by increased capacities of CS and CII (Publication II) and decreased of overall OXPHOS (Publication I). If elevated PCO_2 inhibits CS and CII activity *in vivo* (suggested by the increased capacity as form of compensation, Strobel *et al*., 2013a; and by the lower OXPHOS values), less CCO is needed to maintain ETS in the

proper reduction state and associated catalytic capacity. Thus, the animal could down-regulate the production of CCO to avoid unnecessary (but costly) excess in line with the symmorphosis principles (Weibel *et al.,* 1991).

CCO is often assumed to be present in excess (Brunori *et al.*, 1987; Gnaiger, Lassnig, *et al.*, 1998; Blier and Lemieux, 2001). For example, an excess of CCO capacity relative to OXPHOS (CCO/OXPHOS) of at least 1.5-fold has been reported for the heart mitochondria of three triplefin fish species (*Bellapiscis medius, Forsterygion varium,* and *F. malcolmi* Hilton, Clements and Hickey, 2010). Higher CCO capacity relative to the whole ETS (CCO/ETS) have also been reported in the heart of three species of wrasses (*N. celidotus, N. fucicola* and *T. lunare* Iftikar *et al*., 2014). CCO excess capacity have been suggested to ensure the maintenance of the electron transport system in an oxidized state allowing a sharp electrogenic gradient even with variation in the conditions of substrate availability, temperature and oxygen availability(Gnaiger, Lassnig, *et al.*, 1998; Harrison *et al.*, 2015). However, in the triplefins study (Hilton, Clements and Hickey, 2010)) the intertidal *B. medius* presented a higher CCO/OXPHOS than the two sub-tidal species, and the tropical *T. lunare* (Iftikar *et al*., 2014) showed a greater overall CCO capacity than the two temperate wrasse species indicating an important role of CCO in setting the mitochondrial thermal window with greater CCO excess in the fish living at higher or more variable temperatures (Blier, Lemieux and Pichaud, 2014; Hunter-Manseau *et al.*, 2019).

NEAC juveniles exhibited greater CCO/ETS than juvenile polar cod (Fig.15) despite the absolute CCO capacity values being similar at 3 and 8°C (Publication I and II). The higher CCO/ETS in NEAC compared to polar cod is in line with the hypothesis of a greater CCO excess in fish living in a more challenging environment (Blier, Lemieux and Pichaud, 2014; Hunter-Manseau *et al.*, 2019). NEAC is a boreal eurytherm with a more active lifestyle and less O_2 available in the water than its Arctic relative. Furthermore, since an elevated CCO capacity implies high binding affinity for O_2 even at low tension (Gnaiger, 2003), high CCO/ETS may help NEAC to maintain adequate mitochondrial function during the incursions in hypoxic waters to feed (Skjæraasen *et al.*, 2008; Neuenfeldt, Andersen and Hinrichsen, 2009). Moreover, since elevated CCO activities have been associated with an increase in hyperoxia tolerance in cultured cells through reduced ROS production (Campian *et al.*, 2004), an excess of CCO may also be helpful to avoid excessive ROS formation during tissue reoxygenation after feeding in hypoxic areas. Despite these premises, polar cod is reported to be considerably more hypoxia tolerant (lower P_{O_2} crit) than NEAC (Kempf, 2020) but its tolerance might be mainly related to CI features since polar cod mitochondria have a higher CI/ETS and therefore, maybe, a higher ATP/O.

Atlantic cod embryos have lower CCO/ETS than the conspecific juveniles (Fig.15). The smaller CCO excess suggests that the embryonic mitochondria are optimized to work at their maximum capacity, to produce energy for fast growth. This, however, at the expenses of a broader thermal window. Embryonic

OXPHOS decreased at incubation temperature higher than 9°C and hatching success decreased at temperatures higher than 6°C (Publication IV) while OXPHOS and growth are maintained up to 16°C in juveniles (Publication I; Publication AI). The lower mitochondrial plasticity correlated with smaller CCO/ETS in Atlantic cod embryos support the assumption of embryos being a potential bottleneck for the population acclimation capacity to climate change (Dahlke *et al.*, 2020).

Comparing the excess CCO capacity in the embryos of Atlantic cod and Atlantic herring (Fig.15), CCO/ETS of Atlantic cod was rather constant throughout the temperature range while in Atlantic herring it increased dramatically between 10 and 14°C. Herring mitochondria have higher CCO upon warming possibly to maintain the electron transport system in the oxidized state despite the negative effects of temperature on the ETS complexes and the increase in metabolic rates, paralleled with the decrease of oxygen available in the water. Since 12°C could be considered the upper thermal limit for the Atlantic cod embryos belonging to the North Sea and Skagerrak population (Publication IV) and they did not show any adjustment of CCO/ETS with temperature, although the data are not directly comparable, the increase of CCO/ETS in Atlantic herring at 14°C may suggest a greater mitochondrial plasticity. The ability to increase the CCO capacity could allow Atlantic herring embryos to increase their mitochondrial metabolism upon warming, and therefore have a safety margin for temperature fluctuations. Atlantic herring are spring spawners (such as the Oslo Fjord spawners selected for this thesis) and usually lay benthic eggs in shallow waters (<25m) (Rosenberg and Palmén, 1981; Haegele and Schweigert, 1985), therefore their embryos are more prone to temperature fluctuations compared to Atlantic cod which lays buoyant eggs.

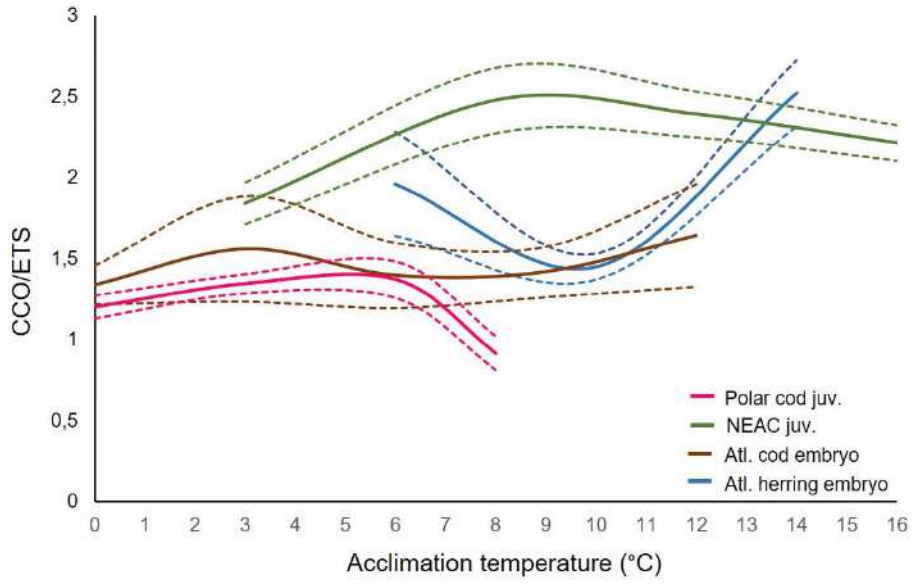


Figure 15: Cytochrome c Oxidase (CCO) capacity relative to the electron transport system (ETS) according to temperature. Continuous lines present the means and dashed lines indicate the S.E.M. range. Control and high PCO_2 groups were pooled as they were not statistically different.

4.4 Synopsis and outlook

This thesis investigated the role of mitochondrial plasticity and acclimation capacity to OAW of Arctic and boreal fish at juvenile and embryonic stage.

4.4.1 Polar cod

In the studies described in this thesis, polar cod presented some traits typical of stenothermal fish. Stenotherms are suggested to have high mitochondrial density with low individual capacity resulting in elevated costs at high temperatures (Pörtner, 2006). Even after four months of incubation at high temperatures, the activity of Citrate synthase (CS), a marker for mitochondrial density, did not change. Moreover, the composition of the cellular membranes did not show any attempt of adjustment to guarantee the appropriate membrane fluidity, with consequent increase in mitochondrial proton leak and associated costs. Above 3°C, stable CS activity is paralleled by stable Complex I (CI) capacity and OXPHOS indicating the little ability of polar cod to modulate the properties of the ATP production machinery in response to temperature (Tab. 4).

Although incubation under elevated PCO_2 increased the CS capacity, probably as compensatory up-regulation, the other aspects of the mitochondrial functioning were not affected, suggesting full compensation of the inhibition of CS. TCA cycle enzymes such as CS are suggested to be the first target of excess cellular PCO_2 (Strobel *et al*., 2013b). Since polar fish metabolism relies mainly on lipids, the direct provision of reduced intermediates ($NADH + H^+$ and $FADH_2$) to the ETS bypassing the TCA cycle may contribute to the high CO_2 tolerance in polar cod mitochondria.

Even if the projected values of OA do not affect the mitochondrial metabolism of juvenile polar cod, reaching a water temperature of 8°C may have dramatic consequences. This temperature could already alter the structure of the mitochondrial membrane increasing proton leak and decreasing the capacity of the embedded enzymes such as Complex IV (CCO). Increased proton leak, if not coupled by increased OXPHOS, decreases the ATP production (Fig.16). At 8°C the cellular energy demand is higher due to increased maintenance costs (increased SMR) but a lower ATP production could cause a decrease in ATP availability and consequent decrease of the animal's growth and eventually higher mortality as described by Kunz *et al.* (2016, Publication AI).

Juvenile polar cod may avoid the 8°C temperature by swimming to colder waters at the bottom of the fjords (Kempf, 2020) preventing the geographical displacement of the population. However polar cod spawn at colder temperatures (-1°C to 2°C) and egg survival decreases at temperatures higher than 3°C (Dahlke *et al.*, 2018) suggesting that the embryonic thermal window could be the key to foresee the

changes in distribution and potential habitat contraction, especially if taking into account also the widespread loss of sea ice (Fig.1j) which serves as protection for developing eggs and feeding habitat for larvae (Christiansen *et al.*, 2016).

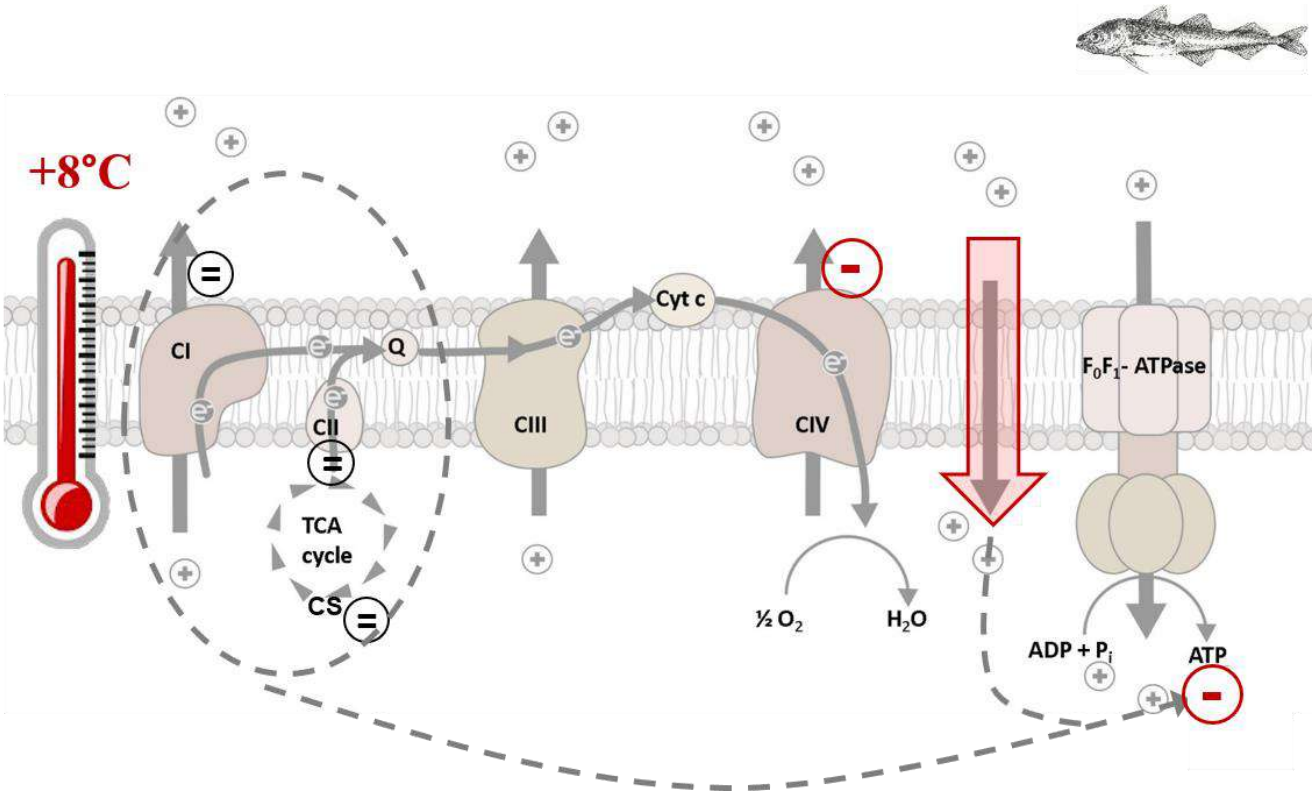


Figure 16: schematic of the suggested effects of high temperature (8°C) on the cardiac mitochondria of polar cod (*B. saida*). Elevated temperature increases proton leak which is not paralleled by an increase in the capacity of the TCA cycle nor of the ETS, leading to lower ATP production.

4.4.2 NEAC

Atlantic cod, mainly represented by the NEAC population, showed the features of a eurytherm fish with fewer mitochondria with high individual capacity (Pörtner, 2006). CS capacity decreased upon warming indicating a decrease in mitochondrial number and/or a shift in metabolic pathways (e.g., from lipid synthesis to carbohydrates catabolism).

The cellular membranes changed their composition towards more stable structures to ensure integrity and function. Proton leak increased with temperature but the OXPHOS coupling efficiency was stable throughout the investigated temperatures range, indicating that proton leak was always under control, thanks to the membrane adjustments. The stability of the OXPHOS coupling efficiency was also guaranteed by the increasing of OXPHOS (and the capacity of all the single complexes) suggesting high plasticity of the mitochondrial machinery to meet the cellular energy demand (Tab.6).

If NEAC mitochondria seemed to deal fairly well with increasing temperature, elevated PCO_2 exerted some negative effects on the entire ATP production mechanism.

CS and CII presented higher capacities *in vitro* under normocapnia after incubation at elevated PCO_2, suggesting compensatory up-regulation. *In vivo*, the increased export of citrate and the inhibited capacity of CII may slow the entire TCA cycle down, decreasing the ETS capacity and therefore the production of ATP (Fig.17). Moreover, lower flux of electrons through CI may enhance the ROS production which could, in return, damage the structure of CI decreasing its capacity, in a detrimental loop.

These effects were visible especially at the highest tested temperature (16°C), probably because of the thermodynamically increased mitochondrial metabolism on the one hand, and thermally induced higher energy demand of the cell on the other hand.

