Intra-specific variation of ocean acidification effects in marine mussels and oysters: integrative physiological studies on tissue and organism responses

Intra-spezifische Unterschiede in Effekten von Ozeanversauerung auf marine Muscheln und Austern: integrative physiologische Studien am isolierten Gewebe und Gesamtorganismus

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"Even if you never have the chance to see or touch the ocean, the ocean touches you with every breath you take, every drop of water you drink, every bite you consume. Everyone, everywhere is inextricably connected to and utterly dependent upon the existence of the sea."

– Sylvia Earle

Preface

This thesis aims at contributing to the understanding of physiological mechanisms underlying the intra-specific variation in sensitivity of marine mussels and oysters to future ocean acidification in order to anticipate their potential for acclimatisation and adaptation. The research was conducted at the Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research (AWI) under supervision of Prof. Dr. Hans-Otto Pörtner, Dr. Gisela Lannig and Dr. Christian Bock as part of the second phase of the German national research project BIOACID (Biological Impacts of Ocean Acidification, sub-project 3.7, grant No. FKZ 03F0655B), funded by the Federal Ministry of Education and Research (BMBF). Parts of this thesis were performed at the Helmholtz Institute for Ocean Research, GEOMAR in Kiel, Germany in collaboration with PD Dr. Frank Melzner and Dr. Jörn Thomsen as well as at the Port Stephens Fisheries Institute (PSFI), NSW, Australia in collaboration with Dr. Laura Parker (University of Sydney), Dr. Wayne O'Connor (New South Wales Department of Primary Industries) and Prof. Dr. Pauline Ross (University of Sydney). Research conducted at the PSFI was supported by a short-term research scholarship of the German Academic Exchange Service (DAAD).

Table of Contents

List of Figures

List of Tables

Abbreviations

AE	Absorption efficiency
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
A_T	Total alkalinity
ATP	Adenosine triphosphate
AWI	Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research
β_{NB}	Non-bicarbonate buffers
BMBF	German Federal Ministry of Education and Research
CI	Condition index
Cl^-	Chloride ion
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
COX	Cytochrome c oxidase
CR	Clearance rate
CS	Citrate synthase
DAAD	German Academic Exchange Service
DIC/C_T	Dissolved inorganic carbon
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithio-bis-[2-nitrobenzoic acid]
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FAO	Food and Agriculture Organization
FMS	Factorial metabolic scope
FSW	Filtered seawater
FW	Fresh weight
GDH	Glutamate dehydrogenase
H^+	Hydrogen ion
HCO_3^-	Bicarbonate ion
H_2CO_3	Carbonic acid
HADH	3-Hydroxyacyl-CoA dehydrogenase
HCl	Hydrogen chloride
IDP	Inosine diphosphate
IDH	Isocitrate dehydrogenase
$KHSO_4$	Potassium bisulfate
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese (II) chloride
MO_2	Oxygen consumption
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide

$NaHCO_3$	Sodium bicarbonate
NH_4^+	Ammonia
NMS	Net metabolic scope
NSW	New South Wales
OA	Ocean acidification
PC	Protein content
PCO_2	Partial pressure of carbon dioxide
PD I/II	Prodissoconch I/II
PO_2	Partial pressure of oxygen
PEP	Phosphoenpolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
pH_{NBS}	pH on the National Bureau of Standards (NBS) scale
pH_e	Extracellular pH
pH_i	Intracellular pH
pH_{Total}	pH on the total scale
PK	Pyruvate kinase
PSFI	Port Stephens Fisheries Institute
PSU	Practical salinity unit
IPCC	Intergovernmental Panel on Climate Change
RCP	Representative Concentration Pathway
RMR	Routine metabolic rate
TCA-cycle	Tricarboxylic acid cycle
Tris	Tris(hydroxymethyl)aminomethane
UNFCC	United Nations Framework Convention on Climate Change
$\Omega_{calcite/aragonite}$	Calcite/aragonite saturation state of seawater

Summary

Uptake of increasing anthropogenic CO_2 emissions by ocean surface waters is causing an increase of seawater PCO_2 accompanied by a decrease of seawater pH and carbonate ion concentrations. This process, termed ocean acidification (OA), is predicted to negatively affect many marine organisms with likely consequences for marine ecosystems and the services they provide.

Calcifying mussels and oysters, and particularly their early life stages, are predicted to be among the most OA sensitive taxa, as OA interferes with the calcification process. In addition, mussels and oysters possess a relatively low ability to compensate for CO_2 induced disturbances in extracellular body fluid pH with potential physiological downstream effects such as elevated metabolic maintenance costs. As mussels and oysters are key habitat forming organisms in many highly productive temperate coastal communities, negative OA effects may translate into deleterious effects at an ecosystem scale. In particular, the relative long generation time of most marine bivalves raises the concern that the rapid rate at which OA occurs may outpace species' ability to genetically adapt, leaving pre-existing genetic variation as a potential key to species resilience under OA. Against this backdrop, this thesis contributes to the understanding of physiological mechanisms that underpin and define the OA vulnerability of ecologically and economically important mussels and oysters. Thereby, emphasis was placed on investigating intra-specific variance as a proxy for potential adaptive capacities.

Kiel Fjord is located in the Western Baltic Sea and is characterised by strong seasonal and diurnal fluctuations in seawater PCO_2. These fluctuations are caused by upwelling events of acidified bottom waters with peak PCO_2 values (>2300 µatm) that are already by far exceeding those projected for open ocean surface waters by the end of this century. Despite these unfavourable conditions, blue mussels (M*ytilus edulis)* dominate the benthic community, which makes this population particularly interesting in the context of metabolic adaptation to OA. Consequently, a long-term multi-generation CO_2 acclimation experiment with different family lines of *M. edulis* from Kiel Fjord formed the first part of this thesis. Offspring of 16 different family lines were transferred to three different PCO_2 conditions, representing present and predicted PCO_2 levels in Kiel Fjord (700 µatm (control), 1120 µatm (intermediate) and 2400 µatm (high)). Larval survival rates were substantially different between family lines at the highest PCO_2 level. Based on these differences, families were classified as either 'tolerant' (i.e. successful settlement at all PCO_2 levels) or 'sensitive' (i.e. successful settlement only

at control and intermediate PCO_2 level). Subsequently, the offspring were raised for over one year at respective PCO_2 levels, followed by measurements of physiological parameters at the whole-animal, tissue (gill and outer mantle) and biochemical level (key metabolic enzymes). The results revealed that routine metabolic rates (RMR) and summed tissue respiration were increased in tolerant families at intermediate PCO_2, indicating elevated homeostatic costs. However, this higher energy demand at the intermediate PCO_2 level was not accompanied by a simultaneous increase in energy assimilation (i.e. clearance rates (CR)), indicating an incipient imbalance in energy demand and supply. Consequently, RMRs at the highest PCO_2 were not different to control RMRs but associated with reduced CRs, which correlated with a lower gill metabolic scope, reduced gill mitochondrial capacities (lower capacities for citrate synthase (CS) and cytochrome c oxidase (COX)) as well as an increased capacity for anaerobic energy production (lower ratio of pyruvate kinase to phospoenolpyruvate carboxykinase). In conjunction with a lower COX to CS ratio observed in outer mantle tissue, this suggested a CO_2-induced shift of metabolic pathways in tolerant families at the highest PCO_2 level. By contrast, sensitive families had an unchanged RMR, tissue respiration and CR at the intermediate CO_2. However, a higher control RMR in sensitive than tolerant families at similar CR suggested a lower, CO_2 independent metabolic efficiency in sensitive families. This was also reflected in their lower gill mitochondrial scope at control conditions compared to tolerant families. These findings suggested that sensitive families lack the metabolic scope to cover OA induced higher maintenance costs and have to rely on energy reallocation and thus, energy trade-offs which may also have prevented survival at the highest experimental PCO_2 level. Accordingly, investigations of 3-hydroxyacyl-CoA dehydrogenase (HADH) capacities, which catalyses a key step in lipid oxidation, suggested an increased reliance on lipids as metabolic fuel in sensitive families at elevated PCO_2. If this was also prevalent during the larval phase, a quicker depletion of lipid reserves before completion of metamorphosis may have contributed to the higher larval mortality at the highest PCO_2 treatment in sensitive compared to tolerant mussels.

The second part of the thesis aimed to clarify whether a higher OA tolerance in Sydney rock oysters (*Saccostrea glomerata*) is directly correlated with an increased capacity to compensate for CO_2 induced extracellular acid-base disturbances, and whether such a capacity is driven by higher metabolic and ion-regulatory costs at the tissue level. Earlier studies focusing on two different populations of Sydney rock oysters demonstrated that oysters that were selectively bred for increased growth and disease resistance ('selected oysters') have a higher CO_2 resilience compared to the wild population ('wild oysters'). To unravel the underlying physiologi-

cal mechanisms, oysters of both populations were acclimated at control and elevated PCO_2 (1100 μatm) levels for seven weeks, followed by determinations of extracellular acid-base parameters (pH_e, P_eCO_2, $[HCO_3^-]_e$), tissue respiration and indirect determination of energy demands of major ion regulatory transport proteins. Indeed, at elevated PCO_2, wild oysters had a lower pH_e and an increased P_eCO_2 whereas extracellular acid-base status of selected oysters remained unaffected. However, differing pH_e values between oyster types were not driven by elevated metabolic costs of major ion regulators at tissue level. Selected oysters rather exhibited an increased systemic capacity to eliminate metabolic CO_2, which likely came through higher and energetically more efficient filtration rates and associated facilitation of gas exchange, suggesting that effective filtration and CO_2 resilience might be positively correlated traits in oysters.

In conclusion, the findings of this thesis contribute to the growing evidence that ongoing OA will likely impair the physiology of marine mussels and oysters with potentially associated downstream consequences for the respective ecosystems. However, the results also suggest adaptive capacities in both species studied. The higher CO_2 resilience of selected Sydney rock oysters was expressed within the – in evolutionary terms – rapid time span of only a few generations of selective breeding, which indicates that rapid adaptation to OA may be possible in marine bivalves. The observed intra-specific variation of OA responses in blue mussels suggests standing genetic variation within this population, which is likely to be key for the persistence of populations under rapidly occurring OA. However, as global change is not limited to OA, future research will have to reassess potential resilience traits and adaptive capacities to OA when combined with changes in other environmental drivers.

Zusammenfassung

Der anthropogen verursachte, rapide Anstieg von atmosphärischen CO_2-Konzentrationen führt zu einem simultanen Anstieg des PCO_2 im Oberflächenwasser der Ozeane, einhergehend mit einer Absenkung des pH-Wertes und der Karbonationenkonzentration. Dieser Prozess, der als „Ozeanversauerung" bezeichnet wird, wirkt sich vermutlich negativ auf viele marine Organismen aus und wird daher potentiell weitreichende Konsequenzen für marine Ökosysteme sowie deren Dienstleistungen haben.

Schalenbildende Muscheln und Austern, insbesondere deren frühen Lebensstadien, reagieren scheinbar besonders empfindlich auf die Ozeanversauerung, da diese sich negativ auf den Kalzifizierungsprozess auswirkt. Zudem besitzen Muscheln und Austern eine relativ geringe Kapazität um CO_2-induzierte Säure-Base-Störungen des extrazellulären Raumes zu kompensieren, was mögliche Folgen in Form von erhöhten ionenregulatorische Kosten, und demnach eines erhöhten Ganztier-Grundumsatzes, nach sich ziehen kann. Riffbildende Muscheln und Austern sind Schlüsselarten in vielen küstennahen Ökosystemen. Daher könnten sich negative Effekte der Ozeanversauerung auf die Physiologie dieser Arten in weitreichenden Konsequenzen auf der Ökosystemebene niederschlagen. Zudem wird befürchtet, dass aufgrund relativ langer Generationszeiten in marinen Bivalven, die Ozeanversauerung rascher voranschreiten wird als die Fähigkeit dieser Organismen sich genetisch anzupassen. Daher könnte bereits bestehende genetische Variation von Arten und Populationen möglicherweise ein Schlüssel zur Resilienz gegenüber der rasch voranschreitenden Ozeanversauerung darstellen. Vor diesem Hintergrund war das Ziel dieser Arbeit zu einem besseren Verständnis der physiologischen Mechanismen beizutragen, welche der hohen Sensitivität von marinen Muscheln und Austern gegenüber der Ozeanversauerung zugrunde liegen. Als Anhaltspunkt für potentielle Anpassungskapazitäten wurde dabei ein besonderes Augenmerk auf intraspezifische Unterschiede von Ozeanversauerungseffekten gelegt.

Die Kieler Förde in der westlichen Ostsee ist, im Vergleich zum offenen Ozean, durch starke diurnale und saisonale PCO_2-Schwankungen geprägt, welche durch den Auftrieb von hypoxischem und mit CO_2 angereichertem, bodennahem Wasser verursacht werden. Dies kann zu PCO_2 Höchstwerten (>2300 µatm) führen, die schon jetzt bei weitem PCO_2 Werte überschreiten, die für Oberflächenwasser des offenen Ozeans für Ende des Jahrhunderts prognostiziert sind. Trotz dieser unvorteilhaften Bedingungen dominiert die Miesmuschel (*Mytilus edulis)* die benthische Gemeinschaft, was diese Population im Hinblick auf metabolische Anpassungen an die Ozeanversauerung besonders interessant macht. Daher wurde im ersten

Teil dieser Arbeit ein Multigenerationen-Experiment mit verschiedenen Familien von Miesmuscheln aus der Kieler Förde durchgeführt. Hierfür wurden die Nachkommen von 16 verschiedenen *M. edulis* Familien auf drei verschiedene experimentelle PCO_2 Szenarien verteilt, welche aktuellen sowie prognostizierten PCO_2-Werten in der Kieler Förde für Ende des Jahrhunderts entsprachen (700 µatm (Kontrolle), 1120 µatm (intermediär) und 2400 µatm (hoch)). Unter dem höchsten Seewasser PCO_2 (2400 µatm) führte dies zu stark schwankenden, familienspezifischen larvalen Überlebensraten. Basierend auf diesem Ergebnis wurden die Familien in „tolerant" (erfolgreiches Siedeln der Larven unter allen drei PCO_2-Bedingungen) und „sensitiv" (Siedeln der Larven nur unter Kontroll- und intermediären PCO_2) eingeteilt. Anschließend wuchsen die Nachkommen unter den jeweiligen PCO_2 Szenarien auf, gefolgt von Messungen physiologischer Parameter auf Ganztier-, Gewebs- (Kieme und marginaler Mantel) sowie auf biochemischer Ebene (metabolische Schlüsselenzyme). Nach einjähriger CO_2-Inkubation zeigten tolerante Familien einen erhöhten Stoffwechsel auf Gewebs- und Ganztierebene unter den intermediären PCO_2-Bedingungen. Dieser erhöhte Energiebedarf wurde jedoch nicht von einer erhöhten Nahrungsaufnahme (Filtrationsrate) begleitet, was auf ein einsetzendes Ungleichgewicht von Energiebedarf und -versorgung hindeutet. Demnach zeigten Muscheln toleranter Familien reduzierte Stoffwechsel- und Filtrationsraten unter den Hoch-CO_2-Bedigungen. Damit einhergehend wurde eine reduzierte metabolische Kapazität des Kiemengewebes, eine reduzierte aerobe mitochondriale Kapazität (Citratsynthase (CS) und Cytochrom-c-Oxidase (COX)) sowie eine potentiell erhöhte anaerobe Energiegewinnung beobachtet (erniedrigtes Verhältnis von Pyruvatkinase zu Phosphoenolpyruvat-Carboxykinase). Zusammen mit einem erniedrigten Verhältnis von CS zu COX im Mantelgewebe, deutet dies auf eine Verschiebung von Stoffwechselwegen in Muscheln toleranter Familien unter dem höchsten experimentellen PCO_2 hin. Hingegen zeigten sensitive Familien unveränderte Stoffwechsel- und Filtrationsraten unter intermediären Seewasser PCO_2. Jedoch deutete ein in, unter Kontrollbedingungen, beobachteter erhöhter Grundumsatz bei ähnlichen Filtrationsraten und einer geringeren Stoffwechselkapazität der Kieme auf eine niedrigere, CO_2-unabhängige Stoffwechseleffizienz in sensitiven im Vergleich zu toleranten Familien hin. Demnach besitzen sensitive Familien möglicherweise nicht ausreichende metabolische Kapazitäten um einen CO_2-induzierten erhöhten Grundumsatz zu decken und müssen womöglich auf eine Umverteilung vorhandener energetischer Ressourcen zurückgreifen. Dies spiegelte sich in einer deutlich erhöhten Kapazität der 3-Hydroxyacyl-CoA-Dehydrogenase wider, was auf eine erhöhte Lipidoxidation hinweist. Falls dies bereits in der Larvenphase auftrat, könnte dies zu der erhöhten Mortalität der Larven unter den Hoch-

PCO_2-Bedingungen in sensitiven Familien beigetragen haben, da begrenzte Lipidreserven möglicherweise vor Vollendung der Metamorphose aufgebraucht wurden.

