Behavioural disturbances and underlying neurophysiological mechanisms during ocean acidification and warming in *Gadus morhua* and *Boreogadus saida*

Matthias Schmidt

ALFRED-WEGENER-INSTITUT
HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG

HELMHOLTZ GEMEINSCHAFT

POLMAR
Helmholtz Graduate School for Polar and Marine Research

Universität Bremen

The cover picture was printed with kind permission of Dr. Kerstin Bilgmann, Macquarie University Sydney, NSW, Australia.

This thesis was funded by the Federal Ministry of Education and Research (BMBF), embedded in the research program “Bioacid”.

Behavioural disturbances and underlying neurophysiological mechanisms during ocean acidification and warming in *Gadus morhua* and *Boreogadus saida*

Verhaltensstörungen und ihre neurophysiologischen Grundlagen während Ozeanversauerung und Erwärmung bei *Gadus morhua* und *Boreogadus saida*

Dissertation zur Erlangung des akademischen Grades

-Dr. rer. nat.-

Dem Fachbereich 2 Biologie/Chemie der Universität Bremen vorgelegt von

Matthias Schmidt, M. Sc.

Göttingen, März 2019

1. **Gutachter: Prof. Dr. Hans-Otto Pörtner**
   Alfred-Wegener-Institut, Helmholtz-Zentrum für Polar- und Meeresforschung
   Sektion Integrative Ökophysiologie
   Am Handelshafen 12, 27570 Bremerhaven
   Und Universität Bremen

2. **Gutachter: Prof. Dr. Michael Koch**
   Universität Bremen, Zentrum für Kognitive Wissenschaften, Institut für Hirnforschung
   Abteilung Neuropharmakologie
   Hochschulring 18, 28359 Bremen

Table of contents

Summary

Ocean acidification as projected for the end of the 21st century has the potential to cause behavioural alterations in fish with unclear consequences for affected species and ecosystems, both in the short and long term. Recent findings indicate that a change in functionality of γ-aminobutyric acid receptors type A ($GABA_A$-receptors) in the brain of fish due to acid-base regulatory processes may be the mechanism underpinning these behavioural disruptions. So far, studies have focused on the effects of CO_2 on tropical and temperate species with no information about the relevance of these observations for polar species. The role of environmental temperature for CO_2-induced behavioural changes is largely unknown, but highly important, as acidification and warming will occur simultaneously in marine ecosystems.

In this thesis, behavioural effects of future CO_2 conditions, the role of environmental temperature and the respective physiological background were analyzed in two cold water adapted fish species collected around Svalbard. Atlantic cod, *Gadus morhua*, is an invasive species currently shifting its distribution northward into colder waters where Polar cod, *Boreogadus saida*, is a native key species in the local food web. Shifting predator prey interactions and the differing potential of species to acclimate and adapt to future temperature and CO_2 conditions will shape the future abundance of each species with concomitant impacts on the polar ecosystem. In manuscript I it is shown that the behaviour of *B. saida* is more sensitive to future environmental CO_2 conditions than the behaviour of *G. morhua*. Nevertheless, the potential for behavioural resilience of *G. morhua* under high CO_2 conditions may be dependent on the experienced environmental temperature and greatest under optimum temperature conditions. In manuscript II, metabolic changes are illustrated, which indicate CO_2-dependent energy limitation in the brain of *B. saida* at 8 °C, but not in *G. morhua*. However, in *G. morhua*, temperature and CO_2-dependent alterations in GABA-metabolism were found potentially increasing this species' behavioural resistance against higher environmental CO_2 partial pressures. In manuscript III, maintenance of intracellular pH in the brain of acutely CO_2-exposed *B. saida* indicating sufficient acid-base regulatory processes is reported. However, long-lasting effects of CO_2 on blood circulation were also observed with unclear relevance for the fitness of this species under expected ocean acidification scenarios. The results of this thesis indicate that *G. morhua* will be more capable to survive in warmer, more acidified waters

around Svalbard than *B. saida*. Furthermore, the potential interplay between behaviour, GABA-metabolism and acid-base physiology with respect to their contribution to metabolic and behavioural resistance against environmental hypercapnia is discussed.

Zusammenfassung

Für das Ende des 21. Jahrhunderts prognostizierte Ozeanversauerung hat das Potential zu Verhaltensänderungen bei Fischen mit unklaren kurz- und langfristigen Konsequenzen für die betroffenen Arten und deren Ökosysteme zu führen. Bisher haben sich Studien vor allem auf CO_2-Effekte bei Spezies aus den Tropen und gemäßigten Breiten fokussiert, allerdings gibt es noch keine Erkenntnisse über die Relevanz dieser Beobachtungen für polare Arten. Aktuelle Ergebnisse legen nahe, dass eine geänderte Funktionalität der γ-Aminobuttersäure Rezeptoren Typ A ($GABA_A$-Rezeptoren) im Gehirn von Fischen durch Mechanismen der Säure-Base Regulation für diese Verhaltensänderungen verantwortlich ist. In Bezug auf die CO_2-induzierten Verhaltensstörungen ist die Rolle der Umgebungstemperatur noch nahezu unbekannt, allerdings von eminenter Wichtigkeit, da eine Versauerung mariner Ökosysteme parallel zu deren Erwärmung stattfindet.

In dieser Dissertation wurden die Effekte zukünftiger CO_2-Bedingungen und der Umgebungstemperatur auf das Verhalten zweier kaltadaptierter Fischarten von Svalbard bestimmt und deren physiologische Mechanismen analysiert. Atlantischer Kabeljau, *Gadus morhua*, ist eine invasive Art, welche momentan ihre Verbreitung nordwärts in kältere Gewässer ausweitet, in denen der Polardorsch, *Boreogadus saida*, als native Schlüsselspezies im lokalen Nahrungsnetz fungiert. Räuber-Beute Interaktionen und Spezies-spezifisches Akklimatisations- und Adaptations-Potential an zukünftige Temperatur- und CO_2-Bedingungen werden die zukünftige Verbreitung dieser Arten beeinflussen, was auch Konsequenzen für das polare Ökosystem erwarten lässt. Die Ergebnisse in Manuskript I zeigen, dass das Verhalten von *B. saida* stärker von Änderungen der CO_2-Konzentration in der Umgebung beeinflusst wird als das Verhalten von *G. morhua*. Diese Widerstandsfähigkeit von *G. morhua* könnte allerdings von der jeweils erfahrenen Umgebungstemperatur abhängen und unter optimalen Temperaturbedingungen am größten sein. In Manuskript II wird über Zeichen für eine CO_2-abhängige Energielimitation im Metabolismus des Gehirns von *B. saida* bei 8 °C berichtet, die bei *G. morhua* nicht auftrat. Allerdings wurde in *G. morhua* eine Temperatur- und CO_2-abhängige Änderung des GABA-Stoffwechsels beobachtet, welche zur Erhöhung der Widerstandsfähigkeit des Verhaltens dieser Spezies gegenüber erhöhtem CO_2 Partialdruck in der Umgebung beitragen könnte. Wie in Manuskript III beschrieben wurde eine Aufrechterhaltung

des intrazellulären pH-Werts im Gehirn von akut CO_2-exponiertem *B. saida* detektiert, was auf eine ausreichend effektive Säure-Base Regulation im Gehirn hinweist. Allerdings wurden zusätzlich auch lang anhaltende Effekte von CO_2 auf die Blutzirkulation dieser Spezies beobachtet. Diese haben eine bisher ungeklärte Relevanz für die Fitness von *B. saida* unter erwarteten Szenarien der Ozeanversauerung. Die Ergebnisse dieser Dissertation legen nahe, dass *G. morhua* einen Vorteil in wärmeren, saureren Gewässern um Svalbard gegenüber *B. saida* hat. Zusätzlich wird der potentielle Zusammenhang zwischen Verhalten, GABA-Stoffwechsel und Säure-Base Regulation in Bezug auf ihren Beitrag zur Resilienz von Stoffwechsel und Verhalten gegen hyperkapnische Umgebungsbedingungen diskutiert.

List of Figures

Manuscript III

List of Tables

Manuscript II

Abbreviations

Accho	Acetylcholine
Ace	Acetate
Ace-coA	Acetyl-coA
AcHis	Acetyl-histidine
AE	Anion exchanger
A-KG	Alpha-ketoglutaric acid
Ala	Alanine
ANOVA	Analysis of variance
Asp	Aspartate
atm	Atmosphere
BA	Benzylamine
Cho	Choline
CPMG	Carr-Purcell-Meiboom-Gill
Cytidylpcho	Cytidylphosphocholine
CV	Coefficient of variation
d	Days
DIC	Dissolved inorganic carbon
D_2O	Deuteriumoxide
DOPAC	3,4-Dihydroxyphenylacetic acid
DPE	Diphenylethylenediamine
e	Extracellular
E_{GABA}	Reversal potential for ion flow through the $GABA_A$-receptor
ESM	Electronic supplementary material
F	Faraday constant
Fig	Figure
FOV	Field of view
GABA	γ-aminobutyric acid
$GABA_A$-receptor	γ-aminobutyric acid receptor
Glc	Glucose
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
Gpcho	Glycerophosphocholine
G-Test	Goodness of fit test
^1H	Hydrogen with one nucleon
h	Hours
HIAA	5-Hydroxyindoleacetic acid
HRMAS	High resolution magnetic angle spinning
HSD	Honestly significant difference

5-HT	5-Hydroxytryptamine (Serotonin)
Hz	Hertz
i	Intracellular
Imax	Maximum electric current
JRES	J-resolved
HPLC	High performance liquid chromatography
IPCC	International Panel on Climate Change
KCC2	K^+/Cl^- cotransporter
Lac	Lactate
L-DOPA	L-3,4-Dihydroxyphenylalanine
M	Mol
MDS	Multidimensional scaling
MI	Myo-inositol
min	Minutes
MRI	Magnetic resonance imaging
MS222	Tricaine methanesulfonate
n	Group size
NA	Number of averages
NAA	N-acetylaspartate
NBC	Na^+/HCO_3^- cotransporter
NCBT	Na^+ coupled HCO_3^- transporter
NKCC1	$Na^+/K^+/Cl^-$ cotransporter
NMR	Nuclear magnetic resonance
OA	Ocean acidification
OAA	Oxaloacetic acid
OCLTT	Oxygen- and capacity- limited thermal tolerance
OW	Ocean warming
OWA	Ocean warming and acidification
^{31}P	Phosphorus with 31 nucleons
P	Relative permeability of a specific ion
Pcho	Phosphocholine
Pco_2	CO_2 partial pressure
ppm	Parts per million
Put	Putrescine
Pyr	Pyruvate
R	Universal gas constant
RCP	Representative concentration pathway
ROI	Region of interest
SD	Standard deviation
s.e.m.	Standard error of the mean
SEM	Standard error of the mean (Manuscript 1)
Suc	Succinate

T	Temperature; In the context of magnetic resonance: Tesla
Tau	Taurine
TCA	Tricarbonic acid
tCr	Total creatine
TE	Echo time
TMAO	Trimethylamine-N-oxide
TR	Repetition time
TSP	Trimethylilylproprionate
Umax	Maximum voltage
V_m	Membrane potential
wk	Weeks

1. Introduction

1.1 Climate change in the marine sub-Arctic and Arctic environment around Svalbard

Since the beginning of the utilization of fossil fuels during the industrial revolution has the human species continuously led to an increase of carbon dioxide (CO_2) levels in the earth's atmosphere. CO_2 acts as a greenhouse gas leading to a rise of environmental temperature; however, around one third of the emitted CO_2 gets absorbed by oceans forming carbonic acid, causing an overall increase in proton activity and thus reduction of pH, which was termed Ocean acidification (OA) (2, 3). The Arctic, which the International Panel on Climate Change (IPCC) refers to as those regions above the 66th latitude (4), is expected to experience a 2.2-2.4 fold stronger warming until the years 2081-2100, in comparison to 1986-2005, than projected for the global average temperature increase, which is known as polar amplification (5). Simultaneously, the Arctic Ocean is projected to experience stronger acidification than other oceans due to increasing freshwater inflow through precipitation and sea ice loss as well as higher air-sea CO_2 fluxes (5). The distribution of ectothermic fish species has been shown to be mainly driven by environmental temperature and each species possesses a specific temperature window which may differ between different development stages (6-9). The temperature window of ectothermic species is according to the oxygen- and capacity- limited thermal tolerance (OCLTT) hypothesis defined by the individuals` potential to maintain oxygen availability in vital organs (10). In addition, environmental drivers such as hypoxia and $CO_2$ concentration may lead to narrowing of the thermal window (10). The rising temperature in the worlds` oceans has been observed to induce a poleward shift of species distributions altering the composition and structure of the respective ecosystems (11). The effects of climate change on species distributions are also visible around the Svalbard archipelago, the study site chosen for this dissertation. Being located between the 74th and 81st Northern latitude, it is surrounded by the Arctic Ocean, Barents Sea, Greenland Sea and Norwegian Sea. Currently, the water temperature around Svalbard ranges between -1.8 °C and 8 °C, with a temperature increase of 2.5 °C being projected until the year 2100 according to the Representative Concentration Pathway 8.5 (5, 12). Descamps *et al.* recently reviewed studies on the observable effects of climate change on marine and terrestrial species distributions and compositions around

Svalbard concluding that several marine bivalve and fish species emerged in the waters around the archipelago accompanied by a reduction in abundance of native species with visible effects on food webs (13).

1.2 Competition between Polar cod and invasive Atlantic cod

One of the fish species which is becoming more abundant around Svalbard as the ocean temperature rises is the temperate Atlantic cod (*Gadus morhua*) which has shifted its habitat further northwards with continuous warming where it temporarily inhabits the waters around Svalbard (13). In this area, the distribution of Atlantic cod overlaps with the distribution of Polar cod (*Boreogadus saida*) with potential for direct and indirect competitive interactions (14). While competition of these species for prey is considered to be rather minor, there is an unclear potential for predator-prey interaction of *G. morhua* on *B. saida* which might negatively affect the population size of *B. saida* (14). As *B. saida* is recognized as one of the key species in the northern polar seas, alteration in habitat and population size may severely change the current marine as well as dependent terrestrial trophic networks in the respective ecosystems (13) with indirect socioeconomic consequences (15-17). The abundance of *G. morhua* around Svalbard is of direct commercial interest as this species is heavily exploited by fisheries (18). However, the fate of these two species in the waters around Svalbard might not only be dependent on environmental temperature and direct species interaction. While environmental temperature is currently considered to be the main driver for species distribution in the discussion of climate change effects on marine ecosystems (9), new research indicates that ocean acidification might act as a separate driver on the fitness of fish species, which is further discussed in 1.3.

1.3 Ocean acidification, fish behaviour and the $GABA_A$-receptor model

Effects of CO_2 on marine fauna were originally considered to be small compared to temperature effects and mostly relevant for calcifying invertebrate species that are demanding the CO_2-dependent saturation of aragonite in sea water for shell development (19). In contrast, highly active ectothermic vertebrate species were considered to be resilient against future CO_2 conditions since their acid-base regulatory processes were shown to be more efficient than in

invertebrate species (20). Surprisingly, recent studies suggest that exactly these efficient acid-base regulatory processes in fish may be disadvantageous under projected CO_2 scenarios. In 2009, Munday *et al.* demonstrated that exposure of the tropical clownfish *Amphiprion percula* to future CO_2 levels for several days leads to behavioural disturbances that can be attributed to impairment in the olfactory system without anatomic alteration of the nasal cavity (21), and they projected detrimental consequences of elevated CO_2-partial pressures (Pco_2) for larvae settlement and predator-prey interactions. In the subsequent years, a growing body of literature confirmed these results and identified that in fish different sensory systems and types of behaviour can be affected by an increase of environmental CO_2. Furthermore, fish species from tropical and temperate environments were screened for CO_2-induced behavioural impairments. While behavioural alterations in fish are wide spread under increased environmental CO_2 partial pressure, there are species-specific differences in the severity of behavioural vulnerability to CO_2 and some temperate species even appear to be resistant to changes in environmental CO_2 (22-24). In order to explain the behavioural differences observed in fish under high CO_2 conditions, Nilsson developed a model on how increases of environmental CO_2 might influence the central nervous system of affected animals (25). The model relates to observations on the functionality of γ-aminobutyric acid receptors type A ($GABA_A$-receptors) in mammals which depends on intra- and extracellular bicarbonate and chloride concentrations. In fish are the concentrations of these ions influenced by the respective acid-base regulatory processes which are used in order to maintain intra- and extracellular pH values at different environmental Pco_2 levels. In the synaptic cleft of neurons, the membrane-bound $GABA_A$-receptor possesses largely inhibitory function via a GABA-dependent net inflow of chloride and bicarbonate ions into the respective neuron. In fish is this net ion current dominated by the inflow of chloride into the cell as the $GABA_A$-receptor of teleosts is approximately 2.3-3 times more permeable for chloride than for bicarbonate ions (26). This net inflow of negatively charged ions into the cell leads to an inhibitory postsynaptic potential which causes a lowering of the membrane potential and therefore reduced excitability of the neuron through excitatory potentials from other presynaptic neurons. The directionality of the net ion flow through the $GABA_A$-receptor is defined through the electrochemical potential V_m inside a neuron (Formula 1) (27) and the electrochemical reversal potential E_{GABA} for this receptor

(Formula 2) (26). The electrochemical potential reflects differences of ion concentrations and their respective electrical charge over a semipermeable membrane and can be calculated through the Goldman equation, a generalization of the Nernst equation. R is defined as the universal gas constant (8.315 J mol^{-1} K^{-1}), T being the temperature in Kelvin, F the Faraday constant (96,485 Coulombs mol^{-1}), *P* the permeability coefficient of a respective ion and in brackets the extracellular ($[\ldots]_e$) and intracellular ($[\ldots]_i$) concentration of each ion.

$$V_M = \frac{RT}{F} ln \frac{P_K[K^+]_e + P_{Na}[Na^+]_e + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_e} \qquad (1)$$

The reversal potential mirrors the ion flux through an ion-channel within the membrane. The selectiveness of a respective channel is reflected in the specific permeability coefficient for each ion. A reversal potential below V_m results in a hyperpolarizing net anion flux into the cell, a reversal potential above V_m results in an excitatory net anion flux out of the cell.

$$E_{GABA} = \frac{RT}{F} ln \frac{P_{Cl}[Cl^-]_i + P_{HCO_3}[HCO_3{}^-]_i}{P_{Cl}[Cl^-]_e + P_{HCO_3}[HCO_3{}^-]_e} \qquad (2)$$

In the model of Nilsson (25) it is suggested that E_{GABA} is in fish under current-day CO_2 conditions well below the neuronal resting potential of -70 mV. However, at least under strongly increased CO_2 partial pressure, fish are assumed to increase the extracellular and intracellular bicarbonate concentration and simultaneously reduce the extracellular chloride concentration at a ratio of 2:1 to 1:1 (bicarbonate/chloride) in order to avoid acidification of body fluids and it is proposed that such a change might also occur under a projected Pco_2 of 1,000 µatm (27-29). This shift in ion composition may lead to an increase of E_{GABA} above the neuronal resting membrane potential, which has recently been verified theoretically by Regan *et al.* (30) and Tresguerres *et al.* (27). This may cause a net outflow of bicarbonate and chloride out of the cell meaning that opening of the $GABA_A$-receptor would result in an excitatory rather than an inhibitory postsynaptic potential (Figure 1). It is important to keep in mind that E_{GABA} represents the combined reversal potential of chloride and bicarbonate depending on their relative permeability through the $GABA_A$-receptor. The equilibrium potential of bicarbonate alone is much more positive than the neuronal membrane potential (31). This means that opening of the $GABA_A$-receptor even for an inhibitory net ion current still results in an outflow of

bicarbonate, but with a stronger simultaneous inflow of chloride generating a net hyperpolarizing anion current into the cell (31). This illustrates the importance of the relative permeability ratios for chloride and bicarbonate with respect to possible acclimation and adaptation processes as discussed later in 3.2. This relationship between the intra- and extracellular ionic composition, the permeability of the $GABA_A$-receptors for these ions and E_{GABA} is visualized in Figure 2.

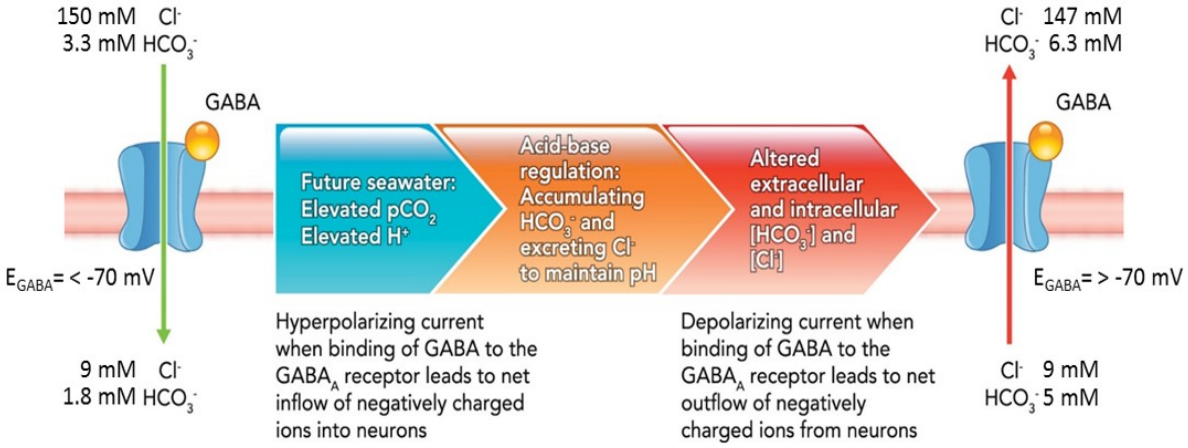


Figure 1: A: $GABA_A$-receptor model for an explanation of the neurophysiological basis of hypercapnia-induced behavioural changes as published by Nilsson *et al.* (32) adjusted after Regan *et al.* (30). Binding of GABA to the $GABA_A$-receptor of the postsynaptic neuron leads to a net inflow of negative ions and hyperpolarization under normocapnic conditions (left). Under increased CO_2 (right), bicarbonate may be accumulated extracellularly in exchange for chloride. While bicarbonate accumulates also intracellularly, the intracellular chloride concentrations are believed to remain unaltered in hypercapnia intolerant species (30). These changes of extra- and intracellular ion concentrations cause an increase of the reversal potential E_{GABA} of the $GABA_A$-receptor with subsequent net outflow of chloride and bicarbonate and thus depolarization after binding of GABA. The displayed ion concentrations of bicarbonate and chloride represent a hypercapnia-intolerant coral reef fish species exchanging extracellular chloride and extracellular bicarbonate in a 1:1 ratio. With an assumed neuronal membrane potential of -70 mV, the activated $GABA_A$-receptor acts inhibitory upon an E_{GABA} of < -70 mV and excitatory upon an E_{GABA} of > -70 mV. Further information for the calculation of E_{GABA} is provided in the text. Only combined net currents of chloride and bicarbonate are shown. Printed with permission of the American Physiological Society.

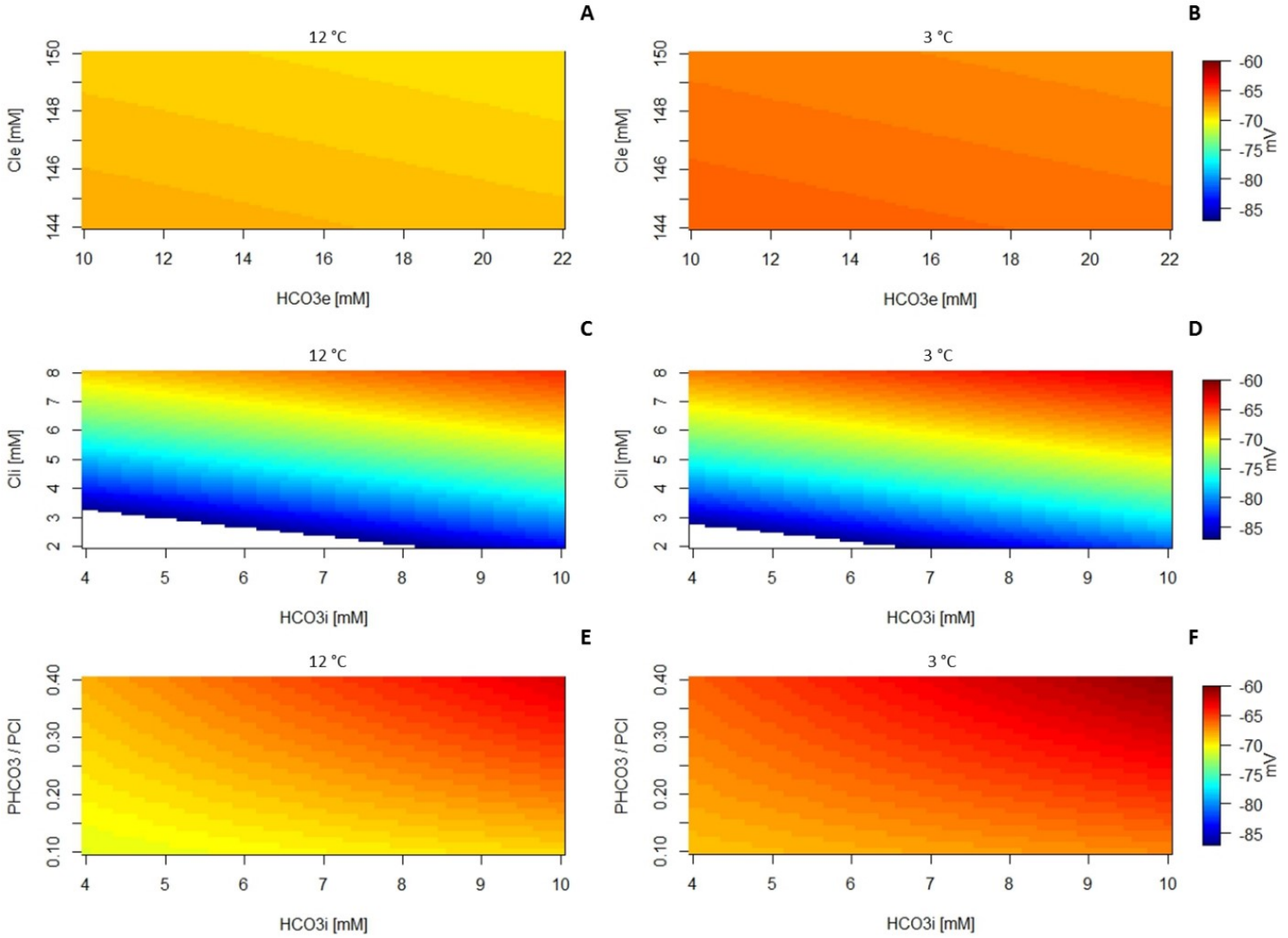


Figure 2: Colors depict the reversal potential of the $GABA_A$-receptor (E_{GABA}) in the brain of a hypothetical Atlantic cod calculated via Formula 2 in dependence of extra- and intracellular bicarbonate (HCO3e and HCO3i) and chloride concentrations (Cle and Cli) as well as the permeability of the $GABA_A$-receptor for bicarbonate relative to chloride (PHCO3 / PCl) at temperatures of 12 °C and 3 °C. Only reversal potentials between -87 mV and -60 mV are displayed.

For the calculation of E_{GABA} in Figure 2 A and B, the intracellular bicarbonate concentration was set to 4 mM, which was determined by Larsen *et al.* in the white muscle of Atlantic cod (33). Utilization of the intracellular bicarbonate concentration of the white muscle for the calculation of E_{GABA} was conducted, as measurements of intracellular bicarbonate in white muscle are suggested to represent the bicarbonate concentration in nervous tissue of the same species (26). The intracellular chloride concentration was set to 8 mM equally to Regan *et al.* (30) for the hypercapnia-tolerant *Pangasianodon hypophthalmus*. The relative permeability of bicarbonate to chloride was set to 0.3 similar to Heuer *et al*. (26) and Regan *et al.* (30).

For the calculation of E_{GABA} in Figure 2 C and D, the extracellular bicarbonate and chloride concentrations were derived from Larsen e*t al.* (33), with extracellular bicarbonate being set to 10 mM and extracellular chloride to 150 mM. The relative permeability of bicarbonate to chloride was set to 0.3 similar to Heuer *et al*. (26) and Regan *et al.* (30).

For the calculation of E_{GABA} in Figure 2 E and F, the intracellular chloride concentration was set to 8 mM equally to Regan *et al.* (30). The extracellular bicarbonate and chloride concentrations were derived from Larsen e*t al.* (33), with extracellular bicarbonate being set to 10 mM and extracellular chloride to 150 mM.

The plots were generated with R (Version 1.1442) and the package "fields". The respective code is listed in the Appendix (6.5).

Intuitively, the model of Nilsson elegantly explains the physiological mechanism of behavioural effects observed in fish under hypercapnic conditions. However, the $GABA_A$-receptor model has only been tested indirectly through treatment of fish under hypercapnic conditions with the $GABA_A$-receptor antagonist Gabazine and the $GABA_A$-receptor agonist Muscimol which led either to a rapid regeneration of different CO_2-impaired types of behaviour (Gabazine, as reviewed by Tresguerres *et al.* (27)) or to a more severe alteration of behaviour under high Pco_2 (Muscimol (34)). Direct measurements of ion-currents under realistic normo- and hypercapnic conditions are still missing just as sufficient data on how some fish species are able to keep up routine behaviour even under projected high CO_2 conditions. First studies indicate that CO_2-dependent changes of the GABA metabolism in the brain of fish may shape an animals` vulnerability to future ocean acidification scenarios (this topic is extensively discussed in 3.1). A deeper understanding on how some fish species are able to maintain their routine behaviour even under high CO_2 conditions while other species struggle will be a valuable factor for the projection of future climate change scenarios on fish species and their ecosystems. Furthermore, the role of the environmental temperature with respect to CO_2-induced behavioural changes is so far poorly understood and requires further investigation (35, 36). Before this thesis was conducted, behavioural studies on CO_2-effects on fish focused mostly on tropical and some temperature species (32), but no information was available on CO_2-induced behavioural effects in fish from polar environments. This gap of knowledge is problematic, since particularly Arctic fish species, as introduced in 1.1, will experience both more severe ocean acidification and ocean warming than fish species from warmer environments (5).

1.4 Thesis objectives

This thesis gathered first data on whether projected ocean acidification scenarios have the potential to alter the behaviour of fish species from polar environments in a similar way as the beahviour of fish species from tropical environments. In addition, the influence of the environmental temperature was investigated. Metabolic adjustments that may form the physiological basis of resistance to high CO_2 conditions, in particular with respect to the GABA-metabolism, were analyzed. In detail, the aims of this thesis were as follows:

1. Investigation of the vulnerability of the behaviour of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*) to projected CO_2 conditions and determination whether environmental temperature acts as a driver in the severity of this vulnerability (Manuscript I). Here it was focused on two types of behaviour, behavioural laterality and routine activity. Behavioural laterality i.e. the preference for conductance of specific behavioural tasks with one preferred side of the body, is an easy to assess proxy for the utilization of the respective specialized brain hemisphere and thus the neuronal networks optimized for the conductance of this task. Behavioural laterality has frequently been measured in ocean acidification research and has been shown to be disrupted by CO_2 in some fish species (37, 38). Routine activity levels can be affected by a variety of inner states such as hunger or anxiety and were altered as well by CO_2 in several fish species (36, 39-42).
2. Subsequent analysis of metabolites involved in GABAergic and serotonergic neurotransmitter systems in the brains of *B. saida* and *G. morhua* as well as quantification of osmolytes and energy metabolites through ^{1}H nuclear magnetic resonance (NMR)-spectroscopy and high performance liquid chromatography (HPLC). These measurements were performed with the brains of those individuals that underwent the prior behavioural testing mentioned above in order to target possible regulatory mechanisms in the central nervous system that might increase behavioural resilience in fish. A possible mediation of these mechanisms through environmental temperature was analyzed (Manuscript II).