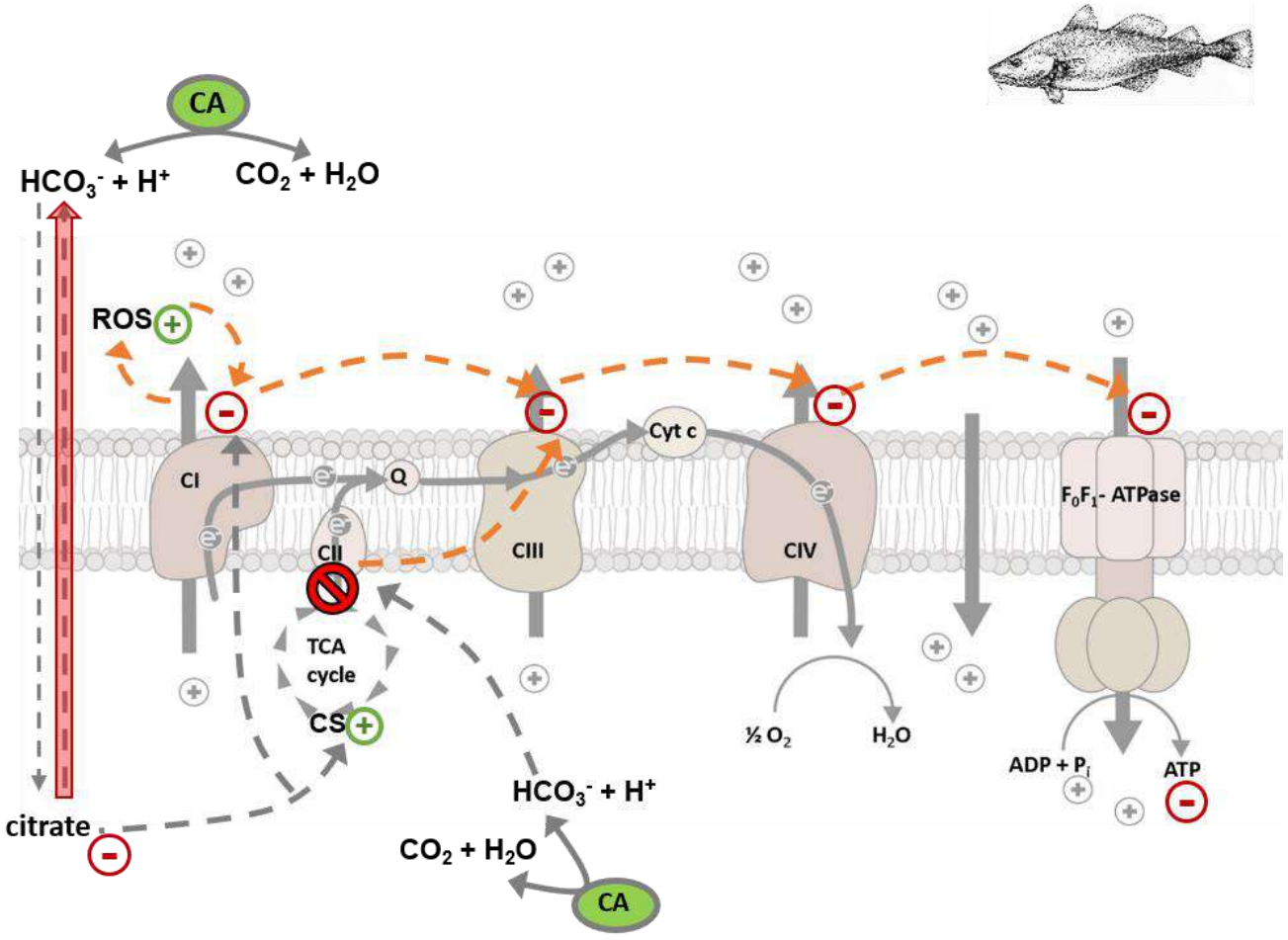
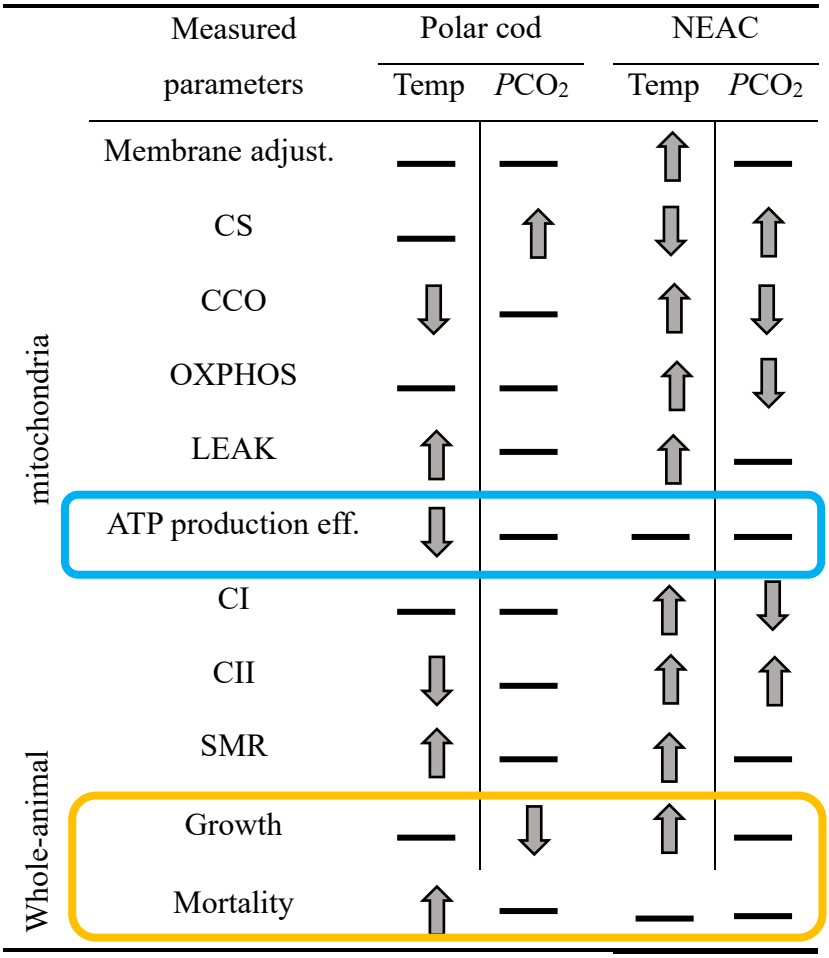


Figure 17: Schematic of the proposed mechanisms to explain the impact of elevated PCO_2 on the cardiac mitochondria of NEAC (*G. morhua*). HCO_3^-, regulated by intra- and extramitochondrial carbonic anhydrase (CA), promote the egress of citrate stimulating the activity of citrate synthase (CS) as compensatory effect and, on the other hand, inhibits directly Complex II (CII) (gray arrows). The higher extrusion of citrate and the inhibition of CII produce secondary effects (orange arrows) such as the decrease the overall ETS capacity leading to lower ATP production. Moreover, the decreased capacity of Complex I (CI) stimulates ROS production, the main target of which is the same CI, decreasing further its capacity.

Table 6: Summary table illustrating the trends in mitochondrial energetics and whole-animal trends in polar cod (*B. saida*) and NEAC (*G. morhua*) according to increasing acclimation temperature and level of PCO_2. The mitochondrial data refer to cardiac cells and were collected for this thesis project, SMR and mortality were collected by K. Kunz (Kunz *et al*., 2016; Publication AI) and discussed in this thesis



4.4.3 Comparison of mitochondrial metabolism between embryos and juveniles of the same species (Atlantic cod).

This thesis presents two datasets on the mitochondrial metabolism of conspecific Atlantic cod individuals at different life-stages. Before comparing the two datasets, it is important to consider that the individuals analysed belonged to different populations presenting genotypic variations.

The main variation concerns the haemoglobin (HbI) polymorphism (Frydenberg, Moeller and Naevdal, 1965; Sick, 1965; Brix, Thorkildsen and Colosimo, 2004). The three main genotypes (HbI-1/1, HbI-1/2, and HbI-2/2) are associated with temperature and latitudinal cline (Frydenberg, Moeller and Naevdal, 1965; Pörtner *et al.*, 2001; Petersen and Steffensen, 2003). In particular, the Northeast Arctic cod (NEAC) population presented a frequency of the Hbl-2 allele of about 90% (Frydenberg, Moeller and

Naevdal, 1965) while individuals in the Øresund area presented about 30% of HbI-1/1, 50% of HbI-1/2 and 20% of HbI-2/2 (Sick, 1965).

Despite their distinct biogeographical distribution and the temperature preferences of the animals according to genotype (Hbl-2/2 cod preferred water temperature of 8.2°C while Hbl-1/1 cod preferred 15.4°C, Petersen and Steffensen 2003), some studies highlighted the lack of differences in the SMR among cod Hbl genotypes at their preferred temperature, suggesting similar haemoglobin carrying capacities (Jordan *et al.*, 2006; Gamperl *et al.*, 2009). Hence, although the datasets presented in this thesis were obtained from specimens of different populations with marked genotypic variations, few general conclusions about aerobic metabolism at the two life stages may be drawn.

In embryos, the CI/ETS was higher than in juveniles (60% and 40% respectively, Fig.13) probably due to the difference in preferred fuel (free amino acids for embryos, carbohydrates and some lipids for juveniles).

Despite the different relative contribution to the ETS, CI activity presented the same behaviour with rising temperature, although with distinct thermal sensitivity, levelling off at 9°C in embryos and at 12°C in juveniles.

In embryos OXPHOS plateaued between 9 and 12°C and the OXPHOS coupling efficiency decreased already at 9°C, whereas in juveniles OXPHOS increased with temperature until the highest experimental temperature (16°C) and the coupling efficiency was stable throughout the whole experimental temperature range (Publication IV and I, respectively). These three aspects together suggest that Atlantic cod embryos have a narrower mitochondrial thermal window than juveniles, especially considering that the embryos came from the Øresund area and therefore might have the more thermal tolerant haemoglobin genotype.

The narrower thermal tolerance of embryos is expressed also by the lower CCO/ETS (Fig.15), since usually a high CCO/ETS indicates individuals living in challenging environments.

Incubation under elevated PCO_2 caused negative consequences in many aspects of embryonic development (decreased MO_2, standard length, hatching success, see above) but not on the mitochondrial functioning, whereas decreased OXPHOS, CI, CII and CCO capacity in juveniles suggested that the combination of temperature and CO_2 acted negatively on the juveniles' mitochondrial machinery directly. Despite the lower mitochondrial capacity, SMR and growth of juveniles did not show any CO_2 effect indicating a well-established ion and acid-base balance system in comparison to embryos and suggesting that at the whole-animal level, elevated PCO_2 has a stronger impact on embryos than on juveniles. Moreover, since the detrimental effects on embryos appeared already between 9 and 12°C, elevated PCO_2 narrowed the embryonic thermal window.

Even if the growth of juvenile cod did not decrease under incubation at elevated PCO_2, the potential

lower ATP production could exert effects on parameters not measured by Kunz *et al.* (2016, Publication AI) such as the reaching of sexual maturity and gamete output due to shifts in energy allocation. Since elevated PCO_2 has a negative impact on hatching success and larval performance (as stated above), a potentially lower number of gametes and of poorer quality might compromise the future reproductive success and eventually the population size.

4.4.4 Atlantic herring

Atlantic herring embryos were tolerant towards PCO_2 levels predicted for the year 2100 in the open ocean (1100µatm, IPCC, 2019). However, herring are benthic spawners and the waters of some of their spawning grounds have already exceeded this value by far (e.g., 2300µatm in the Kiel Fjord; Thomsen *et al.,* 2010) and projected to rise up to 4000 µatm at the end of the century (Thomsen *et al.*, 2010). Therefore, it is unclear if embryos will still be able to develop under the future circumstances or if spawning grounds will be moved to more suitable areas. However, herring being a benthic spawner the new spawning grounds may not be appropriate in terms of depth, substrate, etc. with consequent habitat contraction (Morley, Barnes and Dunn, 2019).

Rising temperature not only negatively influenced the mitochondrial metabolism, with consequences on the size at hatch and on the hatching success, but also accelerates the embryonic development causing a mismatch between hatching events and food availability (Neuheimer, MacKenzie and Payne, 2018; Dahlke *et al.*, 2020). Therefore, OAW may cause a shift in population distribution, shrinkage of the population size and eventually local extinctions due to the loss of spawning grounds.

5. Conclusions

The present thesis contributed to shed light on the role of the mitochondrial metabolism in setting the acclimation capacity to Ocean Acidification and Warming (OAW) in a number of fish species.
In juvenile polar cod (*Boreogadus saida*) the cardiac mitochondria had limited acclimation capacity and their functioning decreased at temperature close to the animal's upper thermal limit (8°C). At this temperature the proton leak and associated costs lowered the ATP production efficiency, decreasing the energy yeld of the cell. At 8°C mortality rates were higher, suggesting a tight link between cardiac mitochondrial metabolism and whole-animal thermal tolerance in this species because of the central role of heart in driving the circulatory siystem and, as consequence, indicating a role of heart in setting the animal's thermal window. On the other hand, polar cod resulted rather tolerant to high PCO_2 with the only increase of Citrate synthase (CS) capacity *in vitro* probably as compensatory up-regulation.
In the juvenile individuals of Northeast- Arctic cod (NEAC, *Gadus morhua*), instead, the mitochondrial metabolism appeared more plastic, feature suggested by the higher Q_{10} for CS and CCO compared to polar cod and the higher CCO/ETS and allowed the fish to cope with the different conditions of temperature and PCO_2 in a broader thermal range (0-16°C) without detrimental effects on growth and survival.
Therefore, with regard to the mitochondrial functioning, NEAC appears more eurytherm and plastic than polar cod and will possibly outperform polar cod as temperatures rise in the overlapping distribution areas such as the Svalbard fjords. Even if the high hypoxia tolerance of polar cod might allow them to find refuge from heat and predators (e.g., the very same NEAC) in the fjord bottom waters, this solution may not be sustainable in the long term due to the high share of aerobic metabolism in polar cod (mainly fueled by fatty-acids). Moreover, the retreat of the winter sea ice may cause the loss of spawning grounds with negative consequences at the population level.
The loss of suitable spawning grounds may constitute the main problem also for Atlantic cod and Atlantic herring. While the mitochondrial metabolism in the embryos of these two species was not affected by high PCO_2, the full exploitation of the CCO capacity by the ETS did not allowed any further adjustment in the respiratory machinery with increasing temperature. A parallel decrease in hatching success and smaller length at hatch, suggested an impairment between energy produced and required. Moreover, although the mitochondrial respiration was not directly affected by elevated PCO_2, the energy requirements increased under this condition, exacerbating the impairment between energy demand and provision. Therefore, if OAW continue, it is likely that spawning grounds will be moved to more suitable

areas (if available) with consequences at the population level and potential habitat contraction.

Moreover, the mitochondrial respiration had a narrower thermal window in embryos, remarking the role of embryos as potential acclimation bottle-neck.

From a mechanistic point of view, one of the main causes of loss of mitochondrial efficiency with temperature was the increase of proton leak, therefore adjustments of the membrane composition and structure as seen in NEAC, may be beneficial and could be considered an acclimation trait. High CI/ETS (as seen in polar cod and in embryos) and high CCO/ETS (as in NEAC) may allow a higher ATP/O ratio and thus, a higher ATP production efficiency under hypoxic conditions. Since, theoretically, Complex I allows a higher ATP/O ratio compared to Complex II but it seemed thermally unstable and more susceptible to ROS, intramitochondrial antioxidant defenses (such as superoxide dismutase and peroxidase) may have a key role in acclimation to elevated temperature.

Since this thesis illustrated the general effects of elevated PCO_2 on the mitochondrial metabolism but not on specific mechanisms, further studies are required to identify the molecular target of Ocean Acidification.

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Appendix

Table A1: Summary table of the seawater parameters measured during the incubation of Polar cod (*Boreogadus saida*) and NEAC (*Gadus morhua*).

Table A2: Summary of the seawater parameters measured during the incubation of Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*) embryos until hatch.

Table A3: List of compounds for the relaxing medium BIOPS (pH 7.4, 380 mOsmol l^{-1}) and the mitochondrial respiration medium MIR05 (pH 7.4, 380 mOsmol l^{-1}).

Table A4: List of chemicals used in the SUIT protocol and their respective concentrations.

Publication AI: Kunz et al., 2016.

Publication AII: Schmidt et al. 2017.

Table A1: Summary table of the seawater parameters measured during the incubation of Polar cod (*Boreogadus saida*) and NEAC (*Gadus morhua*).Values are presented as mean ± S.D. "control" and "high" refer to CO_2 levels. The complete dataset is available in the online Open Access Library PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.866369).

	Acclimation	Measured Temp. (°C)	PCO_2 (µatm)	pH_{tot}
Polar cod	0°C control	0.75±0.41	373.69±34.24	8.10±0.04
	0°C high	0.31±0.44	1056.97±135.56	7.68±0.05
	3°C control	3.30±0.11	425.07±28.93	8.05±0.02
	3°C high	3.01±0.07	1073.06±131.51	7.68±0.05
	6°C control	5.74±0.23	435.27±18.73	8.01±0.02
	6°C high	5.71±0.24	1076.53±73.15	7.64±0.03
	8°C control	8.24±0.14	433.47±16.98	8.04±0.02
	8°C high	8.19±0.16	1026.75±103.48	7.71±0.06
	Acclimation	Measured Temp. (°C)	PCO_2 (µatm)	pH_{tot}
Atlantic cod (NEAC)	3°C control	3.11±0.22	449.89±30.15	7.96±0.03
	3°C high	3.15±0.18	1265.44±144.43	7.54±0.05
	8°C control	8.20±0.09	437.44±10.23	8.02±0.01
	8°C high	8.11±0.13	851.98±109.85	7.77±0.09
	12°C control	12.30±0.43	458.27±45.53	8.03±0.04
	12°C high	12.16±0.35	1137.25±91.64	7.67±0.03
	16°C control	15.73±0.41	515.25±25.84	7.90±0.03
	16°C high	15.71±0.44	1416.11±357.17	7.5±0.12

Table A2: Summary of the seawater parameters measured during the incubation of Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*) embryos until hatch. Values are presented as mean ± S.D. "control" and "high" indicate the CO_2 concentrations. The whole datasets are visible in the Open Access Online Library PANGAEA (Atlantic cod: doi.pangaea.de/10.1594/PANGAEA.884124; Atlantic herring: https:// doi.pangaea.de/10.1594/PANGAEA.884124).