In einer zweiten Studie an der Sydney-Felsenauster (*Saccostrea glomerata*) sollte explizit geklärt werden, ob intraspezifische Unterschiede in der Sensitivität gegenüber Ozeanversauerung mit einer erhöhten Kapazität der extrazellulären Säure-Base-Regulation und damit assoziiert, erhöhten ionenregulatorischen Kosten korrelieren. Frühere Studien an zwei verschiedenen Populationen von *S. glomerata* zeigten, dass Austern, die selektiv auf erhöhtes Wachstum und eine erhöhte Krankheitsresistenz gezüchtet wurden („Zuchtaustern") eine geringere CO_2-Sensitivität im Vergleich zu der natürlich im Feld vorkommenden Population („Wildaustern") besitzen. Um die zugrundeliegenden, physiologischen Mechanismen zu untersuchen wurden Zucht- und Wildaustern bei erhöhtem PCO_2 (1100 µatm) inkubiert und anschließend der extrazelluläre Säure-Base-Status (pH_e, P_eCO_2, $[HCO_3^-]_e$) sowie Geweberespirationsraten und der *in vivo* Energieverbrauch von zentralen zellulären Ionentransportern bestimmt. In der Tat zeigten die Wildaustern einen niedrigeren pH_e und einen erhöhten P_eCO_2 unter erhöhtem Seewasser PCO_2, während der extrazelluläre Säure-Base-Status der Zuchtaustern weitestgehend unbeeinflusst blieb. Jedoch wiesen beide Austerntypen weitestgehend unveränderte ionenregulatorische Kosten auf. Vielmehr deutenden die Ergebnisse auf eine erhöhte systemische Kapazität zur Eliminierung von metabolischem CO_2 bei den Zuchtaustern hin, was vermutlich auf eine gesteigerte und energetisch effizientere Filtrationsleistung und eine damit verbundene, erhöhte Ventilation zurückzuführen ist. Demnach könnte eine effiziente Filtrationsleistung mit einer erhöhten CO_2-Resilienz in Austern positiv korrelieren.

Zusammenfassend lässt sich festhalten, dass sich die fortschreitende Ozeanversauerung vermutlich negativ auf die Physiologie von marinen Muscheln und Austern auswirken wird, was möglicherweise mit negativen Konsequenzen auf der Ökosystemebene einhergeht. Jedoch lassen die Ergebnisse auch auf mögliche Anpassungskapazitäten bei beiden Arten schließen. Die höhere CO_2-Resilienz von den Zuchtaustern (*S. glomerata*) trat in der - evolutionär gesehen – sehr kurzen Zeitspanne von nur wenigen Generationen selektiver Züchtung auf, was darauf hindeutet, dass eine rasche Anpassung an Ozeanversauerung in Austern möglich sein könnte. Zudem zeigte die Langzeitstudie mit *M. edulis* eine hohe Variation von Ozeanversauerungseffekten innerhalb der untersuchten Population, was auf genetische Variation, und somit ebenfalls auf mögliche Anpassungskapazitäten hindeutet. Da sich der globale Wandel jedoch nicht nur auf die Ozeanversauerung beschränkt, müssen zukünftige Studien potenzielle Resilienz-Merkmale und Anpassungskapazitäten gegenüber der Ozeanversauerung im Kontext von weiteren, sich rasch verändernden Umweltfaktoren neu bewerten.

1 Introduction

The world's oceans cover around 71% of the earth's surface. They are vital for regulating the global climate (Rahmstorf 2002), provide about 17% of the animal protein consumed by the world's human population (FAO 2016), host around 25% of all eukaryotic species (Mora et al. 2011) and create around half of the oxygen we breathe (Behrenfeld et al. 2001). Yet, marine habitats and species are in peril due to detrimental impacts caused by ever-increasing human activities. Land-based human activities affect the runoff of freshwater, nutrients and pollutants into coastal waters and thereby remove, alter, or destroy natural habitats as well as affect coastal and marine fisheries (Islam & Tanaka 2004). Ocean-based human activities add to pollution, extract resources and are a vector for the dispersal of alien marine species, all of which may alter trophic cascades and foodwebs and may create changes in species composition of ecosystems (Jackson et al. 2001, Bax et al. 2003, Daskalov et al. 2007). However, due to its global nature, man-made rapid climate change caused by constantly increasing anthropogenic emissions of carbon dioxide (CO_2) and other green house gases (methane, nitrous oxide) indisputably poses the biggest threat to marine ecosystems. In addition, associated changes of environmental drivers due to climate change can exacerbate effects of above-mentioned anthropogenic activities and vice versa. According to the fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC), the ocean has absorbed more than 90% of Earth's additional heat produced through the greenhouse effect since the 1970s. This resulted in an averaged increase of the global ocean surface temperature (upper 75 m) by 0.11 °C per decade between 1971 and 2010, which contributed to the expansion of oxygen minimum zones, the melting of sea ice and sea level rise (IPCC 2013). In addition, the world's oceans represent one of the biggest sinks for anthropogenic CO_2 emissions. This CO_2 uptake drives changes in seawater chemistry and decreases seawater pH; a process referred to as ocean acidification (OA) (see Section 1.1) (Caldeira & Wickett 2003, Orr et al. 2005). Thus, marine ecosystems in the anthropocene are facing rapid changes of multiple abiotic and biotic drivers that are shaping ecological and evolutionary processes, often in non-linear ways (Pörtner 2008, Boyd & Brown 2015, Gattuso et al. 2015). In order to anticipate consequences of anthropogenic impacts on marine species and ecosystems, it is essential that we understand biological responses to changing environmental drivers at physiological and consequently ecological and evolutionary levels (Boyd et al. 2018). The aim of this thesis is to contribute to our understanding of this biological response by studying the effects of ocean acidification on the physiology of ecologically and economically important marine mussels and oysters.

1.1 Anthropogenic CO_2 emissions and ocean acidification

Since the onset of industrial revolution, human use of fossil fuels and changes in land use have led to increased global average atmospheric CO_2 concentrations from around 280 ppm in 1750 to around 407 ppm in 2017 (see Figure 1.1). According to data derived from ice cores, such atmospheric CO_2 concentrations (~400 ppm) are unprecedented in at least the last 800,000 years (IPCC 2013).

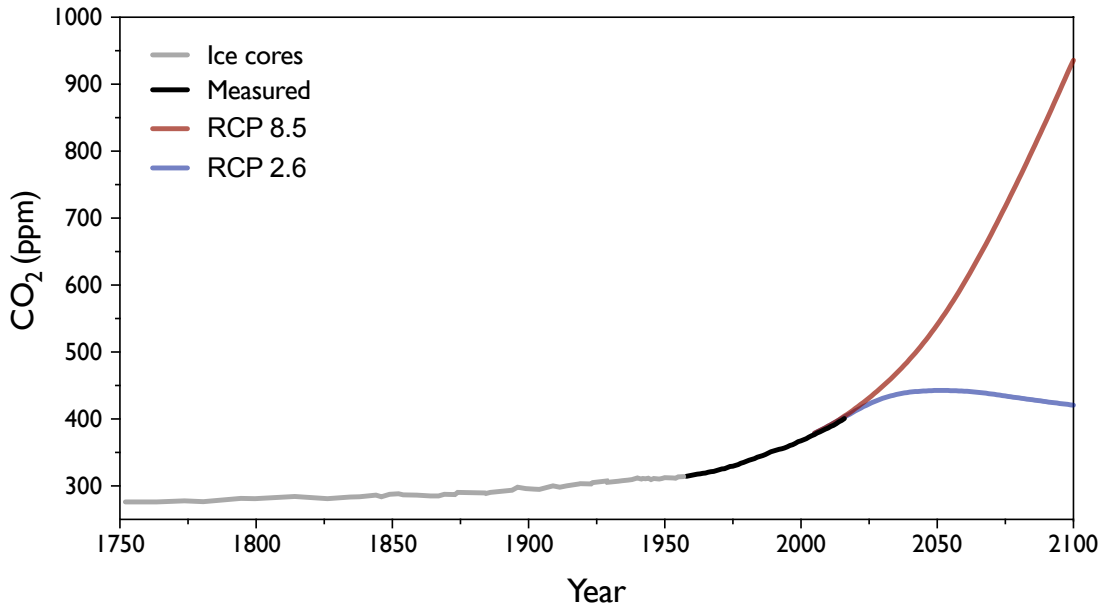

Figure 1.1: Past, present and projected atmospheric CO_2 concentrations. Mean atmospheric CO_2 concentrations from 1750 till today (grey and black line) and scenarios of projected increases of atmospheric CO_2 until 2100 according to the fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC) (RCP 2.6. and 8.5, blue and red line). Past and present atmospheric CO_2 concentrations were downloaded from Scripps Institution of Oceanography (http://scrippsco2.ucsd.edu, R. F. Keeling, S. J. Walker, S. C. Piper and A. F. Bollenbacher, Scripps CO_2 Program). Atmospheric CO_2 concentrations from 1750 until 1958 are derived from ice scores (Etheridge et al. 1996, Meure et al. 2006) data from 1958 till 2017 are yearly averaged *in situ* observations from the Mauna Loa Observatory, Hawaii (Latitude 19.5°N Longitude 155.6°W Elevation 3397m) and the South Pole (Latitude 90.0°S, Elevation 2810m). Projected mean atmospheric CO_2 represent a scenario with lowest increase of radiative forcing (RCP 2.6, van Vuuren et al. 2007) and a scenario with minimal mitigation efforts ('business-as-usual' scenario) (RCP 8.5, Riahi et al. 2007). Data were downloaded from the RCP database (http://www.iiasa.ac.at/web-apps/tnt/RcpDb).

Due to the equilibration of ocean surface waters with the atmosphere and transportation from the surface to the deeper waters by ocean currents, the world's oceans absorb a substantial amount of the atmospheric CO_2 (Le Quéré et al. 2009). Between 1800 and 2007 the oceanic sink accounted for around 31 % of total fossil fuel and cement-manufacturing CO_2

emissions (Sabine et al. 2004, Gruber et al. 2019). When CO_2 enters seawater, it reacts with water molecules leading to the formation of carbonic acid (H_2CO_3). Carbonic acid quickly dissociates and releases a hydrogen ion (H^+) and bicarbonate ion (HCO_3^-). This hydrogen ion (H^+) can react with seawater carbonate ions (CO_3^{2-}) to form another bicarbonate ion (HCO_3). The carbonate species involved in dissolution of CO_2 are related by the following equilibria:

$$CO_2 + H_2O \overset{K0}{\leftrightarrow} H_2CO_3 \overset{K1}{\leftrightarrow} HCO_3^- + H^+ \overset{K2}{\leftrightarrow} CO_3^{2-} + 2H^+ \quad (1)$$

K0, K1 and K2 are the three equilibrium constants (dependent on temperature, salinity and pressure).

Dissolved CO_2, HCO_3^- and CO_3^{2-} form the oceans carbonate system and their sum is referred to as total dissolved inorganic carbon (DIC or C_T). Under the current atmospheric CO_2 concentration of around 400 ppm, the contribution of dissolved CO_2, HCO_3^- and CO_3^{2-} to DIC is approximately 0.5%, 86.5% and 13%, respectively (Zeebe & Wolf-Gladrow 2001) (Figure 2). An increased fraction of dissolved CO_2 uptake of seawater shifts this equilibrium towards formation of HCO_3^- at the expense of CO_3^{2-} and net production of hydrogen ions (H^+) (Figure 1.2).

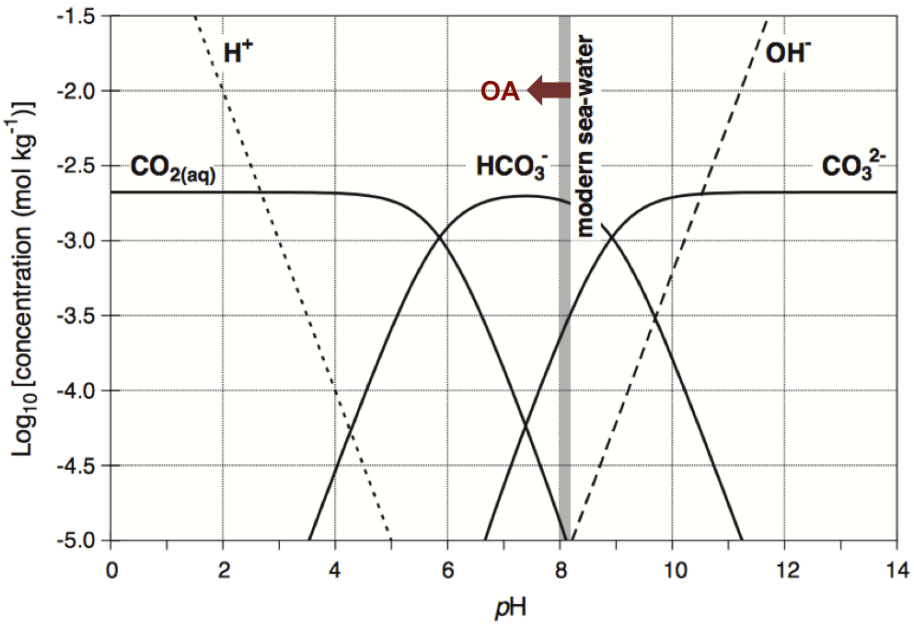

Figure 1.2: Concentrations of the three dissolved carbonate species as a function of seawater pH (Bjerrum plot). An increased concentration of dissolved CO_2 ($CO_{2\,(aq)}$) shifts the equilibrium to the left resulting in a higher bicarbonate concentration (HCO_3^-), a lower carbonate ion concentration (CO_3^{2-}) and a lower seawater pH. The grey area represents present day seawater conditions, the red arrow indicates the direction of shift that occurs due to ocean acidification (OA) (modified after Ridgwell & Zeebe 2005).

As a consequence of the reduction of carbonate ions, the calcium carbonate ($CaCO_3$) saturation state (Ω) decreases. The saturation state is a measure for whether seawater is supersaturated ($\Omega > 1$) or undersaturated ($\Omega < 1$) with respect to particular calcium carbonate polymorphs and is described by the following equation (Zeebe & Wolf-Gladrow 2001):

$$\Omega = \frac{[Ca^{2+}] \times [CO_3^{2-}]}{K_{sp}} \quad (2)$$

With K_{sp} being the stoichiometric solubility product of the respective calcium carbonate polymorph.