3. Observation of physiological parameters involved in acid-base regulation in *B. saida* including intracellular pH, ventilation rate and blood velocity, in order to identify possible shortcomings in the capacity of this species to cope with increased CO_2 conditions. These investigations were conducted through *in vivo* magnetic resonance imaging (MRI), and *in vivo* ^{31}P-NMR spectroscopy (Manuscript III).

Figure 3 shows a map of Svalbard with the locations of sampling sites where specimens of *B. saida* and *G. morhua* were collected. The results obtained as part of this thesis contributed to Consortium 4 of Bioacid II, a German framework to investigate effects of ocean acidification on biological systems. The data add to other studies investigating interactions between different life stages of *B. saida* and *G. morhua* under future temperature and CO_2 conditions in order to project climate change induced ecological changes in the polar seas together with resulting socioeconomic challenges. Further information on Consortium 4 and the other consortia of the Bioacid project is available under https://www.oceanacidification.de/scientific-programme-bioacid-ii/?lang=en.

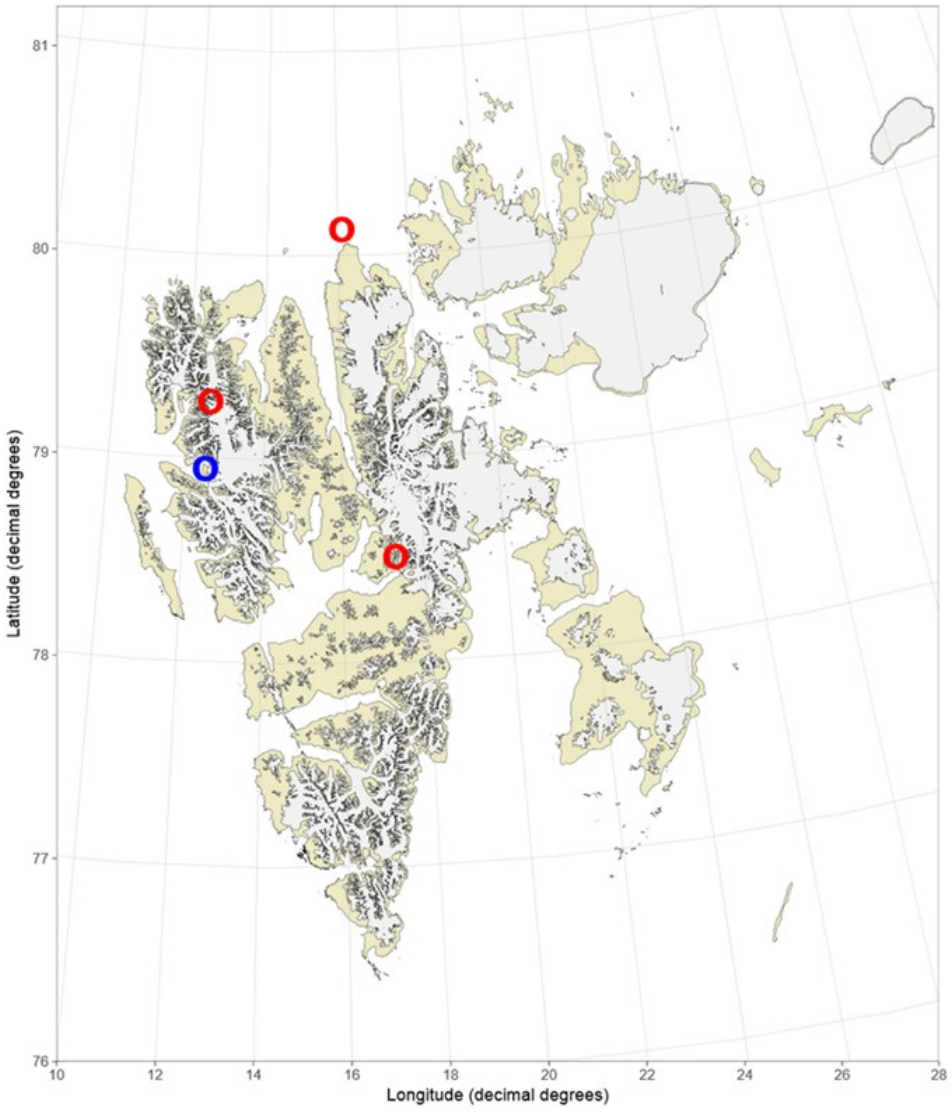


Figure 3: Map of Svalbard indicating the sampling sites of Polar cod *Boreogadus saida* (blue circle) and Atlantic cod *Gadus morhua* (red circles) which were used for this thesis. The map was created using the R-package "PlotSvalbard". Geographic material for this map was acquired from the Basemap provided by the Norwegian Polar Institute (http://geodata.npolar.no/) (1). The R-code for Figure 3 is provided in 6.5.

2. Manuscripts

Three manuscripts were written as part of this thesis. Title, publication status and contributions to the respective manuscripts are listed below followed by abstracts or prints of each manuscript.

2.1 Manuscript outline

Manuscript I

Authors:

Matthias Schmidt, Gabriele Gerlach, Elettra Leo, Kristina Lore Kunz, Steffen Swoboda, Hans-Otto Pörtner, Christian Bock, Daniela Storch

Title:

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

Status:

Published in Marine Ecology Progress Series, 571, 183-191

Doi: 10.3354/meps12130

Contributions:

MS, KK, EL, HP, CB and DS designed the study. KK, EL and MS conducted the incubation of the animals. MS carried out the experiments. Data analysis was done by MS together with SS with participation of DS and GG. MS performed the statistical analysis with contribution of GG. All authors contributed to the interpretation of the results. MS wrote the manuscript which was drafted by KK, EL, SS, GG, HP, CB, and DS. All authors gave final approval for publication.

Manuscript II

Authors:

Matthias Schmidt, Heidrun Sigrid Windisch, Kai-Uwe Ludwichowski, Sean Lando Levin Seegert, Hans-Otto Pörtner, Daniela Storch and Christian Bock

Title:

Differences in neurochemical profiles of two gadid species under ocean warming and acidification.

Status:

Published in *Frontiers in Zoology* (2017) 14:49

Doi: 10.1186/s12983-017-0238-5

Contributions:

MS, HP, HW, CB and DS developed the study design. MS conducted the incubation of the animals. Brains were sampled by MS and HW. SS conducted the NMR measurements with assistance of CB and MS. MS did the HPLC measurements with support of KL. Data analysis and statistical evaluation were performed by MS. All authors contributed to the interpretation of the results. MS drafted the manuscript which was subsequently edited by SS, KL, HW, HP, CB, and DS.

Manuscript III

Authors:

Matthias Schmidt, Hans-Otto Pörtner, Daniela Storch and Christian Bock

Title:

Adjustments of the cardiovascular and acid-base system of Polar cod, *Boreogadus saida*, under elevated CO_2 - An *in vivo* magnetic resonance study.

Status:

Manuscript

Contributions:

MS and CB designed the experiment. MS conducted the incubations and measurements. Data analysis and statistical evaluation were performed by MS. All authors contributed to the interpretation of the results. MS wrote the manuscript which was subsequently drafted by CB, DS and HP. All authors gave final approval for publication.

2.2 Manuscript I

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]

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

Published in Marine Ecology Progress Series, 571, 183-191
Doi: 10.3354/meps12130

[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

Vol. 571: 183–191, 2017
https://doi.org/10.3354/meps12130

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 17

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

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

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 (α = 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).

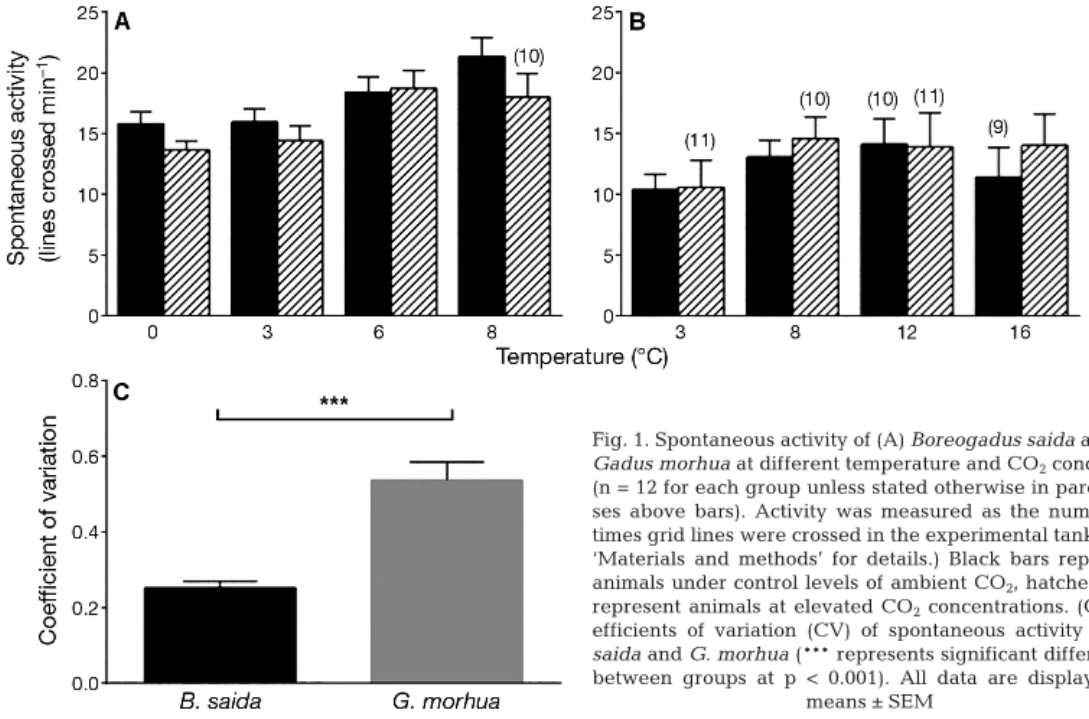


Fig. 1. Spontaneous activity of (A) *Boreogadus saida* and (B) *Gadus morhua* at different temperature and CO_2 conditions (n = 12 for each group unless stated otherwise in parentheses above bars). Activity was measured as the number of times grid lines were crossed in the experimental tanks (see 'Materials 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

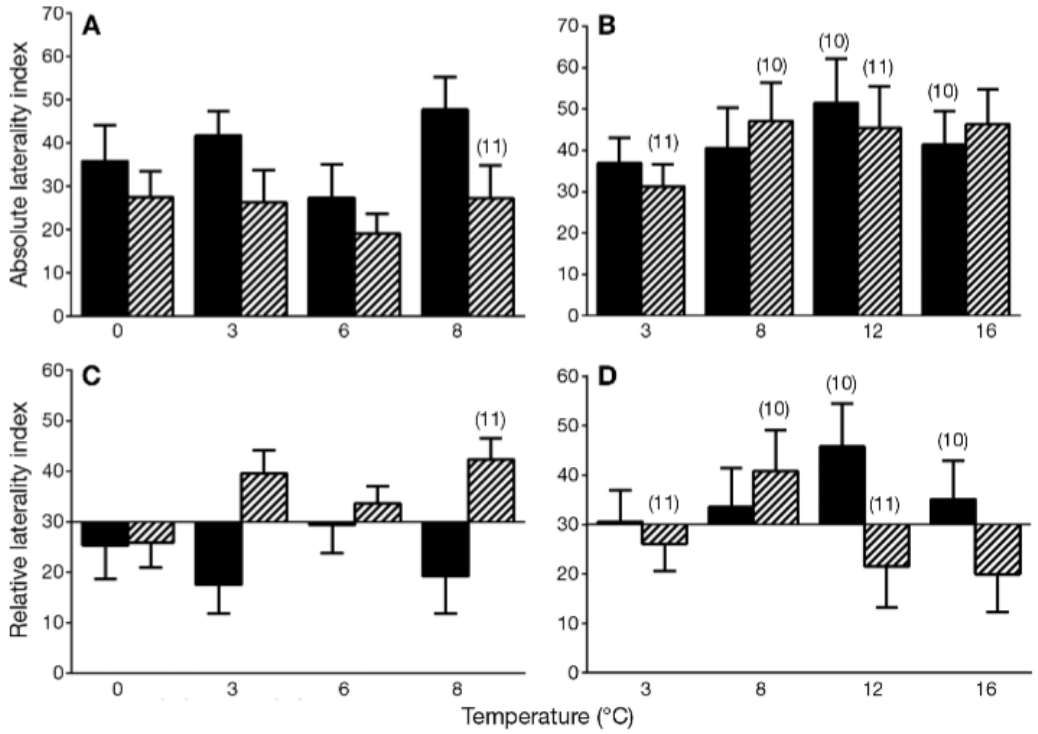


Fig. 2. Absolute laterality index of (A) *Boreogadus saida* and (B) *Gadus morhua* and relative laterality index of (C) *B. saida* and (D) *G. morhua* at different temperature and CO_2 conditions (n = 12 for each group unless stated otherwise in parentheses above bars). Details of laterality indices 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

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.

Acknowledgements. We thank Jasmine Nahrgang for providing the specimens of *B. saida* (funded by the Polarisation grant of the Norwegian Research Council, no. 214184/F20), Janina Popp for her contribution to data analysis and Philip Munday for helpful suggestions for the experimental design. Silvia Hardenberg, Sebastian Berger and Guido Krieten helped with the technical realization of the incubation setup and during the incubation period. We also thank Karim Zanaty, Marcel Machnik, Benjamin Matthei, Fredy Vèliz Moraleda, Anette Tillmann, Isabel Ketelsen and Timo Hirse for their contributions to the measurements of pH and dissolved inorganic carbon. We appreciate the helpful statistical advice of Stephan Frickenhaus and thank Nils Koschnick and Heidrun Windisch for various services they provided throughout the incubation period. This work was funded by the Federal Ministry of Education and Research (BMBF, no. FKZ 03F0655B), Germany. The experiments were conducted in accordance with the ethical standards of the federal state of Bremen, Germany, and were approved under reference number 522-27-11/02-00 (93). The tested animals were used in subsequent experiments that will be published elsewhere and were ultimately sacrificed for organ removal.

LITERATURE CITED

Allan BJM, Miller GM, McCormick MI, Domenici P, Munday PL (2014) Parental effects improve escape performance of juvenile reef fish in a high-CO_2 world. Proc R Soc Lond B Biol Sci 281:20132179

Beierlein L, Salvigsen O, Schöne BR, Mackensen A, Brey T (2015) The seasonal water temperature cycle in the Arctic Dicksonfjord (Svalbard) during the Holocene Climate Optimum derived from sub-fossil *Arctica islandica* shells. Holocene 25:1197–1207

Bignami S, Sponaugle S, Cowen RK (2013) Response to ocean acidification in larvae of a large tropical marine fish, *Rachycentron canadum*. Glob Change Biol 19:996–1006

Bignami S, Sponaugle S, Cowen RK (2014) Effects of ocean acidification on the larvae of a high-value pelagic fisheries species, mahi-mahi *Coryphaena hippurus*. Aquat Biol 21:249–260

Cripps IL, Munday PL, McCormick MI (2011) Ocean acidification affects prey detection by a predatory reef fish. PLOS ONE 6:e22736

Dadda M, Zandonà E, Agrillo C, Bisazza A (2009) The costs of hemispheric specialization in a fish. Proc R Soc Lond B Biol Sci 276:4399–4407

Dadda M, Koolhaas WH, Domenici P (2010) Behavioural asymmetry affects escape performance in a teleost fish. Biol Lett 6:414–417

Domenici P, Allan B, McCormick MI, Munday PL (2012) Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. Biol Lett 8:78–81

Domenici P, Allan BJM, Watson SA, McCormick MI, Munday PL (2014) Shifting from right to left: the combined effect of elevated CO_2 and temperature on behavioural lateralization in a coral reef fish. PLOS ONE 9:e87969

Drinkwater K (2009) Comparison of the response of Atlantic cod (*Gadus morhua*) in the high-latitude regions of the North Atlantic during the warm periods of the 1920s-1960s and the 1990s-2000s. Deep-Sea Res II 56:2087–2096

Ferrari MC, Munday PL, Rummer JL, McCormick MI and others (2015) Interactive effects of ocean acidification and rising sea temperatures alter predation rate and predator

selectivity in reef fish communities. Glob Change Biol 21: 1848–1855

Hamilton TJ, Holcombe A, Tresguerres M (2014) CO_2-induced ocean acidification increases anxiety in rockfish via alteration of $GABA_A$ receptor functioning. Proc R Soc Lond B Biol Sci 281:20132509

Heuer RM, Grosell M (2014) Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am J Physiol Regul Integr Comp Physiol 307:R1061–R1084

Holst JC, McDonald A (2000) FISH-LIFT: a device for sampling live fish with trawls. Fish Res 48:87–91

Hop H, Gjøsæter H (2013) Polar cod (*Boreogadus saida*) and capelin (*Mallotus villosus*) as key species in marine food webs of the Arctic and the Barents Sea. Mar Biol Res 9: 878–894

IPCC (2013) Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge and New York, NY

Ishimatsu A, Hayashi M, Kikkawa T (2008) Fishes in high-CO_2 acidified oceans. Mar Ecol Prog Ser 373:295–302

Jutfelt F, Hedgärde M (2013) Atlantic cod actively avoid CO_2 and predator odour, even after long-term CO_2 exposure. Front Zool 10:81

Jutfelt F, Hedgärde M (2015) Juvenile Atlantic cod behavior appears robust to near-future CO_2 levels. Front Zool 12: 11

Jutfelt F, Bresolin de Souza K, Vuylsteke A, Sturve J (2013) Behavioural disturbances in a temperate fish exposed to sustained high-CO_2 levels. PLOS ONE 8:e65825

Kunz KL, Frickenhaus S, Hardenberg S, Johansen T and others (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 Biol 39:1137–1153

Lai F, Jutfelt F, Nilsson GE (2015) Altered neurotransmitter function in CO_2-exposed stickleback (*Gasterosteus aculeatus*): a temperate model species for ocean acidification research. Conserv Physiol 3:cov018

Maneja RH, Frommel AY, Browman HI, Clemmesen C and others (2013) The swimming kinematics of larval Atlantic cod, *Gadus morhua* L., are resilient to elevated seawater pCO_2. Mar Biol 160:1963–1972

Maneja RH, Frommel AY, Browman HI, Geffen AJ and others (2015) The swimming kinematics and foraging behavior of larval Atlantic herring (*Clupea harengus* L.) are unaffected by elevated pCO_2. J Exp Mar Biol Ecol 466:42–48

Miller GM, Watson SA, Donelson JM, McCormick MI, Munday PL (2012) Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nat Clim Chang 2:858–861

Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MCO, Chivers DP (2010) Replenishment of fish populations is threatened by ocean acidification. Proc Natl Acad Sci USA 107:12930–12934

Munday P, Pratchett M, Dixson D, Donelson J, Endo GK, Reynolds A, Knuckey R (2013) Elevated CO_2 affects the behavior of an ecologically and economically important coral reef fish. Mar Biol 160:2137–2144

Munday PL, Cheal AJ, Dixson DL, Rummer JL, Fabricius KE (2014) Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. Nat Clim Chang 4: 487–492

Neuenfeldt S, Andersen KH, Hinrichsen HH (2009) Some Atlantic cod *Gadus morhua* in the Baltic Sea visit hypoxic water briefly but often. J Fish Biol 75:290–294

Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson SA, Munday PL (2012) Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nat Clim Chang 2:201–204

Nowicki JP, Miller GM, Munday PL (2012) Interactive effects of elevated temperature and CO_2 on foraging behavior of juvenile coral reef fish. J Exp Mar Biol Ecol 412:46–51

Perry AL, Low PJ, Ellis JR, Reynolds JD (2005) Climate change and distribution shifts in marine fishes. Science 308:1912–1915

Renaud P, Berge J, Varpe Ø, Lønne O, Nahrgang J, Ottesen C, Hallanger I (2012) Is the poleward expansion by Atlantic cod and haddock threatening native polar cod, *Boreogadus saida*? Polar Biol 35:401–412

Rogers LJ (1990) Light input and the reversal of functional lateralization in the chicken brain. Behav Brain Res 38: 211–221

Rogers LJ (2010) Relevance of brain and behavioural lateralization to animal welfare. Appl Anim Behav Sci 127:1–11

Rogers LJ, Zucca P, Vallortigara G (2004) Advantages of having a lateralized brain. Proc R Soc Lond B Biol Sci 271(Suppl 6):S420–S422

Sundby S, Nakken O (2008) Spatial shifts in spawning habitats of Arcto-Norwegian cod related to multidecadal climate oscillations and climate change. ICES J Mar Sci 65: 953–962

Sundin J, Jutfelt F (2016) 9–28 d of exposure to elevated pCO_2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (*Ctenolabrus rupestris*). ICES J Mar Sci 73:620–632

Vallortigara G, Rogers LJ (2005) Survival with an asymmetrical brain: advantages and disadvantages of cerebral lateralization. Behav Brain Sci 28:575–589

Welch MJ, Watson SA, Welsh JQ, McCormick MI, Munday PL (2014) Effects of elevated CO_2 on fish behaviour undiminished by transgenerational acclimation. Nat Clim Chang 4:1086–1089

Wittling W, Pflüger M (1990) Neuroendocrine hemisphere asymmetries: salivary cortisol secretion during lateralized viewing of emotion-related and neutral films. Brain Cogn 14:243–265

Wittling W, Block A, Schweiger E, Genzel S (1998) Hemisphere asymmetry in sympathetic control of the human myocardium. Brain Cogn 38:17–35

Wittmann AC, Pörtner HO (2013) Sensitivities of extant animal taxa to ocean acidification. Nat Clim Chang 3:995–1001

Editorial responsibility: Alejandro Gallego, Aberdeen, UK

Submitted: June 6, 2016; Accepted: March 24, 2017
Proofs received from author(s): April 20, 2017

2.3 Manuscript II

Matthias Schmidt[1,2], Heidrun Sigrid Windisch[3], Kai-Uwe Ludwichowski[1], Sean Lando Levin Seegert[1], Hans-Otto Pörtner[1,2], Daniela Storch[1], Christian Bock[1]

Differences in neurochemical profiles of two gadid species under ocean warming and acidification.

Published in *Frontiers in Zoology* (2017) 14:49
Doi: 10.1186/s12983-017-0238-5

[1]Alfred-Wegener-Institute, Helmholtz-Centre for Polar- and Marine Research, Section Integrative Ecophysiology, Am Handelshafen 12, 27570 Bremerhaven, Germany
[2]University of Bremen, Department of Biology/Chemistry, PO 330440; 28334 Bremen, Germany
[3]Heinrich Heine University, Institute for Cell Biology and Zoology, Universitätsstraße 1, 40225 Düsseldorf, Germany

Schmidt *et al. Frontiers in Zoology* (2017) 14:49
DOI 10.1186/s12983-017-0238-5

Frontiers in Zoology

RESEARCH Open Access

Differences in neurochemical profiles of two gadid species under ocean warming and acidification

Matthias Schmidt[1,2], Heidrun Sigrid Windisch[3], Kai-Uwe Ludwichowski[4], Sean Lando Levin Seegert[1], Hans-Otto Pörtner[1,2], Daniela Storch[1] and Christian Bock[1*]

Abstract

Background: Exposure to future ocean acidification scenarios may alter the behaviour of marine teleosts through interference with neuroreceptor functioning. So far, most studies investigated effects of ocean acidification on the behaviour of fish, either isolated or in combination with environmental temperature. However, only few physiological studies on this issue were conducted despite the putative neurophysiological origin of the CO_2-induced behavioural changes. Here, we present the metabolic consequences of long-term exposure to projected ocean acidification (396–548 μatm PCO_2 under control and 915–1272 μatm under treatment conditions) and parallel warming in the brain of two related fish species, polar cod (*Boreogadus saida*, exposed to 0 °C, 3 °C, 6 °C and 8 °C) and Atlantic cod (*Gadus morhua*, exposed to 3 °C, 8 °C, 12 °C and 16 °C). It has been shown that *B. saida* is behaviourally vulnerable to future ocean acidification scenarios, while *G. morhua* demonstrates behavioural resilience.

Results: We found that temperature alters brain osmolyte, amino acid, choline and neurotransmitter concentrations in both species indicating thermal responses particularly in osmoregulation and membrane structure. In *B. saida*, changes in amino acid and osmolyte metabolism at the highest temperature tested were also affected by CO_2, possibly emphasizing energetic limitations. We did not observe changes in neurotransmitters, energy metabolites, membrane components or osmolytes that might serve as a compensatory mechanism against CO_2 induced behavioural impairments. In contrast to *B. saida*, such temperature limitation was not detected in *G. morhua*; however, at 8 °C, CO_2 induced an increase in the levels of metabolites of the glutamate/GABA-glutamine cycle potentially indicating greater GABAergic activity in *G.morhua*. Further, increased availability of energy-rich substrates was detected under these conditions.

Conclusions: Our results indicate a change of GABAergic metabolism in the nervous system of *Gadus morhua* close to the optimum of the temperature range. Since a former study showed that juvenile *G. morhua* might be slightly more behaviourally resilient to CO_2 at this respective temperature, we conclude that the observed change of GABAergic metabolism could be involved in counteracting OA induced behavioural changes. This may serve as a fitness advantage of this respective species compared to *B. saida* in a future warmer, more acidified polar ocean.

Keywords: Ocean acidification, Temperature, ^{1}H–NMR-spectroscopy, Untargeted metabolic profiling, HPLC, GABA

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

Background

Exposure to projected CO_2-induced ocean acidification (OA) scenarios alters the behaviour of some marine teleost species [1]. It has been suggested that these behavioural changes originate as a side effect of acid-base regulatory processes, which include extra- and intracellular bicarbonate accumulation associated with an equivalent reduction of chloride ions [2]. As a consequence, the electrochemical gradient of neurons in the central nervous system alters. This process is believed to affect functioning of γ-aminobutyric acid type A receptors ($GABA_A$-R). Several experimental approaches support this hypothesis of altered $GABA_A$-R activity [1, 3–5]. An altered functioning of the most important inhibitory neurotransmitter within the central nervous system, with great regulatory importance for neuronal circuits may lead to profound changes in neuronal activity and thus energetic demand. Acid-base regulatory processes are suggested to be responsible for altered fish behaviour, but this does not concern all species. Some species have been found to be more resilient to environmental CO_2 than others, with unclear physiological background [6–9].

In this study, we assessed the question how chronic exposure to increased environmental CO_2 affects metabolism in the brain of vulnerable *Boreogadus saida* and more resilient *Gadus morhua* [6, 8, 9]. Investigation of CO_2 in combination with temperature effects should reveal future impacts of climate change in these ecologically and economically relevant species. Around Svalbard, the sea surface temperature is currently between −1.5 °C in winter and 8 °C in summer [10, 11], and is projected to rise by up to 2.5 °C until the year 2100 [12].The distribution of *G. morhua* currently shifts northward [13] and already overlaps with the distribution of *B. saida* in the seas around Svalbard with uncertain ecological consequences [10]. While direct commercial interest in *B. saida* is only minor, compared to that for *G. morhua*, its importance lies mainly in its function as forage for several other utilized fish species [14, 15]. Both, *B. saida* and *G. morhua*, will experience further warming and acidification until the end of the twenty-first century. Behavioural consequences of CO_2 during concomitant warming have so far only been assessed in a few fish species [16–18]. Furthermore, studies of the combined effects of these two factors on brain metabolism have not been conducted at all.

The present study focuses particularly on metabolites and amino acids involved in energy metabolism and regeneration of the neurotransmitter γ-aminobutyric acid (GABA) in order to test whether compensatory mechanisms to a rise of environmental CO_2 are visible on neurotransmitter level. In addition, we studied the neurotransmitter serotonin (5-HT) and its catabolite since serotonergic activity is positively correlated with chronic stress in fish [19]. Compounds involved in the metabolism of phospholipids were also analysed since Leo et al. found that CO_2 might affect proton leakage in mitochondria of *G. morhua* and other teleosts [20, 21]. The networks of tested metabolites involved in GABA and phospholipid metabolism are displayed in Fig. 1. As exposure to increased CO_2 also alters the composition of dissolved extra- and intracellular ion species in the brain of fish [5] we also took osmolyte concentrations into account to fully track changes in brain metabolites under elevated CO_2 and at various temperatures.

Methods

Specimen collection and maintenance

Juvenile *Boreogadus saida* were caught on January 17th 2013 in the inner Kongsfjord (78.97 N, 12.51 E) at an approximate depth of 120 m. They were housed in aquaria of the Tromsø Aquaculture Research Station, in Kårvik, Norway before being transported to the Alfred Wegener Institute in Bremerhaven, Germany (AWI). Juvenile *Gadus morhua* were collected between the 26th and 29th of August 2013 during a cruise of the RV Heincke around Hinlopenstretet (79.30 N, 18.57 E), Rijpfjorden (80.15 N, 22.12 E), and Forlandsundet (78.54 N, 11.3 E) and were subsequently transported to the AWI. Additional information on the cruise is provided under http://doi.pangaea.de/10.1594/PANGAEA.824703. Animals were kept in a recirculating seawater system at the AWI at 5 °C and environmental PCO_2 until onset of the incubations.

Incubations

Extensive information on the incubation and animals has been provided by Kunz et al. [22]. Incubation periods were 102–114 days for *B. saida* and 111–132 days for *G. morhua*. *B. saida* (length: 14.4 ± 1.1 cm; weight: 18.0 ± 4.9 g) was incubated at 0 °C, 3 °C, 6 °C and 8 °C and *G. morhua* (length: 18.0 ± 2.0 cm; weight: 39.5 ± 14.9 g) at 3 °C, 8 °C, 12 °C and 16 °C. Temperatures were chosen in order to represent a wide range of the thermal habitat. At each temperature, animals were kept either at ambient control PCO_2 (396–548 μatm.) or high PCO_2 (915–1272 μatm.), as projected for the year 2100 [12]. The factorial design was comprised of 8 different treatment groups per species. Means and standard deviations of PCO_2 for each treatment group are presented in Additional file 1: Table S1. Temperatures were adjusted by a maximum of 2 °C per day beginning at 5 °C. Afterwards, CO_2 conditions were adjusted during a single day. The incubation started after the desired temperature and CO_2 conditions had been reached. Animals were kept in individual tanks to avoid cannibalism and changes in the neurochemical profile due to dominance hierarchies.