	Acclimation	Duration (days)	Measured Temp. (°C)	Oxygen (%)	PCO_2 (µatm)	pH_F
Atlantic cod	0°C control	45	0.04	95.6	409	8.15
	0°C high	45	0.04	95.6	1114	7.77
	3°C control	30	3.05	95.4	409	8.15
	3°C high	30	3.05	95.5	1057	7.77
	6°C control	18	6.15	94.9	415	8.14
	6°C high	18	6.15	94.7	1122	7.76
	9°C control	13	9.14	94.3	422	8.14
	9°C high	13	9.14	94.6	1091	7.77
	12°C control	9	12.11	94.1	417	8.14
	12°C high	9	12.11	94.3	1097	7.77

	Acclimation	Duration (days)	Measured Temp. (°C)	Oxygen (%)	PCO_2 (µatm)	pH_F
Atlantic herring	6°C control	27	6.15±0.06	94.40±0.71	415±10	8.15±0.02
	6°C high	27	6.15±0.06	94.40±0.61	1101±47	7.77±0.02
	10°C control	16	10.04±0.06	94.00±0.63	408±10	8.17±0.02
	10°C high	16	10.04±0.06	94.40±0.49	1050±46	7.79±0.03
	14°C control	11	14.07±0.20	95.00±0.82	403±12	8.18±0.02
	14°C high	11	14.07±0.20	95.00±0.00	1050±29	7.78±0.02

Table A3: List of compounds for the relaxing medium BIOPS (pH 7.4, 380 mOsmol l^{-1}) and the mitochondrial respiration medium MIR05 (pH 7.4, 380 mOsmol l^{-1}).

	Compound	Concentration
BIOPS	CaK_2EGTA	2.77 mM
	K_2EGTA	7.23 mM
	Na_2ATP	5.77 mM
	$MgCl_2$	6.56 mM
	Taurine	20 mM
	Na_2-Phosphocreatine	15 mM
	Imidazole	20 mM
	Dithiothreitol	0.5 mM
	MES	50 mM
	Sucrose	220 mM
	Compound	**Concentration**
MIR05	EGTA	0.5 mM
	$MgCl_2$	3 mM
	K-lactobionate	60 mM
	Taurine	20 mM
	KH_2PO_4	10 mM
	HEPES	20 mM
	Sucrose	160 mM
	BSA	1 g l^{-1}

Table A4: List of chemicals used in the SUIT protocol and their respective concentrations.

Compound	Concentration
Glutamate – sodium salt	10 mM
Malate – potassium salt	2 mM
Pyruvate – sodium salt	10 mM
Succinate – disodium salt	10 mM
ADP	3* 1 mM
Cytochrome c_{ox}	10 µM
Atractyloside	0.75 mM
Oligomycin	6 µM
FCCP	n* 1 µM
Rotenone	0.5 µM
Malonate	5 mM
Antimycin a	2.5 µM
Ascorbate – sodium salt	2 mM
TMPD	0.5 mM

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ORIGINAL PAPER

New encounters in Arctic waters: a comparison of metabolism and performance of polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*) under ocean acidification and warming

Kristina Lore Kunz[1,2,3] · Stephan Frickenhaus[4,5] · Silvia Hardenberg[6] · Torild Johansen[7] · Elettra Leo[2,3] · Hans-Otto Pörtner[2,3] · Matthias Schmidt[2,3] · Heidrun Sigrid Windisch[8] · Rainer Knust[1] · Felix Christopher Mark[2]

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Abstract Oceans are experiencing increasing acidification in parallel to a distinct warming trend in consequence of ongoing climate change. Rising seawater temperatures are mediating a northward shift in distribution of Atlantic cod (*Gadus morhua*), into the habitat of polar cod (*Boreogadus saida*), that is associated with retreating cold water masses. This study investigates the competitive strength of the co-occurring gadoids under ocean acidification and warming (OAW) scenarios. Therefore, we incubated specimens of both species in individual tanks for 4 months, under different control and projected temperatures (polar cod: 0, 3, 6, 8 °C, Atlantic cod: 3, 8, 12, 16 °C) and PCO_2 conditions (390 and 1170 µatm) and monitored growth, feed consumption and standard metabolic rate. Our results revealed distinct temperature effects on both species. While hypercapnia by itself had no effect, combined drivers caused nonsignificant trends. The feed conversion efficiency of normocapnic polar cod was highest at 0 °C, while optimum growth performance was attained at 6 °C; the long-term upper thermal tolerance limit was reached at 8 °C. OAW caused only slight impairments in growth performance. Under normocapnic conditions, Atlantic cod consumed progressively increasing amounts of feed than individuals under hypercapnia despite maintaining similar growth rates during warming. The low feed conversion

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✉ Kristina Lore Kunz
Kristina.Kunz@awi.de

Stephan Frickenhaus
Stephan.Frickenhaus@awi.de

Silvia Hardenberg
silvia.hardenberg@zmt-bremen.de

Torild Johansen
Torild.Johansen@imr.no

Elettra Leo
Elettra.Leo@awi.de

Hans-Otto Pörtner
Hans.Poertner@awi.de

Matthias Schmidt
Matthias.Schmidt@awi.de

Heidrun Sigrid Windisch
Heidrun.Windisch@hhu.de

Rainer Knust
Rainer.Knust@awi.de

Felix Christopher Mark
Felix.Christopher.Mark@awi.de

[1] Bentho-Pelagic Processes, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Alten Hafen 26, 27568 Bremerhaven, Germany

[2] Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

[3] University of Bremen, Fachbereich 2, NW 2/Leobener Strasse, 28359 Bremen, Germany

[4] Scientific Computing, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany

[5] Hochschule Bremerhaven, An der Karlstadt 8, 27568 Bremerhaven, Germany

[6] Leibniz Center for Tropical Marine Ecology (ZMT), Fahrenheitstrasse 6, 28359 Bremen, Germany

[7] Institute of Marine Research, Sykehusveien 23, 9294 Tromsø, Norway

[8] Institute for Cell Biology and Zoology, Heinrich-Heine-University, Universitätsstrasse 1, 40225 Düsseldorf, Germany

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especially in economically important species like Atlantic cod (e.g. Björnsson et al. 2001; Purchase and Brown 2001; Moran and Støttrup 2011). Knowledge of chronic and synergistic effects of ocean warming and acidification on the performance and fitness of fish species is still scarce, especially with respect to realistic future ocean conditions.

The aim of this study was to investigate the combined effects of projected ocean warming and acidification on growth performance, feed consumption, and standard metabolic rate in populations of two co-occurring gadoids, polar cod and Atlantic cod at their overlapping distribution ranges in the Arctic. The results are used to assess the competitive strength of both polar cod and Atlantic cod under projected water conditions. We discuss our findings in light of their ecology and the conditions faced by the species in their natural habitat.

Materials and methods

Sample collection

The polar cod used in this study were provided by the University of Tromsø, Norway. In January 2013, polar cod were caught from the R/V Helmer Hanssen with a bottom trawl in a depth of 120 m in Kongsfjorden (78° 97′N 12° 51′E) at the western coast of Svalbard. By aid of a fish lift (Holst and McDonald 2000), injuries during trawling were prevented. However, a mortality of approx. 50 % was recorded within the first week after capture. Size at capture was approx. 6–7 cm. The fish were kept in a flow-through seawater tank while being transported to the laboratories of Havbruksstasjonen i Tromsø AS (HiT) and kept until April 2013 at 3.3–3.8 °C under natural light conditions. They were fed three times a week with frozen copepods (*Calanus spec.*). In late April 2013, 150 individuals were transferred to the aquaria of the Alfred Wegener Institute (AWI) in Bremerhaven. In preparation for transportation, the fish were starved for a week.

The recirculating aquaria system at the AWI contained 10 m^3 of seawater originating from the North Sea near Helgoland (German Bight). Nitrification filters, protein skimmers and UV sterilizers as well as additional water changes were used to support high water quality and NO_3^- values below 50 mg L^{-1}. For preconditioning, the fish were kept at 5 °C for another 4 weeks. Starting one day after arrival, the polar cod were weaned onto a daily feeding pattern with high-protein feed pellets (Amber Neptun, 5 mm, Skretting AS, Norway). The light cycle was adjusted to 12 h of light and 12 h of darkness (12L:12D).

In August 2013, Atlantic cod were caught from the R/V Heincke at several locations in the vicinity of Svalbard: Rijpfjorden (80° 15.42′N 22° 12.89′E), Hinlopenstretet (79° 30.19′N 18° 57.51′E), and Forlandsundet (78° 54.60′N 11° 3.66′E) using a pelagic midwater trawl combined with a fish lift (Holst and McDonald 2000) at a depth of 0–40 m. Size at capture was approx. 5–7 cm. The fish were directly transported in a thermostatted recirculating tank system (4 m^3) to the AWI aquaria in Bremerhaven and kept for several months at 5 °C. Mortality during capture was low; however, cannibalism decreased the numbers of specimens substantially. They were fed twice a week with a mixture of frozen copepods, baby krill, and high-protein feed pellets.

Experimental design

The experimental design consisted of eight different stable temperature/PCO_2 treatments, each containing 12 single aquaria (approx. 24 L each). The chosen temperatures (polar cod: 0, 3, 6, 8 °C; Atlantic cod: 3, 8, 12, 16 °C) were based on the range of habitat temperatures and were maintained by the aid of thermostatted rooms. For each temperature, two PCO_2 levels were applied comprising the current conditions [390 µatm = control PCO_2; mean actual values: 430 µatm (polar cod), 480 µatm (Atlantic cod), Tables 1, 2] as well as a future value projected by the turn of the century [1170 µatm = high PCO_2; mean actual values: 1100 µatm (polar cod), 1120 µatm (Atlantic cod), Tables 1, 2] according to the Representative Concentration Pathway (RCP) 8.5 of the Intergovernmental Panel on Climate Change (IPCC) (Pörtner et al. 2014). For equal conditions across individual aquaria within the treatments, the respective PCO_2 conditions were pre-adjusted in a header tank containing approximately 200 L of seawater, supplying the single aquaria. The manipulation of CO_2 partial pressure was achieved by use of a mass flow controller (4 and 6 channel MFC system, HTK, Hamburg, Germany), in which virtually CO_2-free pressurized air was mixed with pure CO_2. This setup was identical for both species. It should be noted that this protocol differs from those traditionally applied in culture systems comprising a group of specimens in a common tank under the same environmental regime.

At the end of May 2013, 96 polar cod with a total length of 10.9–17.3 cm (mean length 14.2 cm ± 1.3 SD) and a weight of 6.7–31.9 g (mean weight 16.8 g ± 5.6 SD) were transferred to the experimental setup. Cooling and warming protocols were applied at a maximum rate of 2 °C per 24 h. To ensure proper technical replicates during allocation to experimental conditions, the distribution of individuals across temperatures, as well as PCO_2 regimes, was random. Each individual was placed into a single aquarium with an individual inflow of 500 mL min^{-1}.

The experiment with 96 Atlantic cod started in the end of April 2014. The size range was 14.2–24.8 cm (mean

Table 1 Polar cod (*Boreogadus saida*)

Treatment (°C/µatm)	Salinity (psu)	TA (µmol/kgSW)	TCO_2 (µmol/kgSW)	pH_{tot}	PCO_2 (µatm)	HCO_3^- (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCa	ΩAr	Temp. (°C)
0/390	30.58 ± 0.98	2490.64 ± 81.02	2365.25 ± 76.27	8.08 ± 0.04	396.25 ± 37.70	2237.39 ± 72.19	103.37 ± 10.47	2.52 ± 0.25	1.57 ± 0.16	0.90 ± 0.37
0/1170	30.70 ± 1.08	2459.25 ± 69.68	2466.79 ± 76.22	7.66 ± 0.05	1091.64 ± 131.67	2357.46 ± 71.32	41.30 ± 3.66	1.01 ± 0.09	0.63 ± 0.06	0.61 ± 0.45
3/390	30.70 ± 1.01	2480.35 ± 45.21	2352.84 ± 39.80	8.05 ± 0.04	431.10 ± 40.41	2223.17 ± 37.20	105.22 ± 8.68	2.56 ± 0.21	1.60 ± 0.13	3.15 ± 0.18
3/1170	30.74 ± 0.98	2447.33 ± 48.03	2447.22 ± 52.89	7.65 ± 0.05	1135.99 ± 147.10	2338.40 ± 49.31	44.35 ± 4.17	1.08 ± 0.10	0.67 ± 0.06	3.06 ± 0.11
6/390	31.47 ± 0.53	2371.73 ± 159.40	2237.56 ± 148.24	8.03 ± 0.02	437.09 ± 21.60	2107.92 ± 137.19	107.16 ± 11.27	2.60 ± 0.28	1.63 ± 0.17	5.63 ± 0.19
6/1170	31.49 ± 0.52	2353.22 ± 165.66	2342.31 ± 164.82	7.64 ± 0.03	1123.50 ± 86.23	2237.51 ± 157.89	46.82 ± 4.79	1.14 ± 0.12	0.71 ± 0.07	5.57 ± 0.21
8/390	32.78 ± 0.82	2528.77 ± 135.07	2357.44 ± 127.60	8.05 ± 0.02	440.90 ± 29.10	2202.98 ± 118.79	133.74 ± 10.36	3.22 ± 0.25	2.03 ± 0.16	8.16 ± 0.13
8/1170	32.71 ± 0.80	2531.76 ± 115.67	2482.53 ± 114.04	7.71 ± 0.05	1052.88 ± 103.87	2364.26 ± 113.98	68.87 ± 17.75	1.66 ± 0.43	1.05 ± 0.27	8.11 ± 0.13

Water chemistry for all treatments as mean ± SD throughout the total experimental period of 4 months

Table 2 Atlantic cod (*Gadus morhua*)

Treatment (°C/µatm)	Salinity (psu)	TA (µmol/kgSW)	TCO_2 (µmol/kgSW)	pH_{tot}	PCO_2 (µatm)	HCO_3 (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCa	ΩAr	Temp. (°C)
3/390	31.62 ± 0.41	2216.73 ± 113.32	2111.36 ± 106.46	7.99 ± 0.04	449.08 ± 39.04	2000.34 ± 99.99	86.36 ± 9.20	2.09 ± 0.22	1.31 ± 0.14	3.00 ± 0.20
3/1170	31.60 ± 0.37	2194.07 ± 112.10	2196.88 ± 101.83	7.61 ± 0.07	1127.75 ± 164.89	2096.49 ± 99.83	38.83 ± 7.14	0.94 ± 0.17	0.59 ± 0.11	2.98 ± 0.20
8/390	33.01 ± 0.35	2267.67 ± 64.73	2123.50 ± 56.92	8.01 ± 0.04	434.51 ± 49.90	1992.24 ± 52.62	110.54 ± 11.08	2.66 ± 0.26	1.68 ± 0.17	8.16 ± 0.11
8/1170	32.70 ± 1.35	2255.23 ± 62.17	2210.37 ± 50.37	7.72 ± 0.08	915.08 ± 127.39	2106.92 ± 46.88	59.73 ± 11.09	1.40 ± 0.22	0.88 ± 0.14	8.07 ± 0.17
12/390	32.14 ± 0.50	2431.18 ± 88.61	2261.45 ± 80.24	8.00 ± 0.08	490.29 ± 112.75	2108.17 ± 78.43	132.63 ± 22.10	3.21 ± 0.54	2.04 ± 0.34	12.03 ± 0.35
12/1170	32.15 ± 0.48	2434.19 ± 39.81	2391.13 ± 37.28	7.66 ± 0.07	1163.71 ± 199.68	2277.80 ± 36.50	64.14 ± 9.36	1.55 ± 0.23	0.98 ± 0.14	11.85 ± 0.37
16/390	32.61 ± 0.37	1930.37 ± 170.37	1809.62 ± 164.23	7.87 ± 0.06	547.87 ± 92.69	1697.86 ± 155.30	91.40 ± 13.81	2.21 ± 0.33	1.41 ± 0.21	15.81 ± 0.25
16/1170	32.61 ± 0.38	1921.64 ± 175.50	1897.43 ± 186.68	7.54 ± 0.10	1271.67 ± 365.09	1803.91 ± 178.67	46.16 ± 9.76	1.12 ± 0.23	0.71 ± 0.15	15.81 ± 0.27

Water chemistry for all treatments as mean ± SD throughout the total experimental period of 4 months

total length 18.5 cm ± 2.2 SD) and weight was 15.3–103.8 g (mean weight 43.4 g ± 17.3 SD). Temperature adjustments to reach final experimental conditions did not exceed 1 °C per 24 h. The protocol of distributing individuals in the single aquaria was identical between both incubations.