Supersaturated seawater favours the formation of calcium carbonate whereas undersaturation leads to dissolution of exposed calcium carbonate polymorphs (Feely et al. 2004). Aragonite and calcite are the two major naturally occurring calcium carbonate polymorphs in seawater and their stability depends on their respective saturation state (Ω_{arag} and Ω_{cal}). Open ocean surface waters are currently supersaturated with both polymorphs (Ω_{arag} ~2-4, and Ω_{cal} ~4-6, Feely et al. 2009). Saturation states for calcite are higher due to higher solubility of aragonite than calcite (1.5 times at 25°C, Riebesell et al. 2010).

In comparison to pre-industrial values, the pH of open ocean waters has already dropped by 0.1 units (Figure 1.3), which corresponds to a 26% increase in the hydrogen ion concentration [H^+] of seawater (Orr et al. 2005, Feely et al. 2009). The magnitude of future ocean acidification will depend on future atmospheric CO_2 concentrations, which, in turn, highly depend on the will and effort of global policy makers to reduce rates of carbon release. The fifth IPCC report (2013) distinguishes between four different scenarios of cumulative greenhouse gas emissions until 2100, representative concentration pathways (RCP) 2.6, 4.5, 6.0 and 8.5. RCP 2.6 represents the scenario with most stringent emission rates and the lowest increase of radiative forcing (2.6 W/m^2) and would reach the target of the Paris Agreement within the United Nations Framework Convention on Climate Change (UNFCCC) to minimise global warming to well below 2°C. By contrast, RCP 8.5 implies a scenario with minimal mitigation efforts ('business-as-usual' scenario) resulting in the highest increase of radiative forcing (8.5 W/m^2). In terms of ocean acidification, atmospheric CO_2 concentrations of 421, 538, 670 and 936 ppm and an associated drop of average ocean surface pH by -0.13, -0.22, -0.28 and -0.42 pH_T (total scale) units are expected at the end of this century for RCP 2.6, 4.5, 6.0 and RCP 8.5, respectively (Figure 1.1 and 1.3).

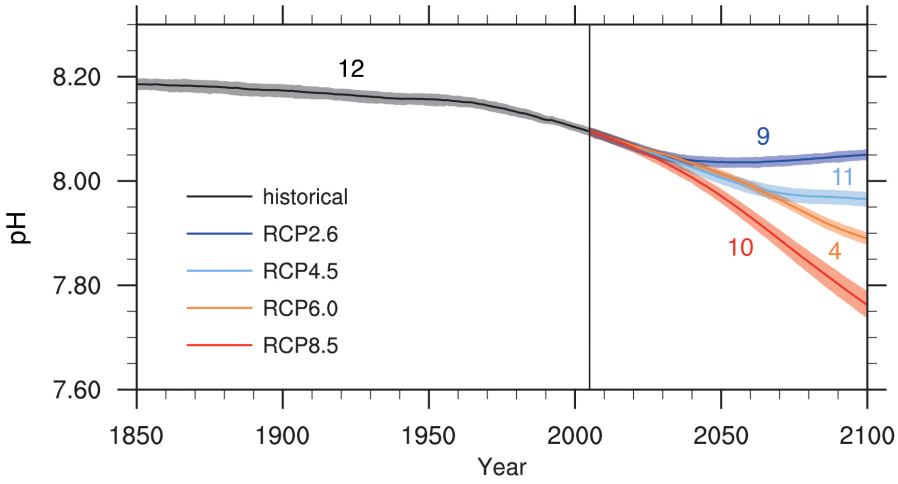

Figure 1.3: Historical and projected decrease of global surface ocean pH over time for representative concentration pathway (RCP) 2.6, 4.5, 6.0 and 8.5. Time series of multi-model means (solid line) and a measure of uncertainty (shaded area) of ocean surface pH in the past (historical) and for the four different RCPs (RCP 2.6, 4.5, 6.0, 8.5 according to the fifth IPCC report (IPCC 2013)). The number of CMIP5 models to calculate the multi-model mean is indicated above each time series. The figure is taken from technical summary (TS) of the working group I (WG I) (Stocker et al. 2013). For further technical details see page 7 and 8 of the supplementary material of the TS of WGI.

1.1.1 Spatio-temporal variability of seawater carbonate chemistry

The above-mentioned values of carbonate chemistry specimens of historical and predicted future ocean acidification (IPCC RCP_S) represent global average values predicted for open ocean surface waters. However, despite extensive equilibration with the atmosphere, seawater pCO_2 levels and associated carbonate chemistry are greatly heterogeneous across the seascape and can both vary in a spatial and temporal manner (Takahashi et al. 2009). Thus, future levels of carbonate chemistry are predicted to vary greatly among regions (e.g. McNeil & Sasse 2016).

This heterogeneity of carbonate chemistry is driven by abiotic factors such as wind, ocean surface temperature (Takahashi et al. 2002), density stratification (Gruber 2011) and ocean currents (DeVries et al. 2017), all of which can affect CO_2 uptake rates, but also by biotic factors such as the biological utilization (photosynthesis) and production of CO_2 (respiration) (Kapsenberg et al. 2015, Baumann & Smith 2017). Furthermore, natural CO_2 seepage from the seafloor through hydrothermal and cold vents can cause localised strong gradients of physico-chemical conditions (Hall-Spencer et al. 2008, Fabricius et al. 2011).

Coastal habitats are subject to particularly strong spatial and temporal fluctuations of carbonate chemistry parameters as coastal biogeochemical dynamics are governed by complex interactions at the land-ocean-atmosphere interface (Gattuso et al. 1998, Waldbusser & Salis-

bury 2014). Terrestrial runoff into coastal zones can directly modulate the carbonate chemistry by changing alkalinity. Total alkalinity, the sum of weak bases, buffers ocean pH and is generally proportional to salinity (Lee et al. 2006). Dilution of coastal waters due to terrestrial freshwater input decreases salinity and, hence, alkalinity, resulting in a stronger shift of carbonate chemistry at a given seawater PCO_2. Moreover, nutrient enrichment of coastal waters via riverine discharge and land runoff can alter rates of primary production in coastal areas which may lead to changes in carbonate chemistry at diurnal and/or seasonal time scales (Waldbusser & Salisbury 2014, Reusch et al. 2018).

In eutrophicated areas, high concentrations of nutrients and dissolved organic matter can lead to a higher production than consumption rate of particulate organic matter at the surface layer. Excess organic matter then sinks to the seabed where its heterotrophic degradation leads to the depletion of oxygen and production of respiratory CO_2 (Gray et al. 2002). Upwelling of such CO_2 enriched bottom waters can then lead to significant perturbations of upper water carbonate chemistry at a seasonal or event-scale (Feely et al. 2008, Mathis et al. 2012). One example is the Kiel Fjord located in the western Baltic Sea. Wind-driven upwelling of CO_2 enriched bottom waters during the summer months causes strong seasonal and diurnal fluctuations of surface water carbonate chemistry and associated seawater pH (Thomsen et al. 2010) (Figure 1.4). Due to the non-linear response of carbonate chemistry, eutrophication related increases of seawater PCO_2 are therefore likely to amplify future ocean acidification in brackish coastal habitats (Cai et al. 2011, Melzner et al. 2013, Reusch et al. 2018).

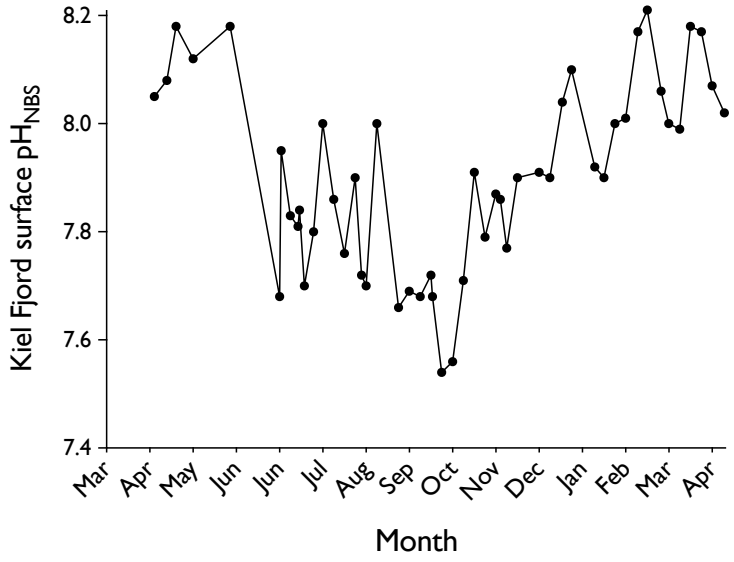

Figure 1.4: Surface pH in Kiel Fjord over one year. Surface pH of Kiel Fjord (54°19.8'N; 10°9.0'E) measured from April 2008 till April 2009 (data taken from Thomsen et al. 2010).

1.2 Biological impacts of ocean acidification

The biological impacts of ocean acidification (OA) have been extensively studied in the last decade (Riebesell & Gattuso 2015) and the results leave no doubt that OA will affect marine life in varied, but mostly negative ways (Pörtner et al. 2014, Gattuso et al. 2015). In order to understand cause and effect of OA-related shifts and constraints that are visible at a community and ecosystem level, it is crucial to understand responses and underlying physiological mechanisms at the organismal level (Pörtner 2012). Hence, direct effects at the species level and their physiology have been the subject to the majority of OA-studies (Riebesell & Gattuso 2015) (including this thesis) and will therefore be the main focus of this section. It is, however, obvious and important to acknowledge that OA will shape community and ecosystem dynamics not only through direct effects at the species level. Indirect effects are mediated by factors such as altered predator prey relationships (e.g. Wright et al. 2018), food abundance (reviewed by Ramajo et al. 2016), changes in habitat structure (e.g. Fabricius et al. 2013) and potentially associated biodiversity shifts (Sunday et al. 2017), all of which then have the potential to offset or exacerbate direct organismal effects. For example, a recent *in situ* mesocosm study reported enhanced survival of herring larvae under elevated PCO_2 due to a CO_2 stimulated increase of primary and, consequently, secondary production providing increased food resources (Sswat et al. 2018), which seems to compensate for the loss in physiological performance of fish larvae reported in single-species laboratory studies (Baumann et al. 2012, Dahlke et al. 2017). Moreover, CO_2 induced shifts of carbonate chemistry can modulate the sensitivity of organisms to other environmental drivers (or vice versa) through additive, synergistic or antagonistic effects (Pörtner & Farrell 2008, Boyd & Brown 2015).

1.2.1 Physiological implications of ocean acidification

There is a high level of confidence that OA will affect the physiology of species from all marine ectothermic taxa (Pörtner et al. 2014) with some life-stages and taxa being more sensitive than others (Kroeker et al. 2013, Wittmann & Pörtner 2013). Marine organisms with large calcified body structures, and particularly the early life stages of these organisms, appear to be most sensitive (Ross et al. 2011, Wittmann & Pörtner 2013). Numerous studies demonstrate that projected changes of the seawater carbonate chemistry drive decreases in calcification processes (e.g. Waldbusser et al. 2015, Thomsen et al. 2015). Besides calcification, OA is predicted to affect other fundamental physiological processes such as metabolic rates and associated aerobic scope (e.g. Schalkhausser et al. 2013), growth (e.g. Baumann et al. 2012), food ingestion and digestion (e.g. Stumpp et al. 2013), behaviour (e.g. Munday et al. 2014),

fecundity and reproduction success (e.g. Parker et al. 2018) as well as morphology (e.g. Chan et al. 2011) and immune functions (e.g. Hernroth et al. 2011).

Physiological implications of OA are mediated by unfavourable shifts of acid-base variables and carbonate system variables (PCO_2, pH, $[HCO_3^-]$, Ω) in organismal body fluids and at the site of calcification (Pörtner 2008, Melzner et al. 2009) (Figure 1.5).

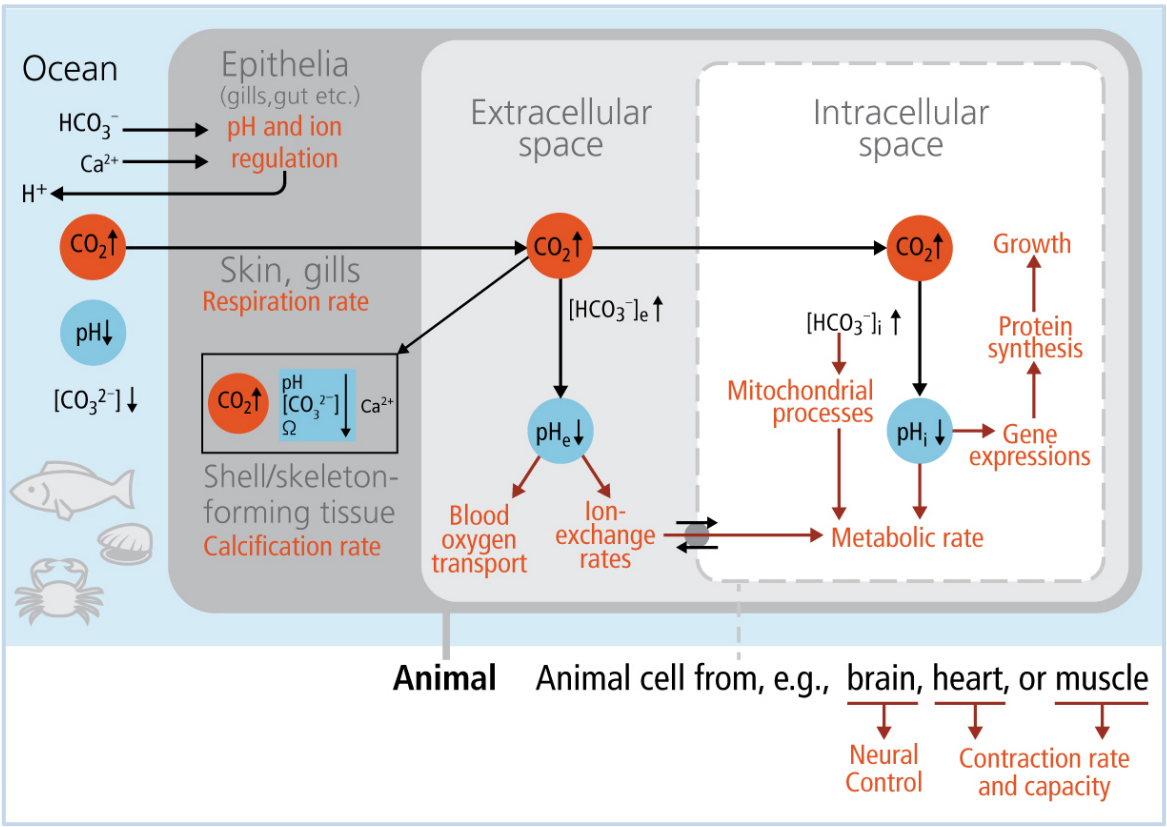

Figure 1.5: Schematic summary of the main effects of ocean acidification on the physiology of marine ectotherms. The increase of seawater PCO_2 results in an increased PCO_2 in the different body and cell compartments (highlighted in red) and associated drop of pH in the respective compartment (highlighted in blue). The red arrows indicate the effects of altered internal acid-base variables on various physiological processes (red text). The figure is after Pörtner (2008) and taken from chapter 6, Ocean Systems (Pörtner et al. 2014), of working group II of the fifth IPCC report.