A gas mixing system (HTK, Germany) was used for adjustment of PCO_2. Temperature, salinity, dissolved

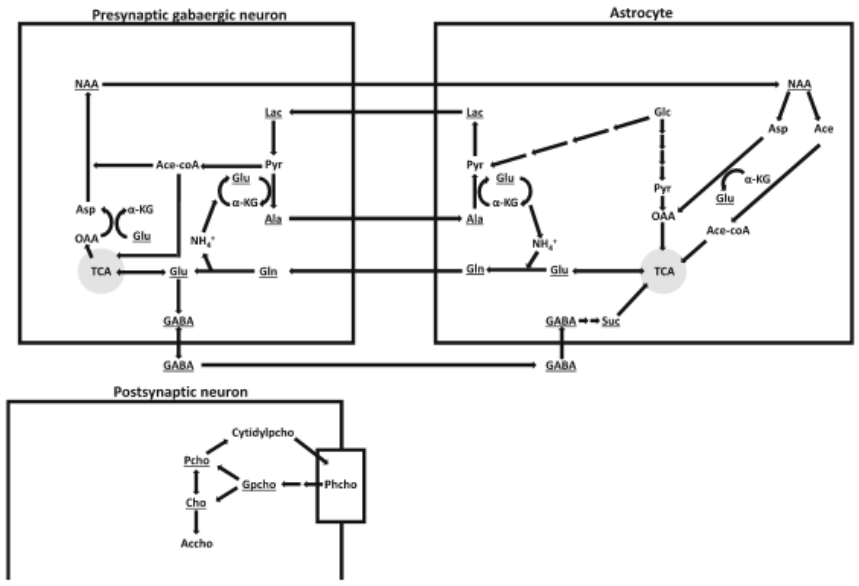


Fig. 1 top: Metabolites involved in the GABA-glutamine cycle for neurotransmitter regeneration and the connected amino acid shuttle for ammonia transfer between presynaptic GABAergic neurons and surrounding astrocytes. Bottom: Metabolism of the membranous phosphatidylcholine as described by Klein [58]. Upon excitation, GABAergic neurons release GABA into the synaptic cleft, from where a minor fraction is taken up by the releasing GABAergic neuron itself and a major fraction by surrounding astrocytes. Within astrocytes it is catabolized to succinate which fuels the tricarbonic acid cycle. α-ketoglutarate of the TCA cycle is metabolized to glutamate and further aminated to glutamine, which is transported transcellularly into the GABAergic neuron. In the neuron, Glutamine is deaminated to glutamate and further decarboxylated to GABA which is again packed into synaptic vesicles. Lactate serves as neuronal energy source and is transported from astrocytes into neurons, where it is oxidized to pyruvate which subsequently enters the TCA cycle after transformation into acetyl-coA. A fraction of cellular pyruvate is aminated to alanine which is transported back to astrocytes in order to avoid accumulation of ammonia inside the neuron. N-acetylaspartate, which is generated in neurons from aspartate and acetyl-coA, can re-enter the TCA cycle of astrocytes as oxaloacetate under energy-deprived conditions. Phosphatidylcholine is present in all cell membranes of neurons and astrocytes but for the sake of clarity, its metabolism is displayed in the postsynaptic neuron only. As described by Bak et al. [35] membranous phosphatidylcholine gets catabolized to dissolved choline or alternatively, phosphocholine via glycerophosphocholine. In cholinergic neurons choline can be utilized for anabolism of the neurotransmitter acetylcholine, while phosphocholine can be used for regeneration of phosphatidylcholine via cytidylphosphocholine. Underlined metabolites were quantified through ^{1}H–NMR spectroscopy. The scheme is adapted after Bak et al. [35] and Klein [58]. Ace = Acetate; Ace-coA = Acetyl-coA; Accho = Acetylcholine; α-KG = Alpha ketoglutaric acid; Ala = Alanine; Asp = Aspartate; Cho = Choline; Cytidylpcho = Cytidylphosphocholine; GABA = Gamma-aminobutyric acid; Glc = Glucose; Gln = Glutamine; Glu = Glutamate; Gpcho = Glycerophosphocholine; Lac = Lactate; NAA = N-acetylaspartate; OAA = Oxaloacetic acid; Pcho = Phosphocholine; Phcho = Phosphatidylcholine; Pyr = Pyruvate; Suc = Succinate; TCA = Tricarbonic acid cycle

inorganic carbon (DIC) and pH_{tot} were measured at least once per week. Extensive information on the methodology of carbonate chemistry measurements and raw data are available under https://doi.pangaea.de/10.1594/PANGAEA.866369. A summary of the carbonate chemistry for the whole incubation period has been published by Kunz et al. [22].

Tissue sampling and preparation

After incubation, animals were exposed to surgical anaesthesia at 200 mg/l MS-222 in a water bath containing seawater from aquaria of the respective treatment group. When the fish did not respond anymore to external stimuli, they were taken out of the water bath and sacrificed though cervical dislocation. Brains were removed, transferred to centrifuge tubes, rapidly frozen in liquid nitrogen and subsequently stored at −80 °C. In order to enable NMR and HPLC analysis on the same tissue samples, each brain was powdered with mortar and pestle under liquid nitrogen. The grind was well mixed and aliquots of ~50 mg were taken for NMR and HPLC analysis. Brains of three to six animals per treatment group were separately analysed via NMR and HPLC using one aliquot for NMR and one aliquot for HPLC measurements. The remaining brains were utilized in experiments not covered by the scope of this paper.

Untargeted metabolic profiling (using ^{1}H–NMR-spectroscopy)

Extraction of brain tissue was conducted after Belle et al. [23]. Briefly, one aliquot of powdered brain tissue was transferred into a cooled glass centrifuge tube on ice. 3 ml ice-cold Dichloromethane/Methanol (2:1 *v*/v) were

added quickly and the suspension mixed. The tube was sonicated for ten minutes at 4 °C in a Branson sonifier with 50% duty cycle. Subsequently, 1 ml 0.88% KCl (*w*/w) was added, the suspension was mixed again and centrifuged for ten minutes at 805 g. The upper methanol phase was transferred to another centrifuge tube and the solution dried overnight in a vacuum concentrator (RVC 2–18 HCl, Christ GmbH Osterode, Germany). The remaining pellet was stored for two to three days at 4 °C in a fridge before being re-suspended in D_2O (Deuterium oxide) containing 0.05% TSP (Trimethylsilylpropionate) as internal standard. Two to four fold the weight of the original grind of D_2O was added in a volume equivalent (depending on the amount of initial brain tissue) to suspend the pellet. Untargeted metabolic profiling based on ^{1}H–NMR spectroscopy was performed on a wide-bore 400 MHz NMR spectrometer (9.4 T WB with Avance III HD electronics, Bruker Biospin, Germany) using a triple tuned ^{1}H-^{13}C-^{31}P-HRMAS NMR probe. A sample volume of ~ 50 μl was filled in a standard zirconium rotor for high-resolution magic angle spinning (HRMAS) NMR spectroscopy. All NMR spectra were conducted at a spinning rate of 3000 Hz and a sample temperature of 10 °C. Four different ^{1}H–NMR measurements were collected for all samples, consisting of a standard one pulse 1D ^{1}H NMR spectroscopy with f1 pre-saturation, a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse train including f1 pre-saturation (Bruker protocol cpmgpr1d), a NOESY sequence and a pseudo 2D ^{1}H-^{1}H J-resolved (JRES) NMR spectroscopy protocol for metabolite identification. All metabolite profiles were analysed from the CPMG NMR protocol with the following acquisition parameters: pulse length 8.4 μs for 90°, time domain 70,656, sweep width of 8802 Hz (22 ppm), acquisition time 4.01 s, relaxation delay 4 s, four dummy scans and 64–256 number of scans depending on the signal-to-noise ratio.

Samples were analysed in randomized order to avoid systematic errors. All spectra were analysed using Chenomx NMR suite 8.1 (Chenomx Inc., Canada). Data were automatically zero filled to at least 128 k and processed with an exponential multiplication of 0.3 Hz. After phase and baseline correction line-shape distortions were eliminated through shim correction. Metabolites were identified using the Chenomx data base and an online spectral data base for organic compounds (http://sdbs.db.aist.go.jp, SDBS, National Institute of Advanced Industrial Science and Technology (AIST)). NMR peak integrals were fitted manually to the specific metabolites for quantification. In total, 24 compounds were identified: Acetate, creatine, lactate, phosphocreatine and succinate are mainly part of cellular energy metabolism; alanine, GABA, glutamine and glutamate are involved in metabolism of GABA either as part of the GABA-glutamine cycle or the lactate-alanine shuttle (lactate is also listed among the metabolites involved in energy metabolism). Compounds involved in the metabolism of phosphatidylcholine are choline, glycerophosphocholine, phosphocholine and putrescine. Acetyl-histidine, myo-inositol (MI), N-acetylaspartate (NAA), taurine and trimethylamine-*N*-oxide (TMAO) serve as osmolytes in the central nervous system. In addition to its function as osmolyte, NAA is further involved in energy metabolism (Fig. 1). Glycine was quantified as it serves together with GABA as important inhibitory neurotransmitter throughout the central nervous system [24]. Peaks of ascorbate, aspartate, threonine and valine were mostly visible, however, either close to detection limit, or strongly overlapping with other, more prominent, compounds. As a reliable quantification of these substances was not possible, they were excluded from subsequent analysis. Methanol is a trace compound from the extraction process and was therefore also not further analysed. The spectrum of acetyl-histidine was not available in the Chenomx data base and was created manually in the Chenomx compound builder prior to analysis. We detected strong differences in metabolite concentrations between samples, which are likely caused by different extraction efficiencies. To compensate for this bias, we calculated for each spectrum total creatine (tCr) - the sum of creatine and phosphocreatine- as internal reference. Afterwards, the concentration of each metabolite relative to the concentration of tCr was calculated and these ratios were used for statistical analysis. For *Boreogadus saida*, the brains of 38 individuals were analysed, for *Gadus morhua*, the brains of 40 individuals. The exact group sizes are document in Additional file 1: Table S1.

HPLC-analysis

Deionized water was utilized for preparation of all buffers. All chemicals used were either of HPLC-grade of the highest purity available. HPLC-analysis of brain tissue was conducted with a modified method of Yoshitake et al. [25] through derivatization with benzylamine (BA) and diphenylethylenediamine (DPE) followed by subsequent fluorescence detection. BA was dissolved 0.3 M in an aqueous solution with 90% methanol (*v*/v). A 0.3 M CAPS buffer was prepared in aqueous 90% methanol (*v*/v) and subsequently adjusted to a pH of 11 with 10 M NaOH. Potassium hexacyanoferrate (III) was prepared in an aqueous 50% methanol (*v*/v). A 0.2 M DPE solution was prepared in methanol and subsequently diluted by 50% with 0.2 M aqueous HCl. Glycine was prepared 0.3 M in H_2O. Two derivatisation reagents were made: First, derivatisation reagent "A" containing the above prepared BA, CAPS, potassium hexacyanoferrate solutions

and methanol in a stoichiometry of 2:6:3:24 (v/v/v/v). Second, derivatisation reagent "B" containing the prepared DPE and glycine solutions in a stoichiometry of 2:1 (v/v).

Powdered brain tissue in a centrifuge cup was extracted by adding 0.1 N ice-cold aqueous perchloric acid with 10^{-7} M ascorbic acid at a volume of ten-fold the weight of the grind. The cup was mixed and sonicated for two minutes at 4 °C in a Branson sonifier with 50% duty cycle. The cup was subsequently centrifuged at 5200 g for 30 min at 4 °C and the supernatant transferred to a different centrifuge cup. The supernatant was brought to a pH of ~4.5 through addition of 0.5 M ice-cold KOH and subsequently centrifuged again for 30 min at 4 °C.

Reversed phase solid phase extraction was conducted with Oasis HLB cartridges (Waters Corporation, Milford, USA), containing 30 mg sorbent per cartridge with 1 ml methanol as solvent for the eluate. The eluate was dried overnight in a centrifuge cup in a vacuum concentrator (RVC 2–18 HCl, Christ GmbH Osterode, Germany). On the next day, the pellet was dissolved in 200 μl H_2O. 200 μl of derivatisation reagent "A" was added and allowed to react for two minutes at room temperature. Subsequently, 200 μl of derivatisation reagent "B" was added and the mixture incubated for 20 min at 50 °C after which it was rapidly cooled on ice. The solution was filtered through a 20 μm filter and transferred into an amber-coloured glass vial. Measurements were conducted overnight in a HPLC system at room temperature with a LaChrom Elite® (Hitachi High Technologies America, USA) on a reversed phase Kinetex C18 column (Phenomenex, length: 150 mm; diameter: 4.6 mm; particle size: 2.6 μm). We achieved separation through a non-isocratic elution with fractions of two media: first, a 15 mM sodium acetate, 1 mM octanesulfonic acid buffer (pH 4.5) that was diluted with acetonitrile (1:2 (*v*/v)) and, second, pure acetonitrile. The used proportions are available in Additional file 2: Table S2. Measurement time was 90 min per sample at a flow rate of 0.6 ml/min. Components were identified through their specific retention times using a fluorescence detector with an excitation wavelength of 345 nm and emission wavelength of 480 nm. Using this method, we were able to quantify norepinephrine, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid (HIAA), serotonin (5-hydroxytryptamine (5-HT)), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and L-3,4-dihydroxyphenylalanine (L-DOPA) in standard solutions. However, in tissue samples, only 5-HT, HIAA and DOPAC were reliably detectable. The other substances were either only visible in very small amounts or not at all. Addition of the respective substances to brain tissue prior to solid phase extraction did not enable their detection suggesting a methodological issue rather than low concentration in the brain tissue. We thus focused our analysis on the serotonergic pathway containing serotonin and its catabolite HIAA. For *Boreogadus saida* and *Gadus morhua*, the brains of 39 individuals per species were analyzed. The exact group sizes are document in Additional file 1: Table S1.

Statistical analysis

Statistical analysis was conducted with "R" (v. 3.2.3). Log-transformed metabolite/tCr ratios from NMR analysis and log-transformed HIAA/5-HT ratios from HPLC analysis of each species were tested separately for temperature-, CO_2- and their interactive effects using ordinate two-way-ANOVAs ($\alpha = 0.05$). Tukey HSD ($\alpha = 0.05$) from the package "agricolae" (v. 1.2–3) was used for post hoc multiple comparison testing. Normality distribution of metabolite/tCr and HIAA/5-HT ratios were evaluated for each treatment group using Shapiro Wilk normality test ($\alpha = 0.05$), variance homogeneity with a Bartlett test ($\alpha = 0.05$). Non-metric multidimensional scaling with stable solution from random starts ("metaMDS") was conducted on metabolite/tCr ratios with the package "vegan" (v. 2.3–3) in order to test for overall temperature- and CO_2-effects among individuals. The stress level of the metaMDS was in an acceptable range (~0.1). Violation of normality distribution was observed in 17 out of 272 groups tested. This may be due to the sheer amount of observations tested with an α of 0.05. The cumulative random chance of observing 17 or more normality violations under this condition is ~14% on the basis of a binomial distribution, which is thus not significantly different from what could be expected by chance. Violation of normality may still yield implications for possible type I and II errors in the applied ANOVAs; however, we did not see justification for removal of the outlying data points, especially in consideration of the small sample size. A list of the groups with violated normality distribution is provided in Additional file 3: Table S3. Variance homogeneity was violated on three occasions: Glycine/tCr and lactate/tCr ratios of *B. saida* and the TMAO/tCr ratio of *G. morhua*.

Results

Table 1 depicts a synopsis of temperature-, CO_2- and interactive effects on the analysed components. Boxplots of those components influenced either by CO_2 or interactively by CO_2 in combination with temperature are shown in Fig. 3 (*Boreogadus saida*) and Fig. 4 (*Gadus morhua*). Complementary boxplots of all compounds, including those affected by temperature only, are available in the Additional file 4: Figure S1 and Additional file 5: Figure S2 for NMR-data, Additional file 6: Figure S3 for HPLC-data.

Table 1 Summary of temperature and CO_2-related effects and their interaction on compounds analysed via ^{1}H–NMR-Spectroscopy and HPLC

		Boreogadus saida			*Gadus morhua*		
Compound	Class	Temperature	CO_2	Interaction	Temperature	CO_2	Interaction
Ace	Energy metabolism	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lac	Energy metabolism	**,↓	n.s.	n.s.	n.s.	*↑	*
Suc	Energy metabolism	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ala	GABA metabolism	***,↓	n.s.	n.s.	***,↓	n.s.	n.s.
GABA	GABA metabolism	***,↓	*,↑	n.s.	***,↓	n.s.	*
Gln	GABA metabolism	*,↓	n.s.	n.s.	*,↑↓	n.s.	n.s.
Glu	GABA metabolism	***,↓	n.s.	***	***,↓	n.s.	n.s.
Cho	Membrane component	n.s.	n.s.	n.s.	n.s.	n.s.	**
Gpcho	Membrane component	***,↓	n.s.	n.s.	***,↓	n.s.	n.s.
Pcho	Membrane component	**,↑↓	n.s.	n.s.	***,↓	n.s.	n.s.
Put	Membrane component	***,↑↓	n.s.	n.s.	***,↓	n.s.	n.s.
AcHis	Osmolyte	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
MI	Osmolyte	***,↓	***,↑	n.s.	***,↓	n.s.	n.s.
NAA	Osmolyte	***,↑	n.s.	n.s.	***,↑	n.s.	n.s.
Tau	Osmolyte	**,↑↓	n.s.	n.s.	***,↓	n.s.	n.s.
TMAO	Osmolyte	***,↓	n.s.	n.s.	***,↓	n.s.	n.s.
Gly	Other	*,↓	n.s.	n.s.	***,↓	n.s.	n.s.
HIAA/5-HT	Other	*,↑	n.s.	n.s.	*,↑	n.s.	n.s.

Compound classes are assigned to match the grouping of each compound as used throughout the discussion of the manuscript
* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. ↑ and ↓ indicate either an increase or a decrease of the respective compound with rising temperature or rising CO_2. ↑↓ indicates apparent uneven effects. Interactive effects are per definition uneven and were therefore not characterized. A plot for each component is available in the (Additional file 4: Figure S1 and Additional file 5: Figure S2 (NMR) and Additional file 6: Figure S3 (HPLC))
Ace Acetate, *AcHis* Acetyl-histidine, *Ala* Alanine, *Cho* Choline, *GABA* Gamma-aminobutyric acid, *Glu* Glutamate, *Gln* Glutamine, *Gpcho* Glycerophosphocholine, *Gly* Glycine, *Lac* Lactate, *MI* Myo-inositol, *NAA* N-acetylaspartate, *Pcho* Phosphocholine, *Put* Putrescine, *Suc* Succinate, *Tau* Taurine, *HIAA* 5-Hydroxyindoleacetic acid, *5-HT* 5-Hydroxytryptamine (Serotonine)

Boreogadus saida - NMR

Figure 2 presents a typical ^{1}H–NMR cpmg spectrum from a methanol/dichloromethane extract of *Boreogadus saida* brain tissue. Temperature strongly affected most compounds tested in this species. Most evident changes were observable among osmolytes, in particular MI, the concentration of which was around 80% lower at 8 °C than at 0 °C ($p < 0.001$, $F_{3,30} = 267.3$, Fig. 3a). A similar reduction with increasing temperature was observed for TMAO ($p < 0.001$, $F_{3,30} = 56.44$). In a striking contrast, NAA increased with rising temperature by about 30% between 0 °C and 8 °C ($p < 0.001$, $F_{3,30} = 39.06$). Taurine exhibited the highest concentration at 6 °C, decreasing above and below this temperature ($p < 0.01$, $F_{3,30} = 5.299$). Acetyl-histidine was not influenced by temperature ($p > 0.05$). All tested substances directly involved in GABA metabolism showed a reduced concentration with increased temperature. This effect was most prevalent for glutamate and GABA ($p < 0.001$, $F_{3,30} = 42.87$ and $p < 0.001$, $F_{3,30} = 52.37$, Fig. 3b and c respectively). The effect of temperature on alanine and glutamine was weaker and mainly caused by a decrease from 6 °C to 8 °C ($p < 0.001$, $F_{3,30} = 14.15$ and $p < 0.05$, $F_{3,30} = 4.417$).

Among compounds representing energy metabolism only lactate was reduced through temperature increase ($p < 0.01$, $F_{3,30} = 4.547$), an effect which was particularly prevalent between 6 °C and 8 °C. Acetate and succinate were not affected by temperature ($p > 0.05$). Most membrane components tested were affected by temperature with the exception of choline ($p > 0.05$). Putrescine and phosphocholine concentrations followed a bell-shaped curve with highest concentrations at 3 °C and 6 °C ($p < 0.001$, $F_{3,30} = 7.892$ and $p < 0.01$, $F_{3,30} = 6.693$). Glycerophosphocholine concentrations decreased significantly with increasing temperature ($p < 0.001$, $F_{3,30} = 87.42$). Glycine was significantly reduced during warming ($p < 0.05$, $F_{3,30} = 3.637$) with a change particularly strong between 6 °C and 8 °C. Simultaneously, glycine displayed a reduction of variance with increasing temperature which was detected with Bartlett test for variance homogeneity.

CO_2-effects were visible for the osmolyte MI and for GABA ($p < 0.001$, $F_{1,30} = 23.58$ and $p < 0.05$, $F_{1,30} = 4.478$). CO_2 caused an increase in the concentrations of these compounds, mainly at 8 °C. However, CO_2-effects were much lower than the observed

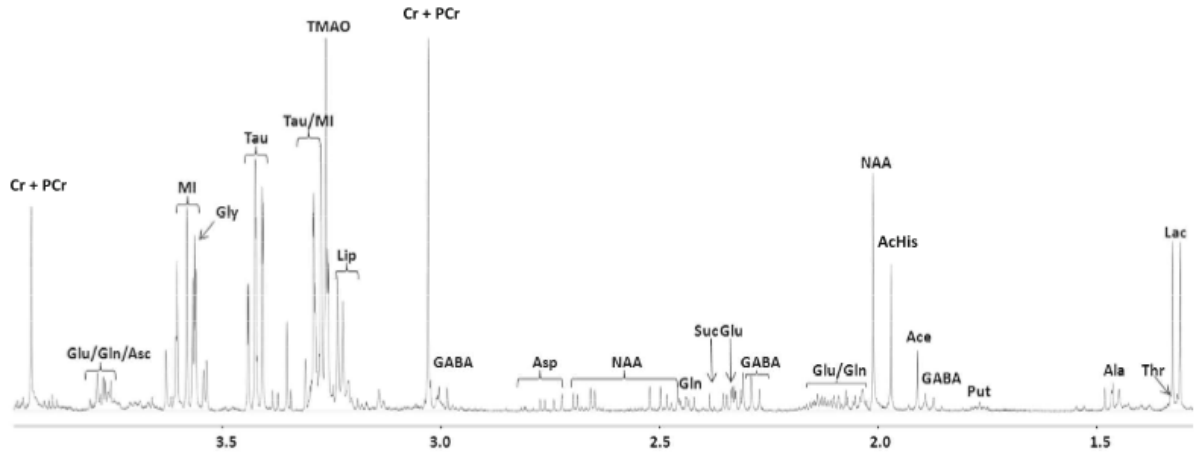


Fig. 2 Exemplary ^{1}H–NMR spectrum of a brain methanol/dichloromethane extract of *Boreogadus saida*. The x-axis represents the chemical shift of the respective compounds in parts per million (ppm). Ace = Acetate; AcHis = Acetyl-histidine; Ala = Alanine; Asc = Ascorbate; Asp = Aspartate; Cr + PCr = Creatine and Phosphocreatine; GABA = Gamma-aminobutyric acid; Gln = Glutamine; Glu = Glutamate; Gly = Glycine; Lac = Lactate; Lip = Lipids including choline, glycerophosphocholine and phosphocholine; MI = Myo-inositol; NAA = N-acetylaspartate; Put = Putrescine; Suc = Succinate; Tau = Taurine; Thr = Threonine; TMAO = Trimethylamine-N-Oxide

temperature-related changes and post-hoc-tests therefore negative.

Interactive effects of temperature and CO_2 were detected in the changes of glutamate content which is involved in GABA metabolism ($p < 0.001$, $F_{3,30} = 7.217$).

Non-metric multidimensional scaling supports the conclusion that the variance in metabolite levels is largely explainable through temperature effects ($p < 0.001$, $R^2 \sim 0.93$, 10^3 permutations, Additional file 7: Figure S4) with only a small contribution by CO_2 ($p \sim 0.6$, $R^2 \sim 0.03$, 10^3 permutations).

Boreogadus saida - HPLC

Warming caused a significant change in serotonin metabolism with an increase of the HIAA/5-HT ratio ($p < 0.05$, $F_{3,31} = 3.525$, Additional file 6: Figure S3A). Neither CO_2, nor interactive effects with temperature were detected.

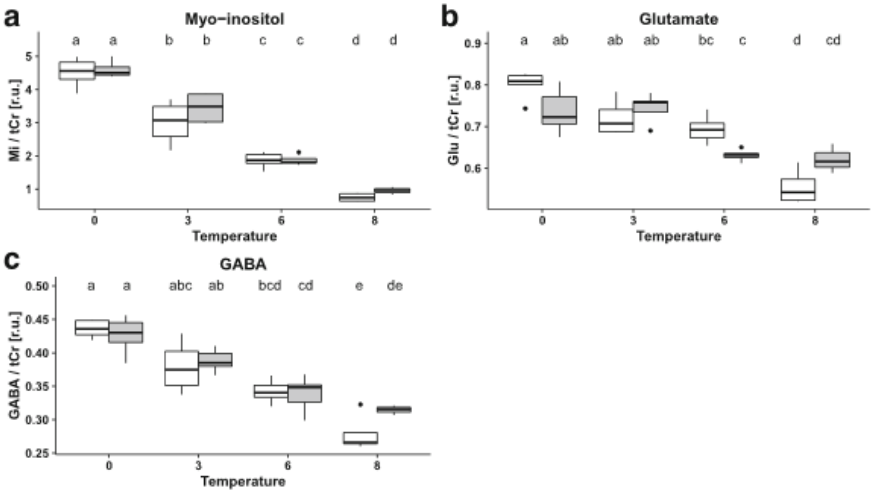


Fig. 3 Boxplots depicting concentrations of metabolites affected by CO_2 (direct or interactive effects, relative to total creatine (tCr), relative units [r.u.]) in the brain of *Boreogadus saida* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile as well as respective standard deviation. Different letters (**a**, **b**, **c**, **d**, **e**) indicate significant differences between treatment groups detected by Tukey HSD post hoc analysis ($p < 0.05$)

Gadus morhua – NMR

As in *B. saida*, most compounds tested were affected by environmental temperature. The strongest changes were again observed among osmolytes. In particular, MI fell by >90% during warming from 3 °C to 16 °C ($p < 0.001$, $F_{3,32} = 986.5$). The greatest reduction by ~75% occurred between 3 °C and 8 °C. A similar reduction by ~90% between 3 °C and 16 °C was observed for TMAO with the greatest drop again between 3 °C and 8 °C ($p < 0.001$, $F_{3,32} = 60.87$). In contrast to MI and TMAO, NAA increased significantly by about 50% between 3 °C and 16 °C ($p < 0.001$, $F_{3,32} = 70.95$). Taurine concentrations were highest at 3 °C and 8 °C and dropped beyond 8 °C ($p < 0.001$, $F_{3,32} = 12.97$). Acetyl-histidine was not affected by temperature ($p > 0.05$). Similar to *B. saida*, concentrations of most compounds involved in GABA metabolism strongly decreased with increasing temperature. This temperature effect was again strongest for glutamate ($p < 0.001$, $F_{3,32} = 87.38$), followed in magnitude by GABA ($p < 0.001$, $F_{3,32} = 32.16$, Fig. 4c). A warming-induced reduction of alanine levels occurred mainly between 3 °C and 8 °C, as observed in *B. saida* ($p < 0.001$, $F_{3,32} = 10.75$). The temperature effect on glutamine was less clear. Means of glutamine at 12 °C and 16 °C were higher than at 3 °C and 8 °C, however, with the exception of the 3 °C control-CO_2 group. Differences in glutamine levels between groups were rather small and post hoc tests negative ($p > 0.05$). The overall temperature effect though, was still slightly significant ($p < 0.05$, $F_{3,32} = 4.370$). Acetate, lactate and succinate levels were not altered by temperature ($p > 0.05$). As in *B. saida*, most components involved in membrane metabolism were affected by temperature, except for choline ($p > 0.05$, Fig. 4b). Putrescine, phosphocholine and glycerophosphocholine level fell during warming ($p < 0.001$, $F_{3,32} = 11.28$, $p < 0.001$, $F_{3,32} = 78.03$ and $p < 0.001$, $F_{3,32} = 121.0$). As in *B. saida*, glycine concentrations decreased with rising temperatures ($p < 0.001$, $F_{3,32} = 40.17$).

A significant CO_2 effect was observed for lactate with an increase under high CO_2 ($p < 0.05$, $F_{1,32} = 7.24$ Fig. 4a). This finding was mainly governed by a CO_2-related increase of lactate at 8 °C ($p < 0.05$ in post hoc analysis) which led to detection of a significant interactive effect of temperature and CO_2 ($p < 0.05$, $F_{3,32} = 2.94$). Interactive effects of temperature and CO_2 were also detected for choline and GABA ($p < 0.05$, $F_{3,32} = 5.870$ and $p < 0.05$, $F_{3,32} = 4.033$ Fig. 4b and c). For these substances post hoc tests revealed a significant CO_2-dependent increase at 8 °C ($p < 0.05$), which was absent at other temperatures.

As in *B. saida*, non-metric multidimensional scaling revealed for *G. morhua* that the vast majority of overall variance was explainable through temperature effects ($p < 0.001$, $R^2 \sim 0.95$, 10^3 permutations, Additional file 8: Figure S5), with only a minor contribution by CO_2 ($p \sim 0.16$, $R^2 \sim 0.09$, 10^3 permutations).

Gadus morhua - HPLC

As in *B. saida*, temperature significantly affected serotonin metabolism of *G. morhua* leading to a rise of the HIAA/5-HT ratio during warming ($p < 0.05$, $F_{3,31} = 3.394$, Additional file 6: Figure S3B). Neither were effects of CO_2, nor interactive effects detected.

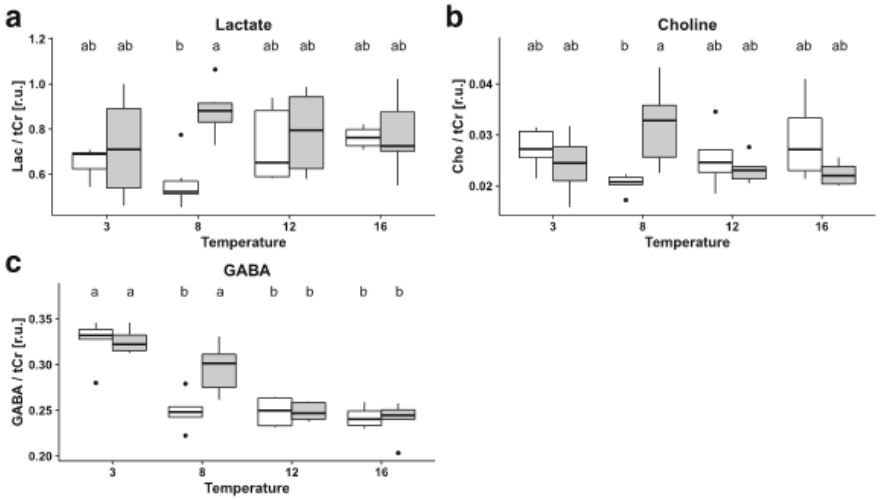


Fig. 4 Boxplots depicting concentrations of metabolites affected by CO_2 (direct or interactive effects, relative to total creatine (tCr)) in the brain of *Gadus morhua* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile as well as respective standard deviation. Different letters (**a**, **b**) indicate significant differences between treatment groups detected by Tukey HSD post hoc analysis ($p < 0.05$)

Discussion

Interference with anaesthesia

It cannot be fully excluded that additional factors such as the anaesthetic procedure may have an interactive effect with temperature or CO_2 contributing to the here presented observations. Effects of MS-222 on the respiratory and cardiovascular system of fish are well documented as well as the rapid MS-222 induced release of cortisol, changing inter alia the ionic composition of blood plasma [26, 27]. Consequences of MS-222 for intracellular osmolytes and neurotransmitter systems have not been reported and would demand further investigation.