Spatial constraints within the culture rooms forced us to isolate individual treatments (polar cod: 0 and 3 °C; Atlantic cod: 12 °C) from the total recirculating water body. Nonetheless, each system contained approx. 1200 L of recirculating seawater preconditioned with respect to temperature and PCO_2. Apart from this, setups were identical to those described above. NH_4^+ tests were conducted twice a week using photometric test kits (Macherey–Nagel, Düren, Germany). The critical threshold for water changes was set to 0.4 mg L^{-1} for NH_4^+. During the incubation with Atlantic cod, water quality was maintained by biological filter systems in combination with protein skimmers (Sander, Germany) and daily water exchanges (600 L).

Water chemistry

The stability of PCO_2 conditions was verified by monitoring pH, temperature, and salinity once to twice a week in triplicate in every treatment. For pH measurements, a pH meter (pH 3310, WTW, Weilheim, Germany) was calibrated with thermally equilibrated NBS-buffers (2-point calibration). The pH values were cross-calibrated to total pH scale using Tris-buffered pH reference material (Batch 4, Marine Physical Laboratory, University of California, San Diego, CA, USA). Temperature and salinity were measured using a WTW LF 197 multimeter (WTW, Weilheim, Germany).

In combination with the total dissolved inorganic carbon (verified in triplicates) determined by a Seal QuAAtro SFA Analyzer (800 TM, Seal Analytical, Mequon, USA), the pH_{tot} values were used to subsequently calculate the seawater carbonate chemistry in the program CO2SYS (Lewis and Wallace 1998), applying the dissociation constants of Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and the $KHSO_4$ constant after Dickson (1990). A summary of water chemistry data is given in Tables 1 and 2.

Growth experiment

In total, the growth experiment lasted 130 days (polar cod) and 133 days (Atlantic cod) under constant conditions. For the determination of individual growth rates, each specimen was measured (total length; to the nearest mm below) and weighed (wet weight; to the nearest 0.1 g) on the third day after feeding, three times during the experiment: (1) prior to the transfer into the experimental setup, (2) after the incubation half-time (polar cod, day 60–68; Atlantic cod, day 44–47), and (3) at the end of the incubation period (polar cod, day 109–130; Atlantic cod, day 108–133). For the precise determination of length and weight, the fish were slightly sedated (0.06 g L^{-1} MS-222) prior to measuring. A light cycle of 12:12 h was maintained. During day time, the light was dimmed except for the feeding and feed-removing events, as well as during water condition measurements.

Each fish was fed ad libitum once every 4 days using a predetermined amount of high-protein feed pellets (Amber Neptun, 5 mm, Skretting AS, Norway). For a better feed acceptance of polar cod, the pellets were soaked with a fixed amount of sea water 24 h before feeding. Remaining feed items were removed after several hours, dried for 24 h at 80 °C, and weighed for the quantification of individual feed intakes. The relative nutrient composition of the pellets was 54.7 % protein, 19.1 % fat, 8.0 % carbohydrates, and 8.5 % moisture. Every single aquarium was cleaned daily from feces and other particles. In the case of feeding days, cleaning happened well before the feeding events.

Respiration measurements

At the end of the long-term incubation, individual oxygen consumption rates [$\dot{M}O_2$; unit: µmol (min*g)$^{-1}$] were determined (polar cod, n = 4–6; Atlantic cod, n = 5–12 per treatment) using automated intermittent-flow respirometry. Two systems comprising six acryl respiration chambers each (polar cod, 1.8 and 2.2 L; Atlantic cod, 3.9 L) were installed. The respiration chambers were submerged in water of the respective temperature/PCO_2 treatments. A circulating water flow was constantly maintained within the respiration chambers by aid of an aquarium pump (8.2 L min^{-1}). A flush pump (5.0 L min^{-1}) was used to fully replenish the O_2 concentration in the chambers after a measurement period of 15 min (polar cod; Atlantic cod, 3 and 8 °C) and 10 min (Atlantic cod, 12 and 16 °C). $\dot{M}O_2$ was measured using optical oxygen probes and recorded with a 10-channel oxygen meter (PreSens Precision Sensing GmbH, Hamburg, Germany; system 1), as well as a four-channel FireStingO2 (Pyro Science GmbH, Aachen, Germany) and two Fibox 3 (PreSens Precision Sensing GmbH, Hamburg, Germany) (system 2). For calibration, the oxygen probes were flushed with nitrogen at room temperature (0 %-calibration), and the 100 %-calibration was performed in fully aerated seawater at the respective experimental temperature prior to each round of measurements. Blank measurements detected bacterial background respiration following $\dot{M}O_2$ measurements at each temperature (polar

cod) or at each temperature/PCO_2 combination (Atlantic cod) during several loops. After subtraction of bacterial respiration (solely detected at 8 °C for polar cod and 16 °C for Atlantic cod), the average of the five lowest, consecutive $\dot{M}O_2$ values per individual was used as an estimate of standard metabolic rate (SMR).

The fish were placed in the respiration chambers on the third day after feeding and remained in the chambers for approx. 48 h to allow for full recovery from handling stress. During the measurement period, non-transparent plastic sheets covered the tanks containing the respiration chambers to reduce potential disturbance. In order to minimize the time of air exposure, weighing was conducted before the fish were placed in the chambers, while length measurements happened after the determination of $\dot{M}O_2$.

Statistical analysis

The specific growth rate (SGR) per day was calculated in percent of the initial weight according to Jobling (1988):

$$\mathrm{SGR} = 100 * (\ln W_{\mathrm{end}} - \ln W_{\mathrm{start}}) * (t_{\mathrm{end}} - t_{\mathrm{start}})^{-1},$$

with W_{start} and W_{end} being the individual weight in gram at the day t_{start} and t_{end}, respectively.

The individual feed intake (F) per meal was calculated according to:

$$F = \mathrm{hl} * F_{\mathrm{in}} - F_{\mathrm{out}}$$

The constant (hl) supports the compensation of different humidity levels of feed pellets before and after drying. The determination of exact humidity levels in feed pellets prior to both incubations revealed slightly divergent correction factors (hl = 0.8820 and 1.0227 during the incubation with polar cod and Atlantic cod, respectively). F_{in} is the amount of feed given to the respective individual per meal, while F_{out} represents the amount of remaining feed items in gram. Subsequently, the feed intake per body weight (weight at experimental midterm) of each individual throughout the whole experimental period was determined. Furthermore, individual stomachs and their content were weighed the third day after the last feeding event in order to determine the degree of stomach filling (SF) in percent of the stomach weight.

Feed conversion ratio (FCR) of each fish was calculated as the individual weight gain divided by the individual feed intake throughout the experiment.The condition factor (CF) was calculated according to Fulton (1911):

$$\mathrm{CF} = 100 * W * L_{\mathrm{t}}^{-3},$$

where W is the wet weight in gram and L_{t} represents the total length in centimeter.

The hepatosomatic index (HSI) was calculated as:

$$\mathrm{HSI} = 100 * W_{\mathrm{L}} * W^{-1},$$

with W_{L} representing the liver weight in gram.

The gonadosomatic index (GSI) was determined according to:

$$\mathrm{GSI} = 100 * W_{\mathrm{G}} * W^{-1},$$

with W_{G} representing the gonad weight in gram.

Prior to statistical analysis, individuals that were heavily infested by parasites or that died during the incubation due to total refusal of feed were excluded from the data set.

All of the following tests were accomplished using the program R version 3.0.2 (R Core Team 2013). $p < 0.05$ is considered as significant. Comparisons of the initial mean weight and length of each fish species between experimental treatments were conducted using a two-way ANOVA. Normal distribution and homoscedasticity were assessed by Shapiro–Wilk tests and Bartlett tests, respectively.

A one-way ANOVA was performed to test for temperature-dependent effects within both PCO_2 levels for each species. In case of significant effects, a subsequent Tukey honest significance test for comparisons of the mean was applied. When the data set was characterized by unequal sample sizes and/or heterogeneous variances (F, SF, FCE, CF, GSI and SMR), a max-t test (Herberich et al. 2010) was conducted to assess temperature effects within the PCO_2 levels. The procedure was performed using a combination of the R packages MULTCOMP (Hothorn et al. 2008) and SANDWICH (Zeileis 2006).

PCO_2-dependent effects on SGR, F, SF, FCE, CF, HSI, GSI, and SMR within each temperature were investigated by aid of Mann–Whitney U tests. In case of significant effects of PCO_2, two linear models (full interaction temperature and PCO_2, and reduced model PCO_2 plus interactive effect temperature: PCO_2, respectively) were fitted in order to characterize the effect of hypercapnia in a two-way approach.

When no effects of PCO_2 were detected within each species, a second Mann–Whitney U test was done for species comparisons in pooled data across PCO_2 treatments for SGR, F, FCE, and SMR at 3 and 8 °C, respectively.

The overall data set from both species was tested for the impact of temperature and PCO_2 with the aid of non-metric multidimensional scaling (NMDS). The parameter GSI was excluded in this analysis, because the sex-specific response was shown to outperform every effect of temperature and PCO_2 in preliminary tests. Furthermore, HSI was excluded, because HSI caused the discard of the 16 °C treatments of Atlantic cod in the NMDS, which entailed a reduction in the power of the NMDS. The NMDS showed stress 0.11 for polar cod and 0.08 for Atlantic cod.

Results

For both species, initial mean fish weight and length did not show any differences between the treatments.

Mortality

Polar cod mortality occurred solely at the highest temperature (8 °C, $n = 3$, ≙13.0 %) at a rather late stage of the incubation (Fig. 1). Dead Atlantic cod ($n = 12$, ≙12.5 %) were recorded at all temperatures and PCO_2 values with a peak of four individuals (33.3 %) at 12 °C/390 µatm. Highest mortality of Atlantic cod ($n = 8$, ≙8.3 %) occurred within the first half of the incubation period (Fig. 1).

Growth

Growth performance of polar cod did not show any statistically significant temperature effect (Fig. 2). However, under control PCO_2 conditions a trend for higher growth at 6 °C was recognizable ($p = 0.07$). No such temperature trend was found under hypercapnia ($p = 0.6$). The growth rate of Atlantic cod was strongly correlated with temperature (control PCO_2: $p < 0.0001$; high PCO_2: $p < 0.0001$) with a high slope at low and a decreasing slope at higher temperatures (Fig. 2). Therefore, no thermal optimum for growth could be recorded between 3 and 16 °C. A comparison of both species revealed no significant difference in growth rates at 3 °C ($W = 361$, $p = 0.07$). At 8 °C, the growth performance of Atlantic cod was higher than that of polar cod ($W = 46$, $p < 0.0001$).

Feed consumption

For both species, the data set of daily feed consumption per gram body weight was influenced by temperature (Fig. 3). The feed intake of polar cod was lowest at 0 °C (mean = 3.5 mg (g BW*day)$^{-1}$) compared to the other experimental temperatures, a difference which was more pronounced under hypercapnia (control PCO_2: 0/3 °C $p = 0.06$, 0/6 °C $p = 0.04$, 0/8 °C $p = 0.1$; high PCO_2: 0/3 °C $p = 0.02$, 0/6 °C $p = 0.02$, 0/8 °C $p = 0.001$). At 3, 6 and 8 °C, the feed consumption plateaued and reached an average of 4.8 mg (g BW*day)$^{-1}$. The degree of stomach filling the third day after feeding in polar cod decreased nonsignificantly with increasing temperature. The daily feed consumption per g body weight of Atlantic cod rose with temperature, covering a range from 5.1 (3 °C) to 11.2 mg (g BW*day)$^{-1}$ (16 °C). Under hypercapnia, the slope was progressively less pronounced at high temperatures compared to normocapnic conditions. Atlantic cod consumed distinctly more feed at 8 °C ($W = 46$, $p < 0.0001$) than polar cod. The third day after feeding, stomach contents of Atlantic cod were low except for nonsignificantly higher amounts at 3 °C.

Feed conversion efficiency

The temperature effect on growth and/or feed intake was also translated into the feed conversion ratio for both

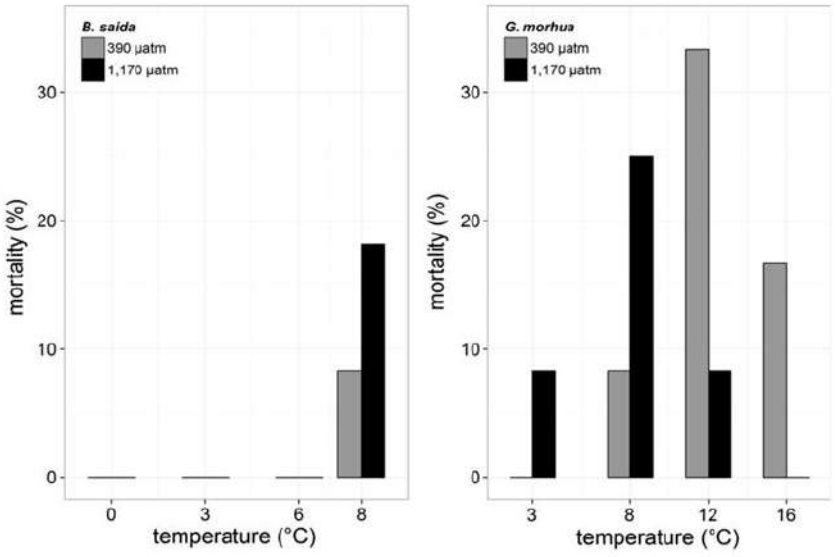

Fig. 1 Total mortality (%) during long-term incubation including specimens that refused feed consumption from the beginning of the experimental period (excluded in the analysis of further parameters)

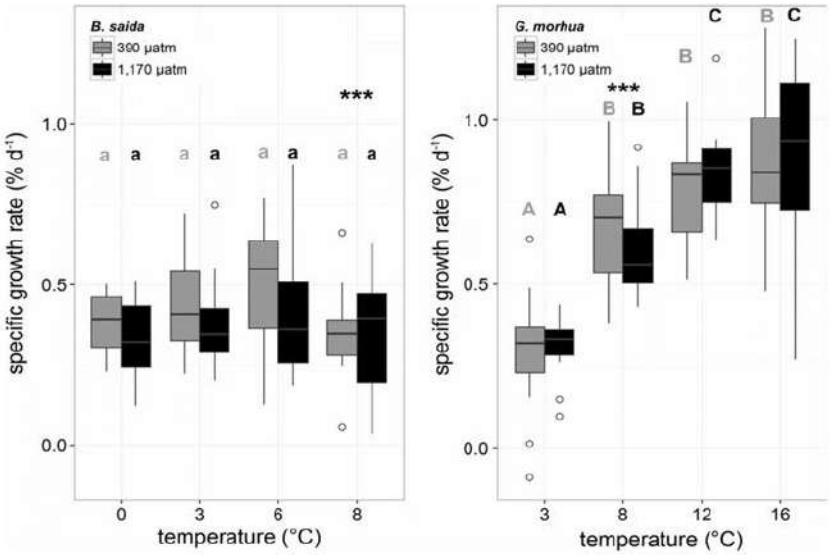

Fig. 2 Specific growth rate (wt% d^{-1}). *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*Boreogadus saida*) (*lowercase*) and Atlantic cod (*Gadus morhua*) (*uppercase*). *Asterisks* significant difference between species at 8 °C; ***: $p < 0.001$

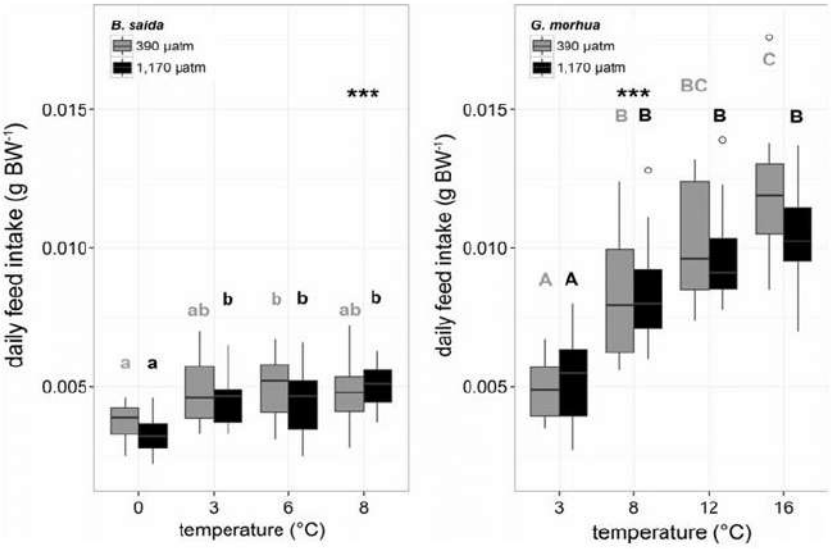

Fig. 3 Daily feed consumption (g BW^{-1}). *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*). *Asterisks* significant difference between species at 8 °C; ***: $p < 0.001$

species (Fig. 4). A nonsignificant trend for feed conversion efficiency of polar cod to be highest was recognizable at 0 °C (mean = 1.18). Feed conversion fell to its lowest value at 8 °C (mean = 0.71; control PCO_2: 0/8 °C $p = 0.001$, 3/8 °C $p = 0.06$, 6/8 °C $p = 0.02$; high PCO_2: 0/8 °C $p = 0.02$, 3/8 °C $p = 0.4$, 6/8 °C $p = 0.4$). Atlantic cod revealed a stable feed conversion ratio at 8, 12 and 16 °C with an average value of 0.97. An exceptionally low feed conversion efficiency was found at 3 °C, which was most distinct under hypercapnia (mean = 0.65; control PCO_2: 3/8 °C $p = 0.04$, 3/12 °C $p = 0.1$, 3/16 °C $p = 0.1$; high PCO_2: 3/8 °C $p = 0.005$, 3/12 °C $p = 0.001$, 3/16 °C $p = 0.001$). Therefore, polar cod showed a higher feed conversion ratio at 3 °C than Atlantic cod ($W = 439$, $p = 0.0005$), while the opposite was the case at 8 °C ($W = 108$, $p = 0.001$).