As marine ectothermic animals release metabolic CO_2 via diffusive gradients into the surrounding seawater, an elevated PCO_2 in the seawater leads, consequently, to an elevated PCO_2 in body fluids, if diffusive gradients are to be maintained (Pörtner et al. 2004, Melzner et al. 2009) (Fig. 1.5). Acute exposure to elevated environmental PCO_2 (also referred to as hypercapnia) therefore leads to a drop of extra– (pH_e) and intracellular (pH_i) pH (see Figure 1.5). The magnitude of the pH drop depends on the buffer capacity of the particular compartment and tissue, provided by non-bicarbonate buffers (β_{NB}) (Melzner et al. 2009). Owing

to higher levels of non-bicarbonate buffers of the intra- compared to the extracellular space, the drop in pH_i is lower compared to the drop of pH_e (Heisler 1989). Nevertheless, passive buffering can only ameliorate acute CO_2 induced pH changes whereas full compensation of pH_i and pH_e under chronically elevated seawater PCO_2 requires the removal of excess proton equivalents as well as the accumulation and/or retention of HCO_3^- via active, and thus, energy consuming ion-regulatory processes (Heisler 1989). The mechanisms involved are not yet fully understood and are likely to vary between species. HCO_3^-/Cl^- ion-exchangers might be involved in the accumulation of HCO_3^-, whereas proton excretion is likely achieved via ATP (energy) consuming H^+-ATPase(s) and/or Na^+/H^+ exchanger(s). The latter is dependent on a Na^+ gradient, actively built up by Na^+/K^+-ATPase(s), a major ATP consumer in most cells (reviewed by Melzner et al. 2009). Thus, OA may constrain and alter energy budgets of marine ectotherms through energy demanding acid–base regulatory processes to compensate for changes of internal acid-base equilibria (Pörtner 2008, Melzner et al. 2009) (Figure 1.5).

While most marine ectotherms experience a pH decrease of internal body fluids when acutely exposed to elevated seawater PCO_2, they vary in their ability to actively compensate for it. Available studies show that pH_i is quickly restored and tightly regulated in most taxa during acute and chronic CO_2 exposure (Pörtner et al. 2000, Michaelidis et al. 2005, Strobel et al. 2012). This is crucial as even small pH_i deviations from physiological optima may impair enzyme kinetics and thus metabolic functioning (Heisler 1989). In contrast, the ability to regulate pH_e under acute and chronic elevated seawater PCO_2 differs between and within taxa and may mirror their different OA sensitivities (Pörtner 2008, Widdicombe & Spicer 2008, Melzner et al. 2009). Species with higher metabolic rates and an active mode of life might be pre-adapted to higher environmental PCO_2 and equipped with a higher acid-base regulatory capacity as they experience *per se* higher internal PCO_2 levels and strongly fluctuating PCO_2 levels during bouts of physical exertion (Pörtner 2008, Melzner et al. 2009). In fact, higher and more active ectothermic taxa such as fish (e.g. Esbaugh et al. 2012, Strobel et al. 2012), cephalopods (Gutowska et al. 2010) and some crustaceans (e.g. Rastrick et al. 2014, Maus et al. 2018) were shown to compensate for OA induced pH_e reductions, whereas pH_e compensation is incomplete in more sluggish or sessile marine invertebrates such as many bivalves (e.g. Thomsen et al. 2010, Lannig et al. 2010).

Uncompensated pH_e can lead to various consequences at different functional levels. For example, in marine bivalves, extracellular acidosis can increase internal shell dissolution (Melzner et al. 2011) and can impair the immune response (Bibby et al. 2008). Moreover, extracellular pH can directly modulate metabolic rates. Depending on the magnitude of change and

individual sensitivity, marine bivalves may respond with either metabolic depression (e.g. Michaelidis et al. 2005, Navarro et al. 2013), increased (e.g. Thomsen & Melzner 2010, Parker et al. 2012) or unchanged metabolic rates (e.g. Lannig et al. 2010). Altered metabolic rates may be related to above-mentioned increased ion-regulatory and homeostatic processes at the cellular/tissue level; defending pH_i at uncompensated pH_e increases the proton flux into the intracellular space and, therefore, requires up-regulation of energy-consuming proton equivalent ion exchange (Boron 2004, Pörtner 2008, Melzner et al. 2009). As a result, exposure to ocean acidification may increase metabolic maintenance costs in marine bivalves, leaving less energy for other, mainly anabolic processes such as protein synthesis, growth, calcification and reproduction (Figure 1.5).

CO_2 induced shifts in allocation of available energy in marine bivalves and other marine ectotherms are likely to vary alongside the PCO_2 level (e.g. Dorey et al. 2013), within species and their life-stages (e.g. Scanes et al. 2014) as well as between tissues depending on their function and sensitivity (e.g. Kreiss et al. 2015, Heuer & Grosell 2016). Alterations of energy metabolism at the cellular and tissue level may include biochemical adjustments of ATP-producing pathways (e.g. mitochondrial capacities, Strobel et al. 2012, 2013a) and ATP-consuming mechanisms (e.g. protein synthesis and ion regulation, Pan et al. 2015) which can impair whole animal functioning (Pörtner 2008, Sokolova et al. 2012). Understanding the mechanisms of OA effects on marine organisms therefore requires knowledge of CO_2 effects at lower levels of biological organisation (i.e. molecular and cellular level) and their consequences for whole organism functioning (Bozinovic and Pörtner 2015). Compensation at the cellular and tissue level may, however, not always be visible at the whole animal level. For instance, compared to control conditions, OA exposure significantly increased the average ATP allocation to protein synthesis and ion regulation from 55 to 85% in feeding in sea urchin larvae (*Strongylocentrotus purpuratus*); an effect that was, however, not visible at the whole animal level (i.e. unchanged morphological characteristics and metabolic rate) (Pan et al. 2015).

Energetic constraints as an underlying cause of impaired organism performance in response to OA is also indirectly evidenced by several studies on marine invertebrates showing that high food (i.e. energy) availability can ameliorate or even outweigh adverse OA effects (Thomsen et al. 2013, Pansch et al. 2014, Towle et al. 2015). The scope for compensatory feeding in the wild may be already limited in terms of resource availability, but in addition elevated PCO_2 can impair performance of the prey and food source itself and thus food availability and quality might be restricted (e.g. Rossoll et al. 2012). Furthermore, OA has

been shown to negatively affect food ingestion, conversion and digestion rates as well as feeding behaviour (for review see Clements & Darrow 2018).

CO_2-induced behavioural impairments have been reported for a range of marine ectothermic species (reviewed by Clements & Hunt 2015) and are thought to be downstream effects of CO_2 induced acid-base regulatory responses mediated by altered gradients of Cl^- and HCO_3^- ions across neuronal membranes, which affect the activity of the GABA-A receptor (Nilsson et al. 2012). Besides behavioural affects, altered set points of ion concentrations and acid-base variables under elevated PCO_2 can have implications for other cellular processes. Elevated intracellular HCO_3^- concentration can modulate mitochondrial functioning (Haider et al. 2016), potentially through direct inhibitory effects on enzymes of the TCA-cycle (Strobel et al. 2012) or mediated through bicarbonate stimulated soluble adenylate cyclase (sAC) (Acin-Perez et al. 2009, Wang et al. 2016, 2017). Such CO_2 effects on mitochondrial enzyme activities and pathways may compromise ATP supply and increase oxidative stress which, in turn, can impair aerobic scope with consequences for overall organismal performance (Pörtner 2008, 2012, Salin et al. 2015, Sokolova 2018).

In summary, ocean acidification drives physiological changes of marine ectotherms not through a single mode of action but via a variety of effects on the different carbonate chemistry variables and their associated cascading effects at different functional levels within the organism. OA sensitivity differs within taxa and their life stages and may be related to their relative capacity to compensate for CO_2 induced disturbances of internal acid-base variables and their respective effect on organismal energy balance and, thus, overall fitness.

1.3 Acclimatisation and adaptation to ocean acidification

With OA already being an ongoing process (Cubasch et al. 2013, Fig. 1.3) it is not the question whether marine species will experience changes of the seawater carbonate chemistry, but how strong these changes will be and, more importantly, the rate at which they occur. The latter is important, as there is increasing concern that the current rate of OA might outpace the ability of marine species to adapt to future conditions (Parmesan 2006, Pörtner et al. 2014). In order to understand and predict how populations of marine organisms will be affected by future changes, it is important to identify physiological traits that confer tolerance or sensitivity towards OA but also to assess the potential of species and the rate at which they can acclimatise and/or adapt (Kelly et al. 2013, Sunday et al. 2014, Stillman & Paganini 2015, Calosi et al. 2016).

Acclimatisation (called acclimation if under laboratory conditions) describes 'the altering of physiological, behavioural, or morphological characteristics through phenotypic plasticity to better suit an environment' (Munday et al. 2013). Phenotypic plasticity is the ability of a genotype to produce different phenotypes in response to a changing environment in order to maintain its fitness (Ghalambor et al. 2007), and can occur within a single life stage (reversible acclimatisation), between life stages (developmental acclimatisation) and also transgenerationally (transgenerational acclimatisation) (Sunday et al. 2014). Adaptation, in contrast, is an evolutionary process and occurs through selection on genetic variation that increases the population tolerance towards the driver of selection. Both processes have been shown to occur in response to elevated PCO_2 (e.g. Calosi et al. 2013) and may be viable strategies for species to cope with future conditions (Kelly & Hoffmann 2013). Furthermore, it has been suggested that acclimatisation may interact with adaptation (Chevin et al. 2010). Phenotypically plastic responses can buffer CO_2 effects, potentially giving time for genetic adaptation to occur (Reusch 2013, Sunday et al. 2014). Conversely, acclimatisation could slow down adaptive responses by decreasing selection gradients (Dam 2013, Sunday et al. 2014).

Acclimatisation responses are comparatively easy to measure as they occur over relatively short time scales (days to a few generations) and have been shown to occur within life stages (e.g. Form & Riebesell 2012) and between life stages (e.g. Parker et al. 2012). One example is a study on green sea urchins (*Strongylocentrotus droebachiensis*) which showed that females exhibited a 4.5 fold reduction of fecundity following four months of exposure to elevated PCO_2 during reproductive conditioning, an effect that was no longer visible after 16 months of exposure (Dupont et al. 2012). By contrast, adaptation usually happens over much longer time scales, especially in long-lived species with long generational turnover times, which complicates its quantification (Sunday et al. 2014). Adaptive responses can either occur through new mutations or selection on pre-existing genetic variation within natural populations. It has been argued that under rapidly occurring environmental changes, such as ocean acidification, adaptation from standing genetic variation might be the prevailing mechanism (Lande & Shannon 1996, Chevin et al. 2010, Pespeni et al. 2013). Measuring the variation of tolerance traits within natural populations and their heritability can therefore help to estimate the potential of a population to adapt (Munday et al. 2013, Reusch 2014, Sunday et al. 2014). However, as a first step, this requires knowledge of traits that are likely to be under selection, i.e. traits that confer tolerance or sensitivity of a species, population or individuals (Kelly and Hoffmann 2013). This can be achieved by comparing responses of pheno- or genotypes with different sensitivities within and between populations to the selective force, i.e. the environ-

mental driver (Applebaum et al. 2014). Populations inhabiting heterogeneous environments, and thereby experiencing a high variability of a given environmental driver, are expected to be most plastic in traits that are adaptive in those conditions (Valladares et al. 2014), making them an interesting model to elucidate physiological key traits that underlie vulnerability or resilience.

1.4 Study organisms: mussels and oysters

Mussels and oysters are bivalves and belong to the phylum mollusca, which is the second largest in the animal kingdom with at least 53,000 described marine species (Bouchet 2006), of which around 8000 are bivalves (Gosling 2003). Marine bivalves are globally distributed and can be found in extreme habitats such as deep-sea hydrothermal vents or under the Antarctic sea ice. However, the majority of species, including mussels and oysters, inhabit the intertidal and sublittoral zones of the world's oceans.

Most mussels and oysters share a similar biology and ecology. They are broadcast spawners and release their gametes into the water column. Around 24 hours after fertilisation, embryos develop into the ciliated planktonic trochophore larvae and a shell gland initiates the secretion of the first larval shell, the D-shaped prodissoconch I (PD I). At this stage, larvae are called veligers and the formation of PDI is followed immediately by the secretion of the second larval shell, the prodissoconch II (PDII). The secretion of the first shell is a critical step in the larval development as it coincides with the formation of the ciliated velum. The velum serves as a feeding and swimming organ and prior to its formation, larvae solely rely on maternally provided endogenous energy reserves (yolk) and passive drift for dispersal. The veliger stage is followed by the pediveliger stage, which is characterised by the development of pigmented eyespots and the ciliated foot. The pediveliger stage marks the end of the planktonic phase as larvae settle and begin with metamorphosis. Metamorphosis occurs usually after several weeks, at a size range from around 200-300 μm, and marks a critical phase in the life history due to a massive re-organisation of body parts, which involves suspension of feeding and therefore reliance on stored nutrients as an energy source (Gosling 2003).

Once settled, mussels and oysters have a purely sessile and benthic lifestyle. They often form extensive reefs, which is why they are referred to as foundation species. Their shells provide hard substrate for attachment, refuges from predation for other species (Gutiérrez et al. 2003) and increase substrate complexity, which supports biodiversity (Maggi et al. 2009, Harley 2011). Moreover, mussels and oysters are considered as ecosystem engineers (e.g. van der

Zee et al. 2012); suspension-feeding bivalves play a crucial role in bentho-pelagic coupling, alter the hydrodynamic regime and reduce turbidity, which can promote growth of submerged vegetation (Newell 2004). Thus, they are keystone species and direct negative OA effects on their physiology could have far-reaching consequences at the ecosystem scale. OA induced loss of biogenic habitat (such as bivalve reefs) was recently shown to be a potential mediator for direct negative effects of OA on coastal biodiversity (Sunday et al. 2017). Focussing research efforts on such keystone species therefore may aid the assessment of OA effects at the community level and on ecosystem functioning (Gaylord et al. 2015).

Besides their ecological importance, marine mussels and oysters are of great economic importance. They represent an important food source, which is reflected in the worldwide farming of oysters and mussels. In 2016, their combined global aquaculture production amounted to approximately 7,600,000 tons, which equals an economic value of around 10.5 billion US dollars (FAO 2018). Thus, negative OA effects on mussels and oysters are expected to lead to economic damage (Narita et al. 2012). As an example, oyster hatcheries along the North American Pacific coast that use the local seawater have experienced great reductions in hatchery production of Pacific oyster larvae (*Crassostrea gigas*) during upwelling of CO_2 acidified water masses (Feely et al. 2008, Barton et al. 2012).

The two species investigated in this thesis are the blue mussel, *Mytilus edulis*, from Kiel Fjord in the western Baltic Sea and the Sydney rock oyster, *Saccostrea glomerata* from the southeast coast of Australia (Figure 1.6).

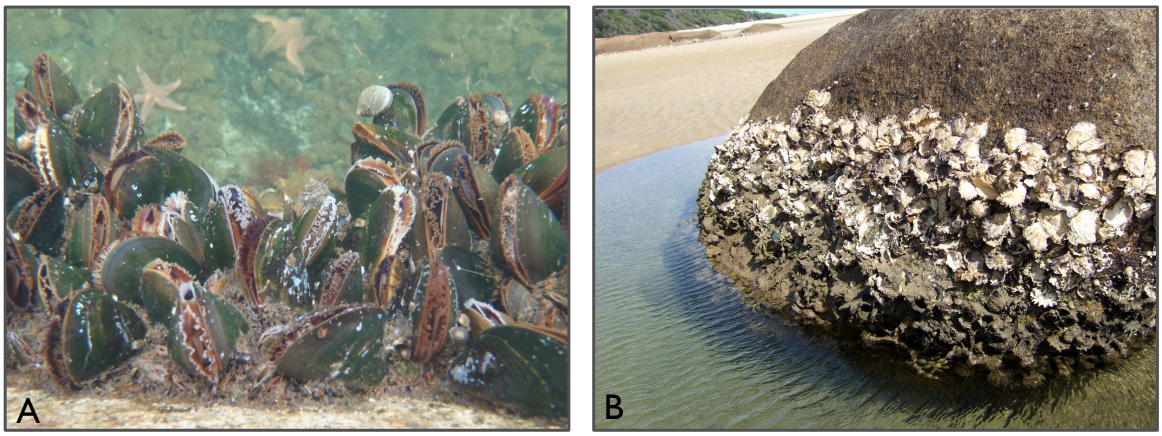

Figure 1.6: (A) Photo of blue mussels, *Mytilus edulis*, in Kiel Fjord and (B) of Sydney rock oysters, *Saccostrea glomerata*, attached to a rock during low tide. Photo credits: (A) Frank Melzner, GEOMAR, (B) Stevage on page 542 in Aquaculture: Farming Aquatic Animals and Plants (2012).