Effect of temperature on brain metabolites

Temperature affected metabolite concentrations in the brain of *Boreogadus saida* and *Gadus morhua* in a very similar manner. The strongest alterations were visible among the osmolytes TMAO and MI, which fell in both species upon warming. This finding is in line with the view that TMAO and MI are cytoprotective in cold environments [28, 29]. In both species TMAO/tCr ratios reacted similarly to warming and underwent a reduction by ~ 70% from 3 °C to 8 °C. A striking difference was visible in the relative amount of TMAO, as at 3 °C and 8 °C the TMAO/tCr ratio in *B. saida* was around sixfold greater than the TMAO/tCr ratio of *G. morhua*, which indicates a greater importance of TMAO in physiological cold adaptation of *B. saida*. While temperature altered taurine concentrations in *B. saida* in a uneven manner, taurine decreased with increasing temperature in *G. morhua*. Osmolytes play a role in cytoprotection, and in both species elevated taurine concentrations may be beneficial below 8 °C [30] and may serve as compensation to the constitutive reduction of TMAO at temperatures above 0 °C. A decrease of osmolytes with increasing temperature was expected as high osmolarity in cold-adapted fish serves freezing point reduction [31]. In contrast, NAA levels increased in both species with increasing temperatures. In addition to its putative functions as osmolyte and potential energy reserve in neurons and oligodendrocytes, concentration changes of this substance might also have implications for membrane composition, as discussed below. However, not all osmotically active substances responded to temperature. Unlike Baslow and Guilfoyle [32] we did not detect temperature-related changes of acetylhistidine concentrations as observed in killifish (*Fundulus heteroclitus*) and goldfish (*Carassius auratus*). However, the temperature range investigated in this study (0 °C - 16 °C) was narrower than the range analysed by Baslow and Guilfoyle (13.3 °C - 30 °C).

In addition to their function as synaptic neurotransmitters, GABA, glutamate and glycine influence osmotic relations [33] which may explain why their concentrations decreased continuously with rising temperatures as observed in the prominent osmolytes as discussed above. This may explain the temperature dependence of glutamate and GABA in both species, and of glycine in *G. morhua*. Their concentrations decreased continuously with rising temperature. Windisch et al. found a strong temperature dependency in the expression of the glycine cleavage system in Antarctic eelpout, which was much higher expressed at cold temperatures below 0 °C indicating importance of glycine metabolism at low temperatures [34]. Whether the glycine cleavage system serves at low temperatures the catabolism of excessive glycine or rather its anabolism demands further experimentation. In *B. saida*, reductions of lactate, alanine, and glutamine levels occurred mainly between 6 °C and 8 °C, potentially indicating energy limitations at 8 °C as not only lactate, but also glutamate, glutamine, GABA are energy sources for the tricarbonic acid (TCA) cycle in brain [35]. This hypothesis is supported by the fact that mortality of *B. saida* only occurred at 8 °C and that this temperature was recently identified to represent the long-term upper thermal tolerance limit for this species [22]. In *B. saida*, glycine concentrations were marginally reduced by increasing temperature, but the variance of concentrations between treatments fell with rising temperature, especially at 8 °C, potentially indicating energy limitations as well. A reduction of alanine levels at 8 °C in *B. saida* may not necessarily be a proxy for energy status, as *G. morhua* also exhibited a reduction of alanine level between 3 °C and 8 °C, but no further alteration of alanine concentrations was observed between 8 °C and 16 °C rather suggesting a temperature-dependent functioning of this amino acid as osmolyte.

Alterations of phosphocholine, glycerophosphocholine and putrescine with temperature may indicate structural changes in membrane metabolism and composition. Phosphocholine and glycerophosphocholine are intermediates of phosphatidylcholine metabolism and putrescine acts as progenitor for other polyamines such as spermidine and spermine, all of which are known to interact with membrane components [36–38]. Temperature-dependent changes in NAA levels as mentioned above might also indicate shifts in lipid metabolism to improve homeoviscous adaptation and oxygen supply. This substance also acts as the major donor of acetyl-groups for the myelinisation of neurons [39, 40].

Temperature had an effect on serotonin metabolism in both, *B. saida* and *G. morhua*, leading to higher HIAA/5-HT ratios at higher temperatures and indicating an increase of serotonin turnover. Similar results were observed in common carp by de Boeck et al., who suggested a Q_{10} effect, but no stress response, as a mechanism [41]. In contrast to our findings, Sebert et al.

measured a reduced HIAA/5-HT ratio with rising temperature in the eel *Anguilla anguilla* [42], a finding which argues against a simple Q_{10} effect. However, serotonin is a modulator of respiration. Increased respiration through a risen O_2 demand at higher temperature may also be reflected in the HIAA/5-HT ratio [43].

Effect of CO_2 on brain metabolites in *Boreogadus saida*

CO_2 effects in *B. saida* were observed for the osmolytes MI and GABA. Interactive effects of temperature and CO_2 were detected for glutamate. All of these observations can mainly be attributed to alterations at 8 °C, where high CO_2 induced an increase in the concentrations of these three metabolites. These changes were quite small compared to temperature effects and were not significant in post hoc analysis. Additionally, CO_2 caused a transient (non-significant) rise of glutamine and glutamate at 8 °C accompanied by a non-significant reduction of NAA. We suggest that these findings are symptomatic for shifts in neural energy metabolism at 8 °C which might be exacerbated by CO_2. In mammals, NAA is coupled to the brain's energy metabolism and may serve as an anaplerotic source of acetate and aspartate [44]. While acetate can enter the TCA cycle after activation with SH-CoA through acetyl-CoA synthase [45], the remaining aspartate can be transformed to oxaloacetic acid and subsequently acetylated to citrate to enter the TCA cycle as well [44]. Also, in a second pathway, aspartate becomes deaminated to oxaloacetic acid generating glutamate from α-ketoglutaric acid. Glutamate, glutamine and GABA concentrations in the brain are tightly coupled through the glutamate/GABA-glutamine cycle [35].

An increase of GABA and glutamate might also indicate greater GABAergic activity at 8 °C and high CO_2, possibly reducing neuronal activity under limited energy conditions. After GABA release by inhibitory neurons, it is partly taken up by astrocytes and converted into succinate by GABA-transaminase and succinic semialdehyde dehydrogenase [46], simultaneously leading to the formation of glutamate from α-ketoglutaric acid. GABA is generated from glutamine via glutamate in neurons. This leads to an accumulation of ammonia, which is transported to astrocytes through the lactate-alanine shuttle [35]. Increased inhibitory activity of GABA is suggested to be responsible for the reduction of brain activity in response to hypercapnia in other vertebrates [47, 48]. A reduced neuronal energy demand may lead to an increase of lactate, as this metabolite serves as the favoured energy resource in neurons and is provided through glycolytic activity in astrocytes as part of the lactate-alanine shuttle [35]. An increase of lactate was not detected at 8 °C under high CO_2 in *B. saida* indicating no surplus of energy substrates. Anaplerotic reactions to refuel the TCA cycle and subsequent oxidative decarboxylation may be crucial for energy supply in cold-adapted species, as these possess only low glycolytic capacity [40]. Strobel et al. found a CO_2-induced reduction in succinate dehydrogenase activity in *Notothenia rossii* [21] and proposed an increased utilization of glutamate and aspartate in order to increase proton consuming decarboxylation processes, thereby supporting pH maintenance. We did not observe an increase in succinate levels at 8 °C in the high CO_2 group of *B. saida*, indicating no inhibition of succinate dehydrogenase. However, glutamate und aspartate catabolism as well as GABA synthesis may still contribute to acid-base regulation.

MI increments at 8 °C in the high CO_2 group remain enigmatic, but may serve as an alternative osmolyte to NAA in order to maintain osmolarity during NAA utilization. As CO_2-induced behavioural alterations in *B. saida* were not affected by environmental temperature [9], we assume that the detected CO_2-dependent metabolic changes at 8 °C are not the primary physiological cause of the CO_2-induced behavioural alterations observed in this species. As an alternative explanation, CO_2-dependent metabolic changes observed in *B. saida* at 8 °C may rather be symptomatic for temperature-induced energy limitation at 8 °C which is further exacerbated by environmental hypercapnia. The upper temperature limit of 8 °C detected by Kunz et al. in the animals used in this study supports this hypothesis [22]. Whether the observed generation of glutamate and GABA serve the formation of utilizable energy metabolites, the reduction of proton concentrations or indicate metabolic depression in order to reduce neuronal energy expenditures remains to be explored.

Effect of CO_2 on brain metabolites in *Gadus morhua*

In *G. morhua*, a CO_2-induced increase of lactate was detected mainly at 8 °C with an additional significant interactive effect of temperature and CO_2. Further interactions of CO_2 and temperature-effects were observed for GABA and choline due to an increase at 8 °C in the high CO_2 group. These changes occurred together with a non-significant increase of alanine, succinate, glutamate and glycine, while NAA remained largely unaffected. Although these findings seem somewhat similar to those in *B. saida* at 8 °C, we nevertheless suggest a different physiological causality involving increased GABAergic activity with unimpaired neuronal energy status. Simultaneous increases of succinate, alanine, glutamate and GABA might indicate an increased GABAergic activity as discussed above for *B. saida*; however, in *G. morhua*, the increase of GABA was detected with a simultaneous rise of lactate indicating an increase in the availability of this energy substrate. NAA as a marker of energy limitation displayed a non-significant trend to increase under

high CO_2, further indicating unimpaired energy status of the brain. Release of GABA and acetylcholine is coupled in some brain regions indicating an additional regulatory function of the latter [49–51] and might explain the significantly increased choline concentration. Interestingly, the high CO_2 group of *G. morhua* at 8 °C not only differed from the high CO_2 groups at other temperatures, but further displayed slightly reduced behavioural CO_2-effects as discussed in Schmidt et al. [9]. Our results indicate that resilience against CO_2-induced behavioural changes may be greatest in the middle of the thermal window; however, the mechanism behind this observation remains obscure.

GABA-metabolism and its putative role for CO_2-resilience

The question arises how an increase of GABAergic activity might protect the behaviour of fish, if altered $GABA_A$-receptor activity is responsible for the behavioural impairments under OA scenarios [2]. At least in mammals, neurophysiological consequences of GABA release are quite flexible and an excitatory function of GABA through $GABA_A$-receptor activity is already observed under normal conditions in the central nervous system [52]. Additionally, whether GABA is excitatory or inhibitory is dependent not only on the electrochemical gradient, but also on the timing and location of GABA release with respect to former excitatory postsynaptic potentials [52]. Thus, electrochemical acclimation or spatiotemporal adjustments of GABA release could sustain regular neuronal processing even under increased CO_2.

One possible alternative explanation arises from the point of view that concentrations of tissue CO_2 might be lower in the centre of an animal's thermal window leading to increased resilience at optimum temperature. Studies allowing to address this hypothesis are scarce. Van Dijk et al. [53] found that CO_2 partial pressure in white muscle of *Zoarces viviparus* rises at temperatures below the thermal optimum for somatic growth of this species [54]. Experimental conditions may also have interfered as the seawater PCO_2 in the 8 °C high CO_2 group was slightly lower than in the other high CO_2 groups in *Gadus morhua* which should have led to a slightly lower tissue CO_2 partial pressure in animals of this specific treatment group. Nonetheless, animals under optimum temperature conditions may be able to maintain the inhibitory function of GABA even under increased CO_2. A simultaneous increase of GABAergic activity might lead to metabolic depression, as indicated by a simultaneous significant increase of lactate and GABA (Fig. 4a and c), and thus further reduction of tissue pCO_2 which might also increase relative resilience as ambient CO_2 rises. While the optimum temperature for growth in the animals used in this study is not known [22], Björnsson et al. found an optimum temperature of ~ 10 °C - 12 °C for Icelandic cod fed ad libitum at similar body weight [55, 56]. The optimum temperature of *G. morhua* depends on several factors including life stage, food supply and population [57]. Limited food supply reduces the optimum temperature for somatic growth [56]. Since animals were not fed ad libitum in this study, 8 °C may thus have been close enough to their thermal optimum. In further studies, blood pCO_2 of cod under increased CO_2 conditions should be investigated over a broad temperature range in order to test this hypothesis and to correlate the findings with their CO_2 induced behavioural alterations.

Conclusion

In conclusion, a temperature increase has similar physiological consequences in both, *Boreogadus saida* and *Gadus morhua* with strong temperature dependent alterations particularly among osmolytes, membrane components and metabolites involved in GABA metabolism. The long-term thermal limit of *B. saida* may have been reached at 8 °C, with energetic constraints exacerbated by an increase of environmental CO_2. In *G. morhua*, no temperature-dependent alteration of the same metabolites has been observed indicating that the upper temperature limit had not been reached at 16 °C. Also, higher environmental CO_2 did not elicit energetic limitation in *G. morhua*. Interestingly, we found a significant CO_2 effect at 8 °C with a CO_2-induced increase of lactate, GABA and choline which could be associated with increased behavioural resistance of *G. morhua* at this temperature. Our data indicate that *B. saida* is more strongly affected than *G. morhua* by the concomitant warming and CO_2 increase expected to occur in the polar ocean until the end of the twenty-first century. In areas where the distribution of these species overlap, *G. morhua* might thus be more resilient to future OWA-related physiological challenges and might out-compete *B. saida* in the long term.

Additional files

Additional file 1: Table S1. Group sizes and environmental CO_2 partial pressure (PCO_2, mean ± standard deviation after Kunz et al. [22]) of *Boreogadus saida* and Gadus morhua that were utilized for NMR and HPLC analysis. (XLSX 10 kb)

Additional file 2: Table S2. Protocol depicting the buffer composition throughout HPLC-analysis. Total measurement time per sample was 90 min. The first column shows the time for onset of the respective composition. (DOCX 11 kb)

Additional file 3: Table S3. List of treatment groups that violated normality-distribution for the respective components. (DOCX 13 kb)

Additional file 4: Figure S1. Boxplots depicting metabolite concentrations (relative to total creatine (tCr)) in the brain of *Boreogadus saida* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third

quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$). Metabolites were sorted functionally in accordance with Table 1. (PDF 176 kb)

Additional file 5: Figure S2. Boxplots depicting metabolite concentrations (s.a.) in the brain of *Gadus morhua* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$). Metabolites were sorted functionally in accordance with Table 1. (PDF 178 kb)

Additional file 6: Figure S3. Boxplots depicting the amount of 5-Hydroxyindoleacetic acid (HIAA) relative to Serotonin (5-HT) in the brain of *Boreogadus saida* (A) and *Gadus morhua* (B) quantified with HPLC. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$). (PDF 19 kb)

Additional file 7: Figure S4. Non-metric multidimensional scaling of metabolite/total creatine ratios in the brain of *Boreogadus saida*. Dots indicate individuals with colours representing the respective treatment temperature. Blue = 0 °C, green = 3 °C, red = 6 °C, yellow = 8 °C. (TIFF 937 kb)

Additional file 8: Figure S5. Non-metric multidimensional scaling of metabolite/total creatine ratios in the brain of *Gadus morhua*. Dots indicate individuals with colours representing the respective treatment temperature. Blue = 3 °C, green = 8 °C, red = 12 °C, yellow = 16 °C. (TIFF 937 kb)

Additional file 9: Raw data. (XLSX 30 kb)

Acknowledgements
The authors are grateful to Jasmine Nahrgang (funded by the Polarisation grant of the Norwegian Research Council, no. 214184/F20) for providing us with the specimens of *B. saida*. Animal incubations were significantly supported by Silvia Hardenberg, Guido Krieten, Nils Koschnick, Sebastian Berger, Kristina Kunz and Elettra Leo. We want to thank Karim Zanaty, Marcel Machnik, Benjamin Matthei, Fredy Vèliz Moraleda, Anette Tillmann, Isabel Ketelsen and Timo Hirse for measurements of DIC, pH, salinity and temperature throughout the incubation periods. Stephan Frickenhaus provided practical support for the multivariate analysis, his contribution is deeply appreciated.

Funding
This study was funded by the Federal Ministry of Education and Research (BMBF, Funding no. FKZ 03F0655B), Germany.

Availability of data and materials
The datasets acquired in this study are available in the Additional file 9: Raw data.

Authors' contributions
MS, HP, HW, CB and DS developed the study design. MS conducted the incubation of the animals. Brains were sampled by MS and HW. SS conducted the NMR measurements with assistance of CB and MS. MS did the HPLC measurements with support of KL. Data analysis and statistical evaluation were performed by MS. All authors contributed to the interpretation of the results. MS drafted the manuscript which was subsequently edited by SS, KL, HW, HP, CB, and DS. All authors read and approved the final manuscript.

Ethics approval
The experiments conducted were in accordance with the ethical standards of the federal state of Bremen, Germany, and were approved under reference number 522–27-11/02–00 (93).

Consent for publication
All authors gave final approval for publication.

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

Publisher's Note

Author details
[1]Alfred-Wegener-Institute Helmholtz-Centre for Polar- and Marine Research, Section Integrative Ecophysiology, Am Handelshafen 12, 27570 Bremerhaven, Germany. [2]Department of Biology/Chemistry, University of Bremen, PO 330440, 28334 Bremen, Germany. [3]Institute for Cell Biology and Zoology, Heinrich Heine University, Universitätsstraße 1, 40225 Düsseldorf, Germany. [4]Alfred-Wegener-Institute Helmholtz-Centre for Polar- and Marine Research, Section Chemical Ecology, Am Handelshafen 12, 27570 Bremerhaven, Germany.

Received: 2 August 2017 Accepted: 13 October 2017
Published online: 30 October 2017

References
1. Heuer RM, Grosell M. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am J Physiol Regul Integr Comp Physiol. 2014; 307:R1061.
2. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson S-A, Munday PL. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nat Clim Change. 2012;2:201–4.
3. Hamilton TJ, Holcombe A, Tresguerres M. CO2-induced ocean acidification increases anxiety in rockfish via alteration of GABAA receptor functioning. Proc R Soc Lond B Biol Sci. 2013;281:20132509.
4. Chung W-S, Marshall NJ, Watson S-A, Munday PL, Nilsson GE. Ocean acidification slows retinal function in a damselfish through interference with GABAA receptors. J Exp Biol. 2014;217:323–6.
5. Heuer RM, Welch MJ, Rummer JL, Munday PL, Grosell M. Altered brain ion gradients following compensation for elevated CO2 are linked to behavioural alterations in a coral reef fish. Sci Rep. 2016;6:33216.
6. Jutfelt F, Hedgärde M. Juvenile Atlantic cod behavior appears robust to near-future CO2 levels. Front Zool. 2015;12:1–7.
7. Sundin J, Jutfelt F. 9–28 d of exposure to elevated pCO2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (Ctenolabrus Rupestris). ICES J Marine Sci. 2016; 73:620–32.
8. Jutfelt F, Hedgärde M. Atlantic cod actively avoid CO2 and predator odour, even after long-term CO2 exposure. Front Zool. 2013;10:81.
9. Schmidt M, Gerlach G, Leo E, Kunz KL, Swoboda S, Pörtner HO, Bock C, Storch D. Impact of ocean warming and acidification on the behaviour of two co-occurring gadid species, Boreogadus Saida and Gadus Morhua, from Svalbard. Mar Ecol Prog Ser. 2017;571:183–91.
10. Renaud P, Berge J, Varpe Ø, Lønne O, Nahrgang J, Ottesen C, Hallanger I. Is the poleward expansion by Atlantic cod and haddock threatening native polar cod, Boreogadus Saida? Polar Biol. 2012;35:401–12.
11. Beierlein L, Salvigsen O, Schöne BR, Mackensen A, Brey T. The seasonal water temperature cycle in the Arctic Dicksonfjord (Svalbard) during the Holocene climate optimum derived from sub-fossil Arctica Islandica shells. The Holocene. 2015;25:1197–207.
12. IPCC. Climate change 2013: the physical science basis. Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. Cambridge and New York: Cambridge University Press; 2013. p. 1535.
13. Kjesbu OS, Bogstad B, Devine JA, Gjøsæter H, Howell D, Ingvaldsen RB, Nash RDM, Skjæraasen JE. Synergies between climate and management for Atlantic cod fisheries at high latitudes. Proc Natl Acad Sci. 2014;111:3478–83.
14. Welch HE, Crawford RE, Hop H: Occurrence of arctic cod (*Boreogadus saida*) schools and their vulnerability to predation in the Canadian high arctic. Arctic. 1993;46:331-9.
15. Harter BB, Elliott KH, Divoky GJ, Davoren GK. Arctic cod (Boreogadus Saida) as prey: fish length-energetics relationships in the Beaufort Sea and Hudson Bay. Arctic. 2013;66:191–6.
16. Nowicki JP, Miller GM, Munday PL. Interactive effects of elevated temperature and CO2 on foraging behavior of juvenile coral reef fish. J Exp Mar Biol Ecol. 2012;412:46–51.
17. Domenici P, Allan BJM, Watson S-A, McCormick MI, Munday PL. Shifting from right to left: the combined effect of elevated CO2 and temperature on Behavioural lateralization in a coral reef fish. PLoS One. 2014;9:e87969.

18. Ferrari MCO, Munday PL, Rummer JL, McCormick MI, Corkill K, Watson S-A, Allan BJM, Meekan MG, Chivers DP. Interactive effects of ocean acidification and rising sea temperatures alter predation rate and predator selectivity in reef fish communities. Glob Chang Biol. 2015;21:1848–55.
19. Winberg S, Nilsson GE. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. Comp Biochem Physiol C: Pharmacol Toxicol Endocrinol. 1993;106:597–614.
20. Leo E, Kunz KL, Schmidt M, Storch D, Pörtner H-O, Mark FC. Mitochondrial acclimation potential to ocean acidification and warming of polar cod (Boreogadus Saida) and Atlantic cod (Gadus Morhua). Front Zool. 2017;14:21.
21. Strobel A, Graeve M, Poertner HO, Mark FC. Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic Nototheniid fish, Notothenia Rossii and Lepidonotothen Squamifrons. PLoS One. 2013;8:e68865.
22. Kunz KL, Frickenhaus S, Hardenberg S, Johansen T, Leo E, Pörtner H-O, Schmidt M, Windisch HS, Knust R, Mark FC. 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 Biol. 2016;39:1137–53.
23. Le Belle JE, Harris NG, Williams SR, Bhakoo KK. A comparison of cell and tissue extraction techniques using high-resolution 1H-NMR spectroscopy. NMR Biomed. 2002;15:37–44.
24. Hernandes MS, Troncone LRP. Glycine as a neurotransmitter in the forebrain: a short review. J Neural Transm. 2009;116:1551–60.
25. Yoshitake T, Kehr J, Yoshitake S, Fujino K, Nohta H, Yamaguchi M. Determination of serotonin, noradrenaline, dopamine and their metabolites in rat brain extracts and microdialysis samples by column liquid chromatography with fluorescence detection following derivatization with benzylamine and 1,2-diphenylethylenediamine. J Chromatogr B. 2004;807:177–83.
26. Topic Popovic N, Strunjak-Perovic I, Coz-Rakovac R, Barisic J, Jadan M, Persin Berakovic A, Sauerborn Klobucar R. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. J Appl Ichthyol. 2012;28:553–64.
27. Wagner KA, Woodley CM, Seaburg AG, Skalski JR, Eppard MB. Physiological stress responses to prolonged exposure to MS-222 and surgical implantation in juvenile Chinook Salmon. N Am J Fish Manag. 2014;34:863–73.
28. Treberg JR, Wilson CE, Richards RC, Ewart KV, Driedzic WR. The freeze-avoidance response of smelt Osmerus Mordax. J Exp Biol. 2002;205:1419–27.
29. Vesala L, Salminen TS, Koštál V, Zahradníčková H, Hoikkala A. Myo-inositol as a main metabolite in overwintering flies: seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. J Exp Biol. 2012;215:2891–7.
30. Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol. 2005;208:2819–30.
31. Scholander PF, van Dam L, Kanwisher JW, Hammel HT, Gordon MS. Supercooling and osmoregulation in arctic fish. J Cell Comp Physiol. 1957;49:5–24.
32. Baslow M, Guilfoyle D. N-acetyl-l-histidine, a prominent biomolecule in brain and eye of Poikilothermic vertebrates. Biomol Ther. 2015;5:635.
33. Gullans SR, Verbalis JG. Control of brain volume during hyperosmolar and hypoosmolar conditions. Annu Rev Med. 1993;44:289–301.
34. Windisch HS, Frickenhaus S, John U, Knust R, Pörtner HO, Lucassen M. Stress response or beneficial temperature acclimation: transcriptomic signatures in Antarctic fish (Pachycara Brachycephalum). Mol Ecol. 2014;23:3469–82.
35. Bak LK, Schousboe A, Waagepetersen HS. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem. 2006;98:641–53.
36. Leite RA, Otaduy MC, Silva GE, Ferreira ML, Aragão Mde F. Diagnostic methods for extra-temporal neocortical focal epilepsies: present and future. Arq Neuropsiquiatr. 2010;68:119–26.
37. Seiler N, Al-Therib MJ. Putrescine catabolism in mammalian brain. Biochem J. 1974;144:29–35.
38. Schuber F. Influence of polyamines on membrane functions. Biochem J. 1989;260:1–10.
39. D'Adamo AF, Yatsu FM. Acetate metabolism in the nervous system. N-acetyl-l-aspartic acid and the biosynthesis of brain lipids. J Neurochem. 1966;13:961–5.
40. Pörtner H, Lucassen M, Storch D. Metabolic biochemistry: its role in thermal tolerance and in the capacities of physiological and ecological function. Fish Physiology. 2005;22:79.
41. Boeck G, Nilsson G, Vlaeminck A, Blust R. Central monoaminergic responses to salinity and temperature rises in common carp. J Exp Biol. 1996;199:1605–11.
42. Sebert P, Barthelemy L, Caroff J. Serotonin levels in fish brain: effects of hydrostatic pressure and water temperature. Experientia. 1985;41:1429–30.
43. Fritsche R, Thomas S, Perry SF. Effects of serotonin on circulation and respiration in the rainbow trout Oncorhynchus Mykiss. J Exp Biol. 1992;173:59–73.
44. Moffett JR, Ross B, Arun P, Madhavarao CN, Namboodiri MAA. N-Acetylaspartate in the CNS: from Neurodiagnostics to neurobiology. Prog Neurobiol. 2007;81:89–131.
45. Starai VJ, Escalante-Semerena JC. Acetyl-coenzyme a synthetase (AMP forming). Cell Mol Life Sci. 2004;61:2020–30.
46. Van Der Laan JW, Boer TD, Brunivels J. DT-n-propylacetate and GABA degradation. Preferential inhibition of succinic semialdehyde dehydrogenase and indirect inhibition of GABA-transaminase. J Neurochem. 1979;32:1769–80.
47. Weyne J, Van Leuven F, Kazemi H, Leusen I. Selected brain amino acids and ammonium during chronic hypercapnia in conscious rats. J Appl Physiol. 1978;44:333–9.
48. Weyne J, Van Leuven F, Demeester G, Leusen I. Metabolism of glutamic acid and related amino acids in the brain studied with 14C-labelled glucose, butyric acid and glutamic acid in hypercapnic rats. J Neurochem. 1977;29:469–76.
49. Bonanno G, Raiteri M. GABA enhances acetylcholine release from hippocampal nerve endings through a mechanism blocked by a GABA uptake inhibitor. Neurosci Lett. 1986;70:360–3.
50. Pittaluga A, Raiteri M. Choline increases endogenous GABA release in rat hippocampus by a mechanism sensitive to hemicholinium-3. Naunyn Schmiedeberg's Arch Pharmacol. 1987;336:327–31.
51. Hasselmo ME, Fehlau BP. Differences in time course of ACh and GABA modulation of excitatory synaptic potentials in slices of rat hippocampus. J Neurophysiol. 2001;86:1792–802.
52. Gulledge AT, Stuart GJ. Excitatory actions of GABA in the cortex. Neuron. 2003;37:299–309.
53. Van Dijk PL, Hardewig I, Pörtner HO. Temperature-dependent shift of pHi in fish white muscle: contributions of passive and active processes. Am J Phys. 1997;272:R84–9.
54. Zakhartsev MV, De Wachter B, Sartoris FJ, Portner HO, Blust R. Thermal physiology of the common eelpout (Zoarces Viviparus). J Comp Physiol B. 2003;173:365–78.
55. Björnsson B, Steinarsson A, Árnason T. Growth model for Atlantic cod (Gadus Morhua): effects of temperature and body weight on growth rate. Aquaculture. 2007;271:216–26.
56. Bjornsson B. Optimal temperature for growth and feed conversion of immature cod (Gadus Morhua L.). ICES J Mar Sci. 2001;58:29–38.
57. Otterlei E, Nyhammer G, Folkvord A, Stefansson SO. Temperature- and size-dependent growth of larval and early juvenile Atlantic cod (Gadus Morhua): a comparative study of Norwegian coastal cod and northeast Arctic cod. Can J Fish Aquat Sci. 1999;56:2099–111.
58. Klein J. Membrane breakdown in acute and chronic neurodegeneration: focus on choline-containing phospholipids. J Neural Transm. 2000;107:1027–63.

2.4 Manuscript III

Matthias Schmidt[1,2], Hans-Otto Pörtner[1,2], Daniela Storch[1], Christian Bock[1]

Adjustments of the cardiovascular and acid-base system of Polar cod, *Boreogadus saida*, under elevated CO_2 - An *in vivo* magnetic resonance study

Manuscript

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

[2]University of Bremen, Department of Biology/Chemistry, PO 330440; 28334 Bremen, Germany

Adjustments of the cardiovascular and acid-base system of Polar cod, *Boreogadus saida*, under elevated CO_2 - An *in vivo* magnetic resonance study

Matthias Schmidt[1,2], Hans-Otto Pörtner[1,2], Daniela Storch[1], Christian Bock[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, Department of Biology/Chemistry, PO 330440; 28334 Bremen, Germany

*Corresponding author

Dr. Christian Bock

Integrative Ecophysiology

Alfred-Wegener-Institute Helmholtz-Center for Polar- and Marine Sciences

Am Handelshafen 12,

27570 Bremerhaven, Germany

E-Mail: Christian.Bock@awi.de

Phone-no.: +49(471)4831-2488

Abstract

Polar cod (*Boreogadus saida*) inhabiting the Arctic Ocean will experience man-made ocean acidification in the near future and has been shown to be behaviourally sensitive to a mild increase of environmental CO_2 which is projected for the end of 21st century. We conducted an *in vivo* MRI study combined with ^{31}P-NMR spectroscopy on *B. saida* in order to observe the effect of an acute mild increase of environmental CO_2 (1668 ± 250 µatm) on ventilation frequency, vascular blood flow and acid-base status in the head of Polar cod for four days. We observed a significant increase of ventilation frequency, which stabilized after one day under hypercapnic conditions. Arterial and venous blood flow both decreased significantly with the increase of environmental CO_2 and were not restored to control values during the observation period. A slight reduction of intracellular pH with the onset of high CO_2 conditions remained insignificant. This study indicates that a rise of environmental CO_2 might lead to long lasting effects in the cardiovascular system of *B. saida*, with unclear long-term implications for the fitness of this species in a warmer, more acidified future ocean.