Condition factor and hepatosomatic index

Polar cod revealed an inverse relationship between CF and temperature. In contrast, the CF of Atlantic cod increased with temperature (Fig. 5).

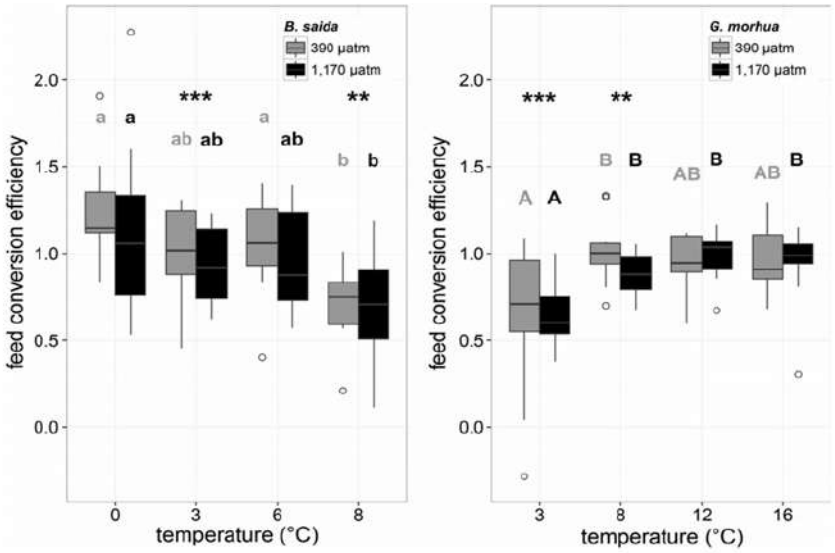

Fig. 4 Feed conversion efficiency. *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*). *Asterisks* significant difference between species at 3 and 8 °C, respectively; **: $p < 0.01$, ***: $p < 0.001$

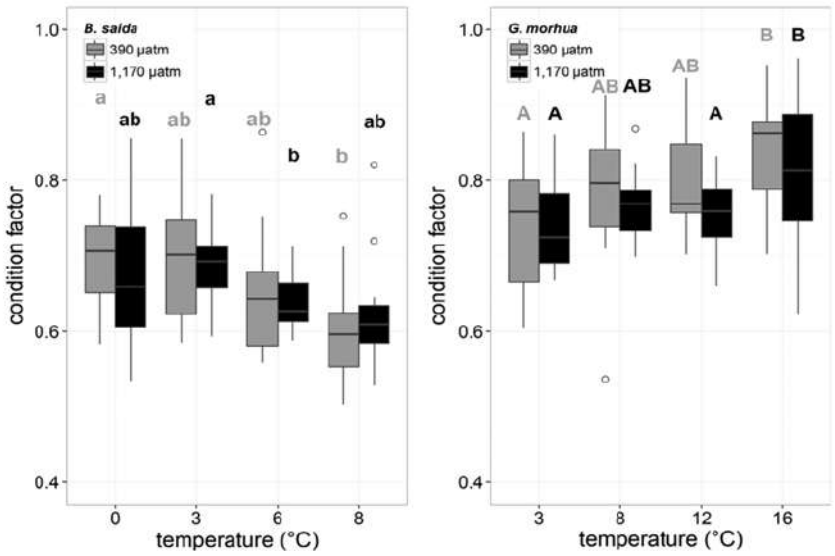

Fig. 5 Condition factor according to Fulton. *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*)

The trend of an increasing HSI at higher temperatures could statistically not be supported for both polar cod and Atlantic cod under normocapnia. Under high PCO_2, the increase in HSI with temperature was more pronounced in Atlantic cod ($p < 0.0001$) (Fig. 6), resulting in a significantly higher HSI under high PCO_2 than at control PCO_2 at 16 °C ($W = 0$, $p = 0.008$). The full linear model showed no significant temperature dependency for both species (polar cod $p = 0.14$, Atlantic cod $p = 0.1$). The reduced linear model supported the findings on PCO_2 impact, taking into consideration the whole range of investigated temperatures and testing the interaction term (polar cod: $p = 0.8$; Atlantic cod: $p = 0.0004$; Online Resource 1). Atlantic cod shows a significant positive slope (0.34151) in its temperature-dependent trend of HSI under elevated PCO_2 matching the trend seen in Fig. 6.

Gonadosomatic index

Sex determination revealed a majority of immature males (85.2 %) among polar cod and a slightly larger fraction of immature females (67.2 %) among Atlantic cod. The GSI

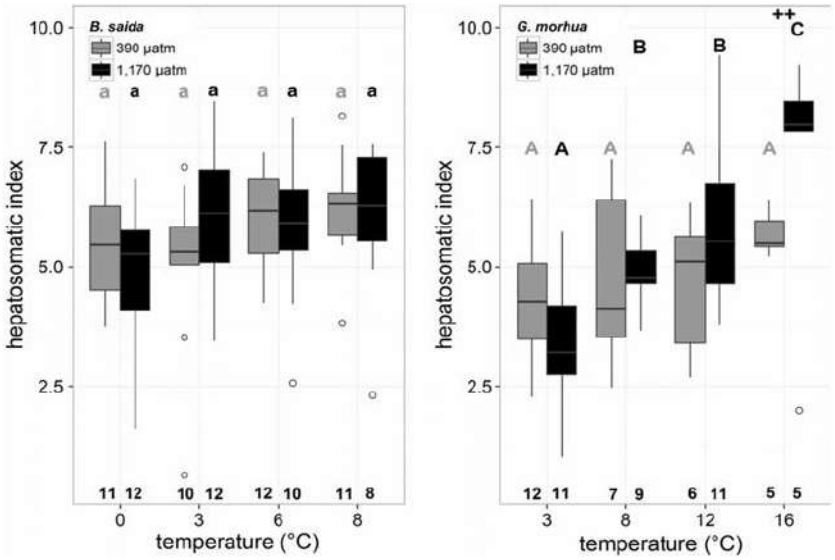

Fig. 6 Hepatosomatic index. Polar cod (n = 8–12); Atlantic cod (n = 5–12). *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*). *Plus sign* significant difference between PCO_2 treatments at the same temperatures within the species; ++: $p < 0.01$. Numbers below = n

of male polar cod and its variability tended to decrease with increasing temperature. At 0 °C, the average value was 7.31 %. The GSI of female Atlantic cod did not show any variation with temperature and appeared to be relatively low (mean = 0.34 %; Fig. 7; GSI data for complementary sexes of both species are shown in the figure in Online Resource 2).

Standard metabolic rate

The SMR of both species was strongly affected by temperature (Fig. 8). Polar cod acclimated for 4 months to the respective temperature showed a similar SMR at 0, 3 and 6 °C (mean = 0.053 µmol $(min*g)^{-1}$), but at 8 °C, SMR was strongly enhanced (mean = 0.080 µmol $(min*g)^{-1}$). This effect was most distinct under normocapnic conditions (control PCO_2: 0/8 °C $p = 0.01$, 3/8 °C $p = 0.002$, 6/8 °C $p = 0.02$; high PCO_2: 0/8 °C $p = 0.02$, 3/8 °C $p = 0.03$, 6/8 °C $p = 0.05$). The SMR of Atlantic cod increased with temperature from 0.030 (3 °C) to 0.068 µmol $(min*g)^{-1}$ (16 °C). At both 3 and 8 °C, SMR was distinctly higher in polar cod than in Atlantic cod (3 °C: $W = 247$, $p < 0.0001$; 8 °C: $W = 189$, $p < 0.0001$).

Effect of temperature and PCO_2 on the global data set

A NMDS analysis of the global data set revealed a significant impact of temperature in both species (polar cod: $p = 0.001$, Atlantic cod: $p = 0.001$; Fig. 9). A significant effect of PCO_2 was only detected in the total data set of polar cod ($p = 0.03$).

Discussion

Few studies are available that investigated growth performance of fish under predicted moderate PCO_2 conditions. In general, vulnerability of marine teleosts under PCO_2 conditions projected for the year 2100 is considered to be low due to excess capacities for acid–base regulation in gill cells (Melzner et al. 2009; Michael et al. 2016). Besides that, the life stages investigated in the present study are less sensitive to changing abiotic conditions than eggs, larvae or spawning life stages (Pörtner and Farrell 2008). Accordingly, neither mortality nor growth performance of polar cod and Atlantic cod were significantly influenced by chronic exposure to hypercapnia in this study. However, nonsignificant trends caused by hypercapnia were found in most of the parameters investigated. As a note of caution, the impact of PCO_2 might be reduced by low growth performance due to low-frequency feeding events. In polar cod, the effect of PCO_2 was visible in a nonsignificant trend for depressed growth performance under high PCO_2 conditions, which was most pronounced at the optimum temperature for growth determined under control conditions (6 °C). Costly compensatory processes in ion and acid–base regulation may cause the decrease in scope for growth. Growth impairment attributed to decreased food consumption under hypercapnic conditions (Smart 1981) may be the result of an uncompensated respiratory acidosis

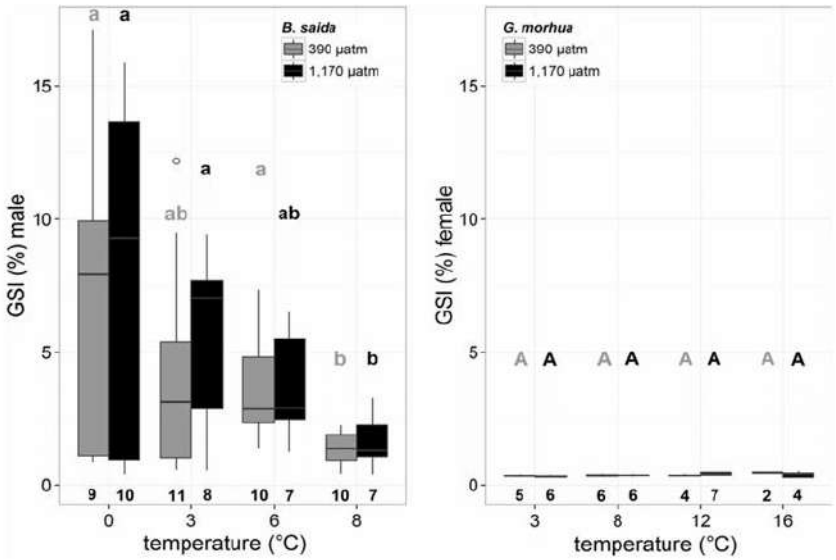

Fig. 7 Gonadosomatic index (%). Polar cod, male ($n = 7$–11); Atlantic cod, female ($n = 2$–7). *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*). Numbers below = *n*

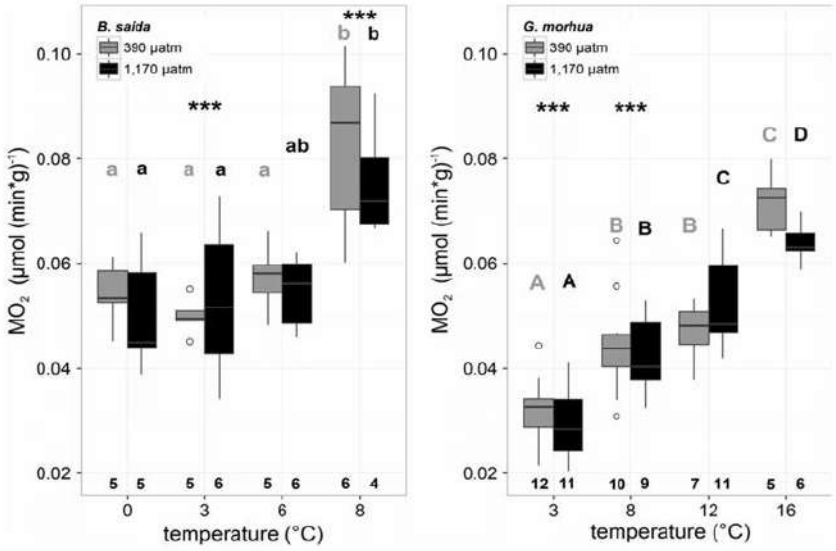

Fig. 8 Standard metabolic rate (µmol $(min*g)^{-1}$). Polar cod ($n = 4$–6); Atlantic cod ($n = 5$–12). *Letters* comparison of temperature treatments at different PCO_2 levels (390 µatm: *gray letters*; 1170 µatm: *black letters*) in polar cod (*lowercase*) and Atlantic cod (*uppercase*). *Asterisks* significant difference between species at 3 and 8 °C, respectively; ***: $p < 0.001$. Numbers below = *n*

(Crocker and Cech 1996). While no such trend was detected in polar cod, a (nonsignificant) trend for depressed feed intake under hypercapnia was recorded for Atlantic cod and became more prominent in the warmth. Nevertheless, a decline in growth performance or feed conversion efficiency was not detected in Atlantic cod of our study. Furthermore, hypercapnia caused a nonsignificant decrease in metabolic rate as well as a significantly enhanced HSI compared to the control treatment at 16 °C. Liver enlargement might be a compensatory response to potentially impaired lipase activity (Yada et al. 2002) under high PCO_2 conditions. An alternative hypothesis attributes the enhanced HSI to an energy surplus, potentially evoked by the amount of energy conserved due to suppressed foraging activity in response to elevated CO_2 levels. This hypothesis indicates that the decline in feed uptake of Atlantic cod is less than the decline in energy demand due to foraging activity such that Atlantic cod is thriving slightly better under hypercapnic conditions close to its thermal optimum for growth. Although this

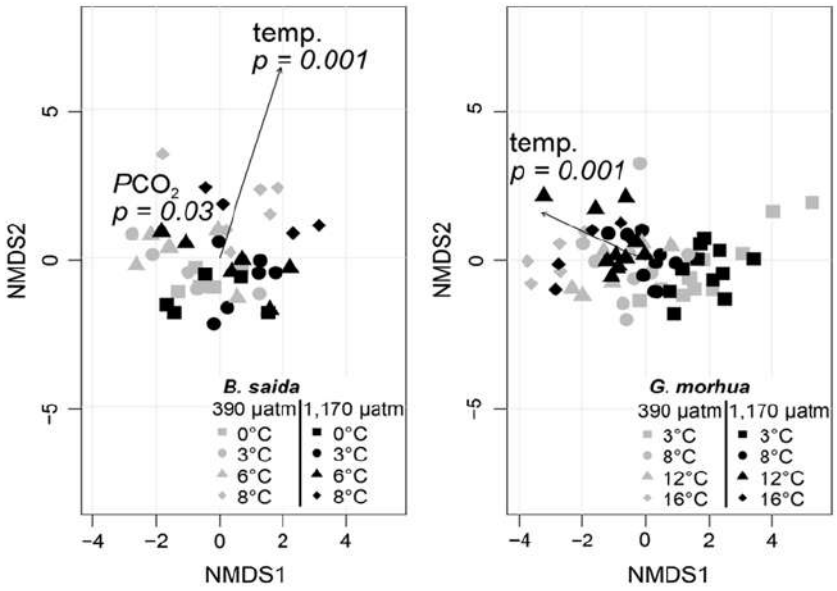

Fig. 9 Result of non-metric multidimensional scaling. Investigation of effects of temperature and PCO_2 on the global data set (excluding GSI and HSI)

explanation remains speculative, if true it may reflect the wide range of abiotic habitat conditions experienced within the lifetime of Atlantic cod (Neuenfeldt et al. 2009). For comparison, polar cod faces less extreme variations in habitat PO_2 and PCO_2. Hence, the onset of growth impairment of polar cod under hypercapnia indicates a higher sensitivity of this species to projected future PCO_2 values, compared to the more tolerant Arctic population of Atlantic cod. This conclusion is supported by the outcome of the NMDS analysis of the overall data set.