1.4.1 Blue mussel, *Mytilus edulis*

Mytilus edulis (Linnaeus, 1758) is a eurythermal and euryhaline species that tolerates salinity conditions from full marine to brackish (Bayne 1976). It can be found from the high intertidal to subtidal coastal zones at middle and higher latitudes of the northern hemisphere. The population studied in this thesis originates from Kiel Fjord in the western Baltic Sea (54°19.8'N; 10°9.0'E). Specimens of Baltic Sea *Mytilus* are considered as hybrids of *M. trossulus* and *M. edulis*. However, as more than 80% of alleles in Kiel Fjord mussels originate from *M. edulis* (Stuckas et al. 2009), Kiel Fjord blue mussels will be referred to as *M. edulis*.

M. edulis dominates the benthic community in Kiel Fjord despite the high variability of seawater pH and PCO_2 (see Section 1.1.1, Fig. 1.4), which particularly occurs during the reproductive phase of the local mussel population (Enderlein & Wahl 2004, Thomsen et al. 2010). Thus, the fact that Kiel Fjord mussels experience already high seawater PCO_2 levels and, in particular, a high variability of seawater carbonate chemistry during the critical phase of larval development makes this population an interesting model to study the potential for metabolic adaptation towards OA. Previous laboratory and field experiments showed that Kiel Fjord mussels are able to maintain metabolic and calcification rates after long term exposure to PCO_2 levels of up to 3000 µatm, despite a chronic reduction of their extracellular pH (Thomsen et al. 2010, Thomsen & Melzner 2010, Thomsen et al. 2013, Hüning 2014). This seems possible due to high food (energy) availability in eutrophicated Kiel Fjord underpinning the energetic component for the effects of OA on mussels. However, maintained metabolic rates were associated with a reduced scope for growth (Hüning 2014) and potentially tissue-specific, reallocation of available metabolic energy. The latter is indicated by previous work showing adjustments of tissue specific gene expression (Hüning et al. 2013) and proteomic responses (Hüning 2014) in mantle and gill tissue of Kiel Fjord mussels following long-term CO_2 exposure.

1.4.2 Sydney rock oyster, *Saccostrea glomerata*

The Sydney rock oyster, *Saccostrea glomerata* (Gould 1850) (formerly also known as *Saccostrea commercialis*) is a native Australian oyster which occupies intertidal and shallow subtidal estuarine habitats along the New South Wales (NSW) and southeast Queensland coasts. It forms the basis of a large aquaculture industry with a long-standing tradition (Schrobback et al. 2014). However, while up until the late 1970s, the Sydney rock oyster industry grew steadily, production rates have since declined (O'Connor & Dove 2009). This production decline is

attributed to various factors, including competition with the faster growing Pacific oyster (*C. gigas*) and the outbreak of oyster diseases, namely QX disease (caused by parasitic *Marteilia sydneyi,* Perkins & Wolf 1976) and winter mortality (cause currently under debate, see Spiers et al. 2014) (O'Connor and Dove 2009). To tackle this issue, a breeding program was established in Port Stephens in 1990 with the aim to increase growth rates and resistance against winter mortality. In 1997, it was expanded to include QX disease resistance (Nell et al. 2000).

Due to the economic and ecological importance of *S. glomerata*, potential impacts of OA on this species have been previously addressed in a series of studies. In 2009, a study showed OA induced impairment of larval growth and survival of *S. glomerata* (Watson et al. 2009). Subsequent studies by Parker et al. (2010, 2012) confirmed these results. Furthermore, they also showed that larval growth impairment was significantly less in larvae of the selectively bred oyster lines oysters originating from the Port Stephens breeding programme compared to the wild population (termed 'selected' and 'wild' oysters hereinafter, respectively). Moreover, exposure to elevated PCO_2 levels of adult wild and selected oysters during reproductive conditioning enhanced larval growth and survival compared with offspring from parental oysters kept at ambient PCO_2. Still, larval performance of selected oysters was higher compared to wild oysters' larvae (Parker et al. 2012). These results not only show intraspecific variation in OA responses, but also transgenerational acclimation and, thus, a potential adaptive capacity (Parker et al. 2010, 2012). However, the underlying physiological mechanisms of the higher CO_2 resilience in selected oysters remained unknown. Higher whole animal metabolic rates in selected compared to wild oysters under control and even more so under elevated PCO_2 suggested an energetic component (Parker et al. 2012).

1.5 Research questions and approaches

In light of ongoing ocean acidification and the particularly high sensitivity of marine bivalves the first aim of this thesis was to study the effects of elevated seawater PCO_2 on physiological traits of economically and ecologically important mussels and oysters at different levels of biological organisation. In a second step these physiological OA responses were examined for intraspecific and intra-population variability in order to identify potential adaptive physiological traits and to assess the capacity of the investigated species to metabolically adapt to future ocean acidification.

Working with the Kiel Fjord *M. edulis* as a model organism, the following research questions were addressed in the first (Publication I) and second part (Publication II) of this thesis:

1. How does long-term exposure to elevated PCO_2 affect the energy supply and the energy allocation at the whole animal and tissue level?
2. How does OA affect major pathways of energy metabolism in different tissues of *M. edulis?*
3. Do physiological OA responses vary within the Kiel Fjord *M. edulis* population?

To answer these questions, a long-term multi-generation experiment with 16 family lines of *M. edulis* was collaboratively conducted (see Publication A4). After 1 year of CO_2 exposure, physiological responses of F1 (generation 1) mussels were determined at the whole animal level (metabolic rates and filtration rates), tissue level (metabolic rates of filtrating gill and calcifying mantle tissue, energy allocation to protein synthesis, gill tissue metabolic scope) (Publication I) and biochemical level (maximal activities of key metabolic enzymes) (Publication II). As CO_2 sensitivity differed between family lines (i.e. different larval survival rates at highest experimental PCO_2), physiological responses of 'sensitive' families were compared with those more 'tolerant' families.

In the third part of this thesis (Publication III) wild and selected Sydney rock oysters (*S. glomerata*) were compared to answer the following research questions:

4. Is the higher resilience of selected compared to wild oysters due to a higher capacity to compensate for CO_2 induced extracellular acid-base disturbances?
5. If so, is this higher capacity driven by metabolic and ion-regulatory processes at the cellular/tissue level?

To do so, wild and selected oysters were exposed for seven weeks to a present and projected seawater PCO_2 level. Subsequently, their extracellular acid-base status was determined as well as respiration rates and *in vivo* metabolic costs of prominent ion regulators (Na^+/K^+-ATPase, H^+-ATPase, Na^+/H^+-exchange) of isolated gill and mantle tissues.

2 Material and Methods

This section summarises the material and methods that were used to collect the data presented in Publication I, II and III. Two different CO_2 acclimation experiments were performed; a long-term, multi-generational CO_2 acclimation with Baltic blue mussel, *Mytilus edulis* (Experiment 1) as well as a medium-term CO_2 acclimation experiment of Sydney rock oysters, *Saccostrea glomerata* (Experiment 2) (see Sections 2.1 and 2.2). Table 2.1 provides an overview over of physiological parameters that were measured at the whole-animal, tissue and biochemical level (see Sections 2.3 to 2.5).

Table 2.1: Summary of physiological parameters measured during Experiment 1 and 2. ✓: measured, -: not measured. CS: citrate synthase, COX: cytochrome-c-oxidase, PK: pyruvate kinase, PEPCK: phosphoenolpyruvate carboxykinase, HADH: 3-hydroxyacyl-CoA dehydrogenase, GDH: glutamate dehydrogenase. * only outer mantle tissue used for *Mytilus edulis*.

		Exp. 1	**Exp. 2**
Species		*M. edulis*	*S. glomerata*
Seawater PCO_2 level (µatm)		700, 1120, 2400	500, 1120
Whole animal parameters	Condition index (CI)	✓	✓
	Clearance rate (CR)	✓	-
	Routine metabolic rate (RMR)	✓	-
Extracellular acid-base	pH_e	✓	✓
	PCO_2	-	✓
	HCO_3^-	-	✓
Gill parameters	Oxygen consumption (MO_2)	✓	✓
	Metabolic scope	✓	-
	MO_2 ion-regulators (Na^+/K^+-ATPase, H^+-ATPase, Na^+/ H^+-exchange)	-	✓
	MO_2 protein synthesis	✓	-
	Maximal activities of metabolic enzyme (CS, COX, GDH, HADH, PK, PEPCK)	✓	-
	Protein content	✓	-
Mantle* parameters	MO_2	✓	✓
	MO_2 ion-regulators (Na^+/K^+-ATPase, H^+-ATPase, Na^+/H^+-exchange)	-	✓
	MO_2 Protein synthesis	✓	-
	Maximal activities of metabolic enzyme (CS, COX, GDH, HADH, PK, PEPCK)	✓	-
	Protein content	✓	-

2.1 Experimental animals

2.1.1 Collection, treatment and breeding design of *Mytilus edulis*

Mid June 2012, mature specimens of Baltic blue mussels, *M. edulis,* were collected in subtidal depths from Kiel Fjord in front of the GEOMAR building (54°19.8'N; 10°9.0'E). Mussel shell lengths ranged between 53 to 73 mm. Collected mussels were brought directly to a temperature controlled room at GEOMAR where they were cultured in flow-through aquaria supplied with filtered seawater (FSW, 5µm, ~ 18°C and 15 PSU) from the fjord.

The following day, mussels were spawned in order to generate different family lines. Each mussel was placed in a beaker filled with FSW (0.2 µm) and induced to spawn by gradually increasing the water temperature (Δ 5–7 °C). Subsequently, eight sires (1-8) and eight dams (A-H) (F0 generation) were crossed at control conditions in a reduced North Carolina I design in order to generate 16 full-sib families within four half-sib groups (Figure 2.1). After 2-3 h, the fertilisation rate was checked (>90%) and 3×5000 embryos (F1 generation) per family and PCO_2 level line randomly divided among experimental units containing FSW (0.2 µm) with a nominal PCO_2 level of either 700 (control), 1120 (intermediate) or 2400 µatm (high).

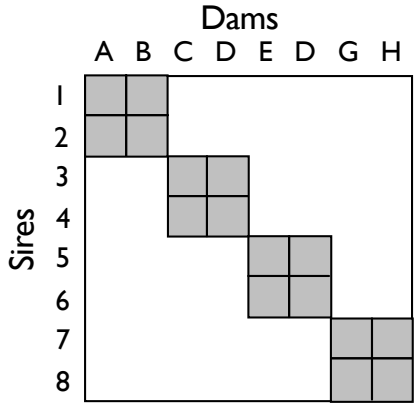

Figure 2.1: Crossing scheme to create F1 generations of *Mytilus edulis*. F0 *M. edulis* (eight dams and eight sires) were crossed pairwise to generate 16 families (F1 generation).

Around three weeks later, dependant on larval settlement success, families were classified as either 'tolerant' or 'sensitive'. Families whose larvae successfully settled at the highest experimental PCO_2 were classified as tolerant (5 out of 16), the remaining as sensitive (11 out of 16) (Figure 2.1). Subsequently, F1 mussels of tolerant and sensitive families were cultured at the respective seawater PCO_2 level (see Section 2.2.1). During the next spawning season

(~1 year, July 2013), F1 mussels were spawned again as described above for further crossing experiments (for details see Publication A4) as well as to minimise side effects of uneven gonadal status of mussels on subsequent measurements of metabolic rate. Following a two-week recovery phase, physiological experiments (see Sections 2.3 to 2.5) of tolerant and sensitive mussels commenced. A flow chart of the experimental procedure can be found in Publication I.

2.1.2 Collection and treatment of *Saccostrea glomerata*

Two different types of Sydney rock oysters, *S. glomerata*, were used for Experiment 2; oysters of the local population ('wild oysters') (Cromarty Bay in Port Stephens, 152°03′E, 32°42′S) as well as oysters of an aquaculture population (line B2, 7th generation) that were selected for faster growth and increased immune competence ('selected oysters') in the course of a breeding program of the Port Stephens Fisheries Institute (PSFI), Department of Primary Industries, NSW, Australia (see Section 1.4.2). The base population for the mass selected line of the breeding program consisted of wild oysters that were collected from the major Sydney rock oyster growing estuaries in NSW including Wallace Lake (32°30′S, 152°29′E), Port Stephens (32°45′S, 152°10′E), the Hawkesbury (33°30′S, 151°12′E) and Georges Rivers (34°00′S, 151°10′E). Collected oysters were mass spawned in the hatchery and the fastest growing returned to the field after reaching a shell length of at least 12 mm. Subsequently, mature oysters that survived a respective disease outbreak were brought back to hatchery, mass spawned again and the growth selection repeated, for over seven generations in total (see Nell et al. 2000).

In order to avoid potential side effects caused by different environments that wild and selected oysters experienced during ontogenesis, oysters of both types were treated identically throughout their complete life history. Selected and wild oysters originated from broodstocks that were mass-spawned (750 oyster per oyster type) in 2012 in the oyster hatchery of the PSFI. Spawning was induced using a standardized spawning protocol established for Sydney rock oysters at the PSFI (O'Connor et al. 2008). Briefly, oysters were placed in spawning trays filled with FSW (1µm) at a temperature of around 24°C. Next, water temperature was steadily increased by 4-5°C over 30 minutes using a submersible heater. Oysters were held at the high temperature for 15 minutes before freshwater was added in order to decrease salinity from around 35 to 22. If spawning did not commence after 15 minutes, the FSW was exchanged and the procedure repeated (for a more detailed description see O'Connor et al. 2008).

Following the spawning procedure, larvae of wild and selected oysters were kept in the hatchery up until they reached a size of around 3 mm. Then, wild and selected oysters were moved into cultivation trays and both were deployed at the same lease in Cromarty Bay. After approximately three years (March 2015), trays with adult selected and wild oysters were brought back to the PSFI and 100 individuals of each oyster type picked at random (shell length between 70 to 100 mm). Fouling organisms were cleaned from the shells of all oysters before each group was divided in half and allocated to one of two identical acclimation tanks (see Section 2.2.2). Two days later, all oysters were exposed to the spawning procedure described above in order to avoid potential side effects of different gonadal status on subsequent physiological measurements. Afterwards, oysters were acclimated to the laboratory for another three weeks at control conditions before they were transferred into CO_2 acclimation tanks with either a control seawater PCO_2 or elevated PCO_2 level (see Section 2.2.2). After a seven-week acclimation period, sampling and physiological measurements started (see Sections 2.3, 2.4 and 2.5).

2.2 Acclimation set-up

2.2.1 *Mytilus edulis* (Experiment 1)

The long-term CO_2 acclimation experiment of *Mytilus edulis* was conducted in a temperature controlled room at GEOMAR in Kiel, Germany. This room was equipped with a seawater pipeline that provides three-stage FSW (50, 20 and 5 μm) directly from Kiel Fjord. Furthermore, it is connected to a central automatic custom-made CO_2 mixing-facility (Linde Gas & HTK Hamburg, Germany) that allows manipulation of seawater carbonate chemistry by directly bubbling the seawater with CO_2 enriched air.