Keywords: Ocean acidification, Hypercapnia, Ventilation, Cardiovascular system, Acid-base regulation

Introduction

Since the beginning of the industrial revolution has a large fraction of anthropogenic emitted CO_2 been absorbed by the oceans, leading to the formation of carbonic acid and subsequent ocean acidification (1). The progressively increasing seaweater CO_2 content has been shown to elicit behavioural alterations in marine teleost species (2). While observed effects were strongest in tropical species, new research found behavioural changes also in *Boreogadus saida*, a cold water adapted species which inhabits the Arctic Ocean (3). It is suggested that behavioural alterations are caused by a shift in the electrochemical gradient in GABAergic neurons, which arises through accumulation of extra- and intracellular bicarbonate during compensation for hypercapnia-induced intra- and extracellular respiratory acidosis (4, 5). In teleosts, an acute increase of CO_2 partial pressure can affect also other physiological parameters involved in acid-base regulation, in oxygen supply through modification of the hemoglobin oxygen binding curve as well as in cardiocirculatory performance (6, 7). In this study, we investigated to what extent such physiological alterations occur in CO_2-sensitive *B. saida* under a mild and acute increase of environmental CO_2 using *in vivo* magnetic resonance imaging combined with ^{31}P-NMR spectroscopy. In order to test whether acid-base regulation or cardiovascular functioning of *B. saida* are sensitive to minor increases of environmental CO_2, we focused on CO_2-induced acute changes of breathing rate, arterial and venous blood velocity as well as intracellular pH. These variables were analyzed in the experimental animals for three subsequent days in order to detect the onset of compensatory processes.

MATERIAL AND METHODS

Specimen collection and maintenance

Juvenile specimen of *B. saida* were caught on a polar night trawl in the inner Kongsfjord (78.97 N, 12.51 E). They were initially kept in the Tromsø Aquaculture Research Station in Kårvik, Norway and were subsequently transported to the Alfred Wegener Institute in Bremerhaven, Germany, where they were housed at 5 °C and ambient CO_2 before experimentation. Detailed

information on specimen collection and housing prior to experimentation has been provided by Kunz *et al.* (8).

Seawater manipulation and carbonate chemistry

A 4-channel MKS GSV-19 system was used for generation of low and high CO_2 partial pressures (Pco_2) in seawater. Two seawater reservoirs were prepared, one with low (Control Pco_2, 252 ± 100 µatm), and the other with high (1668 ± 250 µatm) CO_2 concentration. Control seawater Pco_2 was set slightly below current atmospheric CO_2 partial pressure of about 400 µatm in order to compensate for a possible minor accumulation of CO_2 inside the Perspex chamber in which the animal was placed for experimentation. The seawater carbonate chemistry was determined three times for each animal: first under control conditions, second directly after the onset of high CO_2 conditions and third on the last day of the experiments. Carbonate chemistry parameters were determined as described in Schmidt *et al.* (9). Means ± SD of carbonate chemistry parameters are available in Table 1 of the electronic supplementary material (ESM).

Animal fixation and experimental setup

Four juvenile animals were briefly sedated in 200 mg/l Tricaine methanesulfonate (MS222) and fixed inside a Perspex chamber (length = 408 mm, inner diameter = 100 mm) with one flattened end (180 mm length) for placement of a surface coil (Figure 1). Clamps with two diagonal and one horizontal slide were used to restrict animal movement and the fixed animal was placed with the head under the flattened end of the chamber. The chamber was closed and a 20 mm $^{1}H/^{31}P$ surface coil was fixed to the outside of the chamber above the animals` head and neck. The chamber was connected to a low-CO_2 seawater circle with a temperature of 3 ± 1 °C and was afterwards placed inside the bore of a 4.7 Tesla animal scanner (Biospec 47/40 DBX, Bruker BioSpin MRI GmbH, Ettlingen, Germany) containing a gradient system with Imax = 100 A, Umax = 160 V and 50 mT/m (BGA 12, Bruker BioSpin GmbH, Ettlingen, Germany). The bore was closed on both ends with plastic foam slides to reduce illumination. The water flow through the

chamber was set to 300-400 ml/min. Experiments started ~ 24 hours after the animals` restriction inside the chamber in order to enable recovery from sedation.

Magnetic resonance imaging and spectroscopy

The experimental protocol was as follows: After the acclimation period, vascular blood flow, intracellular pH and ventilation rate were measured at least three times under control CO_2. Afterwards, the seaweater supply was switched to high CO_2 conditions (Day 1). Measurements of vascular blood flow, intracellular pH and ventilation rate were continuously acquired for the next three days (Day 2-4) and the experiment was conducted until the end of Day 4. The experiment was terminated for one animal on Day 2 and for a second animal in the morning of Day 4 for technical reasons. For each animal, the median for each day was calculated for vascular blood flow, intracellular pH and ventilation rate.

Ventilation rate

The ventilation of an animal was determined from coronal Intragate FLASH images of the gills with a flip angle of 45°; hermite pulse shape (pulse length: 1.0 ms; pulse width: 5,400 Hz); repetition time (TR): 8.0 ms; echo time (TE): 3.1 ms; field of view (FOV) 60x60 mm; slice thickness: 2 mm; number of averages (NA): 64; matrix 256x256 pixel; scan duration: 1 minute and 7 seconds.

Vascular blood flow

Prior to quantification of the vascular blood flow, a 2D Angio with 40 slices was performed (FC2DAngio) with the following scan parameters: flip angle: 60°; gauss pulse shape (pulse length: 1.7 ms); TR: 19.27 ms; TE: 8.1 ms; NA: 2; FOV: 80x60 mm; slice thickness: 1 mm; matrix: 256x192 pixel; scan duration: 4 minutes and 55 seconds. Afterwards, vascular blood flow was determined through velocity mapping. The slice was placed 3 ± 2 mm caudal to formation of the single dorsal aorta out of the paired dorsal aortae returning from the respiratory tract. Velocity

mapping was conducted via flow compensated FLASH imaging (Flowmap-Velocity mapping) using a flip angle of 30°; hermite pulse shape (pulse length: 1.8 ms; pulse width: 3,000 Hz) a minimum flow rate of 0.4 cm/s (velocity encoding); TR: 29.52 ms; TE: 11 ms; NA: 8; FOV: 70x70 mm; slice thickness: 1 mm; matrix: 512x256 pixel; scan duration: 1 minute and 30 seconds. Regions of interest (ROIs) were manually placed around the dorsal aorta and caudal vein. The mean velocity calculated from each pixel within the ROIs was later used for statistical analysis. Figure 2 displays an exemplary anatomical image (Figure 2 A) with the corresponding velocity map, showing the ROIs (Figure 2 B).

Intracellular pH

Intracellular pH was calculated from *in vivo* ^{31}P-NMR spectra (NSPECT) acquired with a flip angle of 60°, Bp 32 pulse shape (pulse length: 200 µs; pulse width: 6,400 Hz); TR: 1.4 s; NA: 800; scan duration: 18 minutes and 40 seconds. Intracellular pH was determined through the chemical shift difference between phosphocreatine and free phosphate using the formula after Kost (10) with buffer values taken from Pörtner (11). As the intracellular concentration of phosphate is considered much greater than the extracellular concentration it can be assumed that the detected phosphate signal and thus the measured pH represents intracellular conditions (see basic physiology considerations; for comparison: plasma phosphate was measured in Tilapia by van Waarde *et al*. (~1.2 mmol/L, (12)) and intracellular phosphate in epithelial cells of brown trout by Morgan *et al.* (~88 mmol/L, (13))). Signal peaks were fitted using an in-house custom designed software (CTpv, (14)).

Statistical analysis

For each animal, the median for each day was calculated for vascular blood flow, intracellular pH and ventilation rate. Missing values were filled through mean values of the remaining animals in order to enable a subsequent nonparametric Friedman test (α=0.05) for repeated measures. Post-hoc analysis was not conducted due to low sample size and thus low statistical power.

RESULTS

An escape response of the animals was visible particularly right after fixation inside the Perspex chamber, but diminished during the 24 h pre-experimental period. Nevertheless, some animal motion was observed also during the experimental procedure. In order to reduce the influence of movement on the results, we excluded those MRI measurements that showed movement artifacts from the dataset. Peak locations of creatine phosphate and free phosphate were checked and corrected manually.

The ventilation rate increased on Day 1 after onset of high CO_2 conditions (Figure 3, $p < 0.01$) and normalized from Day 2 onwards. Arterial and venous blood flow were both significantly dependent on CO_2 conditions (Figure 4 A and B respectively, $p < 0.01$ each) with velocities decreasing one to two days after the onset of high CO_2 conditions. The decrease of arterial and venous blood velocity lasted until the end of incubation. However, the venous blood velocity showed a trend for some recovery on Day 4. A non-significant reduction of intracellular pH was observed under high CO_2-conditions (Figure 5, $p = 0.479$).

DISCUSSION

The here presented set-up successfully enabled the simultaneous observation of ventilation rate, blood flow and intracellular pH in an *in vivo* experimental design during manipulation of environmental conditions. Velocity-mapping could be conducted reliably despite flow artefacts of the surrounding sea water, since these artefacts did not overlap with those vessels chosen for the quantification of blood flow. While Intragate is used in human for cardiac visualization and beating frequency, the cine movie created by this tool confirmed that Intragate can be used to measure the frequency of ventilation in fish. Localization of the acquired ^{31}P-spectra was conducted through placement of the surface coil and was verified through reference images. Thus, partial volume effects contribute to the detected ^{31}P signal and the observation of intracellular pH refers not only to the brain, but also to closely surrounding other tissues.

An increase of environmental CO_2 leads to a smaller CO_2 gradient between the animal and surrounding sea water causing a reduced CO_2 excretion and thus CO_2-accumulation in intra- and extracellular compartments where it dissociates into bicarbonate and protons (5). While in this study an increase of environmental CO_2 did not significantly reduce intracellular pH, this may be different, at least on a short term scale, for extracellular pH, as the extracellular compartment contains only ~1/5 of effective non-bicarbonate buffer systems of the intracellular compartment (15).

We detected first a rise of the ventilation frequency after onset of environmental hypercapnia, but a normalization within one day. In teleosts, the ventilation frequency is mainly dependent on the availability of oxygen, however some species also show a higher ventilation frequency after increase of environmental CO_2 (7). While CO_2-induced ventilation leads to rapid reduction of the CO_2 partial pressure in mammals, such a mechanism is less efficient in aquatic animals, since the CO_2 gradient between animal and surrounding water is much lower to start with than in air breathing animals. Therefore, aquatic animals that regulate their intra- and extracellular pH rely strongly on other mechanisms such as active extrusion of protons over gill tissue or accumulation of bicarbonate (5). In contrast to mammals which possess central CO_2 sensory neurons in the medulla oblongata, teleosts exhibit neuroepithelial cells in their gills that enable them to detect alterations of CO_2 in their environment (16). Atlantic cod (*Gadus morhua*) display a strong aversive behaviour towards water with increased CO_2 partial pressure, even during a mild increase of environmental CO_2 (17). Thus, one might suggest that exposure to high CO_2 conditions might also serve as an aversive stimulus, possibly leading to an increased release of catecholamines. However, circulating catecholamines may not directly be involved in the regulation of ventilation (18) and might thus only play a minor role in this respect.

The cardiovascular response to increased environmental CO_2 is species-specific. For example, a hypercapnia-induced increased sympathetic activity in rainbow trout affects peripheral resistance and blood pressure, ventricular stroke volume and heartbeat frequency, either directly through sympathetic nerve endings, or indirectly through catecholamines (19, 20). Severe hypercapnia of up to 15% CO_2 is known to reduce the cardiac output in *Gadus morhua* through a negative inotrope effect of excessive H^+ ions (21), which is however unlikely to contribute to the results observed in this study, since the onset of the mild hypercapnic

conditions did not lead to a significant reduction of intracellular pH. All of those cardiovascular parameters mentioned above have the potential to alter the arterial and venous blood flow, and the results presented in this study indicate that the circulatory system of *B. saida* might be affected by a mild increase of environmental CO_2 even though we were not able to determine the exact origin of this observation. Also, whether CO_2-induced alterations in the cardiovascular system contribute to the behavioural sensitivity of this species to future ocean acidification conditions remains to be investigated. Recent physiological studies analyzed the effect of future climate change scenarios on behaviour, energy metabolism and aerobic performance of *B. saida* in order to predict the fate of this species in the future polar oceans (3, 8, 22, 23). So far, these studies indicate that *B. saida* will experience impairments in all of these parameters in a warmer, more acidified environment that are only incompletely compensated for even after several months of exposure period. This short term study indicates that the cardiovascular system may be involved in these challenges. Adaptation may occur on multi-generational timescales, which is however difficult to assess in cold water species with slow growth and reproduction rates.

Ethical approval

The experiments conducted were in accordance with the ethical standards of the federal state of Bremen, Germany, and were approved under reference number 522-27-11/02-00 (93).

Data accessibility

The datasets acquired in this study are available in the ESM.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors` contributions

MS and CB designed the experiment. MS conducted the incubations and measurements. Data analysis and statistical evaluation were performed by MS. All authors contributed to the interpretation of the results. MS wrote the manuscript which was subsequently drafted by CB, DS and HP. All authors gave final approval for publication.

Acknowledgements

This study was funded by the Federal Ministry of Education and Research (BMBF, Funding no. FKZ 03F0655B), Germany. Jasmine Nahrgang (funding by Polarisation grant no. 214184/F20 of the Norwegian Research Council,) contributed the specimens of *B. saida* to the experiments. The authors want to thank Anette Tillmann for measurements of carbonate chemistry throughout the incubation periods and Rolf Wittig for his contribution to the post processing of intracellular pH raw data.

Figures

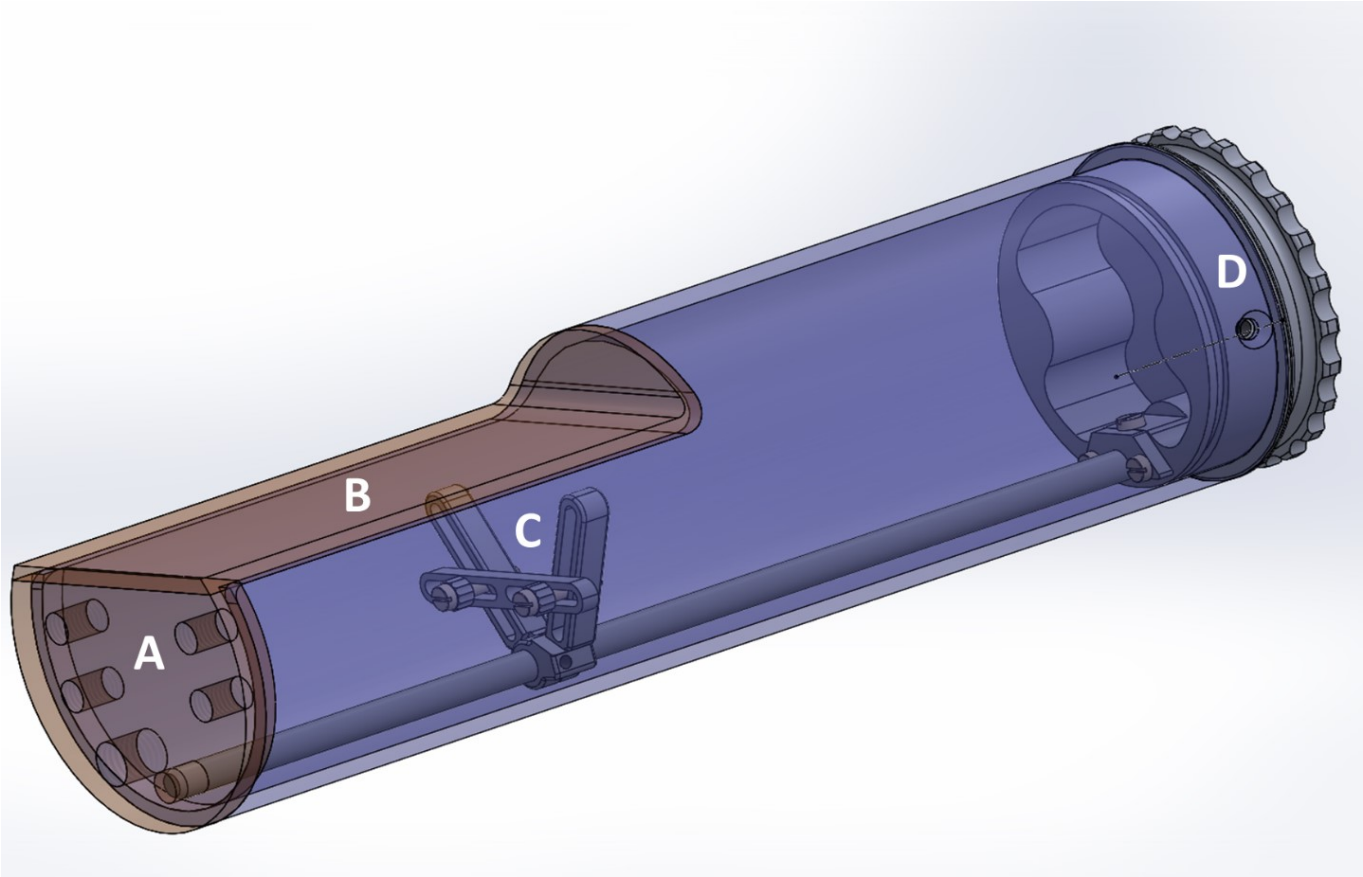


Figure 1: Perspex chamber for animal fixation throughout experimentation. A: Anterior end with boreholes for water inflow and a glass fiber thermometer. B: Flattened end of the fixation chamber on which a 20 mm $^{1}H/^{31}P$ surface coil was placed. The animal was restricted in movement through several clamps with two vertical and one horizontal Perspex slides (C, only one clamp demonstrated for clarity means). D: posterior end of the fixation chamber with a boreholes for a lateral fixation screw and for water outflow (not visible from this perspective).

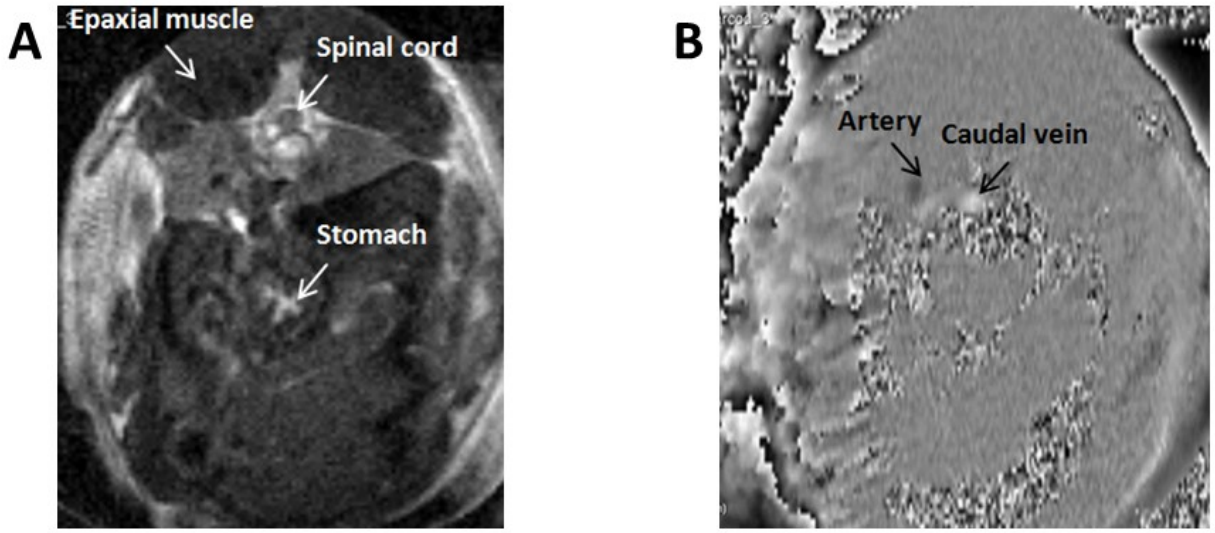


Figure 2: Anatomic image (A) and corresponding flow compensated FLASH image (Velocitymap, B) as used for quantification of vascular blood flow. Dark grey scales in B indicate flow in caudal direction, bright grey scales in anterior direction.

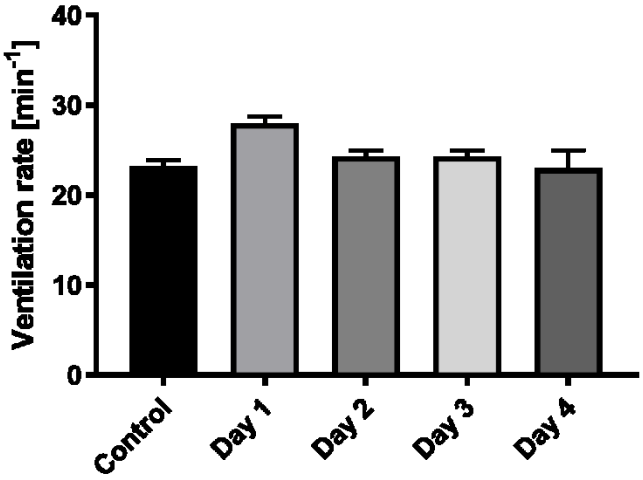


Figure 3: Ventilation rate of *Boreogadus saida* under environmental (Control, *252 ± 100* µatm CO_2) and increased CO_2 conditions (Day 1-4, *1668 ± 250* µatm CO_2). Means ± s.e.m. are depicted. n = 4 for the Control and Day 1, n = 3 for Day 2 and Day 3, n = 2 for Day 4.

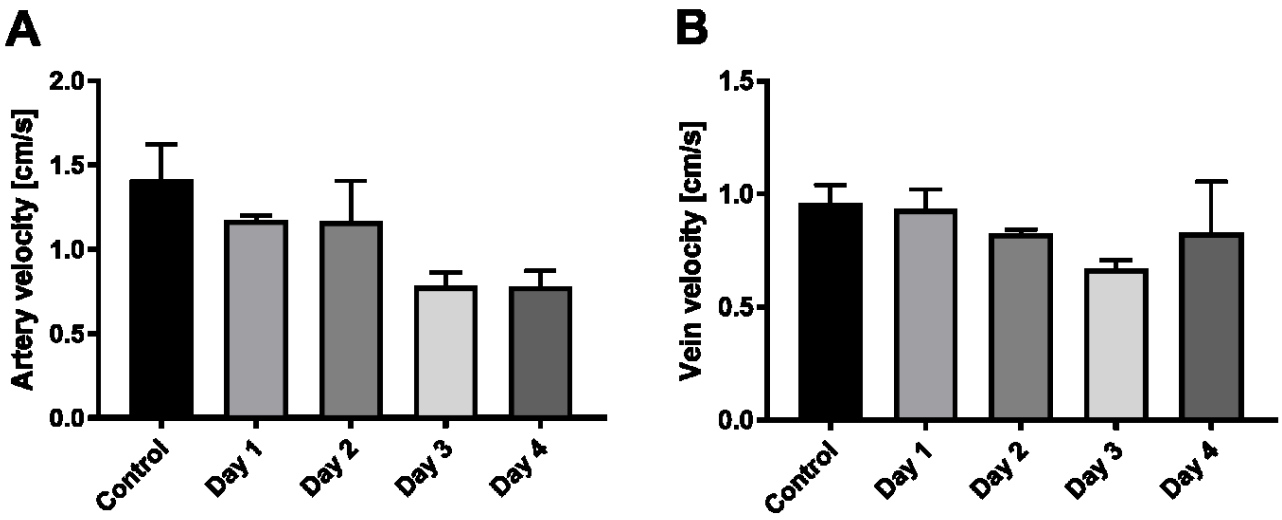


Figure 4: Arterial (A) and venous (B) blood velocity of *Boreogadus saida* under environmental (Control, *252 ± 100* µatm CO_2) and increased CO_2 conditions (Day 1-4, *1668 ± 250* µatm CO_2). Means ± s.e.m. are depicted. n = 4 for the Control and Day 1, n = 3 for Day 2 and Day 3, n = 2 for Day 4.

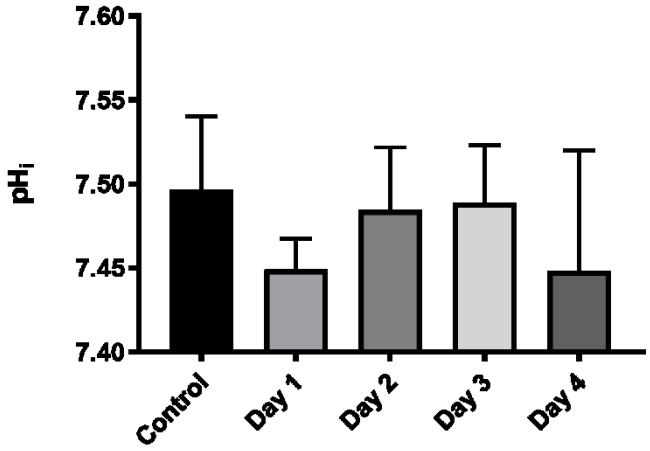


Figure 5: Intracellular pH in cranial tissues of *Boreogadus saida* under environmental (Control, 252 ± 100 µatm CO_2) and increased CO_2 conditions (Day 1-4, 1668 ± 250 µatm CO_2). Means ± s.e.m. are depicted. n = 4 for the Control and Day 1, n = 3 for Day 2 and Day 3, n = 2 for Day 4.

References

1. IPCC. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, 2013 ISBN 978-1-107-66182-0.

2. Heuer RM, Grosell M. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 2014;307(9):R1061.

3. Schmidt M, Gerlach G, Leo E, Kunz KL, Swoboda S, Pörtner HO, et al. 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. 2017;571:183-91.

4. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson S-A, et al. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nature Clim Change. 2012;2(3):201-4.

5. Tresguerres M, Hamilton TJ. Acid–base physiology, neurobiology and behaviour in relation to CO_2-induced ocean acidification. The Journal of Experimental Biology. 2017;220(12):2136.

6. Ishimatsu A. Physiological effects on fishes in a high-CO_2 world. Journal of Geophysical Research. 2005;110(C9).

7. Pörtner HO. Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view. Marine Ecology Progress Series. 2008;373:203-17.

8. Kunz KL, Frickenhaus S, Hardenberg S, Johansen T, Leo E, Pörtner H-O, et al. 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. 2016:1-17.

9. Schmidt M, Leo E, Kunz KL, Lucassen M, Windisch HS, Storch D, et al. (Table 1 + Table 2) Time series of seawater carbonate chemistry calculated throughout incubation periods of

Boreogadus saida and Gadus morhua during exposure to different CO2 and temperature conditions. In supplement to: Kunz, Kristina Lore; Frickenhaus, Stephan; Hardenberg, Silvia; Torild, Johansen; Leo, Elettra; Pörtner, Hans-Otto; Schmidt, Matthias; Windisch, Heidrun Sigrid; Knust, Rainer; Mark, Felix Christopher (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, 39(6), 1137-1153, https://doiorg/101007/s00300-016-1932-z: PANGAEA; 2016.

10. Kost GJ. pH standardization for phosphorus-31 magnetic resonance heart spectroscopy at different temperatures. Magnetic Resonance in Medicine. 1990;14(3):496-506.

11. Pörtner HO. Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. Respiration Physiology. 1990;81(2):275-88.

12. Van Waarde A, Van Dijk P, Van Den Thillart G, Verhagen M, Erkelens C, Bonga SW, et al. 31P-NMR Studies on Acid-Base Balance and Energy Metabolism of Acid-Exposed Fish. The Journal of Experimental Biology. 1990;154(1):223.

13. Morgan I, Potts W, Oates K. Intracellular ion concentrations in branchial epithelial cells of Bown trout (*Salmo trutta* L.) determined by X-ray microanalysis. J Exp Biol. 1994;194(1):139-51.

14. Bock C, Sartoris FJ, Wittig RM, Pörtner HO. Temperature-dependent pH regulation in stenothermal Antarctic and eurythermal temperate eelpout (Zoarcidae): an *in-vivo* NMR study. Polar Biology. 2001;24(11):869-74.

15. Pörtner HO, Langenbuch M, Reipschläger A. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. Journal of Oceanography. 2004;60:705-18.

16. Qin Z, Lewis JE, Perry SF. Zebrafish (Danio rerio) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO_2. The Journal of Physiology. 2010;588(5):861-72.

17. Jutfelt F, Hedgärde M. Atlantic cod actively avoid CO_2 and predator odour, even after long-term CO_2 exposure. Frontiers in Zoology. 2013;10:81-.

18. Perry SF, Kinkead R, Fritsche R. Are circulating catecholamines involved in the control of breathing by fishes? Reviews in Fish Biology and Fisheries. 1992;2(1):65-83.

19. Ishimatsu A, Kikkawa T, Hayashi M, Lee KS, Kita J. Effects of CO_2 on marine fish: larvae and adults. Journal of Oceanography. 2004;60(4):731-41.

20. Perry SF, Fritsche R, Hoagland TM, Duff DW, Olson KR. The control of blood pressure during external hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology. 1999;202(16):2177.

21. Gesser H, Poupa O. Acidosis and cardiac muscle contractility: Comparative aspects. Comparative Biochemistry and Physiology Part A: Physiology. 1983;76(3):559-66.

22. Leo E, Kunz KL, Schmidt M, Storch D, Pörtner H-O, Mark FC. Mitochondrial acclimation potential to ocean acidification and warming of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*). Frontiers in Zoology. 2017;14(1):21.

23. Schmidt M, Windisch HS, Ludwichowski K-U, Seegert SLL, Pörtner H-O, Storch D, et al. Differences in neurochemical profiles of two gadid species under ocean warming and acidification. Frontiers in Zoology. 2017;14(1):49.

3. Discussion

3.1 The physiological interplay between CO_2, temperature and GABA-metabolism on behavioural disturbances in fish

Manuscript I is the first study which assessed behavioural effects of ocean acidification scenarios on fish species in polar environments (43). However, ocean acidification does not occur in an isolated manner but simultaneously to ocean warming, and therefore, the effects of these two drivers need to be analyzed from an integrative perspective. We detected significant effects of CO_2 on behavioural laterality and significant effects of temperature on routine activity of *B. saida*, while no such changes were observed in *G. morhua* (43). The temperature-related increase in routine activity of *B. saida* was interpreted as a symptom for increased energy metabolism similar to Nowicki *et al.* and Munday *et al.* (36, 40). The CO_2-dependent reduction of absolute and relative behavioural laterality in *B. saida* was interpreted as a reduction of neuronal functionality, as discussed by Domenici *et al.* (37). We did not observe interactive effects of temperature and CO_2 on behavioural laterality and routine activity in *B. saida* and *G. morhua;* however, in *G. morhua*, we found under high CO_2 conditions a non-significant trend for a shift from right to left lateralization at 3, 12 and 16 °C and a trend for a shift from left to right lateralization at 8 °C and therefore concluded that an interactive behavioural effect was probably missed due to low sample size (43).