We kept individuals isolated from each other due to cannibalism recorded in the common tanks prior to the start of the experiment. Thereby, their feeding efforts (including foraging and competition) were minimized to below levels typical for their natural environment and aquaculture conditions. This may have improved their growth performance overall and at higher temperatures. The temperature profiles detected here can thus not easily be transferred to the natural environment but provide relative information on the competitive strength of the two species with respect to growth.

For polar cod, mortality occurred exclusively at the highest investigated temperature (8 °C) at a late stage of the experiment. This indicates that moderate constraints on the animal's well-being had long-term effects, classifying 8 °C as the species' long-term upper thermal tolerance limit. Protective mechanisms such as heat-shock proteins, use of anaerobic metabolism, and antioxidative defense are likely to only support time-limited periods of passive tolerance as outlined by the OCLTT concept (Pörtner 2012). Elevated baseline energy turnover leads to reduced growth and, finally, via whole organism and cellular functional constraints and stress, reduced survival rates in polar cod at 8 °C. This contrasts earlier findings by Christiansen et al. (1996) who reported that polar cod survives temperatures up to 14 °C under laboratory conditions. Unfortunately, the authors neither specified the initial acclimation temperature nor the exact duration in the warming protocol. Higher lethal temperatures during shorter-term warming protocols, however, seem to be a general phenomenon and were also found by Peck et al. (2009) in polar species.

Polar cod is known to be a slow growing species (Hop et al. 1997a; Gjøsæter 2009), explaining the limited maximum body length of approx. 30 cm despite a maximum lifespan of 7 years (Bradstreet et al. 1986; Hop et al. 1997b). Furthermore, growth rates decrease with increasing body weight and age (e.g. Jobling 1983, 1988; Björnsson and Steinarsson 2002; Björnsson et al. 2007). Size-at-age data (Falk-Petersen et al. 1986) at the start of the experiment as well as the maturation stage indicate an age of 2 years for polar cod, whereas Atlantic cod were approx. 1 year old. Thus the comparison of growth rates and further characteristics of the two species in our experiment may be affected by their slightly different positions in the life cycle. The slow growth rate of polar cod (potentially amplified by low-frequency feeding events) likely prevented the detection of statistical differences in body weight increments between temperature treatments. However, due to a trend for increasing growth rates from 0 to 6 °C under control PCO_2 conditions, we assume a growth

APPENDIX

optimum between 3 and 6 °C that coincides with the temperature preference of this species (Schurmann and Christiansen 1994). At 0 °C/390 µatm, we recorded a specific growth rate of 0.39 % d^{-1}, in line with the findings by Christiansen (1995) for polar cod from the Pechora Sea. The initial average weight as well as the feeding frequency was identical in both Christiansen's and our study. In contrast, Hop et al. (1997a) found a relative growth rate (~0.7 % d^{-1}) at 0 °C for polar cod from Canadian waters, slightly exceeding the one in our study (0.58 % d^{-1}, according to the formula in Hop et al. 1997a) despite similar initial body weight. Divergent growth rates relate to the substantially higher daily energy uptake measured by Hop et al. (1997a) (2.058 kJ d^{-1}) compared to the present study (1.436 kJ d^{-1}). Although lower feeding frequencies may have prevented individuals from exploiting their maximum growth capacity in this study, positive growth clearly demonstrates that the feed intake was sufficient to cover more than baseline costs. It remains to be explored whether growth rates differ between various polar cod populations across the Arctic as well as between fjord and oceanic stocks (Madsen et al. 2015), in similar ways as they differ between Atlantic cod populations (Pörtner et al. 2008).

A depressed feed intake of polar cod at 0 °C compared to the other experimental temperatures likely contributes to their lower growth rates at cold temperature (Drinkwater 2005). The limits of voluntary feed intake are mirrored by temperature-dependent stomach evacuation rates (Brett 1979; Hop and Tonn 1998). Low temperatures are associated with low stomach evacuation rates (evacuation half times at -1.42 ± 0.07 °C = 36–70 h) (Hop and Tonn 1998), high assimilation rates, and low energetic cost of processes competing with growth, resulting in an enhanced feed conversion efficiency at low feed intake. Hence, the thermal optimum for feed conversion results lower than the thermal optimum for growth. Árnason et al. (2009) detected a difference of 3 °C between these two optima for turbot (*Scophthalmus maximus*), while our data indicate a difference of as large as 3–6 °C for polar cod. Although digestion rates, and thus appetite, are known to rise with increasing temperatures (Brett 1979), the daily feed intake reached a plateau at all temperatures investigated above 0 °C, indicating that 18.8 mg (g wet BW*meal)$^{-1}$ may represent maximal stomach filling in polar cod from Kongsfjorden (average BW = 23.3 g). This hypothesis implies complete stomach emptying between feeding events above 0 °C; however, stomach inspection confirmed incomplete emptying at the end of the experiment. Based on identical amounts of consumed feed per meal for polar cod at 3–8 °C and enhanced metabolic rates at 8 °C, we conclude that elevated baseline costs were the constraining factor for both growth and feed conversion at 8 °C. Furthermore, faster stomach evacuation rates at 8 °C and accordingly shortened assimilation periods potentially caused decreasing exploitation efficiency of consumed feed in similar ways as described for brown trout (*Salmo trutta*) by Elliott (1976).

Another parameter interfering with somatic growth is gonadal development. In late September, male polar cod kept at 0 °C developed a mean GSI of 7.31 %, whereas no such trend was visible for the females at the same temperature (mean GSI = 2.09 %). Hop et al. (1995) reported that gonadal development in male polar cod from the Canadian Arctic maintained at 1 °C started in August and reached GSI values similar to those in our fish in September. Reproducing males exhibited a GSI of approx. 30 % from December onwards (ibid.). Females show a slow initial increase in gonadal tissue and an exponential gonadal development with a peak in gonad size approximately 2 months after males are ready to spawn (Hop et al. 1995). However, a substantial difference in specific growth rates between sexes before the winter months was not found (Christiansen 1995). The variability in GSI between temperature treatments as well as the mean GSI decreased with increasing temperatures, indicating an unfavorable shift in the energy budget. This trend is also reflected in a continuous decrease in the condition factor at higher temperatures. The lipids needed for gonadal development are very likely mobilized from liver tissue. Therefore, the increasing trend of HSI with temperature might be attributed to a decreasing ability to exploit liver energy reserves in the warmth. Furthermore, gonad development might cause a slight underestimation of growth rate at 0 °C, considering the lower weight of gonadal tissue compared to muscle tissue of identical energy content.

In contrast to the findings for polar cod, mortality among Atlantic cod occurred predominantly at an early stage of the experiment, mainly due to the total refusal of feed, followed by a few occasional deaths until the end of the incubation. As mortality was scattered across treatments, neither temperature nor PCO_2 seemed to be the cause for the recorded mortality.

The growth rates of Atlantic cod were directly correlated with temperature and no distinct thermal optimum was found within the investigated temperature range (3–16 °C). Growth optima are size- (Björnsson et al. 2007) and ratio-dependent (Brett 1979) and differ slightly between populations (Pörtner et al. 2001, 2008). Björnsson et al. (2007) found an optimum temperature for growth in similar sized (56.9 g) Islandic cod at 12.1 °C. The low frequency of feeding events may have caused a feed limitation during our experiments with potential consequences for the thermal optimum for growth. According to Brett (1979), the optimum temperature for growth shifts to lower temperatures under restricted food supply. We therefore conclude

that the temperature optimum for growth for the investigated population of Atlantic cod in the present study is unlikely above 16 °C. The limited scope for locomotion for individuals in the present study may have modified the priorities in energy allocation such that growth takes priority, especially at high temperatures where activity is known to be most pronounced and costly (e.g. Brown et al. 1989). Investigations of spontaneous activity in our fish revealed no significant differences between the temperatures (pers. obs.) supporting the hypothesis of shifted priorities in energy allocation in favor of growth. Nevertheless, Björnsson et al. (2007) recorded an optimum growth rate of 1.75 % d^{-1} (12.1 °C), which exceeds the rate in the present study (0.82 % d^{-1}, 12 °C) more than twofold for cod of similar size. Björnsson et al. (2007) fed their fish several times per day, potentially explaining the difference in growth rates. Despite the potential feed limitation in our experiment, we recorded similar daily feed consumption rates compared to a study with higher feeding frequencies: Peck et al. (2003) detected a daily feed intake of 1.62 % (wet BW*day)$^{-1}$ for Atlantic cod of 7.57 g at 12 °C fed one to three times per day with artificial diet (19.84 kJ (g wet wt.)$^{-1}$). The fish in the present study consumed 1.44 % (wet BW*day)$^{-1}$ (15.865 kJ (g wet wt.)$^{-1}$) at the same temperature and a mean body weight of 40.4 g. Although the percent feed consumption is considered to decrease with increasing body weight, the comparable amounts consumed in both studies indicate a compensation for low feeding frequency by high feed consumption per meal. High feed consumption is supported by complete stomach emptying between the feeding events at this temperature as confirmed by stomach inspections at the end of the experiment. Despite higher rates of stomach emptying (Tyler 1970) and rising metabolic costs with increasing temperatures, both condition factor and HSI increased continuously, indicating an energy surplus. Accordingly, feeding was sufficient to support positive growth at all temperatures. Along similar lines of reasoning, the increasing feed consumption rates per unit body weight at higher temperatures were likely sufficient to cover the higher maintenance costs, indicated by constant feed conversion efficiencies at increasing temperatures. Solely at 3 °C, the feed conversion efficiency appeared to be reduced [by 34.2 % (390 µatm) and 30.6 % (1170 µatm)]. At this temperature, the feed intake is very likely limited by low stomach evacuation rates and low digestion rates. Accordingly, 3 °C approaches the lower thermal limit of this species' metabolic efficiency, potentially evoked by insufficient capacity of the digestive system (Pörtner 2001). However, cod are still able to exploit this temperature range as the habitat temperatures experienced by adult Atlantic cod from the Barents Sea were found to fall temporarily below 3 °C (Michalsen et al. 2014). At all comparable temperatures, Björnsson et al. (2001) found higher feed conversion efficiencies for Atlantic cod than recorded in this study, possibly indicating a high plasticity of this species to adapt to different feeding regimes.

Further considerations include a potential functional and genetic difference between different stocks of Atlantic cod. The fish in Björnsson et al. (2007) were hatchery reared and originated from an Icelandic broodstock, while our fish were caught in the area around Svalbard. Growth performance of different cod stocks has been shown to decrease with increasing latitude (Fischer 2002). The selection of specific phenotypes from the larval population at high mortality may cause such functional differences. Although genetic differences in growth performance cannot be eliminated by acclimatization processes (Pörtner et al. 2001), the differences in growth rates of different populations reared under similar conditions appear to be small (Purchase and Brown 2001, cf. Pörtner et al. 2008). Therefore, Björnsson and Steinarsson (2002) considered a genetic contribution to Atlantic cod growth to be less than the influence of environmental factors.

Although the specimens of both species originate from an environment with temperatures well below their optimum for growth, the stenothermal polar cod is very well adapted to the low temperatures, whereas the eurythermal Atlantic cod appears to reach the lower boundaries of its thermal window. Despite higher maximal growth capacity in Atlantic than in polar cod, the better growth performance at low temperatures emphasizes the higher degree of cold adaptation of polar cod than of Atlantic cod coexisting in the same area. Consequently, the majority of polar cod in the present study were caught at temperatures between −1.5 and 3.0 °C in bottom waters, whereas juvenile Atlantic cod were caught in surface waters at temperatures between 4.0 and 6.0 °C. Hence, the benefit of low predation pressure for polar cod at low temperatures (Brown et al. 1989) seems to outweigh the physiological restrictions. The high feed conversion efficiency at low temperatures enables polar cod to reveal positive growth even with little or temporally restricted food supply. Furthermore, reduced foraging activity involves less exposure to potential predators. In contrast to polar cod, Atlantic cod is well adapted to handle high food abundances (Jensen et al. 1991), indicated by greatly varying feed consumption rates with temperature. Therefore, its growth performance, as well as the temperature optimum for growth in nature, are strongly correlated with food availability (Jobling 1994). The physiology of Atlantic cod seems to be specialized in efficient and fast growth in order to reduce predation pressure at small body size. The feed conversion efficiency of Atlantic cod decreases remarkably in the cold, evoked by a stronger impairment of feed intake compared to the

decrease in maintenance costs. Nevertheless, adult Atlantic cod can experience and tolerate subzero temperatures for short periods (Michalsen et al. 2014), making them likely candidates to prey upon polar cod in deep, cold water layers in the fjords around Svalbard.

Under projected climate conditions for the year 2100, growth performance of polar cod is expected to remain unchanged. However, from the present perspective, its feed conversion efficiency can be assumed to decrease progressively. Considering that other temperate fish species are also extending their distribution range to the North, such competition might cause a northward displacement of polar cod. Hop and Gjøsæter (2013) expect the pelagic capelin (*Mallotus villosus*) and herring (*Clupea harengus*) as likely invader species replacing polar cod as a key species in the area of investigation. Atlantic cod is expected to thrive under future ocean conditions in the area around Svalbard, provided that its prey species also adapt to the environmental changes or that it is able to switch the species preyed upon. However, the distribution of demersal Atlantic cod is likely constrained by the shelf edge (Hop and Gjøsæter 2013), whereas ice-associated polar cod is found throughout the Arctic Ocean. Ultimately, it is not the increase in annual mean temperature but rather the change in extreme temperatures that will be crucial for a species' settlement (Stachowicz et al. 2002).

In conclusion, the combined drivers ocean acidification and warming (OAW) caused nonsignificant trends in growth performance and SMR of polar cod and in feed consumption and HSI of Atlantic cod. While the performance of polar cod will be impaired under projected water conditions by year 2100, Atlantic cod will thrive, visible in lower amounts of feed necessary for the maintenance of growth performance. The higher tolerance of Atlantic cod to changing water conditions is likely attributed to the wide range of abiotic habitat conditions experienced within its lifetime. Temperature is the predominant environmental factor causing the shift in relative performance by influencing the energy allocation between individual processes in the energy budget of both species. The stenothermal polar cod is specialized in temperatures well below its thermal optimum for growth and can thrive with little food supply, thereby escaping competitive pressure. Atlantic cod is eurythermal and rather displays high plasticity under changing abiotic conditions, but is less competitive in the cold. Hence, we argue that the competitive strength of polar cod is expected to decrease dramatically under future warming and acidification. Therefore, a northward displacement and thereby a decreasing distribution range of polar cod will be the likely consequence of the ongoing distribution shift of Atlantic cod to the north. However, the reported effects may become less pronounced over time depending on potential trans-generational adaptation effects.

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Compliance with ethical standards

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

Ethical approval All procedures performed in the present study were in accordance with the ethical standards of the federal state of Bremen, Germany, and were approved under the reference number 522-27-22/02-00 (113).

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PUBLICATION AII

Impact of ocean warming and acidification on the behaviour of two co-occurring gadid species, *Boreogadus saida* and *Gadus morhua*, from Svalbard.

Matthias Schmidt, Gabriele Gerlach, **Elettra Leo**, Kristina L. Kunz, Steffen Swoboda, Hans-O. Pörtner, Christian Bock, Daniela Storch

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Impact of ocean warming and acidification on the behaviour of two co-occurring gadid species, *Boreogadus saida* and *Gadus morhua*, from Svalbard

Matthias Schmidt[1,2], Gabriele Gerlach[3], Elettra Leo[1,2], Kristina Lore Kunz[1,2], Steffen Swoboda[1], Hans-Otto Pörtner[1,2], Christian Bock[1], Daniela Storch[1,*]

[1]Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Section Integrative Ecophysiology, Am Handelshafen 12, 27570 Bremerhaven, Germany

[2]University of Bremen, Fachbereich 2, NW 2 / Leobener Strasse, 28359 Bremen, Germany

[3]Carl von Ossietzky University Oldenburg, Institute for Biology, Group Biodiversity and Evolution of Animals, Carl von Ossietzky Str. 9-11, 26111 Oldenburg, Germany

ABSTRACT: Ocean acidification induces strong behavioural alterations in marine fish as a consequence of acid–base regulatory processes in response to increasing environmental CO_2 partial pressure. While these changes have been investigated in tropical and temperate fish species, nothing is known about behavioural effects on polar species. In particular, fishes of the Arctic Ocean will experience much greater acidification and warming than temperate or tropical species. Also, possible interactions of ocean warming and acidification are still understudied. Here we analysed the combined effects of warming and acidification on behavioural patterns of 2 fish species co-occurring around Svalbard, viz. polar cod *Boreogadus saida* and Atlantic cod *Gadus morhua*. We found a significant temperature effect on the spontaneous activity of *B. saida*, but not of *G. morhua*. Environmental CO_2 did not significantly influence activity of either species. In contrast, behavioural laterality of *B. saida* was affected by CO_2 but not by temperature. Behavioural laterality of *G. morhua* was not affected by temperature or CO_2; however, in this species, a possible temperature dependency of CO_2 effects on relative laterality may have been missed due to sample size restrictions. This study indicates that fish in polar ecosystems may undergo some, albeit less intense, behavioural disturbances under ocean acidification and in combination with ocean warming than observed in tropical species. It further accentuates species-specific differences in vulnerability.