During the larval phase, mussels were acclimated in one litre experimental units (polyvinylchloride, KAUTEX, Germany) filled with 500 ml of 0.2 μm FSW (18°C, 15 PSU). Each unit contained 5000 larvae and was continuously bubbled with respective gas mixtures. Experimental animals were acclimated to three different seawater PCO_2 level (700 (control), 1120 (intermediate), 2400 μatm (high) that correspond to present day and projected future regularly occurring PCO_2 levels in Kiel Fjord by the end of the century (Thomsen et al. 2010, 2013, Melzner et al. 2013). Per family line, the number of replication for the different experimental seawater PCO_2 level was three, resulting in a total of 144 experimental units. The water was changed weekly with pre-equilibrated FSW and animals were fed daily as described in Section 2.2.3.

At the end of September 2012, at a size or around 1-2 mm, the now juvenile mussels from each of the three larval replicates for each family and respective PCO_2 level were combined into a 20 l flow-through aquarium within an acclimation set-up (Figure 2.2). In this set up, acclimation of sensitive families was only continued at the control and intermediate PCO_2 level (no larvae survived the high PCO_2 level), whereas tolerant families were continued to acclimate at all three PCO_2 level. When transferring mussels, the three larval replicates of each family and respective PCO_2 level were combined into one 20 l flow-through aquarium (37 aquaria in total). Aquaria were equipped with a mesh-covered overflow and were gravity fed with FSW (5 µm) from a reservoir tank. The reservoir tank itself was supplied with seawater from a large storage tank (300 l) connected to the seawater pipeline. Each 20 l aquarium was individually and continuously bubbled with respective gas mixtures to ensure stable seawater PCO_2 level. After two months, mussel density per aquarium was randomly reduced down to 20 animals in order to achieve optimised feeding conditions (see Section 2.2.3). This resulted in negligible mortality rates of juvenile F1 mussels during the whole acclimation period

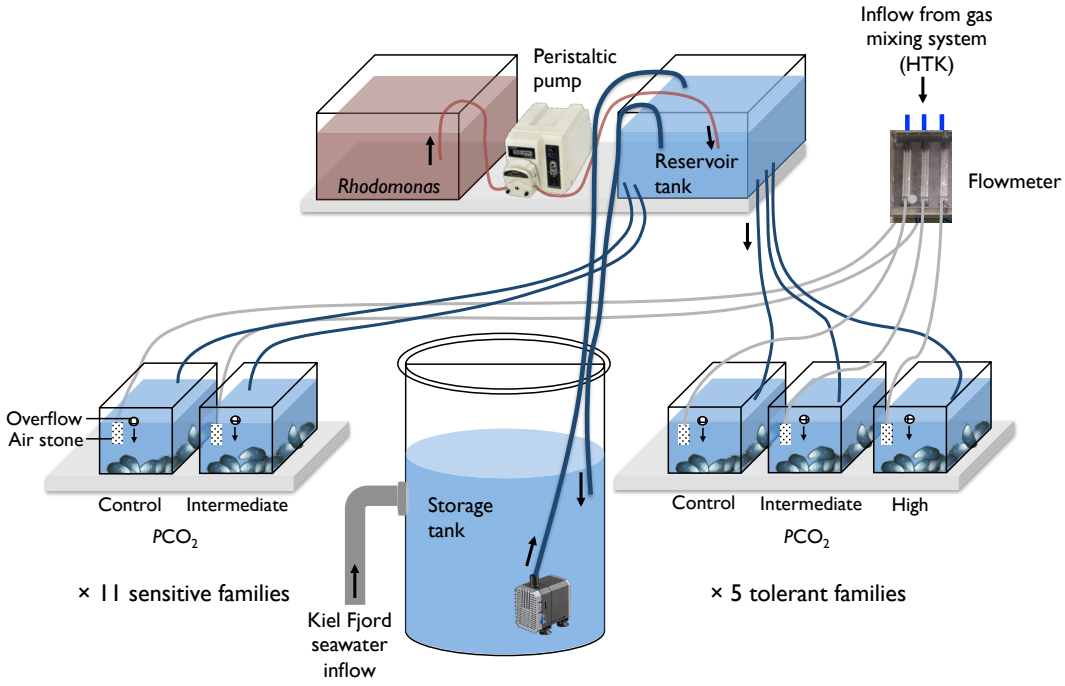

Figure 2.2: CO_2 acclimation set-up from Experiment 1 (*Mytilus edulis*). Water from the storage tank was pumped into the reservoir tank, which was constantly supplied with *Rhodmonas* suspension from a separate tank. Aquaria were fed from the reservoir tank by gravity. A return line into the storage tank avoided overfilling the reservoir tank. Different PCO_2 levels were maintained by constantly bubbling aquaria with respective gas mixtures provided by the gas mixing system (HTK). The five tolerant families were acclimated at all three PCO_2 level, the eleven sensitive families only at the control and intermediate PCO_2. Water hoses are in dark blue; gas lines in grey and peristaltic pump tubing in red. Black arrows indicate flow directions.

2.2.2 *Saccostrea glomerata* (Experiment 2)

The CO_2 acclimation experiment with wild and selected Sydney rock oysters was conducted at the PSFI, Taylors Beach, NSW, Australia from March to June 2015. Oysters were acclimated for seven weeks at a control (500 µatm) and an elevated seawater PCO_2 (1000 µatm). The latter represents a value similar to those predicted for ocean surface waters at the end of this century according to the RCP 8.5 of the fifth report of the Intergovernmental Panel on Climate Change (Pörtner et al. 2014). The number of replication per PCO_2 level was three, resulting in a total of six individual recirculating CO_2 acclimation systems that were randomly placed in a temperature controlled room set to 24°C. Each system consisted of a large 750 l reservoir and two 40 l tubs that were mounted on top of the reservoir tank. Spray bars connected to a submersible aquarium pump supplied FSW (1 µm, 24°C, 35 PSU) from the reservoir tank to the tubs from where it overflowed back into the reservoir tank (Figure 2.3).

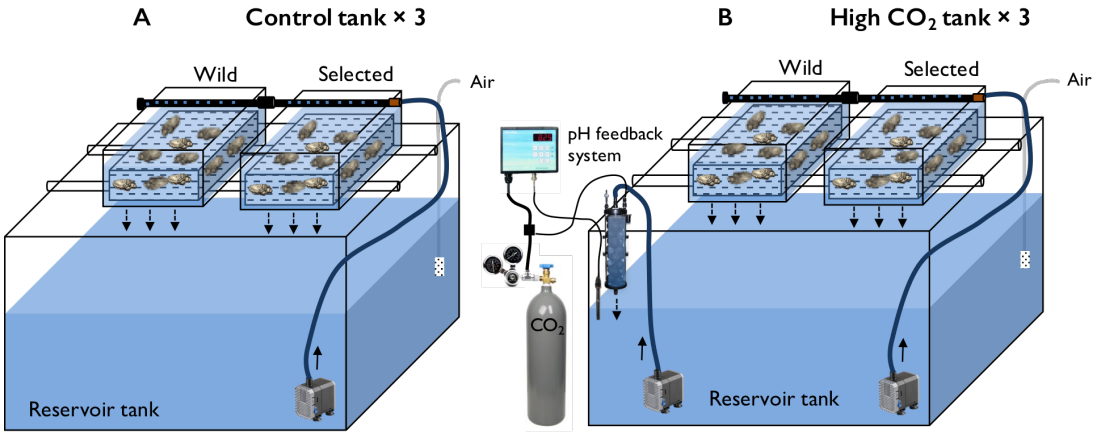

Figure 2.3: CO_2 acclimation set-up from Experiment 2 (*Saccostrea glomerata*). (A) Set-up for the three control tanks. (B) Set-up for the three high CO_2 tanks. Water was pumped from the reservoir tank via spray bars into two tubs, which would overflow back into the reservoir tank. Tubs contained either wild or selected oysters. High CO_2 tanks were additionally equipped with a pH probe feedback system to maintain the elevated seawater PCO_2 level. Water hoses are in dark blue, gas lines in grey. Black arrows indicate flow directions.

One tub contained wild oysters and the other selected oysters (maximum density of 13 oysters per tub). As subsequent physiological measurements were time consuming, oysters were transferred successively into the CO_2 acclimation system over a period of 2.5 weeks in order to assure a standardised incubation period of seven weeks. Within each tub, oysters were placed in mesh baskets in order to reduce handling stress during water changes (see below). All reservoir tanks were bubbled with pressurised air whereas high CO_2 tanks were addition-

ally equipped with a computerised pH probe feedback system (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia) in order to establish and maintain the elevated seawater PCO_2 level (Figure 2.3 B). This system consists of a computer connected to a pH probe, which continuously measures seawater pH (NIST, accuracy ± 0.01). A connected CO_2 cylinder injects gaseous CO_2 which gets into the seawater via a CO_2 reactor (to ensure proper equilibration) until the set seawater pH is reached. Then, CO_2 injection is stopped through a solenoid valve. Every second or third day, the water of each reservoir tank was changed completely. To do so, reservoir tanks were drained and tubs containing oysters were quickly moved to a second set of reservoir tanks which were previously filled with FSW (1 µm, 24°C, 35 PSU) and, in the case of the high CO_2 tanks, equilibrated to the elevated PCO_2 level. During this process, all pH probes of the feedback systems were calibrated (NIST buffer) and the mesh baskets containing the oysters were quickly rinsed with freshwater. The tubs and now empty reservoirs were then prepared for the next water change by scrubbing and rinsing with Virkon S solution (Antec Corp, North Bend, WA, USA). Seawater used in this experiment was locally collected at either Little Beach (152°07′E, 32°72′S) or Shoal Bay Beach (152°10'E, 32°43'S). Oysters were fed twice daily (see Section 2.2.3) and mortality did not exceed 5 % in any of the tanks.

2.2.3 Nutrition of experimental animals

All animals were fed at least once daily with fresh algae suspension in order to minimise side effects of malnutrition or starvation, which are known to modify physiological responses to ocean acidification (e.g. Thomsen et al. 2013, Ramajo et al. 2016).

During the larval phase of Experiment 1, mussels were fed daily with fresh *Isochrysis* suspension starting 2 days post-fertilisation. After one week, fresh *Rhodomonas* suspension was added. As food uptake per larvae increases with age, the total numbers of algal cells added per experimental unit were adjusted weekly (*Isochrysis*: 40,000 (week 1); 60,000 (week 2); 80,000 (week 3); *Rhodomonas:* 10,000 (week 2); 15,000 (week 3)). Once in the flow through system, mussels were continuously fed by steadily pumping fresh *Rhodomonas* suspension into the reservoir tank by means of a peristaltic pump (Figure 2.2). To assure sufficient nutrition, *Rhodomonas* densities in aquaria (mean ± SD; 1030 ± 417 cells ml^{-1}) and the reservoir tank (mean ± SD; 2778 ± 936 cells ml^{-1}) were continuously monitored throughout the experiment using a particle counter (Z2 Coulter® Particle count and size analyser, Beckman CoulterTM, Germany).

During Experiment 2, oysters were fed following the standard hatchery routine (O'Connor et al. 2008). Twice daily a mixture of fresh *Chaetoceros calcitrans* (50%), *Pavlova lutheri* (25%) and *Tisochrysis lutea* (25%) was added to each reservoir tank at a concentration of approximately 2×10^9 cells oyster^{-1} day^{-1}.

2.2.4 Monitoring of seawater carbonate chemistry

During Experiment 1, temperature, salinity and pH_{NIST}, were monitored daily in one-third of the culture jars (48 in total) and weekly in the flow-through system using a WTW 330i pH meter equipped with a Sentix81 electrode and Cond 340i salinometer with a TetraCon 325 electrode. Dissolved inorganic carbon (DIC) was determined weekly by means of an AIRICA autoanalyser (Marianda GmbH, Germany). Additionally, total scale pH was measured weekly during the larval phase using a 626 Metrohm pH meter equipped with a glass electrode that was calibrated with Tris/HCl and 2-aminopyridine/HCl seawater buffers mixed for a salinity of 15 PSU according to Dickson et al. (2007).

Throughout Experiment 2, pH_{NIST}, temperature and salinity were checked twice daily in each tank using a daily-calibrated pH probe (InLab Routine Pt1000, Mettler Toledo GmbH, Germany) connected to a pH meter (WTW 3310,Weilheim, Germany) and a salinometer (WTW LF 197, Germany), respectively. Furthermore, total alkalinity (TA) was quantified at each water change using triplicate Gran-titration (Gran 1952).

For both experiments, corresponding seawater carbonate system speciation were calculated with CO2SYS (Lewis & Wallace 1998) using the dissociation constants for $KHSO_4$ K1 and K2 after Dickson et al. (2007) and Mehrbach et al. (1973) refitted by Dickson and Millero (1987), respectively. Tables summarising measured and calculated carbonate chemistry of Experiment 1 and 2 can be found in Publication I and III, respectively.

2.3 Whole animal physiological parameters

2.3.1 Condition index

For both species and experiments, the condition index (CI) was calculated at the end of the respective acclimation phase to see whether the overall condition of mussels and oysters differed between the different CO_2 treatments as well as between mussel families and oyster lines, respectively. In addition, condition indices of wild and selected oysters were determined before the start of the CO_2 acclimation in order to see whether laboratory culture

affected oysters condition. Condition indices were calculated after Lawrence and Scott (1982) using the following equation:

$$CI = \left(\frac{body_{DW}}{shell_{DW}}\right) \times 100 \quad (3)$$

DW: dry weight in gram.

In order to determine body and shell dry weight from mussels and oysters, soft tissues were separated from the shells and both dried at 80°C for at least 24 h and then weighed. Condition indices of both species are summarised in Table 2.2.

Table 2.2: Condition indices of experimental animals from Experiment 1 and 2. Values are given as mean ± SD. N = 8-13 for *Mytilus edulis*, N= 9-12 for *Saccostrea glomerata.*

Species	Family/oyster type	Treatment	Condition index
M. edulis	Sensitive	Control PCO_2	15.1 ± 1.9
		Intermediate PCO_2	16.9 ± 3.0
	Tolerant	Control PCO_2	15.1 ± 2.1
		Intermediate PCO_2	15.5 ± 1.2
		High PCO_2	14.8 ± 1.8
S. glomerata	Wild	Pre-exposure	4.4 ± 0.7
		Control PCO_2	4.0 ± 0.9
		High PCO_2	4.5 ± 1.3
	Selected	Pre-exposure	4.0 ± 0.6
		Control PCO_2	3.8 ± 0.8
		High PCO_2	4.1 ± 1.2

2.3.2 Clearance rates

Clearance rates were only assessed during Experiment 1. A day before, mussels were individually placed in 800 ml glass beakers that were submerged in the respective flow through aquarium. This procedure reduced the handling stress at the day of experimentation and allowed bysall attachment of the mussels. The following day, beakers were carefully taken out of aquaria and the water replaced with 700 ml of 0.2 µm FSW (at 18°C) equilibrated to the respective seawater PCO_2. Following a 1 h recovery period, *Rhodomonas* algae were added at a concentration of 5000 cells ml^{-1} and a water sample (12 ml) taken every 5 minutes over a

total period of 25 minutes. Algae concentration of each sample was directly determined in duplicates by means of a Z2 Coulter® Particle count and size analyser (Beckman CoulterTM, Germany). Clearance rates (CR) were then calculated as the decrease of *Rhodomonas* concentration over time (verified as a straight line in a semi-log plot, R_2 = 0.91-0.99) using the following equation (according to Coughlan 1969):

$$CR = \left(\frac{V}{n \times t}\right) \ln\left(\frac{C_0}{C_t}\right) \quad (4)$$

V: volume of the suspension in ml
n: number of mussels (1 in this case)
t: time in minutes
C_0: algae concentration before time 't' in cells ml^{-1}
C_t: algae concentration after time 't' in cells ml^{-1}

Clearance rates were determined twice for each mussel and the average rate then standardised to shell length (in ml min^{-1} cm shell $length^{-1}$). During the whole measurement, each beaker was constantly bubbled with air containing the respective PCO_2 to maintain PCO_2 level as well as to avoid algae sedimentation. A beaker without mussel always served as control to account for potential algae sedimentation. *Rhodomonas* concentration of the final sample never fell below 1000 cells ml^{-1}, a concentration known to decrease filtration activity in *Mytilus edulis* (Riisgård & Randløv 1981).