An interaction between CO_2 and temperature effects on the behaviour of *B. saida* and *G. morhua* would not have been surprising given the proposed mechanistic underpinning of the CO_2-induced behavioural disturbances (refer to 1.3 for further information). The physiological consequences of changes in environmental temperature and CO_2 partial pressure are interrelated as temperature shapes the standard metabolic rate, oxygen demand and generation of CO_2 in an interplay with the respective species` temperature window making it difficult to distinguish between mere CO_2 and temperature effects (44, 45). In addition, temperature is a direct factor in the calculation of the reversal potential of the $GABA_A$-receptor (E_{GABA}, Formula 2) causing a linear reduction of E_{GABA} as temperature rises. Interactive effects of temperature and CO_2 with respect to foraging and behavioural laterality have already been

reported in coral reef fish by Domenici *et al.* and Nowicki *et al.* (35, 36) confirming that the consequences of environmental CO_2 and temperature need to be addressed in combination.

Overall, *Boreogadus saida* appeared to be more vulnerable to future CO_2-conditions than *Gadus morhua*. This is in line with recent studies by Jutfelt *et al.*, Sundin *et al.* and Kwan *et al.* (23, 24, 46) who also observed behavioural resilience in temperate *G. morhua*, temperate damselfish *Chromis punctipinnis* and relative behavioural resilience in temperate wrasse *Ctenolabrus rupestris* to increased CO_2 partial pressure. Species-specific differences in the severity of CO_2 effects on fish behaviour indicate that some fish might possess the ability to rapidly adapt their behavior to future CO_2 conditions. Considering that *G. morhua* frequently forages in hypoxic and hypercapnic waters (47), it may sound intuitive that this species is better adapted to these challenging conditions. It does, however, not answer the question on how a fish species is actually able to overcome such environmental challenges.

The observation of a possible interaction between temperature and CO_2 effects on the behavioural laterality in *G. morhua* becomes relevant in combination with manuscript II, which indicated an altered GABA-metabolism in *G. morhua* under increased environmental CO_2 at 8 °C, but not at 3, 12 or 16 °C (48). Changes in GABA-metabolism upon CO_2-exposure are supported by a recent study by Schunter *et al.* who found an increased transcription rate of several proteins involved in the GABA-metabolism in the brain of *Acanthochromis polyacanthus* during acute and long term (within-generation) CO_2 exposure indicating an increase of GABAergic activity (49). From the current perspective, it is not clear, whether an altered GABA-metabolism under increased environmental CO_2 may be the cause or rather the consequence of resilience against increased environmental CO_2. In this thesis, the alteration of the GABA-metabolism was in *G. morhua* especially visible in the treatment group which was discussed to be potentially more resistant against environmental hypercapnia and therefore, a beneficial effect through elevated GABAergic activity was suggested (43, 48). In contrast, the increased GABAergic metabolism observed during CO_2 exposure in offspring of both CO_2 sensitive and CO_2 resistant individuals of *A. polyacanthus* was interpreted by Schunter *et al.* as a detrimental self-amplifying cycle through an overall reduced inhibitory potential under increased CO_2 conditions (49). Interestingly, the alteration of the GABA-metabolism was not visible in the first-generation offspring of *A. polyacanthus*.

But how could an increase of GABAergic activity be beneficial for the CO_2-response of *G. morhua*? This question is an important issue, particularly as in Californian rockfish *Sebastes diploproa*, an exposure to high CO_2 conditions in combination with the $GABA_A$-receptor agonist Muscimol led to a worsening of behavioral CO_2-effects (34). It is important to consider that a reversed net ion flux through the $GABA_A$-receptor is unlikely absolute. As discussed in manuscript II, whether or not a GABA release acts in an inhibitory or excitatory way is dependent on the location of the GABAergic synapse as well as the exact time of GABA release with respect to former excitatory postsynaptic potentials (48, 50). Furthermore, excitatory GABAergic activity may under certain conditions still result in a reduced excitability of the postsynaptic neuron through reduction of the membrane resistance, a process termed "shunting" (51, 52). Also, neurons by no means possess a resting membrane potential between -70 and -80 mV, but can rather switch physiologically between a more polarized inactive down-state and a more depolarized up-state, either through intrinsic ion currents or associated neuronal networks (27, 53). Whether a GABA release acts in an inhibitory or excitatory manner on a postsynaptic neuron would therefore additionally be dependent on the exact state of this neuron. These observations indicate that, even under high CO_2 conditions, a part of the neuronal GABAergic activity may still act inhibitory. Hypothetically, if this fraction of remaining neuronal GABAergic activity under high CO_2 conditions is large enough, i.e. that the majority of GABAergic activity still acts inhibitory, an increase of GABAergic activity may have the potential to compensate for some excitatory GABAergic activity. Vice versa, if the majority of GABAergic activity acts excitatory under high CO_2 conditions, an increase of GABAergic activity may even be detrimental and could cause a vicious cycle, as suggested by Schunter *et al.* (49). This hypothesis is speculative, but it may explain the conflicting viewpoints with respect to the consequences of increased GABAergic activity (protective or detrimental) in fish under high CO_2 conditions (48, 49). Adjustments of the utilization of neurotransmitters under certain conditions are not uncommon. At least mammals are very well able to modify their neuronal GABAergic activity under certain conditions (51). For example, a rise of inhibitory GABAergic activity for the generation of a metabolic depression may be an important mechanism for neuronal protection against hypoxic events and hypoglycaemia in rodents (54-56), which is suggested to reduce energy-demanding excitatory glutamatergic neuronal activity in

postsynaptic neurons. As *G. morhua* commonly experiences hypoxic, hypercapnic conditions (47), the assumption that this species possesses a similar potential to regulate GABAergic activity may be reasonable.

From the current perspective, it remains unclear why a CO_2-induced altered GABAergic activity was observed in *G. morhua* only at 8 °C, but not at 3, 12 or 16 °C. 8 °C may resemble thermal optimum conditions of the studied animals (48) potentially indicating that behavioural resilience is greatest at optimum temperature. This may be a hint that an animals` aerobic scope and ability to allocate energy to compensatory processes may be an important determinant of the potential to withstand high environmental CO_2 conditions. In *A. polyacanthus*, changes of the GABAergic metabolism were observed in offspring of both, CO_2 sensitive and CO_2 resistant individuals (49). This indicates that other physiological mechanisms may contribute to the potential of fish to cope with future ocean acidification conditions, which will be addressed in the following.

3.2 Additional possible acclimation and adaptation processes shaping the resilience of fish behaviour to future climate change scenarios

CO_2-dependent adjustments of the GABAergic activity as discussed in 3.1 are only one possible mechanism which could increase a species` behavioural resilience against high environmental CO_2 conditions. In the following, the current state of the literature on possible compensatory mechanisms which may act complementary to the in 3.1 discussed adjustment of the GABA metabolism will be reviewed.

Whether a $GABA_A$-receptor acts excitatory or inhibitory is dependent on the membrane potential V_m and the reversal potential E_{GABA}. E_{GABA} is defined by the extra- and intracellular bicarbonate and chloride concentrations, the permeability coefficients of the $GABA_A$-receptor for both of these ions and the environmental temperature (refer to 1.3 for further information) (25, 27). Adjustments of the extra- and intracellular ionic composition as well as the permeability of the $GABA_A$-receptor for bicarbonate and chloride have the potential to alter E_{GABA} and thus to act compensatory to CO_2-related behavioural changes. This principle is displayed in Figure 2 in section 1.3. Adjustment of the extra- and intracellular chloride and bicarbonate concentrations may serve to keep E_{GABA} below a neurons` membrane potential. In

summary, mechanisms that lead to an increase of extracellular bicarbonate and chloride concentrations, or a decrease of intracellular bicarbonate and chloride have the potential to lower E_{GABA} and vice versa (27, 57). The hypothesis that a species-specific potential for the regulation of extra- and intracellular bicarbonate and chloride concentrations may be one reason why the behaviour of some species happens to be more robust against environmental hypercapnia than the behaviour of CO_2 sensitive species was recently strengthened by a study of Regan *et al.* (30). The authors state that fish species that have the potential to accumulate extracellular bicarbonate in exchange for chloride with a rate of 2:1 would at high CO_2 concentrations possess a lower E_{GABA} than animals that accumulate extracellular bicarbonate in exchange for chloride with a rate of 1:1 (30). This relationship is visible in Figure 2 A and B of chapter 1.3. Similarly, fish species that accumulate in neurons intracellular bicarbonate in exchange for chloride would at high CO_2-conditions possess a lower E_{GABA} than species that accumulate bicarbonate without such an intracellular reduction of chloride, which is visible in Figure 2 C and D of chapter 1.3 (30). Regan *et al.* argue that such ion-regulatory mechanisms may on the one hand be helpful in order to cope with high CO_2 conditions, but that such adaptations may on the other hand lead to an increase of E_{GABA} and thus excitatory $GABA_A$-receptor activity at low CO_2 concentrations (30). This hypothesis was subsequently verified theoretically and experimentally for the CO_2-tolerant freshwater species *Pangasianodon hypophthalmus* indicating that an adjustment of extracellular and intracellular chloride and bicarbonate concentrations may indeed serve as an adaptation mechanism to high environmental CO_2. Whether these results are transferable to marine fish species remains to be investigated. It is important to notice that the proposed transporters involved in the extracellular accumulation of bicarbonate in exchange for chloride at projected CO_2 increases are not yet identified (27). Further, actual measurements of the activity of neuronal transporters which are suggested to be relevant for regulation of E_{GABA} in the brain of fish have not been conducted to date and have so far only been studied in mammals (58). These transporters include the $Na^+/K^+/Cl^-$ cotransporter (NKCC1), the K^+/Cl^- cotransporter KCC2 (KCC2), the Na^+/HCO_3^- cotransporter (NBC), the Na^+ dependent Cl^-/HCO_3^- transporters (NCBT), and the Cl^-/HCO_3^- antiporters (AE3) with the latter two exchanging bicarbonate with chloride at a ratio of 2:1 and 1:1 respectively (31, 59). At least for KCC2, a CO_2 related change on

transcription level has been recorded in the brain of spiny damselfish *Acanthochromis polyacanthus*, which may serve a reduction of intracellular chloride and thus also a reduction of E_{GABA} (49). In mammals, the activity of KCC2, which transports chloride from the cytoplasm into the extracellular space, relative to the activity of NKCC1, which transports chloride ions into the cytoplasm of neurons, is relevant for the adjustment of E_{GABA} (52). Changes in the relative activity of KCC2 and NKCC1 determine that $GABA_A$-receptors may be excitatory during embryonic development and in neonatals, but inhibitory during delivery and from early childhood on (31, 56). Contrary to *A. polyacanthus* did the three-spined stickleback *Gasterosteus aculeatus* not exhibit a CO_2 related alteration in the expression of KCC2 or NKCC1 in the brain (58). Interesting in this respect are recent findings that *G. aculeatus* displays disrupted behavioural patterns in response to increased environmental CO_2 despite its resistance against other environmental stressors (38). As in *A. polyacanthus* an increase of KCC2 was observed during acute CO_2 exposure in offspring of both CO_2 sensitive and resistant animals, the compensatory KCC2 increase alone may in this species not be sufficient to prevent a detrimental increase of E_{GABA} (49).

Current behavioural studies indicate that marine eurythermal species may be more resilient against future CO_2 conditions than stenothermal species (23, 24, 46). This is in line with the hypothesis of Regan *et al.* that the behavioural resilience may be dependent of an animals` acid base regulatory system (30) as eurythermal animals rely on more active mechanisms for intracellular pH regulation than stenothermal animals in order to maintain protein functioning at different temperature regimes (60). In any case, acid–base regulatory mechanisms in neurons would necessarily occur in both, CO_2-sensitive and CO_2 insensitive species, since neuronal glutamatergic and GABAergic activity both lead to a reduction of intracellular pH, which was reviewed by Chesler *et al.* and Ruffin *et al.* (31, 59): In the case of glutamate-dependent intracellular acidification, activation of excitatory glutamate receptors causes an increase of intracellular Ca^{2+} though activation of postsynaptic ionotropic Ca^{2+}-permeable NMDA-receptors or postsynaptic metabotropic G_q-coupled glutamate receptors. The intracellular Ca^{2+} is subsequently transported out of the cell through Ca^{2+}/H^+ antiporters leading to a reduction of intracellular pH. Furthermore, an increase of glucose catabolism upon excitatory stimuli with a concomitant rise of intracellular lactate and CO_2 might add to the intracellular acidification. In

contrast, the intracellular acid shift after $GABA_A$-receptor activity of neurons is caused by the conductivity of this receptor for bicarbonate. As discussed in 1.3 does the activation of the $GABA_A$-receptor lead to an outflow of intracellular bicarbonate simultaneously to the inflow of chloride resulting in a reduction of intracellular bases and thus a lowering of pH_i. Such changes of the intracellular pH need to be regenerated in both, CO_2-sensitive and CO_2-resilient fish species in order to maintain neuronal functionality (59). In manuscript III, we did not find a significant reduction of pH_i in the head of *B. saida* upon onset of hypercapnic conditions of 1,700 µatm CO_2 indicating that also those fish species with a behaviour sensitive to CO_2 possess acid-base regulatory mechanisms in the brain. Recent observations made by Heuer *et al.* also suggest the regulation of intracellular pH in the brain of CO_2-sensitive fish species as they even measured overcompensation of pH_i in the brain of the CO_2-sensitive spiny damselfish *A. polyacanthus* in response to a rise of environmental CO_2 to ~1,900 µatm (57). So far, studies that specifically analyze differences in the acid-base regulatory system of CO_2-sensitive and CO_2-resilient fish species are still lacking and would have to be addressed in future research.

Apart from ion- and acid-base regulatory systems, several other mechanisms have been identified which may shape an animals` potential to acclimate or adapt to future CO_2-scenarios. One of these mechanisms may occur on the basis of composition of the $GABA_A$-receptor, which consists of 5 subunits, with so far 19 known different homologous subunit types (27). Altered subunit composition affects not only pharmacological properties, but also channel kinetics and the permeability for bicarbonate and thus E_{GABA}. Differences in the composition of the $GABA_A$-receptor are plausible, particularly since the $GABA_A$-receptor inhibitor Gabazine does not restore CO_2-impaired behaviour equally in all species investigated (27). A CO_2-dependent expression $GABA_A$-receptor subunits has been observed in *G. aculeatus* and *A. polyacanthus* suggesting that adjustment of the composition of the $GABA_A$-receptor may be relevant at least for intragenerational acclimation to high CO_2 conditions (49, 58).

So far, research on CO_2-effects on fish behaviour has focused solely on the functionality of the $GABA_A$-receptor. However, all considerations discussed above, i.e. intra- and extracelluar ion concentrations, permeability coefficients and reversal potentials, also apply to the glycine receptor, which is permeable to chloride and bicarbonate, like the $GABA_A$-receptor (27). In the central nervous system, activity of the ligand mediated glycine receptor causes an inhibitory

postsynaptic potential through net inward current of bicarbonate and chloride into the postsynaptic neuron, which could be reversed under high CO_2 conditions (27, 61). Recent work on *A. polyacanthus* support the potential role of glycine for acclimation and adaptation processes to CO_2 related behavioural changes (62). Schunter *et al.* found that if offspring of CO_2 resistant individuals of *A. polyacanthus* were exposed to high CO_2 conditions, they expressed the glycine neurotransmitter transporter protein (Sc6A5) at a significantly higher level compared to CO_2-exposed offspring of CO_2-sensitive animals of this species (62).

Another relevant aspect for CO_2 acclimation and adaptation emerged with a recent study by Ferrari *et al.* who detected that animals under high predation risk exhibit less CO_2-impaired behaviour than those animals with low predation risk, indicating that some impairments may be compensated for by the use of other sensory modalities as suggested by Regan *et al.*, too (30, 63). In addition, recent manuscripts of Lai *et al.* and Schunter *et al*. discuss neuronal restructuring as a potential acclimation mechanism as these authors found a CO_2-dependent species-specific response in the expression of genes involved in neurogenesis (49, 64). However, the three species investigated all display disrupted behaviour under increased CO_2 conditions and it can thus be assumed, that, if neurogenesis serves as a coping mechanism, it only provides insufficient protection.

Schunter *et al.* further found adjustments in the circadian rhythm and melatonin production to be a driver of behavioural resilience (62) and the authors suggest that these traits link to adjustments in acid-base regulation. However, the exact mechanistic background of this observation remains obscure.

All aspects discussed above have the potential to be involved in the physiological basis of acclimation to an acute CO_2 increase if the required genetic preconditions exist in the affected individuals. Studies that analyzed CO_2-induced behavioural disruptions after long term CO_2 exposure indicate that such potential for acclimation may be limited (43). Transgenerational acclimation and adaptation processes might create the physiological background needed to increase behavioural resilience to elevated CO_2. So far, only few multigenerational studies on this issue have been conducted with partially conflicting results. While current work indicates that detrimental effects of CO_2 on metabolic rate, growth and survival of fish might be compensated for within a few generations (65, 66), the picture might look different with respect

to CO_2-induced behavioural effects. Welch *et al.* report no intergenerational potential of *A. polyacanthus* to restore CO_2-impaired behaviour (67) and Munday *et al.* observed CO_2-induced behavioural disruptions in wild coral reef fish near natural CO_2 vents and consequently concluded that fish may yield only low potential for intergenerational adaptation to high CO_2 conditions (41). Additional findings by Welch *et al.* may further complicate this picture as they observed in *A. polyacanthus* a heritable variability of resistance to acute CO_2-induced behavioural changes (68) which was no longer visible when the animals were chronically exposed to high CO_2 conditions. In contrast, Allan *et al.* found that some types of CO_2-impaired behaviour of the anemone fish *Amphiprion melanopus* can become partially or even fully restored over one generation indicating at least some potential for paternal or intergenerational acclimation mechanisms (69). Furthermore, Regan *et al.* argue that thriving fish populations can be found in hypercapnic tropical freshwater habitats and their findings on *P. hypophthalmus* indicate that fish might be very well able to adapt their behaviour to high CO_2 conditions, if the needed gene variants exist in the respective populations (30). However, the authors also question whether the potential for intergenerational acclimation and adaptation might be sufficient to keep up with the current pace of acidification (30). Multigenerational studies were conducted so far only over one generation and therefore mostly account for paternal and epigenetic effects. Thus, it is not known yet, whether the benefits of transgenerational acclimation processes can persist for several following generations (70). Nevertheless, mechanisms for transgenerational acclimation create phenotypic variation between generations and therefore possess the potential to shift the mean phenotype in a population and in this way alter the speed of selection and multigenerational adaptation (70). As cold water adapted species display relatively slow growth and extended generation time, studies on the adaptive potential of tropical stenothermal reef fishes with shorter generation times may provide the most applicable reference available. This discrepancy of generation times must be kept in mind when discussing the adaptive potential of different teleosts to future CO_2 conditions i.e. that the adaptive potential of cold water species might be significantly lower than the adaptive potential of their tropical counterparts. Possibly the most integrated data on the potential for acclimation and adaptation of fish species to hypercapnia can be drawn from the fossil record and palaeo- high CO_2 events (71), when fish adapted efficiently to high CO_2

conditions (72, 73). However, actual ocean acidification occurs at a rate about 10 times faster than any event within at least the last 65 million years and will probably exceed the adaptive potential of many sensitive species, especially of those with long generation times (26, 74).

3.3 Ecosystem implications

Many of the observed behavioural and sensory patterns disrupted by environmental CO_2 are directly or indirectly involved in predator avoidance, and thus link to potential changes in predator prey interactions. Such changes bear the potential to propagate throughout ecosystems as CO_2 has been observed to affect the behaviour of both prey and predatory fish species (75), including invertebrates (76, 77) and elasmobranchs (78, 79) altering predation rates and prey preference (80). In contrast to these considerations Ferrari *et al.* found in a recent study that impaired behaviours or senses may be compensated for through increased utilization of other senses (63) potentially reducing the actual effect of impaired behaviour at ecosystem level. Interestingly, Munday *et al.* reported, that fish communities at CO_2 vents were nearly identical to fish communities in nearby control reefs despite the innate behavioural sensibility of these fish species to environmental CO_2 (41). However, this does not necessarily mean that ecosystem structures remain unaffected by the rise in environmental CO_2 since this observation might have also been caused by a continuous replenishment of fish from surrounding low-CO_2 areas (41). Recently, Nagelkerken *et al.* showed that CO_2-altered behavioural patterns indeed have the potential to affect fish populations leading to a reduction of biodiversity and a homogenization of the species composition (81). However, additional effects of warming will further change this picture since recent work indicates that temperature may be much more potent driver for the mediation of predator-prey interactions than environmental CO_2 with CO_2-effects being slightly additive to temperature effects (82). However, a generalization of the combined effects of temperature and CO_2 was challenging with complex interaction patterns of temperature and CO_2 demonstrating the difficulty of the prediction of combined future temperature and CO_2 changes in the marine ecosystems (82).

In the assessment of how *G. morhua* and *B. saida* will strive in the waters around Svalbard in a warmer and more acidified ocean, our findings indicate that *G. morhua* is more prepared to face higher temperatures and CO_2 conditions than *B. saida* (43, 48). Adding to findings by Leo *et al.*,

Kunz *et al.* and the current observation of the northward shift of fish species (6, 83, 84), it appears likely, that *G. morhua* will continuously inhabit the areas around Svalbard with *B. saida* shifting its distribution patterns into more northern, colder waters with sea ice coverage, leading to concomitant challenges for dependent species and the ecosystems of the northern polar seas. These suggestions are supported by a recent integrative model by Koenigstein *et al.* which predicts that *G. morhua* will successfully populate the Barents Sea within the first half of the 21st century, but with the current speed of warming and acidification, the combined physiological consequences of temperature and CO_2 may even exceed this species` potential for adaptation in the long term (85).

4. Conclusions and future perspectives

This thesis provides evidence that the behaviour of *Boreogadus saida* may be more sensitive to environmental hypercapnia compared to the behaviour of *Gadus morhua* which adds to the emerging body of literature that *B. saida* will indeed fare poorly under future climate change scenarios. Furthermore, it was found that an adjustment of the GABA metabolism may be inolved in the physiological mechanisms to achieve resistance of the nervous system and thus resistance of the behaviour to high CO_2 conditions.

Related to the results obtained in this thesis, four approaches might be particularly promising in order to understand the overarching principles of CO_2-induced behavioural disruptions. First, in order to test the role of acid-base regulation for the sensitivity to environmental CO_2, it is mandatory to determine the actual intra- and extracellular chloride and bicarbonate concentrations in exemplary neurons of CO_2-sensitve and CO_2-resistant fish species under different CO_2-conditions. Such measurements could, for example, be conducted through utilization of ion-sensitive microelectrodes for the determination of extra- and intracellular chloride concentrations (86) together with pH and carboselective microelectrodes for the determination of extra- and intracellular bicarbonate concentrations (87, 88). In addition, Patch clamp techniques may be used in order to estimate actual permeability coefficients of the $GABA_A$-receptor in the brain of fish (27). Based on these data, CO_2-dependent shifts of the reversal potential of the $GABA_A$-receptors could be calculated under realistic conditions. Second, more clarification with respect to the regulation of the extra- and intracellular bicarbonate and chloride concentrations is needed. The abundance of the NKCC1, KCC2, NBC, NCBT and AE3 should be tested immunohistochemically in the brain of CO_2-sensitive and CO_2-resistant fish species. Third, in this study it was proposed that an increase in GABAergic activity may in Atlantic cod *(Gadus morhua)* be a mechanism for resistance against high environmental CO_2-conditions, potentially in a temperature dependent manner. This hypothesis could be tested through behavioural analysis of Atlantic cod during exposure to control and high CO_2-conditions at different temperature regimes in addition to the $GABA_A$-receptor agonist Muscimol or the $GABA_A$-receptor antagonist Gabazine. If the resilience of *G. morhua* is caused by an increase of the GABAergic activity in a temperature-dependent manner, addition of Muscimol to fish exposed to high CO_2 conditions may lead to detrimental behavior at

temperatures above or below the optimum temperature. At the optimum temperature, the behaviour should not be impaired through elevated environmental CO_2 in addition to Muscimol. Vice versa, addition of Gabazine to fish exposed to high CO_2 conditions may cause behavioural disturbances at optimum temperature conditions but not at temperatures above or below. Fourth, the question remains whether fish may possess the ability to adapt to high CO_2 conditions. Incubation of CO_2-sensitive species to ocean acidification scenarios and behavioural testing over multiple generations, favorably at different temperature regimes, would be valuable in order to predict the fate of fish species and their ecosystems in the future oceans.

5. References

1. Vihtakari M. PlotSvalbard: PlotSvalbard - plot research data from Svalbard on maps. . R package version 0.1.16. ed2017.
2. Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL, et al. The oceanic sink for anthropogenic CO2. Science. 2004;305(5682):367-71.
3. Caldeira K, Wickett ME. Anthropogenic carbon and ocean pH. Nature. 2003;425:365.
4. IPCC. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA: 2014.
5. IPCC. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, 2013 ISBN 978-1-107-66182-0.
6. Pörtner HO, Knust R. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science. 2007;315(5808):95-7.
7. Pörtner HO, Peck MA. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. Journal of Fish Biology. 2010;77(8):1745-79.
8. Holt RE, Jørgensen C. Climate change in fish: effects of respiratory constraints on optimal life history and behaviour. Biology Letters. 2015;11(2).
9. Pörtner HO, Farrell AP. Physiology and climate change. Science. 2008;322:690-2.
10. Pörtner HO. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ectotherms. J Exp Biol. 2010;213:881-93.
11. Parmesan C. Ecological and evolutionary responses to recent climate change. Annual Review of Ecology, Evolution, and Systematics. 2006;37:637-69.
12. Beierlein L, Salvigsen O, Schöne BR, Mackensen A, Brey T. The seasonal water temperature cycle in the Arctic Dicksonfjord (Svalbard) during the Holocene Climate Optimum derived from sub-fossil Arctica islandica shells. The Holocene. 2015;25(8):1197-207.
13. Descamps S, Aars J, Fuglei E, Kovacs Kit M, Lydersen C, Pavlova O, et al. Climate change impacts on wildlife in a High Arctic archipelago – Svalbard, Norway. Global Change Biol. 2016;23(2):490-502.
14. Renaud P, Berge J, Varpe Ø, Lønne O, Nahrgang J, Ottesen C, et al. Is the poleward expansion by Atlantic cod and haddock threatening native polar cod, Boreogadus saida? Polar Biology. 2012;35(3):401-12.
15. Welch HE, Crawford RE, Hop H. Occurrence of Arctic Cod (Boreogadus saida) Schools and Their Vulnerability to Predation in the Canadian High Arctic. 1993. 1993;46(4):9. Epub 1993-01-01.
16. Harter BB, Elliott KH, Divoky GJ, Davoren GK. Arctic Cod (Boreogadus saida) as Prey: Fish Length-Energetics Relationships in the Beaufort Sea and Hudson Bay. Arctic. 2013;66(2):191-6.
17. Koenigstein S, Ruth M, Gößling-Reisemann S. Stakeholder-Informed Ecosystem Modeling of Ocean Warming and Acidification Impacts in the Barents Sea Region. Frontiers in Marine Science. 2016;3:93.
18. Drinkwater KF. The response of Atlantic cod (Gadus morhua) to future climate change. ICES Journal of Marine Science. 2005;62(7):1327-37.
19. Kleypas JA, Langdon C. Coral reefs and changing seawater chemistry. In: Phinney J, Hoegh-Guldberg O, Kleypas J, Skirving W, Strong AE, editors. Coral Reefs and Climate Change: Science and Management. Washington, D.C., USA: American Geophysical Union; 2006. p. 73-110.
20. Widdicombe S, Spicer JI. Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? Journal of Experimental Marine Biology and Ecology. 2008;366(1-2):187-97.

21. Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, et al. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(6):1848-52.
22. Jutfelt F, Hedgärde M. Atlantic cod actively avoid CO2 and predator odour, even after long-term CO2 exposure. Frontiers in Zoology. 2013;10:81-.
23. Jutfelt F, Hedgärde M. Juvenile Atlantic cod behavior appears robust to near-future CO2 levels. Frontiers in Zoology. 2015;12(1):1-7.
24. Kwan GT, Hamilton TJ, Tresguerres M. CO_2-induced ocean acidification does not affect individual or group behaviour in a temperate damselfish. Royal Society Open Science. 2017;4(7).
25. Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson S-A, et al. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nature Clim Change. 2012;2(3):201-4.
26. Heuer RM, Grosell M. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 2014;307(9):R1061.
27. Tresguerres M, Hamilton TJ. Acid–base physiology, neurobiology and behaviour in relation to CO2-induced ocean acidification. The Journal of Experimental Biology. 2017;220(12):2136.
28. Brauner CJ, Baker DW. Patterns of acid-base regulation during exposure to hypercarbia in fishes. In: Glass ML, Wood SC, editors. Cardio-Respiratory Control in Vertebrates. Berlin2009. p. 43-63.
29. Damsgaard C, Gam LTH, Dang DT, Van Thinh P, Huong DTT, Wang T, et al. High capacity for extracellular acid-base regulation in the air-breathing fish *Pangasianodon hypophthalmus*. The Journal of Experimental Biology. 2015.
30. Regan MD, Turko AJ, Heras J, Andersen MK, Lefevre S, Wang T, et al. Ambient CO_2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO_2-altered behaviour by taking a hypercapnia dweller down to low CO_2 levels. The Journal of Experimental Biology. 2016;219(1):109-18.
31. Chesler M. Regulation and Modulation of pH in the Brain. Physiological Reviews. 2003;83(4):1183-221.
32. Nilsson GE, Lefevre S. Physiological Challenges to Fishes in a Warmer and Acidified Future. Physiology. 2016;31(6):409-17.
33. Larsen BK, Pörtner HO, Jensen FB. Extra- and Intracellular Acid-Base Balance and Ionic Regulation in Cod (*Gadus morhua*) During Combined and Isolated Exposures to Hypercapnia and Copper. Mar Biol. 1997;128(2):337-46.
34. Hamilton TJ, Holcombe A, Tresguerres M. CO_2-induced ocean acidification increases anxiety in Rockfish via alteration of $GABA_A$ receptor functioning. Proceedings of the Royal Society B: Biological Sciences. 2014;281(1775).
35. Domenici P, Allan BJM, Watson S-A, McCormick MI, Munday PL. Shifting from Right to Left: The Combined Effect of Elevated CO2 and Temperature on Behavioural Lateralization in a Coral Reef Fish. PLoS ONE. 2014;9(1):e87969.
36. Nowicki JP, Miller GM, Munday PL. Interactive effects of elevated temperature and CO2 on foraging behavior of juvenile coral reef fish. Journal of Experimental Marine Biology and Ecology. 2012;412(0):46-51.
37. Domenici P, Allan B, McCormick MI, Munday PL. Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. Biology Letters. 2012;8(1):78-81. Epub 2011/08/19.
38. Jutfelt F, Bresolin de Souza K, Vuylsteke A, Sturve J. Behavioural Disturbances in a Temperate Fish Exposed to Sustained High-CO2 Levels. PLoS ONE. 2013;8(6):e65825.
39. Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MCO, Chivers DP. Replenishment of fish populations is threatened by ocean acidification. Proceedings of the National Academy of Sciences. 2010;107(29):12930-4.