KEY WORDS: Ocean acidification · Climate change · Fish behaviour · Laterality · Activity · Polar habitat · Atlantic cod · Polar cod

INTRODUCTION

Ocean acidification (OA), i.e. the perturbation of seawater carbonate chemistry by accumulating CO_2, has the potential to strongly alter the behaviour of various marine teleosts and elasmobranchs, affecting for example their activity, boldness, predator avoidance, learning and behavioural laterality, and interfering with their sensory processes (Heuer & Grosell 2014). While behavioural alterations have mostly been observed under acute exposure to increased CO_2 partial pressure (pCO_2), there also appears to be a species-specific potential to adapt behaviour across generations (Miller et al. 2012, Allan et al. 2014, Munday et al. 2014, Welch et al. 2014). However, OA develops in parallel to ocean warming (OW), but to date, interactive effects of OA and OW on the behaviour of teleosts remain understudied and have been

*Corresponding author: daniela.storch@awi.de

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analysed only in a few tropical species (Nowicki et al. 2012, Domenici et al. 2014, Ferrari et al. 2015). In cold-adapted fish species, OA-induced behavioural changes have not been assessed, although the polar ocean of the northern hemisphere is expected to experience the greatest changes in both temperature and pCO_2-induced acidification in the near future (IPCC 2013).

Here we analysed the combined effects of OA (as projected for the year 2100) and temperature on spontaneous activity and behavioural laterality of 2 co-occurring teleost species from Svalbard, Norway. These types of behaviour have been shown to be affected by CO_2 in tropical (activity and laterality) and temperate (laterality) fish species. CO_2-induced effects on activity are species dependent, with activity being either increased or reduced by a predicted rise in CO_2 (Munday et al. 2010, 2013, 2014, Cripps et al. 2011, Nowicki et al. 2012). Behavioural lateralization is defined as the side preference of an animal conducting a certain task (e.g. 'handedness') or, in this study, the tendency to turn to one side at the end of an experimental runway (Domenici et al. 2012). Earlier studies observed a reduction in the behavioural laterality of tropical and temperate fish species after acclimation to OA scenarios (Domenici et al. 2012, 2014, Jutfelt et al. 2013) with the exception of temperate Atlantic cod *Gadus morhua* and the temperate wrasse *Ctenolabrus rupestris* (Jutfelt & Hedgärde 2015, Sundin & Jutfelt 2016).

Increased spontaneous activity can lead to higher energetic demands, requiring more food uptake, which can subsequently lead to greater exposure to predators (Munday et al. 2013). Conversely, an increase in activity could be the consequence of reduced foraging success or increased energetic demand leading to intensified foraging behaviour to fill this energetic gap (Cripps et al. 2011). The potential effects of behavioural lateralization on animal fitness are not fully resolved. Lateralized behaviour reflects functional asymmetry of the brain, where one brain hemisphere specializes in conducting a certain task. Specialization may be useful to increase the speed of sensory processing when several different stimuli must be analysed simultaneously (Rogers et al. 2004). This is especially important in fish, such as Gadidae, that possess lateral eyes and no mobile neck, so that each eye (and thus each brain hemisphere) perceives an almost entirely different set of visual information (Vallortigara & Rogers 2005). Furthermore, fish lack the corpus callosum that accelerates information transfer between brain hemispheres in placental mammals (Dadda et al. 2009). Dadda et al. (2010) found a correlation between the degree of behavioural lateralization and escape performance in teleost prey fish. As a trade-off, non-lateralized animals performed better at cognitive tasks than lateralized fish when relevant similar stimuli occurred simultaneously on both sides of the body (Dadda et al. 2009). While these findings explain why non-lateralized animals are also commonly found in the wild, they complicate prediction of ecological consequences caused by changes in laterality on a population level. Here, we interpret a change in behavioural laterality as a proxy for disturbance in nervous system functioning, in similar ways as reported by Domenici et al. (2012).

The polar cod *Boreogadus saida* has a circumpolar distribution in Arctic and subarctic waters and is considered a key species in the Arctic ecosystem (Hop & Gjøsæter 2013). The Atlantic cod *Gadus morhua* is a temperate fish species which has shifted its distribution farther north with recent warming (Sundby & Nakken 2008, Drinkwater 2009). At present, the distribution areas of *B. saida* and *G. morhua* overlap for most of the year in the coastal waters around Svalbard, where sea surface temperature fluctuates between −1.8°C in winter and up to 8°C in summer (Renaud et al. 2012, Beierlein et al. 2015). The surface water temperature of this area is predicted to increase further by 2.5°C until the year 2100 according to the Representative Concentration Pathway (RCP) 8.5 scenario (IPCC 2013). The consequences of further temperature-driven northward migration of *G. morhua* and its interaction with *B. saida* on the Arctic ecosystem are unknown, especially as simultaneous OA might alter the usual behaviour of each species. We sought to document the species-specific vulnerability of the behaviour of both *B. saida* and *G. morhua* in response to combined OW and OA. We therefore incubated *B. saida* and *G. morhua* for 6 wk under present-day and future pCO_2, with the latter being set close to the maximum pCO_2 value projected by RCP 8.5 for the year 2100 (IPCC 2013). Animals were incubated at 4 different temperatures, between 0 and 8°C for *B. saida* and between 3 and 16°C for *G. morhua*, to cover a broad overlapping range of temperatures from the thermal window of each species.

MATERIALS AND METHODS

Animal collection

Juvenile *Boreogadus saida* were caught at 120 m depth in the inner part of the Kongsfjord on a polar night trawl on 17 January 2013 (78.97° N, 12.51° E).

Subsequently, the animals were kept in facilities of the Tromsø Aquaculture Research Station, in Kårvik, Norway. Juvenile *Gadus morhua* were caught in various locations of western Svalbard between 26 and 29 August 2013 on a cruise of the RV 'Heincke' in Rijpfjorden (80.15° N, 22.12° E), Hinlopenstretet (79.30° N, 18.57° E) and Forlandsundet (78.54° N, 11.3° E). A fish lift combined with a pelagic mid-water trawl was used to catch the animals (Holst & McDonald 2000). Further information on the cruise is available at http://doi.pangaea.de/10.1594/PANGAEA.824703. Specimens of both species were transported to the Alfred Wegener Institute in Bremerhaven, Germany, and kept in aquaria at a water temperature of 5°C in a re-circulating seawater system prior to the start of the incubation.

Incubation

Experiments on *B. saida* and *G. morhua* started in June 2013 and June 2014, respectively. *B. saida* and *G. morhua* were incubated at 0, 3, 6 and 8°C and at 3, 8, 12 and 16°C, respectively. pCO_2 was either 374–515 µatm (control CO_2) or 852–1416 µatm (high CO_2) in a full factorial approach with a group size of 12 animals treatment^{-1}, resulting in a sample size of 96 animals for each species. Animals were transferred into individual tanks (height: 35 cm, diameter: 30 cm, volume: ~24 l with a flow-through of ~500 ml min^{-1}) and randomly distributed among treatment groups. The animals were kept separately in order to enable quantification of feed consumption of each individual, which was published separately (Kunz et al. 2016). Water supply occurred through a re-circulating aquarium system with a total volume of 10 m^3. The seawater for the system was collected in 'Tiefe Rinne', close to Heligoland (Helgoland), Germany, in the North Sea. Adequate water quality was ensured through nitrification filters, UV-sterilizers and protein skimmers, and the nitrate concentration was kept at <50 mg l^{-1} at all times. Temperature was adjusted in 4 temperature-controlled rooms (1 room for each temperature treatment) by a maximum change of 2°C d^{-1} for each group, starting from 5°C. pCO_2 in high-CO_2 groups was increased within 1 d after the temperatures were adjusted. The incubation period started after the desired temperature and CO_2 condition had been reached for each treatment group. The animals were fed ad libitum with a commercial pellet food (Amber Neptun, Skretting) every fourth day. Day/night cycle was 12:12 h, with lights on at 08:00 h. Oxygen concentration in fish tanks was measured occasionally throughout the incubation period and was always found to be ~100%. Apart from temperature, room conditions were kept as similar as possible, with similarly dimmed light and a small distance between shelves containing the tanks with different CO_2-treatments (~1 m). Opaque walls of the tanks shielded external stimuli effectively, and activities inside the rooms were kept to a minimum. Behavioural experiments were conducted 6 wk after onset of the incubation and lasted 8 d in total. Length and weight of each animal were measured at the beginning of the incubation and 1 d after the end of the behavioural experiments. Mean lengths and weights (±SD) of individuals in each treatment group and species are available in Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/m571p183_supp.pdf. One out of 96 *B. saida* and 8 out of 96 *G. morhua* died during the incubation period for unknown reasons. The 8 casualties among *G. morhua* occurred in 5 different treatment groups at all temperatures as well as at control and high pCO_2, with no more than 2 specimens dying per treatment group. These mortalities were thus considered independent of the treatment conditions. A representative image of the incubation system of one treatment group is provided in Fig. S1 in the Supplement.

CO_2 and carbonate chemistry

Seawater was aerated with an air/CO_2 mixture from a gas-mixing pump (HTK) before flowing into the tanks holding the animals. Temperature, salinity, dissolved inorganic carbon and pH_{tot} were determined at least once weekly in order to calculate the seawater carbonate parameters. Means were calculated for each week; Tables S1 & S2 list the means ± SD over the whole incubation period for each treatment group and species. Detailed methodological information and the raw data are provided at https://doi.pangaea.de/10.1594/PANGAEA.866369.

Behavioural testing

Spontaneous activity

Spontaneous activity was tested 2 d after feeding. A camera was installed in the centre above the housing tank of an animal next to a white LED lamp for better illumination. Recordings were started manually 10 min after camera installation and illumination. The recordings lasted for at least 10 min, and the last

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5 min were used for quantification of spontaneous activity. For post-processing of the video, a grid was placed centrally over the tank, dividing it into 4 equally sized rectangles using the software packages ImageJ and Dartfish®. The frequency of grid lines crossed was counted for each individual within a 5 min period of recording. A crossing was counted when the whole head of an animal crossed a grid line (ending right before the pectoral fins). For each animal, the total number of grid lines crossed was divided by 5 to obtain the number of lines crossed min^{-1}. Operator-controlled analysis of behaviour was performed in a randomized order for each species without knowing animal or treatment to avoid any observer bias. Videos were recorded throughout the whole day, whereby only animals of one temperature treatment were observed per day. The sequence of video recordings alternated between the 2 CO_2 treatments to compensate for possible daytime-related differences in activity. In total, data from 94 *B. saida* and 87 *G. morhua* were used to quantify spontaneous activity. Video recordings for 2 animals had to be discarded for technical reasons.

Behavioural laterality

On the same day, after recording activity, each fish was transferred into a 125 × 50 cm aquarium containing a 2-sided T-maze, similar to the maze used by Domenici et al. (2012), to investigate combined effects of temperature and CO_2 on behavioural laterality via a detour test. The opaque maze, with a runway length of 70 cm and width of 8 cm, was placed in the centre of the aquarium. Perpendicular to each of the maze's ends was a dark grey, opaque barrier with a length of 25 cm leaving a gap of 5.5 cm on each side so that the animal could leave the maze on either left or right (see Fig. S2 in the Supplement for a scheme of the setup). The sides of the aquarium were shielded with a dark grey cover. The aquarium was filled with 10 cm of seawater according to the test animal's treatment conditions. After an acclimation period of 10 min, the animal was gently encouraged to swim through the maze by approaching it from behind with a meshed plastic slide until the animal reached the end of the runway where it escaped to the left or to the right. The side on which the individual left the maze was noted. This procedure was repeated 14 times for each fish, whereby the swimming direction through the chamber was reversed after each trial to compensate for the potentially disturbing influence of the fish's orientation towards existing room-related structures. Absolute and relative laterality indices were calculated as described by Domenici et al. (2012). Absolute laterality quantifies the preference of an animal for one side over the other; thus an animal that turned to the same side every time was allocated an absolute laterality index of 100. In contrast, the relative laterality index takes the side preference of each animal into account. An animal that turned to the left every time was allocated a relative laterality index of −100 and an animal that turned to the right every time was assigned a relative laterality index of +100. All trials were conducted by the same experimenter and lasted about 10 min for each animal. In total, data from 95 *B. saida* and 88 *G. morhua* were tested for behavioural laterality.

Statistical analysis

Spontaneous activity and absolute and relative laterality were analysed by an ordinary 2-way ANOVA to test for significant effects of temperature, CO_2 and possible interactions of these 2 factors. Normality of each group was investigated via D'Agostino and Pearson omnibus normality tests and the homogeneity of variances via a Brown-Forsythe test with $\alpha = 0.05$. A significant deviation from a normal distribution was detected in 3 out of 48 groups tested (*B. saida*: spontaneous activity at 8°C and high pCO_2; absolute laterality at 6°C and low pCO_2. *G. morhua:* spontaneous activity at 8°C and high pCO_2). However, using an α of 0.05 sets the chance of a false positive Type 1 error of each normality test to 5%, which may account for the deviation from normality in those 3 out of 48 tested groups. Furthermore, in 2 out of 3 cases, the observed violation of normality was caused by a single animal, and an exclusion of these animals did not lead to disappearance of the observed significant findings. We thus concluded that it is still acceptable to use the 2-way ANOVA under these conditions. A coefficient of variation (CV) was determined for each treatment group of spontaneous activity data by calculating the ratio of standard deviation and mean values, and the difference between the 2 species was analysed using a 2-sided Mann-Whitney test ($\alpha = 0.05$). Correlation between animal length and spontaneous activity was tested with a 2-tailed non-parametric Spearman r-test. The CO_2 effect on side preference on a population level and a possible CO_2-induced change from a non-random to a random distribution of left and right turns were tested for each species by pooling relative laterality data of all temperatures in accordance to control or high pCO_2, as we had not detected a significant

temperature effect on behavioural laterality in either *B. saida* or *G. morhua*. Subsequently, 2-sided 1-sample *t*-tests were conducted ($\alpha = 0.05$) for each CO_2 treatment of each species testing for significant differences from the hypothetical mean of 0. Deviation from a random binomial distribution was tested for via a log likelihood ratio goodness of fit test (*G*-test) using the software 'R' (v. 3.2.3) and the R-package 'DescTools'. GraphPad Prism® 6 was used for all other statistical tests and for generation of figures.

RESULTS

Spontaneous activity

Spontaneous activity of *Boreogadus saida* increased significantly with rising ambient temperature between 0 and 8°C ($p < 0.001$, $F_{3,86} = 7.064$, Fig. 1A). No significant difference in spontaneous activity of *B. saida* was detected between control and high CO_2 concentrations ($p = 0.0700$, $F_{1,86} = 3.368$). In contrast, spontaneous activity of *Gadus morhua* did not significantly depend on temperature ($p = 0.3172$, $F_{3,79} = 1.195$, Fig. 1B) or CO_2 concentration ($p = 0.5024$, $F_{1,79} = 0.4540$). *G. morhua* displayed a non-significant trend towards greater mean activity with increasing temperature which was strong between 3 and 8° but levelled off at higher temperature and even decreased in the group at 16°C under normal CO_2 levels. A significant interaction between temperature and CO_2-related effects was not detected in either species (all $p > 0.05$). The CV of spontaneous activity of *G. morhua* was significantly higher than the CV of *B. saida* ($p < 0.001$, Fig. 1C). Spontaneous activity was not significantly correlated with body length in either species ($p > 0.05$).