2.3.3 Routine metabolic rate

Routine metabolic rate (RMR) were assessed during Experiment 1 using an intermittent-flow set-up. The set-up consisted of four identical Plexiglas® respiration chambers (100 ml volume) that were submerged in a 100 l water tank filled with 0.2 µm FSW equilibrated to the respective PCO_2 level. A peristaltic pump (ISMATEC, Switzerland) and gastight tubing system (Tygon®) was connected to each respiration chamber ensuring a continuous water circulation. Oxygen saturations within chamber circuits were monitored by means of oxygen micro-optodes (needle-type, Presens GmbH, Germany) that were inserted into tubing systems via a y-shaped plastic connector and previously calibrated at 18°C using oversaturated sodium sulfite solution for 0% and aerated seawater for 100% air saturation. Prior to the start of each measurement, mussels were allowed to acclimate to the respiration chambers and to recover from handling stress for at least 1 h. During this recovery phase, respiration chambers were constantly flushed with water from the tank using small submersible aquarium pumps that were connected to each chamber. Subsequently, the flushing pump was turned off and the decline in oxygen saturation measured for 30 minutes. Within a stop

phase, the oxygen content never fell below 90%. After 30 minutes, a 15 minutes flushing period ensured a re-saturation of the water back to 100% air saturation. To account for the relatively high variability of oxygen consumption measurements, respiration of each mussel was determined in triplicates (three stop phases) and then averaged. During all measurements, the opening status of each mussel was monitored and measurements excluded if mussel valves were closed. Following completion of the measurements, mussels were placed in individual mesh cages and were transferred into respective aquaria in order to determine tissue respiration (see Section 2.5.1) after a recovery phase of 24-48 hours. After tissue respiration had been determined (Section 2.5), mussels' body tissue and shells were separately dried at 80°C for 48 hours and oxygen consumption rates calculated using the following equation:

$$M_{O2} = \frac{\Delta P_{O2} \times \beta_{O2} \times V}{SFDW} \quad (5)$$

M_{O2}: oxygen consumption rate (µmol O_2 g shell free dry weight $^{-1}$ h^{-1})
ΔPO_2: change in oxygen partial pressure over time (kPa h^{-1})
β_{O2}: oxygen capacity of seawater at 18°C (µmol O_2 l^{-1} kPa^{-1}) according to Boutilier (1984)
V: water volume in the respiration chamber and tubing system (l)
SFDW: shell free dry weight (g).

2.4 Extracellular acid-base status

In order to determine the extracellular acid-base status, hemolymph samples of oyster and mussels showing signs of filtration activity (i.e. open shells) were taken using gastight syringes (Hamilton, Switzerland). To do so, animals were immediately shucked and hemolymph withdrawn by pericardial puncture for oysters, whereas hemolymph samples of the smaller-sized mussels were extracted from the adductor muscle. Following extraction, a hemolypmph subsample was quickly transferred into 2 ml vials and extracellular pH (NIST scale) determined at the respective acclimation temperature using a micro electrode (InLab Ultra-Micro, Mettler Toledo GmbH, Germany) connected to a pH meter (WTW 3310, Germany), that was calibrated with NIST buffers at acclimation temperature (18°C or 24°C for mussels and oysters, respectively).

In order to determine extracellular PCO_2 (P_eCO_2) and bicarbonate levels $[HCO_3^-]_e$ for oysters, total extracellular CO_2 (C_eCO_2) of a 100 µl hemolymph subsample was measured using a Ciba-Corning 965 CO_2 analyser (Olympic Analytical Service, UK). The CO_2 analyser was

calibrated daily in a linear range (0.71 to 11.36 mM) using a $NaHCO_3$ standard solution (1 g l^{-1}). In order to account for potential instrument drift, 100 µl of distilled water was measured before, and standard solution after each hemolymph sample. Extracellular P_eCO_2 and $[HCO_3^-]_e$ were calculated using the following modified Henderson-Hasselbalch equations:

$$P_eCO_2 = C_eCO_2 \times (10^{pH-pK'''} \times \alpha CO_2 + \alpha CO_2)^{-1} \quad (6)$$

$$[HCO_3^-]_e = C_eCO_2 - \alpha CO_2 \times P_eCO_2 \quad (7)$$

αCO_2: CO_2 solubility coefficient at 24°C (0.0346 mmol l^{-1} kPa^{-1})
pK''': the negative logarithm of the dissociation constant at 24 °C.

αCO_2 and pK''' were calculated after Heisler (1986) using the ionic strength (*I*) and the molarity of dissolved species (*M*) as they occur in seawater at a salinity of 35 (to represent these values in the oyster hemolymph, (Hammer et al. 2011), a hemolymph protein concentration of 0.05 g l^{-1} (adopted from Peters & Raftos 2003) and a sodium concentration of 468 mM (measured using a blood gas analyser, ABL80, Radiometer Medical, Brønshøj, Denmark).

2.5 Physiological and biochemical parameters of isolated tissues

Physiological and biochemical parameters were determined for gill and mantle tissue (outer mantle for *M. edulis*, see below).

2.5.1 Structure and function of gill and mantle tissue

Lamellibranch bivalves, such as mussels and oysters, have two gills, or ctenidia (see Figure 2.4). Each gill is formed by numerous double V-shaped filaments, which are joined at the ctenidal axis. Each V-shaped filament is called a demibranch and each demibranch consists of two lamellae. The lamellae are either connected by ciliary junctions (fillibranch gill type, mussels) or by tissue connections, the socalled interfilament junctions (eulamellibranch gill type, oysters) (Gosling 2003). Bivalve gills have a dual function; they draw water into the mantle cavity, which serves for ventilation and feeding. This water current is produced by the lateral cilia, which sit along the sides of filaments (Gosling 2003). Food particles in the water are then captured by the latero-frontal cilia and transported to the food grove by the frontal cilia (Gosling 2003). The movement of the cilia is under nervous control and can be artificially modulated by applying the respective neurotransmitter (see Section 2.5.3).

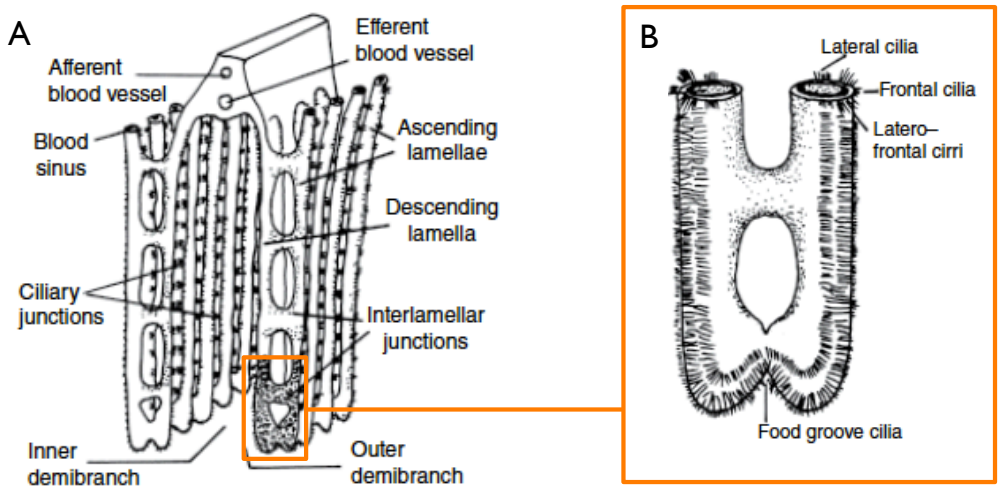

Figure 2.4: (A) Schematic drawing of a gill section of a fillibranch gill (*Mytilus edulis*) and (B) a transverse section through one gill filament (orange frame) showing the ciliation pattern. The drawing is modified after Gosling (2003).

The mantle tissue completely encloses the animal within the shell. It mainly consists of connective tissue with hemolymph vessels, nerves and muscles and can be broadly divided into the inner mantle and outer mantle margin with the three mantle folds (Gosling 2003). These two mantle regions are separated by the pallial line, which marks the area where the pallilal muscle fibres of the inner fold attach the mantle to the shell (Gosling 2003). Each of the mantle folds has a special function: the muscular inner fold is important for controlling the water flow into the mantle cavity; the middle one has mostly sensory functions whereas the epithelia of the outer mantle fold secrete, in concert with the outer epithelia of the inner mantle, the different parts of the shell (Figure 2.5).

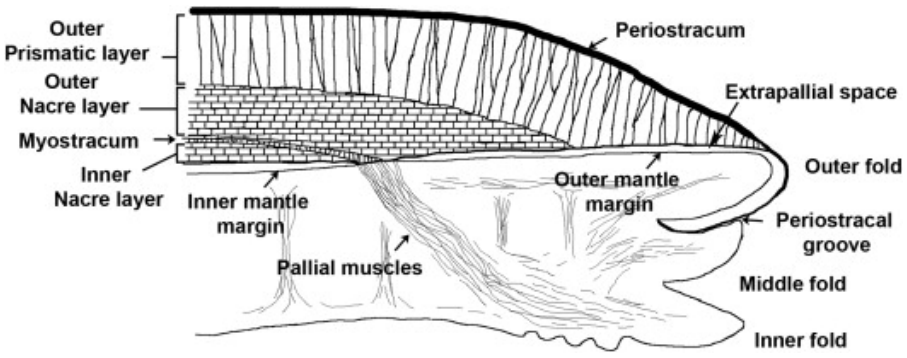

Figure 2.5: Schematic drawing of a transverse section through bivalve mantle tissue and shell. The picture shows the different shell components as well as the inner mantle and the mantel edge with the three mantle folds. Taken from Génio et al. (2012) after Taylor et al. (1969).

Besides its role in producing the shell, the mantle is an important organ for nutrient storage (mainly glycogen) and in mussels, but not in oysters, the inner mantle is the site of gametogenesis (Gosling 2003). For this reason, only the outer mantle was used for experiments with *M. edulis*.

2.5.2 Respiration rates

Respiration rates of isolated gill and mantle tissue were determined during both experiments. Shells of mussels and oysters were opened, gill and mantle tissue (mantle margin with mantle folds for *M. edulis*) carefully dissected and chopped into small similar-sized pieces using sterile forceps and scissors. Subsequently, tissue pieces were transferred into Petri dishes containing respective buffers where they recovered from handling stress for at least 20 minutes.

Buffers were designed to closely mimic respective *in vivo* hemolymph conditions of *M. edulis* and *S. glomerata,* respectively, when acclimated to the respective seawater PCO_2 level, temperature and salinity. For a detailed description of experimental buffer composition please refer to Table S1 and Section 2.5 of Publication I, and Publication III, respectively. Table 2.3 summarises the physicochemical conditions of used buffers.

Table 2.3: Physicochemical conditions of experimental buffers.

Species	Treatment	Temp. (°C)	pH_{NIST}	PCO_2 (kPa)	PO_2 (kPa)	Osmolarity (mOsm)
M. edulis	Control PCO_2	18	7.50	0.16	16	494
	Intermediate PCO_2	18	7.44	0.22	16	494
	High PCO_2	18	7.31	0.33	16	494
S. glomerata	Control PCO_2	24	7.51	0.18	18	1100
	High PCO_2	24	7.47	0.30	18	1100

Equilibration to required PCO_2 and PO_2 levels were achieved by directly bubbling buffers with custom-made gas mixtures (Air liquide, Germany, Experiment 1 or BOC, Australia for Experiment 2) at the respective experimental temperature. Following equilibration, buffer pH was adjusted by adding the respective amount of fresh $NaHCO_3$ solution calculated from the Henderson-Hasselbalch equation by use of pK‴ solubility coefficient αCO_2 determined according to Heisler (1986). Buffer pH was checked before and after each respiration exper-

iment using an InLab® micro-electrode (Mettler Toledo®) connected to pH-meter (WTW 3310, Germany) and was found to be stable for all experiments conducted.

After a recovery phase, respiration rates of tissue pieces were determined using closed water-jacketed custom-made glass respiration chambers whose temperature was controlled by external thermostats (Lauda or Haake, Germany) connected via Tygon® tubing (Figure 2.6). Each chamber was equipped with a small glass agitator and was mounted on magnetic stirrers to avoid stratification. Chambers were filled with respective buffers and tissue pieces were inserted and placed with sterile forceps onto a custom-made spacer (1 mm gauze attached to a piece of gas-tight Tygon® tubing) to avoid mechanical damage from the agitator. Chambers were then closed airtight using a custom-made Plexiglas® plug equipped with an O-ring and small central channel that accepts the needle-type oxygen sensor (micro-optodes, Presens GmbH, Germany) (Figure 2.6). After air bubbles were removed through the central channel, oxygen sensors were inserted and tissue oxygen consumption measured for at least 30 minutes. Prior to measurements, oxygen sensors were calibrated in chambers at experimental temperature using oversaturated sodium sulfite solution for 0% and aerated buffer for 100% air saturation. Respiration rates per tissue type were determined at least in duplicates and standardised to tissue dry weight using equation 5 (Section 2.3).

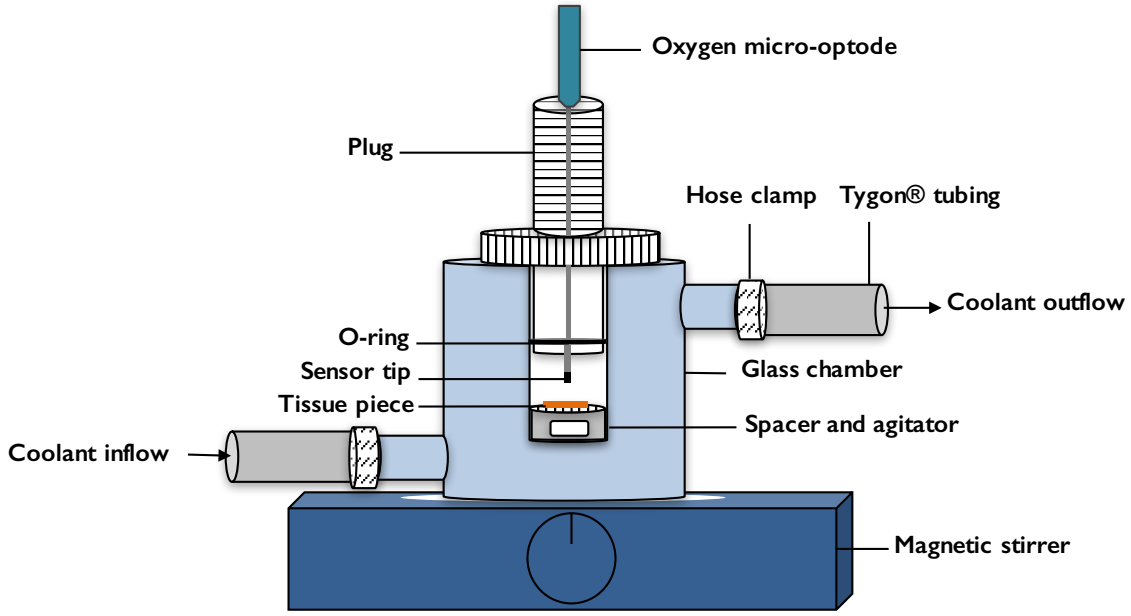

Figure 2.6: Schematic drawing of the tissue respiration set-up. The oxygen micro-optode was inserted into the chamber through a channel in the plug, which closed the glass chamber gas-tight. The tissue piece was placed on a spacer within the chamber to avoid damage from the agitator. The agitator was added to prevent stratification of the buffer in the chamber. Coolant was pumped through the double-walled glass chamber via an external thermostat (not shown) to control experimental temperature.