40. Munday P, Pratchett M, Dixson D, Donelson J, Endo GK, Reynolds A, et al. Elevated CO2 affects the behavior of an ecologically and economically important coral reef fish. Marine Biology. 2013;160(8):2137-44.
41. Munday PL, Cheal AJ, Dixson DL, Rummer JL, Fabricius KE. Behavioural impairment in reef fishes caused by ocean acidification at CO2 seeps. Nature Clim Change. 2014;4(6):487-92.
42. Cripps IL, Munday PL, McCormick MI. Ocean Acidification Affects Prey Detection by a Predatory Reef Fish. PLoS ONE. 2011;6(7):e22736.
43. Schmidt M, Gerlach G, Leo E, Kunz KL, Swoboda S, Pörtner HO, et al. 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. 2017;571:183-91.
44. Pörtner HO. Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. Marine Ecology Progress Series. 2012;470:273-90.
45. Van Dijk PL, Hardewig I, Pörtner HO. Temperature-dependent shift of pHi in fish white muscle: contributions of passive and active processes. Am J Physiol. 1997;272(1 Pt 2):R84-9.
46. Sundin J, Jutfelt F. 9–28 d of exposure to elevated pCO2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (Ctenolabrus rupestris). ICES Journal of Marine Science. 2015;73(3):620-32.
47. Neuenfeldt S, Andersen KH, Hinrichsen HH. Some Atlantic cod Gadus morhua in the Baltic Sea visit hypoxic water briefly but often. Journal of Fish Biology. 2009;75(1):290-4.
48. Schmidt M, Windisch HS, Ludwichowski K-U, Seegert SLL, Pörtner H-O, Storch D, et al. Differences in neurochemical profiles of two gadid species under ocean warming and acidification. Frontiers in Zoology. 2017;14(1):49.
49. Schunter C, Welch MJ, Nilsson GE, Rummer JL, Munday PL, Ravasi T. An interplay between plasticity and parental phenotype determines impacts of ocean acidification on a reef fish. Nature Ecology & Evolution. 2018;2(2):334-42.
50. Gulledge AT, Stuart GJ. Excitatory Actions of GABA in the Cortex. Neuron. 2003;37(2):299-309.
51. Roth FC, Draguhn A. GABA metabolism and transport: effects on synaptic efficacy. Neural plasticity. 2012;2012:805830-. Epub 2012/02/23.
52. Wright R, Raimondo JV, Akerman CJ. Spatial and temporal dynamics in the ionic driving force for GABA(A) receptors. Neural plasticity. 2011;2011:728395-. Epub 2011/06/27.
53. Wilson C. Up and down states. Scholarpedia. 2008;3:1410. Journal Article.
54. Weyne J, Van Leuven F, Kazemi H, Leusen I. Selected brain amino acids and ammonium during chronic hypercapnia in conscious rats. Journal of Applied Physiology. 1978;44(3):333-9.
55. Weyne J, Van Leuven F, Demeester G, Leusen I. Metabolism of glutamic acid and related amino acids in the brain studied with 14C-labelled glucose, butyric acid and glutamic acid in hypercapnic rats. Journal of Neurochemistry. 1977;29(3):469-76.
56. Tyzio R, Cossart R, Khalilov I, Minlebaev M, Hübner CA, Represa A, et al. Maternal Oxytocin Triggers a Transient Inhibitory Switch in GABA Signaling in the Fetal Brain During Delivery. Science. 2006;314(5806):1788.
57. Heuer RM, Welch MJ, Rummer JL, Munday PL, Grosell M. Altered brain ion gradients following compensation for elevated CO2 are linked to behavioural alterations in a coral reef fish. Scientific Reports. 2016;6:33216.
58. Lai F, Fagernes CE, Jutfelt F, Nilsson GE. Expression of genes involved in brain GABAergic neurotransmission in three-spined stickleback exposed to near-future CO(2). Conservation Physiology. 2016;4(1):cow068.
59. Ruffin V, Salameh A, Boron W, Parker M. Intracellular pH regulation by acid-base transporters in mammalian neurons. Frontiers in Physiology. 2014;5:43.

60. Pörtner HO, Sartoris FJ. Invasive studies of intracellular acid-base parameters: environmental and functional aspects. In: Taylor, EW, Egginton, S and JA Raven (eds) Regula-tion of tissue pH in plants and animals: A reappraisal of current techniques Cambridge University Press. 1999:69-98.
61. Zhao P, Qian H, Xia Y. GABA and glycine are protective to mature but toxic to immature rat cortical neurons under hypoxia. European Journal of Neuroscience. 2005;22(2):289-300.
62. Schunter C, Welch Megan J, Ryu T, Zhang H, Berumen Michael L, Nilsson Göran E, et al. Molecular signatures of transgenerational response to ocean acidification in a species of reef fish. Nature Climate Change. 2016;6:1014.
63. Ferrari MCO, McCormick MI, Watson S-A, Meekan MG, Munday PL, Chivers DP. Predation in High CO2 Waters: Prey Fish from High-Risk Environments are Less Susceptible to Ocean Acidification. Integrative and Comparative Biology. 2017;57(1):55-62.
64. Lai F, Fagernes CE, Bernier NJ, Miller GM, Munday PL, Jutfelt F, et al. Responses of neurogenesis and neuroplasticity related genes to elevated CO_2 levels in the brain of three teleost species. Biology Letters. 2017;13(8).
65. Murray CS, Malvezzi A, Gobler CJ, Baumann H. Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. Marine Ecology Progress Series. 2014;504:1-11.
66. Miller GM, Watson S-A, Donelson JM, McCormick MI, Munday PL. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nature Clim Change. 2012;2(12):858-61.
67. Welch MJ, Watson S-A, Welsh JQ, McCormick MI, Munday PL. Effects of elevated CO2 on fish behaviour undiminished by transgenerational acclimation. Nature Clim Change. 2014;4(12):1086-9.
68. Welch Megan J, Munday Philip L. Heritability of behavioural tolerance to high CO2 in a coral reef fish is masked by nonadaptive phenotypic plasticity. Evolutionary Applications. 2017;10(7):682-93.
69. Allan BJM, Miller GM, McCormick MI, Domenici P, Munday PL. Parental effects improve escape performance of juvenile reef fish in a high-CO2 world. Proceedings of the Royal Society of London B: Biological Sciences. 2014;281(1777):20132179.
70. Munday PL. Transgenerational acclimation of fishes to climate change and ocean acidification. F1000Prime Reports. 2014;6:99.
71. Wittmann AC, Pörtner H-O. Sensitivities of extant animal taxa to ocean acidification. Nature Clim Change. 2013;3(11):995-1001.
72. Bush AM, Bambach RK. Paleoecologic Megatrends in Marine Metazoa. Annual Review of Earth and Planetary Sciences. 2011;39(1):241-69.
73. Chen Z-Q, Benton MJ. The timing and pattern of biotic recovery following the end-Permian mass extinction. Nature Geoscience. 2012;5:375.
74. IPCC. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA: 2014.
75. Allan BJM, Domenici P, McCormick MI, Watson S-A, Munday PL. Elevated CO2 Affects Predator-Prey Interactions through Altered Performance. PLoS ONE. 2013;8(3):e58520.
76. Watson S-A, Lefevre S, McCormick MI, Domenici P, Nilsson GE, Munday PL. Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. Proceedings of the Royal Society B: Biological Sciences. 2014;281(1774).
77. Watson S-A, Fields JB, Munday PL. Ocean acidification alters predator behaviour and reduces predation rate. Biology Letters. 2017;13(2).
78. Green L, Jutfelt F. Elevated carbon dioxide alters the plasma composition and behaviour of a shark. Biology Letters. 2014;10(9):20140538.

79. Dixson DL, Jennings AR, Atema J, Munday PL. Odor tracking in sharks is reduced under future ocean acidification conditions. Global Change Biol. 2015;21(4):1454-62.
80. Ferrari MCO, McCormick MI, Munday PL, Meekan MG, Dixson DL, Lonnstedt Ö, et al. Putting prey and predator into the CO2 equation – qualitative and quantitative effects of ocean acidification on predator–prey interactions. Ecology Letters. 2011;14:1143–8.
81. Nagelkerken I, Goldenberg SU, Ferreira CM, Russell BD, Connell SD. Species Interactions Drive Fish Biodiversity Loss in a High-CO_2 World. Current Biology. 2017;27(14):2177-84.e4.
82. Allan BJM, Domenici P, Watson SA, Munday PL, McCormick MI. Warming has a greater effect than elevated CO_2 on predator–prey interactions in coral reef fish. Proceedings of the Royal Society B: Biological Sciences. 2017;284(1857).
83. Kunz KL, Frickenhaus S, Hardenberg S, Johansen T, Leo E, Pörtner H-O, et al. 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. 2016:1-17.
84. Leo E, Kunz KL, Schmidt M, Storch D, Pörtner H-O, Mark FC. Mitochondrial acclimation potential to ocean acidification and warming of Polar cod (Boreogadus saida) and Atlantic cod (Gadus morhua). Frontiers in Zoology. 2017;14(1):21.
85. Koenigstein S, Dahlke Flemming T, Stiasny Martina H, Storch D, Clemmesen C, Pörtner HO. Forecasting future recruitment success for Atlantic cod in the warming and acidifying Barents Sea. Global Change Biol. 2017;24(1):526-35.
86. Hansen AJ. Effect of anoxia on ion distribution in the brain. Physiological Reviews. 1985;65(1):101-48.
87. Chesler M, Chen JCT, Kraig RP. Determination of extracellular bicarbonate and carbon dioxide concentrations in brain slices using carbonate and pH-selective microelectrodes. Journal of Neuroscience Methods. 1994;53(2):129-36.
88. Pörtner HO, Bickmeyer U, Bleich M, Bock C, Brownlee C, Melzner F, et al. Measurements of CO_2 sensitive processes: studies of acid-base status and regulation. In: Riebesell U, Fabry VJ, Gattuso JP, editors. Guide for Best Practices in Ocean Acidification Researchand Data Reporting 2010.

6. Appendix

6.1 Other thesis-related articles and conference contributions

Schmidt M., Storch D., Bock C., Pörtner H.-O. (2013) Ocean acidification and warming affect the behaviour of the Polar cod *Boreogadus saida.* Bioacid Phase II Annual meeting (Conference talk)

Schmidt M., Storch D., Bock C., Gerlach G., Munday PL., Pörtner H.-O. (2013) Behavioural changes in response to temperature and CO_2 in Polar cod. YouMaRes 4 Conference 2013 (Conference talk)

Cohen-Rengifo M., Crafton R.E., Hassenrück C., Jankowska E., Koenigstein S., Sandersfeld T., Schmid M.S., **Schmidt M.**, Simpson R., Sheward R.M.(2013) Module 1 Marine Ecosystems and Climate Change. in: Dummermuth, A. and Grosfeld, K. (eds.): Climate change in the marine realm: an international summer school in the framework of the European Campus of Excellence, Berichte zur Polar- und Meeresforschung = Reports on polar and marine research, Bremerhaven, Alfred Wegener Institute for Polar and Marine Research, 662, 75 p., hdl:10013/epic.41554

Schmidt M., Bock C., Pörtner H.-O., Storch D. (2014) Effects of ocean acidification and warming on the behaviour of Polar cod *Boreogadus saida.* ESSAS annual meeting (Conference poster)

Schmidt M., Pörtner H.-O., Bock C. (2014) A new set-up to investigate neurophysiological effects of CO_2-induced ocean acidification on the brain of fish via MR imaging and spectroscopy. Joint Annual Meeting ISMRM-ESMRMB 2014 (Peer reviewed conference poster)

Schmidt M., Pörtner H.-O., Storch D., Bock C. (2015) Combined behavioural effects of ocean acidification and warming on two Gadid species and their physiological investigation. Bioacid Phase II Annual meeting (Conference poster)

Schmidt M., Pörtner H.-O., Bock C., Storch D. (2016) Behaviour of Atlantic cod (*Gadus morhua*) is more resilient to ocean warming and acidification than that of Polar cod (*Boreogadus saida*). 4th International Symposium on the Ocean in a High-CO2 World (Conference poster)

Schmidt M., Pörtner H.-O., Storch D., Bock C. (2016) Exploratory analysis of neurophysiological changes under ocean warming and acidification (OWA) in two Gadid species using 1H-NMR spectroscopy. 4th International Symposium on the Ocean in a High-CO2 World (Conference talk)

Kunz K., Frickenhaus S., Hardenberg S., Johansen T., Leo E., Pörtner HO., **Schmidt M.**, Windisch HS., Knust R., Mark FC. (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*, 39 (6), pp. 1137-1153. doi: 10.1007/s00300-016-1932-z

Leo E., Kunz KL, **Schmidt M.**, Storch D., Pörtner HO., Mark FC. (2017) Mitochondrial acclimation potential to ocean warming and acidification of Polar cod (*Boreogadus saida*) and Atlantic cod (*Gadus morhua*), *Frontiers in Zoology*, 14:21, doi: 10.1186/s12983-017-0205-1

The following supplement accompanies the article

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 Lore Kunz, Steffen Swoboda, Hans-Otto Pörtner, Christian Bock, Daniela Storch*

*Corresponding author: daniela.storch@awi.de

Marine Ecology Progress Series 571: 183–191 (2017)

Table S1: Carbonate chemistry, temperatures as well as animal lengths and weights of the incubation of *Boreogadus saida*. The table depicts mean and standard deviation for the parameters of each treatment group over the entire incubation period. "low" and "high" indicate control and high CO_2 concentrations. Lengths and weights are given for beginning/end of the incubation period.

	Salinity (PSU)	Alkalinity (µmol/kgSW)	CO_2 (µmol/kgSW)	pH_{tot}	pCO_2 (µatm)	HCO_3^- (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCa	ΩAr	Temperature (°C)	Length (cm)	Weight (g)
0 °C low												
Mean	30.03	2445.27	2318.66	8.10	373.69	2192.05	103.22	2.52	1.57	0.75	13.7/14.3	16.3/19.7
SD	1.23	83.92	73.07	0.04	34.24	66.79	13.81	0.33	0.21	0.41	1.5/1.6	6.5/7.0
0 °C high												
Mean	29.97	2449.60	2456.20	7.68	1056.97	2348.37	41.15	1.01	0.63	0.31	13.8/14.2	15.3/18.6
SD	1.30	76.69	83.62	0.05	135.56	77.83	2.39	0.06	0.04	0.44	1.3/1.4	5.2/5.5
3 °C low												
Mean	29.96	2465.76	2340.26	8.05	425.07	2212.11	103.97	2.54	1.58	3.30	14.0/14.8	17.1/22.5
SD	1.02	52.12	49.53	0.02	28.93	46.97	6.05	0.14	0.09	0.11	1.4/1.5	5.7/6.0
3 °C high												
Mean	29.91	2440.40	2435.75	7.68	1073.06	2328.78	45.56	1.11	0.69	3.01	14.8/15.6	20.9/26.1
SD	0.91	52.68	54.26	0.05	131.51	50.84	4.18	0.10	0.06	0.07	1.1/1.1	5.8/5.8
6 °C low												
Mean	31.76	2253.07	2128.06	8.01	435.27	2006.99	98.81	2.39	1.50	5.74	13.9/15.0	15.1/21.8
SD	0.64	34.30	31.71	0.02	18.73	30.05	5.39	0.13	0.08	0.23	0.8/0.6	4.1/2.9
6 °C high												
Mean	31.77	2224.45	2213.15	7.64	1076.53	2113.71	44.27	1.07	0.67	5.71	14.8/15.8	18.2/25.8
SD	0.62	39.56	40.90	0.03	73.15	38.95	3.10	0.07	0.05	0.24	1.3/1.2	5.0/6.1
8 °C low												
Mean	33.43	2443.40	2275.64	8.04	433.47	2126.43	128.99	3.10	1.96	8.24	14.3/15.1	15.6/21.2
SD	0.68	27.98	22.26	0.02	16.98	19.07	5.16	0.12	0.08	0.14	1.5/1.5	4.6/5.8
8 °C high												
Mean	33.43	2436.57	2381.74	7.71	1026.75	2263.12	70.90	1.70	1.08	8.19	14.6/15.2	18.7/21.7
SD	0.69	31.43	25.46	0.06	103.48	40.78	25.30	0.61	0.38	0.16	2.4/2.2	11.9/10.0

Table S2: Carbonate chemistry, temperatures as well as animal lengths and weights of the incubation of *Gadus morhua*. The table depicts mean and standard deviation for the parameters of each treatment group over the entire incubation period. "low" and "high" indicate control and high CO_2 concentrations. Lengths and weights are given for beginning/end of the incubation period.

	Salinity (PSU)	Alkalinity (µmol/kgSW)	CO_2 (µmol/kgSW)	pH_{tot}	pCO_2 (µatm)	HCO_3^- (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	ΩCa	ΩAr	Temperature (°C)	Length (cm)	Weight (g)
3 °C low												
Mean	31.55	2092.48	1996.12	7.96	449.89	1893.19	78.64	1.91	1.20	3.11	18.4/18.9	43.3/47.4
SD	0.25	94.64	90.42	0.03	30.15	85.44	5.55	0.14	0.08	0.22	2.1/2.2	14.7/17.9
3 °C high												
Mean	31.59	2079.98	2098.94	7.54	1265.44	1999.10	31.82	0.77	0.48	3.15	19.5/20.0	51.9/58.6
SD	0.25	99.30	102.86	0.05	144.43	98.31	4.06	0.10	0.06	0.18	1.7/1.8	16.5/18.1
8 °C low												
Mean	32.93	2327.67	2179.15	8.02	437.44	2043.75	114.60	2.76	1.74	8.20	18.4/19.3	44.2/57.7
SD	0.26	60.41	49.68	0.01	10.23	42.25	8.31	0.20	0.13	0.09	2.7/3.3	24.2/36.7
8 °C high												
Mean	32.19	2316.33	2258.62	7.77	851.98	2150.19	67.75	1.53	0.96	8.11	17.6/18.3	39.0/46.8
SD	2.12	49.46	41.91	0.09	109.85	41.40	11.60	0.25	0.16	0.13	2.2/2.4	16.0/21.0
12 °C low												
Mean	31.67	2474.28	2292.30	8.03	458.27	2131.77	141.34	3.43	2.17	12.30	18.0/19.9	38.4/56.1
SD	0.22	64.54	54.46	0.04	45.53	49.87	14.96	0.36	0.23	0.43	2.7/2.1	14.1/23.8
12 °C high												
Mean	31.74	2462.05	2415.41	7.67	1137.25	2301.65	66.02	1.60	1.02	12.16	17.5/19.5	36.9/58.2
SD	0.26	27.34	26.68	0.03	91.64	25.41	5.34	0.13	0.08	0.35	2.2/2.6	14.9/26.6
16 °C low												
Mean	32.58	1970.21	1840.24	7.90	515.25	1723.54	97.41	2.36	1.51	15.73	17.8/19.4	37.8/55.4
SD	0.26	99.13	88.19	0.03	25.84	79.57	9.70	0.23	0.15	0.41	2.2/2.4	14.3/24.5
16 °C high												
Mean	32.55	1961.32	1947.82	7.50	1416.11	1851.16	43.52	1.05	0.67	15.71	18.8/20.8	44.8/75.2
SD	0.30	94.98	74.37	0.12	357.17	71.66	13.36	0.32	0.21	0.44	2.8/3.4	23.2/41.4

Figure S1: Representative incubation system of one treatment group with 12 animals. Each grey tank contains a single individual. Water supply is given from one header tank (white container on top) for each treatment group, in which water is pre-gassed in accordance to the respective CO_2 treatment (Photograph courtesy of Kristina Kunz).

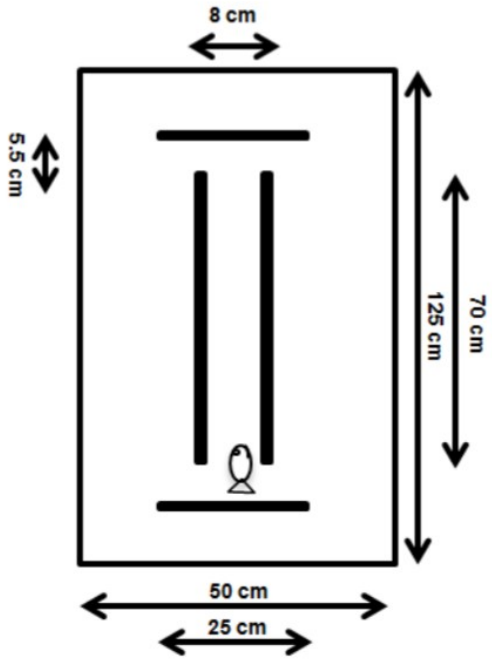


Figure S2: Sketch of two sided T-maze for a Detour test. Relations in this image are not to scale.

6.3 Electronic supplementary material of Manuscript II

Species	Temperature [°C]	CO_2-Group	Group size NMR	Group size HPLC	pCO_2 [µatm]
Boreogadus saida	0	Low	5	6	396.25 ± 37.7
Boreogadus saida	0	High	6	5	1091.64 ± 131.67
Boreogadus saida	3	Low	4	5	431.1 ± 40.41
Boreogadus saida	3	High	5	5	1135.99 ± 147.1
Boreogadus saida	6	Low	6	6	437.09 ± 21.6
Boreogadus saida	6	High	5	5	1123.5 ± 86.23
Boreogadus saida	8	Low	4	4	440.9 ± 29.1
Boreogadus saida	8	High	3	3	1052.88 ± 103.87
Gadus mohua	3	Low	5	5	449.08 ± 39.04
Gadus mohua	3	High	4	5	1127.75 ± 164.89
Gadus mohua	8	Low	6	5	434.51 ± 49.9
Gadus mohua	8	High	6	5	915.08 ± 127.39
Gadus mohua	12	Low	5	5	490.29 ± 112.75
Gadus mohua	12	High	5	5	1163.71 ± 199.68
Gadus mohua	16	Low	4	4	547.87 ± 92.69
Gadus mohua	16	High	5	5	1271.67 ± 365.09

Additional file 1: Table S1: Group sizes and environmental CO_2 partial pressure (pCO_2, mean ± standard deviation after Kunz *et al.* [22]) of *Boreogadus saida* and *Gadus morhua* that were utilized for NMR and HPLC analysis.

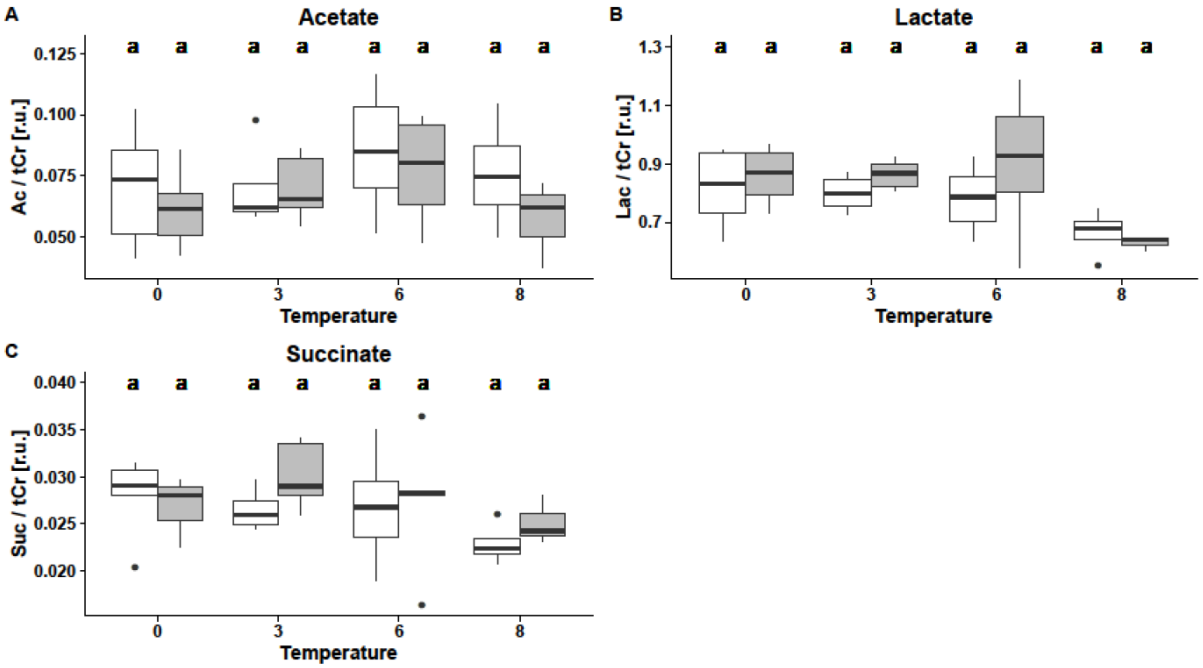
Minute	Acetate-buffer/Acetonitrile [%]	Acetonitrile [%]
0	88	12
60	80	20
62	60	40
64	40	60
66	20	80
68	0	100
72	20	80
74	40	60
76	60	40
78	80	20
80-90	88	12

Additional file 2: Table S2: Protocol depicting the buffer composition throughout HPLC-analysis. Total measurement time per sample was 90 minutes. The first column shows the time for onset of the respective composition.

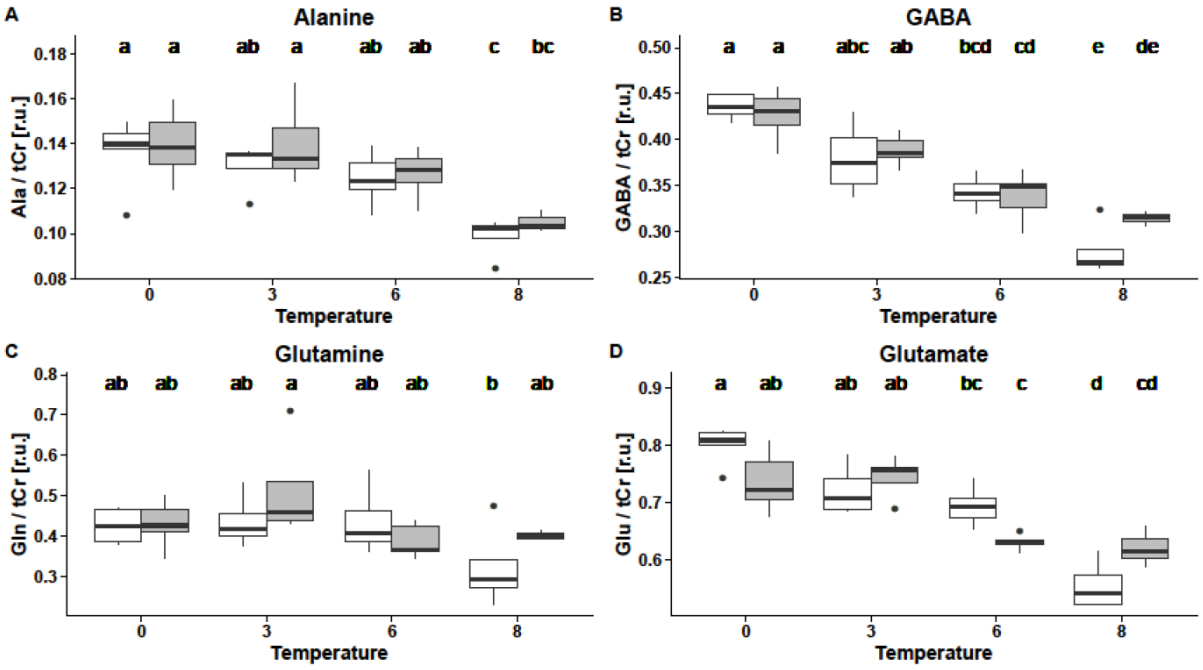
Compound	*Boreogadus saida*	*Gadus morhua*
Acetate	3 °C Control CO_2	
Acetyl-histidine		
Alanine	3 °C Control CO_2 8 °C Control CO_2	
Choline	3 °C Control CO_2 8 °C Control CO_2	
Gamma-aminobutyric acid	8 °C Control CO_2	
Glutamine		3 °C High CO_2
Glycerophosphocholine		8 °C High CO_2 16 °C High CO_2
Glutamate		3 °C Control CO_2 16 °C Control CO_2
Glycine	3 °C High CO_2	
Lactate		
Myo-inositol		
N-acetylaspartate		
Posphocholine		
Putrescine	8 °C Control CO_2	
Succinate		3 °C Control CO_2
Taurine		16 °C High CO_2
Trimethylamine *N*-oxide	6 °C High CO_2	8 °C High CO_2

Additional file 3: Table S3: List of treatment groups that violated normality-distribution for the respective components.