Behavioural laterality

Absolute laterality of *B. saida* was significantly reduced by CO_2 ($p < 0.01$, $F_{1,87} = 7.152$, Fig. 2A), but was not affected by temperature ($p = 0.2156$, $F_{3,87} = 1.518$). Also, in this species, relative laterality was dependent on CO_2 ($p < 0.01$, $F_{1,87} = 10.26$, Fig. 2C), but not on temperature ($p = 0.7020$, $F_{3,87} = 0.4728$), with a shift from left to right orientation under increased CO_2 concentrations. Side preference of *B. saida* was significantly left biased under control CO_2 ($p < 0.05$, $t = 2.242$, df = 47), significantly right biased under high CO_2 ($p < 0.05$, $t = 2.260$, df = 46) and significantly differed from a random binomial distribution under both CO_2 conditions ($p < 0.001$, $G = 13.761$, χ^2 df = 1 for low pCO_2 and $p < 0.01$, $G = 7.0399$, χ^2 df = 1 for high pCO_2).

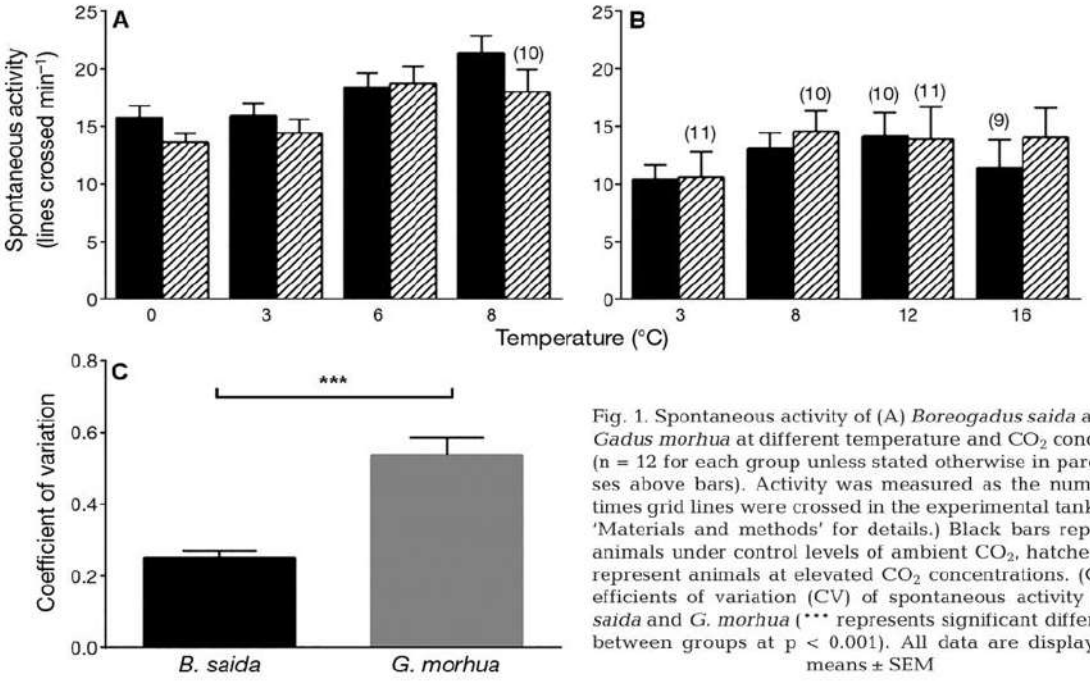

Fig. 1. Spontaneous activity of (A) *Boreogadus saida* and (B) *Gadus morhua* at different temperature and CO_2 conditions (n = 12 for each group unless stated otherwise in parentheses above bars). Activity was measured as the number of times grid lines were crossed in the experimental tanks (see 'Materials and methods' for details.) Black bars represent animals under control levels of ambient CO_2, hatched bars represent animals at elevated CO_2 concentrations. (C) Coefficients of variation (CV) of spontaneous activity for *B. saida* and *G. morhua* (*** represents significant differences between groups at $p < 0.001$). All data are displayed as means ± SEM

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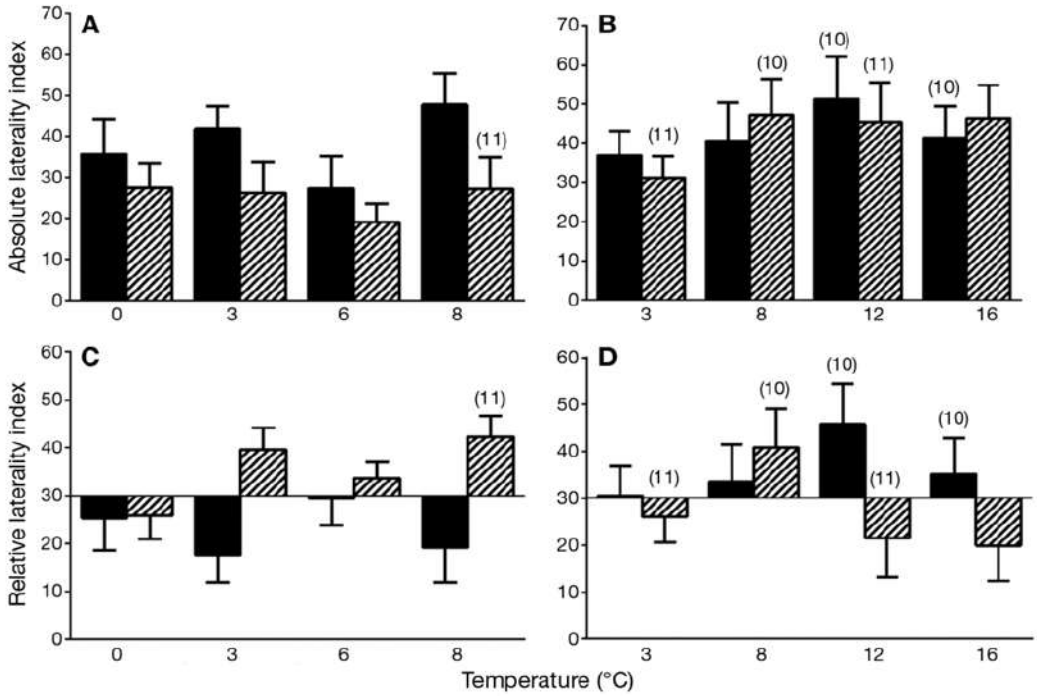

Fig. 2. Absolute laterality index of (A) *Boreogadus saida* and (B) *Gadus morhua* and relative laterality index of (C) *B. saida* and (D) *G. morhua* at different temperature and CO_2 conditions (n = 12 for each group unless stated otherwise in parentheses above bars). Details of laterality indices are given in the 'Materials and methods'. Black bars represent animals under control; hatched bars represent animals at elevated CO_2 concentrations. Data are displayed as means ± SEM

In *G. morhua*, absolute laterality was not affected by CO_2 ($p = 0.9949$, $F_{1,80} = 4.086 \times 10^{-5}$, Fig. 2B) or temperature ($p = 0.3966$, $F_{3,80} = 1.002$). Relative laterality also did not significantly depend on CO_2 or temperature ($p = 0.0913$, $F_{1,80} = 2.920$ and $p = 0.5375$, $F_{3,80} = 0.7293$, respectively, Fig. 2D). *G. morhua* did not exhibit a significant side preference under control or under high CO_2 conditions ($p = 0.1272$, $t = 1.556$, df = 43 and $p = 0.3792$, $t = 0.8886$, df = 43, respectively). The side preference differed significantly from a binomial random distribution under low, but not quite under high CO_2 conditions ($p < 0.01$, $G = 8.4349$, χ^2 df = 1 and $p = 0.09$, $G = 2.8659$, χ^2 df = 1, respectively). In both species, no interactive effects were detected between CO_2 and temperature effects on absolute and relative laterality (all $p > 0.05$).

DISCUSSION

This is the first study analyzing and comparing the combined effects of CO_2 and temperature on the behaviour of 2 gadid fish species, one polar and cold adapted (*Boreogadus saida*), the other (*Gadus morhua*) temperate and invasive to the high polar environments due to global warming. We demonstrated that the behavioural vulnerability of fish, even if they are related, may be species-dependent in response to temperature and CO_2.

While we observed a significant influence of temperature on spontaneous activity of *B. saida*, no such significance was detected in *G. morhua*. However, in the latter, a possible temperature effect may have been masked by high inter-individual variability, which was significantly more pronounced in *G. morhua* than in *B. saida*. We found no CO_2-related effect on the spontaneous activity of *B. saida* and *G. morhua*, in contrast to strong alterations that were observed in tropical cardinalfish (*Apogon cyanosoma* and *Cheilodipterus quinquelineatus*) and damselfish (*Pomacentrus wardi*) (Munday et al. 2010, 2014), which either showed an increase or a decrease in activity in response to elevated pCO_2 as predicted for future OA scenarios. However, CO_2-effects on swimming behaviour of fish appear to be strongly species-dependent, and our results are consistent with findings of several

studies that observed largely resilient routine swimming activity and kinematics particularly in temperate species (including *G. morhua* larvae) (Maneja et al. 2013, 2015, Sundin & Jutfelt 2016), but also in tropical species (Nowicki et al. 2012, Bignami et al. 2013, 2014). As our study was conducted with juvenile specimens of *G. morhua*, it can be concluded that swimming behaviour at least in this species appears to be robust to an increase in environmental CO_2 across different life stages.

Interestingly, the effect of CO_2 on laterality was different in *B. saida* and *G. morhua*. In *B. saida*, absolute lateralization was significantly reduced and paralleled by a shift from left to right lateralization, whereas in *G. morhua*, we found no changes in absolute lateralization or side preference. These results conform with recent experiments on temperate fish species, that found a CO_2-induced reduction of absolute laterality in three-spined stickleback *Gasterosteus aculeatus* (Jutfelt et al. 2013, Lai et al. 2015), but not in wrasse *Ctenolabrus rupestris* or in juvenile *G. morhua* of similar age compared to the specimens in our study (Jutfelt & Hedgärde 2015, Sundin & Jutfelt 2016). Contrary to findings in *Pomacentrus wardi* (Domenici et al. 2014), we did not detect any interaction of temperature- and CO_2-related effects on behavioural laterality. However, potential interactive effects of CO_2 and temperature on the relative laterality of *G. morhua* may have been missed because of low statistical power resulting from a relatively small sample size. At 3, 12 and 16°C, there was a CO_2-induced trend from right to left lateralization in *G. morhua*, with the opposite at 8°C, and we suggest this to be the main reason why the turning directions of *G. morhua* were not significantly different from a random binomial distribution at high CO_2. Inter-individual variability of behavioural lateralization is by definition very high, and one must thus be quite cautious with interpretation of these findings. Based on our results, the possibility of interactive effects on the behaviour of *G. morhua* should not be strictly ruled out. Furthermore, for changes in absolute and relative laterality, high inter-individual variability and low effect sizes may have given rise to potential type I errors, which must be considered when comparing differences in CO_2 effects between *B. saida* and *G. morhua*. A definitive answer to these issues requires further experimental investigation.

Domenici et al. (2014) found a CO_2-induced shift in turning preference from right to left, which was interpreted as a change in task processing from the left to the right brain hemisphere. Across taxa, the right brain hemisphere is associated with stress-related endocrine responses and reactive behavioural patterns (Rogers 2010). In humans, the right brain hemisphere is the predominant driver of the pituitary–adrenal axis and of sympathetic cardiac control (Wittling & Pflüger 1990, Wittling et al. 1998). In contrast, the left brain hemisphere is associated with the execution of routine behaviour (Rogers 2010). A shift in laterality from right to left preference under future OA scenarios as observed by Domenici et al. (2014) would thus indicate a shift in the stress-related cognitive state of the fish, i.e. a CO_2-induced shift from a low to a high stress level. Those explanations (inverted function or changing stress level) may not be mutually exclusive, as Hamilton et al. (2014) found a CO_2-induced increase of anxiety in rockfish, which could be an indication for a shift to a more active right brain hemisphere. In the study of Hamilton et al. (2014), the mentioned increase in anxiety was related to altered $GABA_A$-receptor functioning. Speculatively bringing these hypotheses together, an inversion of the $GABA_A$-receptor function could also be the cause of a shift in brain hemispherical usage which could then be responsible for the shift in side preference.

The question arises why the shift in side preference was opposite in *B. saida*. Hemispheric laterality is generated during ontogenesis and can be inversed, as shown in domestic chicken by Rogers (1990). This could also be the case for *B. saida*; thus, our findings might still have the same implications as in coral reef fishes. However, this explanation remains speculative, and its verification requires further exploration. The CO_2-induced reduction in absolute lateralization of *B. saida* may indicate reduced fitness under future OA scenarios, as the degree of lateralization may correlate with other behavioural parameters such as efficiency of predator avoidance (Dadda et al. 2010). However, predictions about the ecological consequences of our findings need to be made with care, as the animals in our study were kept separate from each other. Furthermore, the sudden availability of more space during laterality tests may have had an unknown effect on the observed outcome. It cannot be excluded that the fish may have behaved differently if they had been incubated under more natural conditions in schooling groups with social hierarchies. Nevertheless, both species were treated similarly and thus comparison of temperature and CO_2 effects between these species remains meaningful.

Overall, elevated CO_2 levels may affect some behavioural patterns of cold-adapted teleosts, but our findings also indicate species-specific differences in behavioural resilience to OA. Our results are similar

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to those obtained in 3 other studies on temperate fish species. A significant CO_2 effect on behaviour, including behavioural laterality and activity, was found in three-spined stickleback, but again, not in temperate Atlantic cod, indicating a reduced vulnerability of behaviour in this species to an increase in environmental CO_2 (Jutfelt et al. 2013, Jutfelt & Hedgärde 2013, 2015). This may be an adaptive trait reflecting its demersal mode of life and repeated exposure to hypoxia and hypercapnic water layers (Neuenfeldt et al. 2009). Due to preadaptation to different environments and levels of variability, the degree of alterations of behaviour under increased pCO_2 may vary strongly between species. The mechanisms causing the disturbance of behaviours may include accumulation of bicarbonate in the body fluids (Nilsson et al. 2012) which results from acid–base regulation compensating for CO_2-induced acidification (Ishimatsu et al. 2008). The physiological systems supporting behaviour to be insensitive to elevated CO_2 (and possibly, bicarbonate accumulation) remain to be investigated. Such understanding will be crucial for projecting teleost resilience under future CO_2 scenarios (Wittmann & Pörtner 2013). As discussed above, it may be possible that CO_2 effects on the behaviour of *G. morhua* are dependent on the environmental temperature. This would make Atlantic cod a useful species for elaboration of the physiological mechanisms determining behavioural vulnerability or resistance of fish species in a future, more acidified ocean.

In summary, this study indicates that the behaviour of *B. saida* is more vulnerable to future OA than the behaviour of *G. morhua*. We did not observe significant temperature-driven modulation in the extent of behavioural alteration; however, in *G. morhua*, interactive effects of temperature and CO_2 might have been missed due to the small size of treatment groups. Nevertheless, the temperature-independent reduction in the behavioural laterality of *B. saida* may indicate reduced fitness of this species in a high CO_2 world, which might place it at a disadvantage in competitive and predator–prey interactions with *G. morhua* in the waters around Svalbard. Future warming of the area can lead to an increasing population size of *G. morhua* and a further northward shift of species distribution areas (Perry et al. 2005). In a warmer, more acidified, open ocean, *G. morhua* may outcompete *B. saida* in the long term. However, the potential of species to acclimate or adapt their behaviour under combined OA and OW over generations has received little attention (Allan et al. 2014) and urgently demands further investigation.

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Declaration on the contribution of the candidate to a multi-author article/manuscript which is included as a chapter in the submitted doctoral thesis

Chapter: Publication I (Leo *et al.* 2017)

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 15%
Experimental work and/or acquisition of (experimental) data:	ca. 80%
Data analysis and interpretation:	ca. 90%
Preparation of Figures and Tables:	ca. 100%
Drafting of the manuscript:	ca. 100%

Chapter: Publication/Manuscript II (Leo *et al.* 2019)

Contribution of the candidate in % of the total work load (up to 100% for each of the following categories):

Experimental concept and design:	ca. 20%
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Data analysis and interpretation:	ca. 90%
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Drafting of the manuscript:	ca. 100%

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Data analysis and interpretation:	ca. 70%
Preparation of Figures and Tables:	ca. 100%
Drafting of the manuscript:	ca. 75%

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Experimental concept and design:	ca. 15%
Experimental work and/or acquisition of (experimental) data:	ca. 30%
Data analysis and interpretation:	ca. 30%
Preparation of Figures and Tables:	ca. 10%
Drafting of the manuscript:	ca. 30%

Trieste, den 30.032021
Signature:

Elettra Leo
Viale Raffaello Sanzio 13
34128 Trieste, Italien

Trieste, den 30.03.2021

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

(vom 14. März 2007)

Hiermit erkläre ich, Elettra Leo, dass ich die Arbeit mit dem Titel

Cellular metabolism of different life stages of marine teleosts during Ocean Acidification and Warming

1. Ohne unerlaubte fremde Hilfe angefertigt habe.
2. Keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.
3. Die in den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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Elettra Leo