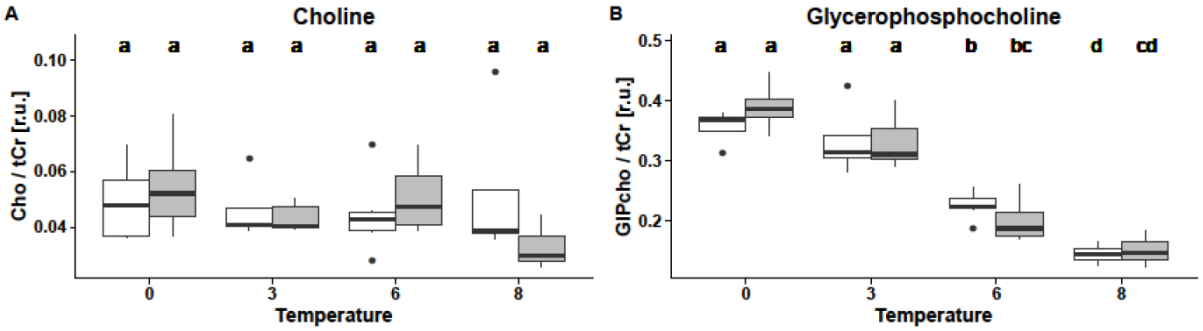
Energy metabolism

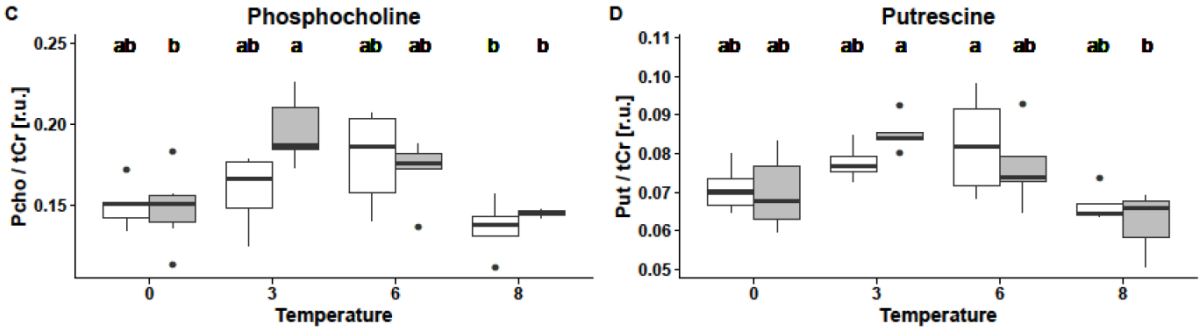


GABA metabolism

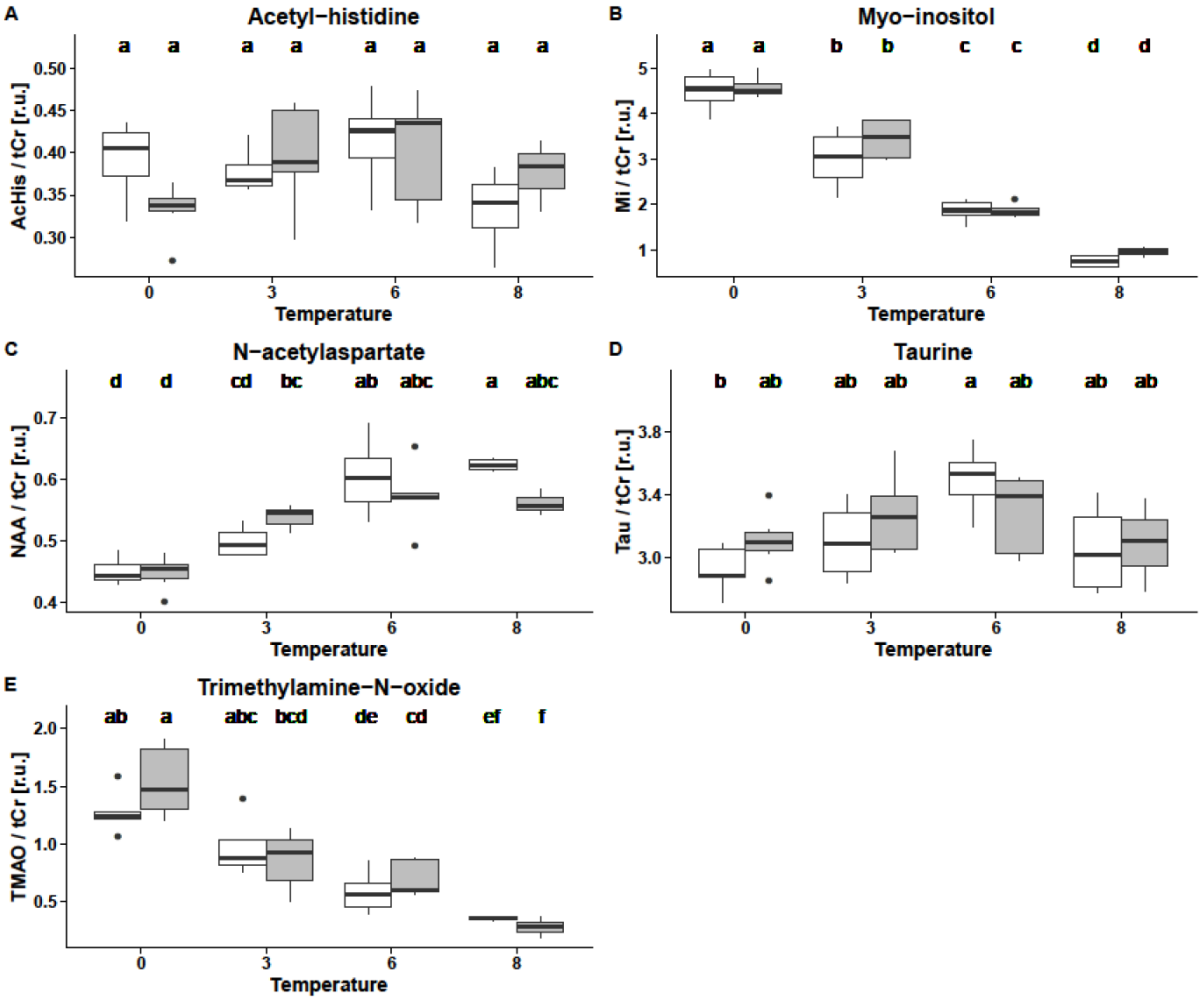


Membrane components

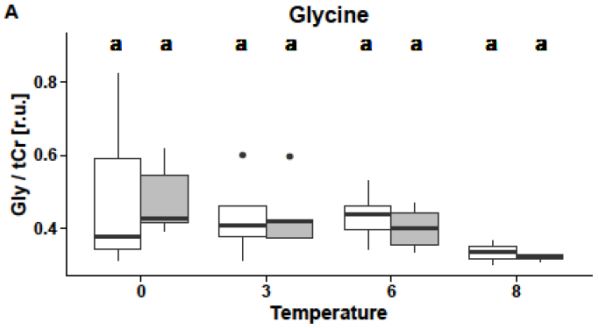




Osmolytes

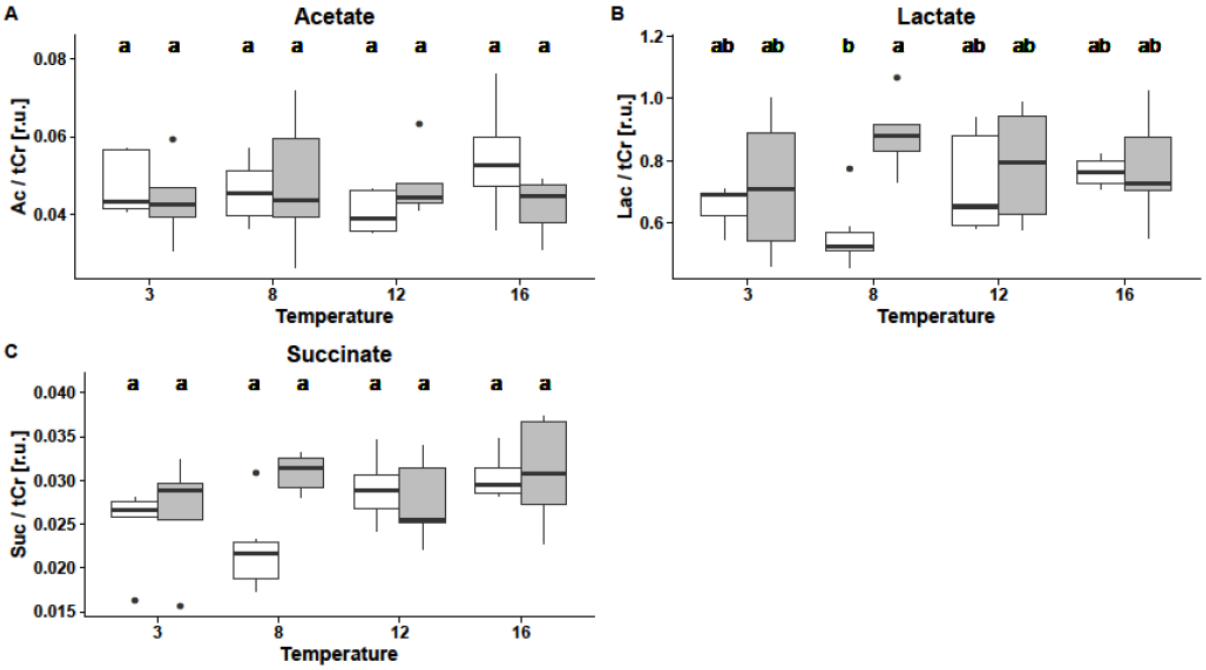


Other

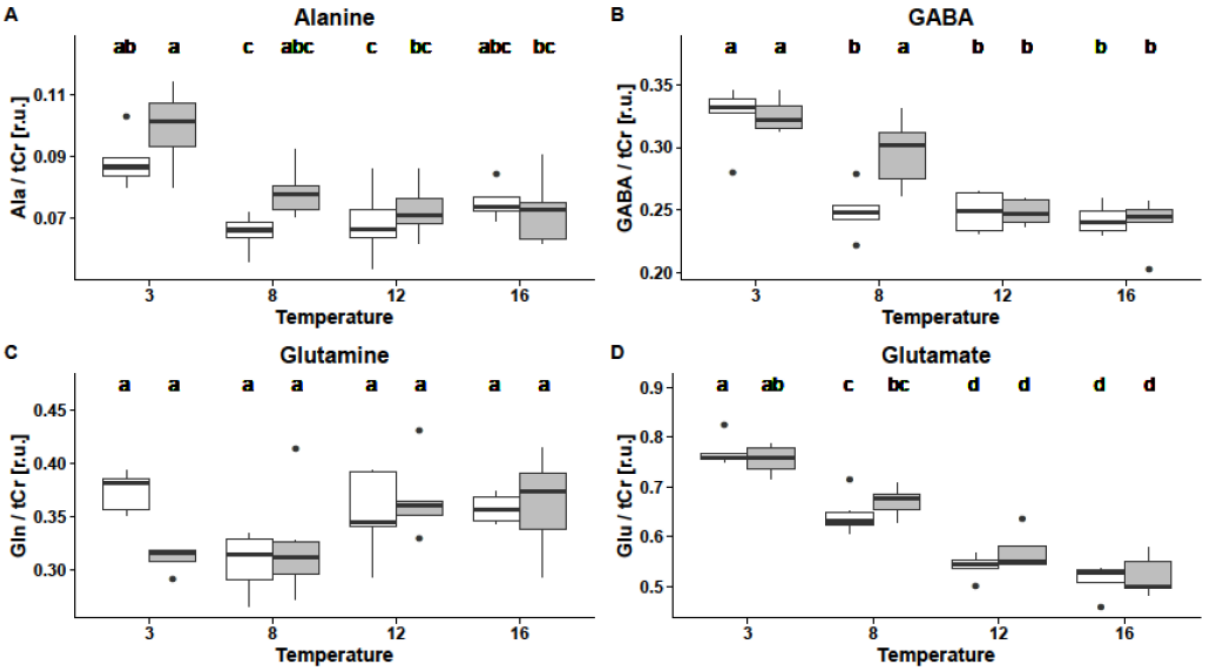


Additional file 4: Figure S1: Boxplots depicting metabolite concentrations (relative to total creatine (tCr)) in the brain of *Boreogadus saida* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$). Metabolites were sorted functionally in accordance with Table 1.

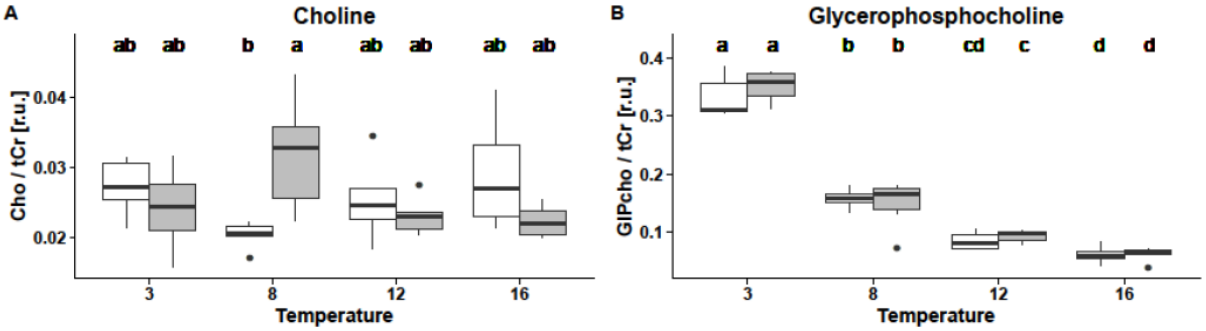
Energy metabolism

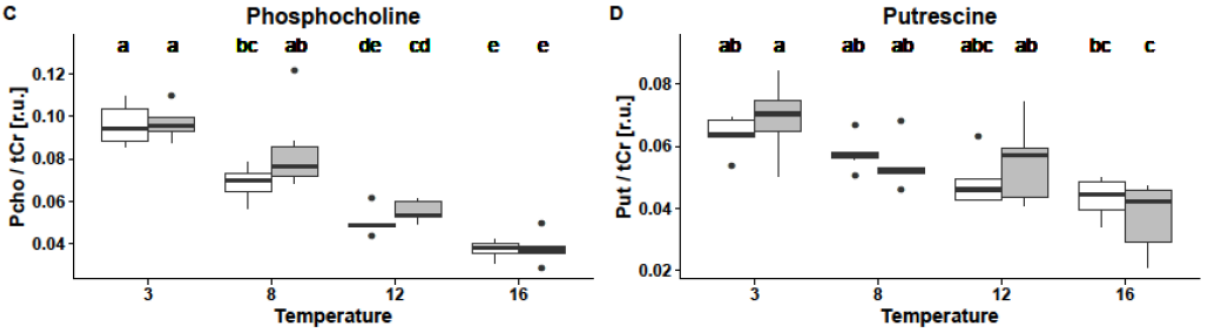


GABA metabolism

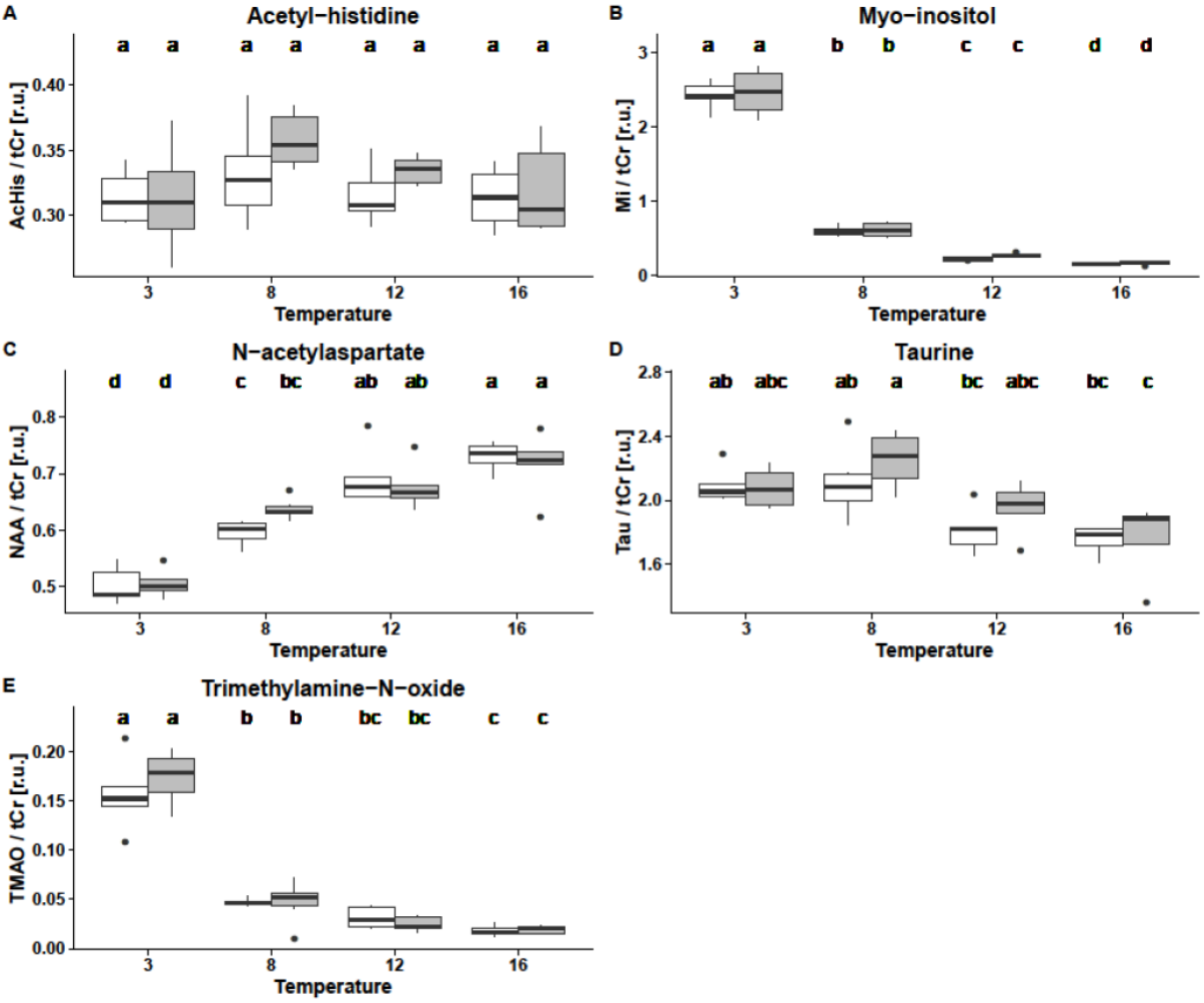


Membrane components

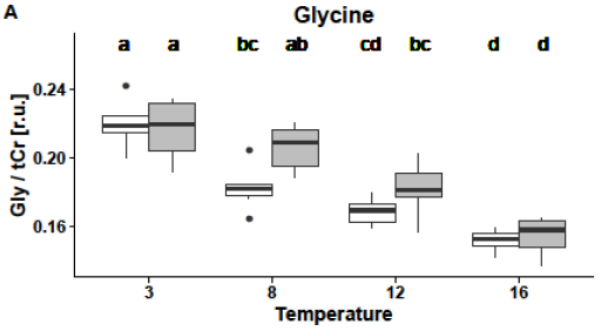




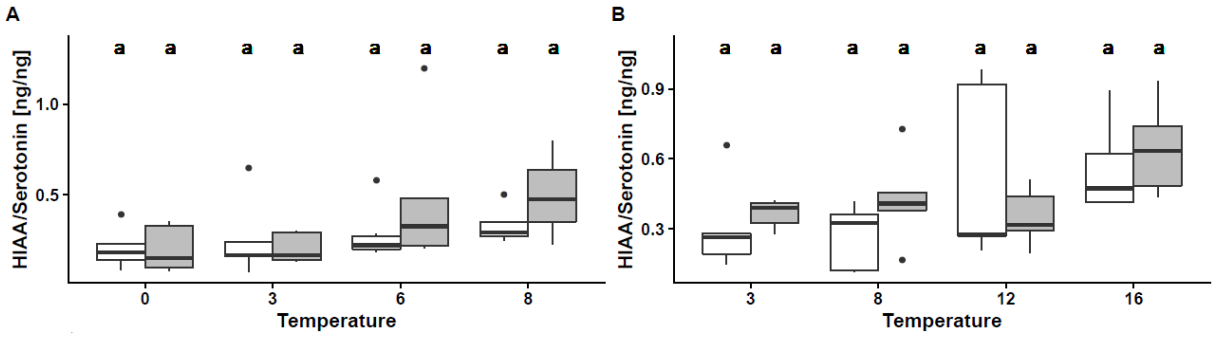
Osmolytes



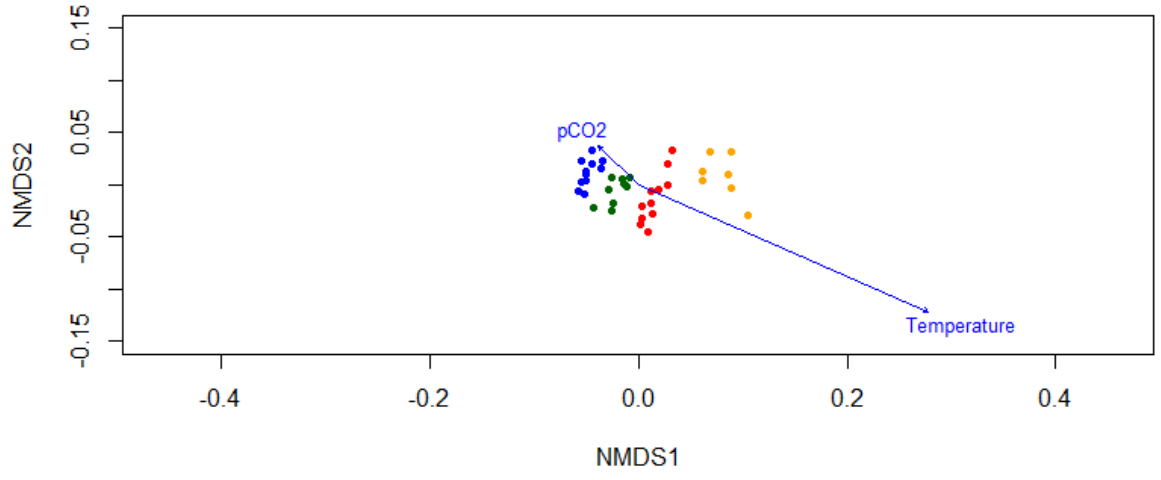
Other



Additional file 5: Figure S2: Boxplots depicting metabolite concentrations (s.a.) in the brain of *Gadus morhua* at different temperatures and CO_2 partial pressures. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$). Metabolites were sorted functionally in accordance with Table 1.

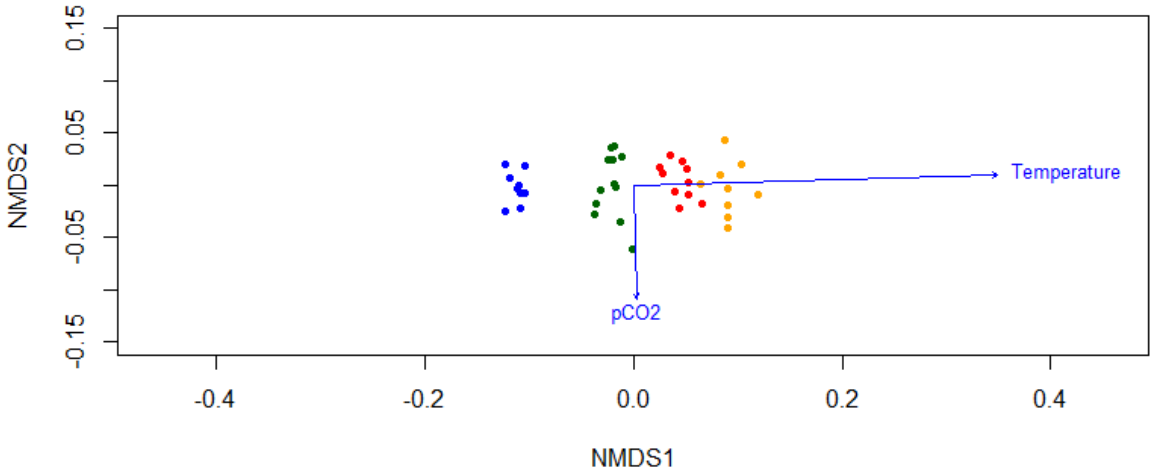


Additional file 6: Figure S3: Boxplots depicting the amount of 5-Hydroxyindoleacetic acid (HIAA) relative to Serotonin (5-HT) in the brain of *Boreogadus saida* (A) and *Gadus morhua* (B) quantified with HPLC. White shading indicates control, grey shading high CO_2 partial pressure. Each box contains median, first and third quartile. Different letters indicate significant differences detected with Tukey HSD post hoc analysis ($p < 0.05$).



Additional file 7: Figure S4: Non-metric multidimensional scaling of metabolite/total creatine ratios in

the brain of *Boreogadus saida*. Dots indicate individuals with colours representing the respective treatment temperature. Blue = 0 °C, green = 3 °C, red = 6 °C, yellow = 8 °C.



Additional file 8: Figure S5: Non-metric multidimensional scaling of metabolite/total creatine ratios in the brain of *Gadus morhua*. Dots indicate individuals with colours representing the respective treatment temperature. Blue = 3 °C, green = 8 °C, red = 12 °C, yellow = 16 °C.

Additional file 9: Rawdata: Available online under
https://frontiersinzoology.biomedcentral.com/articles/10.1186/s12983-017-0238-5

6.4 Electronic supplementary material of Manuscript III

	Salinity	TA (µmol/kgSW)	TCO_2 (µmol/kgSW)	pH_{tot}	pCO_2 (µatm)	HCO_3^- (µmol/kgSW)	CO_3^{2-} (µmol/kgSW)	CO_2 (µmol/kgSW)	ΩCa	ΩAr
Low CO_2										
Mean	31.43	2644.3	2415.16	8.3	251.89	2226.57	172.41	16.17	4.2	2.62
SD	0.06	111.62	15.91	0.19	99.51	65.65	73.98	6.3	1.8	1.12
High CO_2										
Mean	31.68	2391.4	2452.1	7.47	1668.02	2323.12	27.46	101.51	0.67	0.42
SD	0.21	79.4	77.31	0.06	249.52	74.74	3.75	12.37	0.09	0.06

Additional file 1: Table S1: Carbonate chemistry of seawater under low (= Control) and high CO_2 conditions (Mean ± SD).
TA: Total alkalinity, TCO2: Total CO_2 species, SD: Standard deviation.

Additional file 2: Rawdata: Will be published together with the manuscript.

6.5 R-codes

Figure 2 A+B:

```
require(fields)

R<-8.315 # Ideal gas constant

T<- 285.15 # Temperature in Kelvin, Figure 2B was calculated with T<-276.15

F<-96.485 # Faraday constant


Cle<-seq(144, 150, by=0.1) # Extracellular chloride concentration in mM

Cli<-8 # Intracellular chloride concentration in mM

HCO3i<-4 # Intracellular bicarbonate concentration in mM

HCO3e<-seq(10,22, by=0.1) # Extracellular bicarbonate concentration in mM

PHCO3<-0.3 # Relative permeability of the GABA_A-receptor for bicarbonate

PCl<-1.0 # Relative permeability of the GABA_A-receptor for chloride


E<-function(HCO3e,Cle)

{

R*T/F*log((PCl*Cli+PHCO3*HCO3i)/(PCl*Cle+PHCO3*HCO3e))

}

EGABA<-outer(HCO3e,Cle,E)


image.plot(HCO3e, Cle, EGABA, xlab="HCO3e [mM]", ylab="Cle [mM]", legend.lab="mV",
breaks=seq(-87, -60, length.out=65), col=tim.colors(64))
```

Figure 2 C+D:

```
require(fields)
R<-8.315 # Ideal gas constant
T<- 285.15 # Temperature in Kelvin, Figure 2D was calculated with T<-276.15
F<-96.485 # Faraday constant

Cle<- 150 # Extracellular chloride concentration in mM
Cli<-seq(2,8, by=0.1) # Intracellular chloride concentration in mM
HCO3e<- 10 # Extracellular bicarbonate concentration in mM
HCO3i<- seq(4,10, by=0.1) # Intracellular bicarbonate concentration in mM
PHCO3<-0.3 # Relative permeability of the GABA_A-receptor for bicarbonate
PCl<-1.0 # Relative permeability of the GABA_A-receptor for chloride

E<-function(HCO3i,Cli)
{
R*T/F*log((PCl*Cli+PHCO3*HCO3i)/(PCl*Cle+PHCO3*HCO3e))
}
EGABA<-outer(HCO3i,Cli,E)

image.plot(HCO3i, Cli, EGABA, xlab="HCO3i [mM]", ylab="Cli [mM]", legend.lab="mV",
breaks=seq(-87, -60, length.out=65), col=tim.colors(64))
```

Figure 2 E+F:

```
require(fields)
R<-8.315 # Ideal gas constant
T<- 285.15 # Temperature in Kelvin, Figure 2F was calculated with T<-276.15
F<-96.485 # Faraday constant

Cle<- 150 # Extracellular chloride concentration in mM
Cli<- 8 # Intracellular chloride concentration in mM
HCO3e<- 10 # Extracellular bicarbonate concentration in mM
HCO3i<- seq(4,10, by=0.1) # Intracellular bicarbonate concentration in mM
PHCO3<-seq(0.1,0.4, by =0.01) # Relative permeability of the GABA_A-receptor for bicarbonate
PCl<-1.0 # Relative permeability of the GABA_A-receptor for chloride

E<-function(HCO3i,PHCO3)
{
R*T/F*log((PCl*Cli+PHCO3*HCO3i)/(PCl*Cle+PHCO3*HCO3e))
}
EGABA<-outer(HCO3i,PHCO3,E)

image.plot(HCO3i, PHCO3, EGABA, xlab="HCO3i [mM]", ylab="PHCO3 / PCl", legend.lab="mV",
breaks=seq(-87, -60, length.out=65), col=tim.colors(64))
```

Figure 3:

```
require(PlotSvalbard)

Species = c("o", "o", "o", "o") #defines the marks on the map

lat.utm = c(8767759.9, 8898534.2, 8804506.2, 8719638.4) # sampling latitudes

lon.utm = c( 446833, 521388.4, 449647.1, 551001.3) # sampling longitudes

df = data.frame(Species, lon.utm, lat.utm)


basemap("svalbard", round.lat = 1, round.lon = 2) + geom_text(data = df, aes(x = lon.utm, y =
lat.utm, label = Species), color = c("blue", "red", "red", "red"), fontface = 2, size=35.4/72.27*30)
```

7. Eigenständigkeitserklärung nach §6 (5) der Promotionsordnung Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche (vom 14. März 2007)

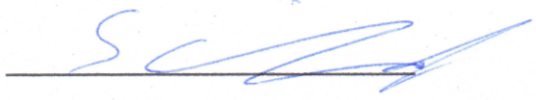
ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Behavioural disturbances and underlying neurophysiological mechanisms during ocean acidification and warming in *Gadus morhua* and *Boreogadus saida*

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.



8. Contributions to the first-author manuscripts of this thesis

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:

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

Experimental concept and design:	ca. 70%
Experimental work and/or acquisition of (experimental) data:	ca. 100%
Data analysis and interpretation:	ca. 80%
Preparation of Figures and Tables:	ca. 100%
Drafting of the manuscript:	ca. 80%

Chapter:

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

Experimental concept and design:	ca. 70%
Experimental work and/or acquisition of (experimental) data:	ca. 80%
Data analysis and interpretation:	ca. 85%
Preparation of Figures and Tables:	ca. 95%
Drafting of the manuscript:	ca. 80%

Chapter:

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

Experimental concept and design:	ca. 80%
Experimental work and/or acquisition of (experimental) data:	ca. 95%
Data analysis and interpretation:	ca. 80%
Preparation of Figures and Tables:	ca. 95%
Drafting of the manuscript:	ca. 80%

Date: 02.04.19

Signatures: 

9. Acknowledgements

An dieser Stelle möchte ich mich bei Prof. Dr. Hans-Otto Pörtner sowie bei Dr. Christian Bock und Dr. Daniela Storch für die Betreuung dieser Doktorarbeit bedanken. Für ihr Vertrauen, mich eigene Ideen und Ansätze entwickeln und umsetzen zu lassen.

Prof. Dr. Michael Koch danke ich sehr herzlich für die Übernahme der Rolle des Zweitgutachters.

Der Graduiertenschule Polmar mit Dr. Claudia Hanfland und Dr. Claudia Sprengel möchte ich für ihre Unterstützung danken.

Jasmine Nahrgang who provided the specimens of *Boreogadus saida* needed for this thesis.

Bei Dr. Ekkehard Küstermann der Universität Bremen für zahlreiche fachliche Gespräche insbesondere zu Beginn der Dissertation.

Kai-Uwe Ludwichowski und der Abteilung Marine Chemie für die Einarbeitung, Beratung und zweitweise Überlassung ihrer HPLC und insbesondere für ihre Geduld.

Dem Team der Institutscafeteria „Alfreds“, die mir bei intensiven Arbeitsphasen häufig eine besonders große Portion gegeben haben.

Allen Mitgliedern der Sektion Integrative Ökophysiologie für ihre Menschlichkeit, fachliche und technische Beratung.

Dem Bundesministerium für Bildung und Forschung, welches diese Arbeit zu großen Teilen finanziert hat.

Anna-Carla Raddatz, dafür, dass Du immer an mich glaubst.

Meine Familie für ihre durchgängige Unterstützung in allen Lebenslagen in jeglicher Hinsicht ohne die diese Arbeit nicht möglich gewesen wäre. Günter, Konni, Stefan, Nicola, Bettina: Danke schön!

The thesis contains corrections of spelling mistakes