During Experiment 1, the proportion of the gill and outer mantle tissue compared to total body weight was determined for twelve mussels collected from Kiel Fjord by dissecting, drying (80 °C for 48 h) and then weighing both tissues as well as the remaining body tissues. Mean dry weight proportion of the gill was 10.4 ± 0.8 and 15.3 ± 0.8% for the outer mantle. Using these values, the fractional contribution of gill and outer mantle in per cent to whole animal oxygen consumption (% $MO_{2\ Tissue}$) was calculated using the following equation:

$$\%\ MO_{2\ Tissue} = \left(100/MO_2\right) \times \left(MO_{2\ Tissue}/WP_{Tissue}\right) \quad (8)$$

MO_2: whole animal MO_2 (µmol O_2 g shell free dry weight^{-1} h^{-1})
$MO_{2\ Tissue}$: tissue MO_2 (µmol O_2 g dry weight^{-1} h^{-1})
WP_{Tissue}: weight proportion tissue (in % to whole animal dry weight)

2.5.3 Energy allocation and gill metabolic scope

To assess metabolic energy allocation to protein biosynthesis and different ion-regulators, specific drugs inhibiting respective processes (see Table 2.4) were injected into respiration chambers using gas-tight syringes (Hamilton, Switzerland). This was done directly after completion of tissue respiration measurements (see Section 2.5.2) by quickly removing and reinserting the oxygen sensor. By applying specific inhibitors, oxygen consumption of tissues dropped and the difference in respiration before and after the inhibition was considered as the oxygen demand of the respective cellular process (Figure 2.7A). Oxygen demands were then converted into % fractions of the uninhibited total oxygen consumption of respective tissues.

Table 2.4: Summary of inhibitors applied during tissue respiration experiments.

Species	Tissue	Cellular process	Drug	Concentration (mM)
M. edulis	Gill, outer mantle	Protein biosynthesis	Cycloheximide	0.1
S. glomerata	Gill, inner mantle	Na^+/K^+-ATPase	Ouabain	1
	Gill, inner mantle	H^+-ATPase	Bafilomycin A1	0.0001
	Gill, inner mantle	Na^+/H^+-exchanger	EIPA	0.1

In addition to inhibitor experiments, metabolic scope for filtration in gill tissue was determined during Experiment 1 by means of serotonin stimulation. Serotonin is an excitatory nerve transmitter in *M. edulis,* which regulates, in conjunction with its antagonist dopamine, the beat frequency of the water-flow generating lateral cilia in gill tissue (Paparo & Aiello 1970). In excised *Mytilus* gills as well as *in vivo*, serotonin application stimulates beating of lateral cilia and associated oxygen consumption in a dose-dependent manner (Clemmesen & Jørgensen 1987), whereby 10 µM marks the concentration known to induce the maximal beat frequency (Riisgård & Larsen 2007). After measuring unstimulated gill respiration, serotonin (final concentration 10 µM) was injected into respective respiration chambers as described above and stimulated gill respiration was considered as the metabolic scope for filtration (termed 'metabolic scope' hereafter) (Figure 2.7B). Subsequently, net metabolic scope (NMS) and factorial metabolic scope (FMS) were calculated using the following equations:

$$NMS = \text{Gill } MO_{2\ \text{stimulated}} - \text{Gill } MO_{2\ \text{unstimulated}} \quad (9)$$

$$FMS = \text{Gill } MO_{2\ \text{stimulated}} / \text{Gill } MO_{2\ \text{unstimulated}} \quad (10)$$

NMS: in µmol O_2 g dry weight^{-1} h^{-1}

Inhibitors and serotonin were dissolved in DMSO. Final DMSO concentration did not exceed 0.2% (v/v), which showed only minor effects on tissue respiration (± 0.2–5%).

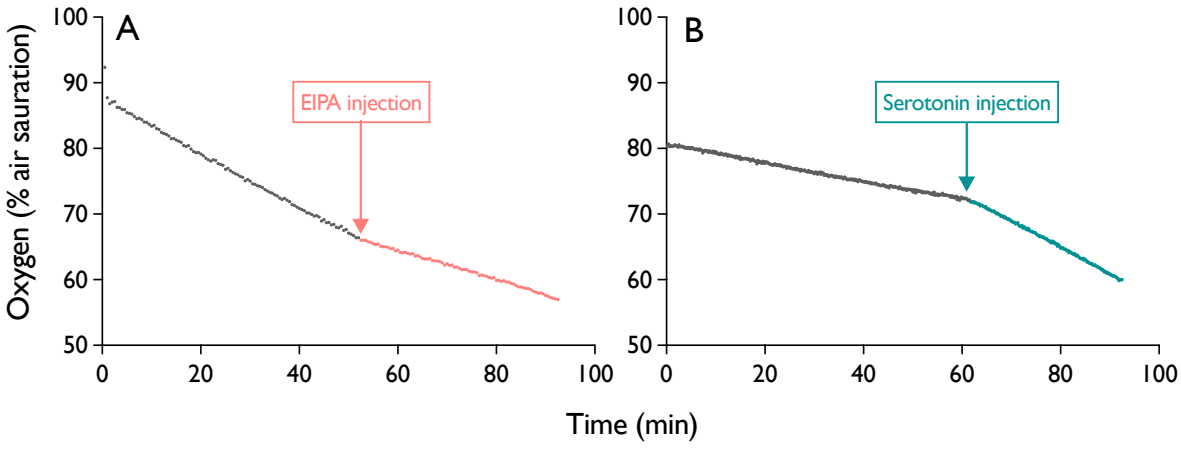

Figure 2.7: Example traces from tissue respiration experiments. A Decrease of oxygen saturation in respiration chamber due to gill tissue (*S. glomerata*) respiration before (in grey) and after EIPA (in red) was applied. Note the difference in slope due to the inhibitory effect of EIPA. B Decrease of oxygen saturation in respiration chamber due to gill tissue (*M. edulis*) respiration before (in grey) and after serotonin (in blue) was applied. Note the difference in slope due to the stimulatory effect of serotonin.

2.5.4 Maximal activities of metabolic enzymes

Maximal enzyme activities of cytochrome-c-oxidase (COX), citrate synthase (CS), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), 3-hydroxyacyl-CoA dehydrogenase (HADH), as well as glutamate dehydrogenase (GDH) were measured spectrophotometrically *in vitro* in crude gill and outer mantle extracts of *M. edulis*. Respective tissues were sampled from sensitive and tolerant F1 mussels after they had been acclimated for 15 months to the different PCO_2 level (October 2013). Mussels were dissected, gill and outer mantle tissue immediately snap frozen in liquid nitrogen and then stored at -80°C. At the day of experimentation, frozen tissue samples were homogenised (8 seconds for 2 times) in extraction buffer (20 mM Tris-HCl buffer, pH 7.5 at 4°C, supplemented with 1mM EDTA and 0.1% Triton X-100) at a ratio of 10 µl buffer per mg tissue by means of a Precellys tissue homogenizer (Precellys24, Bertin Technology, France). Subsequently, cell debris was removed by centrifugation (11 min, 1000 g, 4°C) and the supernatant used as crude extracts for enzyme assays. Assay components, start reagents and wavelength (λ) for each of the measured enzymes are summarised in Table 2.5.

All assays were performed at 18°C (acclimation temperature of mussels at sampling day) in 96-well microplates at a final volume of 200 µl. Microplates were filled with respective assay components (without starter reagent) and were placed on a custom-made, thermostatted aluminium block set to 18°C. Following thermal equilibration, tissue homogenates were added and microplates were transferred into the microplate reader (PowerWave HT Biotek, Germany) and absorbance was measured at the respective wavelengths until stabilisation of the signal. Subsequently, adding the respective start reagent started the reaction (Table 2.5). Each sample was measured in duplicates for two amounts of crude extract (single and double amount, four measurements in total).

Maximum enzyme activities were calculated referring to tissue fresh weight (U/g FW) as well as per mg tissue protein (U/mg protein). Protein concentration of each sample was determined according to Bradford (1976) using bovine serum albumin as protein standard (Sigma-Aldrich, Germany).

Table 2.5: Assay composition, start reagents, buffer pH and wavelength (λ) for the different metabolic enzyme assays. CS: citrate synthase (after Sidell et al. 1987), COX : cytochrome-c-oxidase (modified after Moyes et al. 1997), PK: pyruvate kinase (modified after Driedzic & Fonseca de Almeida Val 1996), PEPCK: phosphoenolpyruvate carboxykinase (after Lockwood and Somero 2012 based on Harlocker et al. 1991), HADH: 3-hydroxyacyl-CoA dehydrogenase (modified after McClelland et al. 2005), GDH: glutamate dehydrogenase (modified after Sánchez-Muros et al. 1998).

Enzyme	Assay composition	Start reagent	pH (18°C)	λ (nm)
COX	20 mM Tris-HCl buffer, 0.5% Tween 20	0.05 mM Cyto-chrome-$c_{(red)}$	7.8	550
CS	75 mM Tris-HCl buffer, 0.25 mM DTNB, 0.4 mM Acetyl-CoA	0.5 mM Oxaloace-tate	8.0	412
PK	80 mM Tris-HCl buffer; 80 mM KCl; 5 mM $MgSO_4$; 5 mM ADP; 0.2 mM NADH; 5 µg/ml lactate dehydrogenase	1 mM PEP	7.5	340
PEPCK	65 mM imidazole-HCl buffer, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM PEP, 1.5 mM IDP, 0.15 mM NADH, 6 U/ml malate dehydrogenase	20 mM $NaHCO_3$	6.6	340
HADH	50 mM imidazole buffer, 0.15 mM NADH, 2.5µg/ml Antimcyin A	0.1 mM Aceto-acetyl-Co-A	7.5	340
GDH	40 mM phosphate buffer, 100 mM NH_4^+, 1 mM ADP, 0.2 mM NADH	5 mM α-Ketoglutarate	7.4	340

2.6 Statistics

Statistical analyses were performed using Sigma Plot 12.0 (Systat Software Inc.) for Publication I and 'R' (software version 3.2.3, R Development Core Team 2015) for Publication II and III. At first, normality and homogeneity of variance were assessed by Shapiro-Wilk and Levene's tests, respectively. For data presented in Publication I and II, this was followed by a full two-way ANOVA in order to test for the effects of 'family type' (sensitive or tolerant) and 'seawater PCO_2' on measured physiological parameters at the control and intermediate PCO_2 level. Additionally, a one-way ANOVA was performed to assess PCO_2 effects within the tolerant families. For data presented in Publication III, linear mixed effect models

(LMMs) with 'oyster type' and 'seawater PCO_2' as fixed factors and 'replication tank' as random factor nested within fixed factors were used to determine significant effects on physiological parameters (LMERTEST package). As no significant 'tank' effect ($P > 0.1$) was detected for any measured parameter (RAND function of LMERTEST), data were re-analysed using a full factorial ANOVA (CAR package) with the fixed factors 'oyster' and 'seawater PCO_2'. If any significant family/oyster-type or PCO_2 effects were detected by ANOVA, a Holm–Sidak (Publication I) or TukeyHSD (Publication II and III) post hoc test was performed for pairwise comparisons of treatments. $P < 0.05$ was accepted to indicate significant differences. If not stated otherwise, all data are presented as mean ± SEM.

3 Publications

List of peer-reviewed publications and declaration of the candidate's contribution towards them.

Publication I

Laura S. Stapp, Jörn Thomsen, Hanna Schade, Christian Bock, Frank Melzner, Hans-Otto Pörtner and Gisela Lannig (2017). Intra-population variability of ocean acidification impacts on the physiology of Baltic blue mussels (*Mytilus edulis*): integrating tissue and organism response. *Journal of Comparative Physiology Part B - Biochemical, Systems, and Environmental Physiology*, 187: 529–543. doi: 10.1007/s00360-016-1053-6

The multi-generation acclimation experiment of *M. edulis* was designed by JT and FM while all physiological experiments were designed by myself with help of GL, CB, JT and FM. I helped JT with the mussel acclimation and I performed all physiological experiments with help of HS for filtration measurements. I analysed all data except for the carbonate chemistry data which was provided by JT. Data interpretation was done by myself and discussed with GL, JT, FM, CB and HOP. I wrote the manuscript, which was revised by all authors.

Publication II

Laura S. Stapp, A. Tillmann, J. Thomsen, C. Bock, H. O. Pörtner and G. Lannig CO_2-dependent adjustments of metabolic pathways differ within a population of blue mussels (*Mytilus edulis*) in revision for the *Journal of Comparative Physiology Part B - Biochemical, Systems, and Environmental Physiology*

I designed the concept of the study and experimental design with help of AT and GL. AT and I collected the data. The analysed samples originated from the multi-generation acclimation experiment of Publication I, which was designed by JT and others (see above). I analysed all data and wrote the manuscript, which was revised by all co-authors.

Publication III

Laura S. Stapp, Laura M. Parker, Wayne A. O'Connor, Christian Bock, Pauline M. Ross, Hans O. Pörtner and G. Lannig (2018) Sensitivity to ocean acidification differs between populations of the Sydney rock oyster: Role of filtration and ion-regulatory capacities. *Marine Environmental Research,* 135: 103-113 doi: 10.1016/j.marenvres.2017.12.017

The idea and concept for this study was developed by myself and GL with advice from HOP and CB. I performed all experimental work with support of LMP and WAO. Data analyses and interpretation was done by myself and discussed with GL, CB and HOP. I wrote the manuscript, which was revised by all co-authors.

Contributions to additional peer-reviewed publications over the duration of the PhD. Publication A1-A4 are listed in chronological order and are included in the Appendix.

Publication A1

Ben P. Harvey, Balsam Al-Janabi, Stefanie Broszeit, Rebekah Cioffi, Amit Kumar, Maria Aranguren-Gassis, Allison Bailey, Leon Green, Carina M. Gsottbauer, Emilie F. Hall, Maria Lechler, Francesco P. Mancuso, Camila O. Pereira, Elena Ricevuto, Julie B. Schram, **Laura S. Stapp**, Simon Stenberg and Lindzai T. Santa Rosa (2014) Evolution of Marine Organisms under Climate Change at Different Levels of Biological Organisation, *Water* (2014) 6: 3545-3574. doi: 10.3390/w6113545

Publication A2

Laura S. Stapp, Cornelia M. Kreiss, Hans-Otto Pörtner and Gisela Lannig (2015). Differential impacts of elevated CO_2 and acidosis on the energy budget of gill and liver cells from Atlantic cod, *Gadus morhua, Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology*, 187: 160-167. doi: 10.1016/j.cbpa.2015.05.009

Publication A3

Elliot Scanes, Laura M. Parker, Wayne A. O'Connor, **Laura S. Stapp** and Pauline M. Ross (2017). Intertidal oysters reach their physiological limit in a future high-CO_2 world*, Journal of Experimental Biology*, 220: 765-774. doi:10.1242/jeb.151365

Publication A4

Jörn Thomsen, **Laura S. Stapp**, Kristin Haynert, Hanna Schade, Maria Danelli, Gisela Lannig, K. Mathias Wegner and Frank Melzner (2017). Naturally acidified habitat selects for ocean acidification–tolerant mussels. *Science advances*, 3: e1602411.doi:10.1126/sciadv.1602411

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Publication I

Intra-population variability of ocean acidification impacts on the physiology of Baltic blue mussels (*Mytilus edulis*): integrating tissue and organism response

Laura S. Stapp, Jörn Thomsen, Hanna Schade, Christian Bock, Frank Melzner, Hans-Otto Pörtner and Gisela Lannig

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ORIGINAL PAPER

Intra-population variability of ocean acidification impacts on the physiology of Baltic blue mussels (*Mytilus edulis*): integrating tissue and organism response

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Abstract Increased maintenance costs at cellular, and consequently organism level, are thought to be involved in shaping the sensitivity of marine calcifiers to ocean acidification (OA). Yet, knowledge of the capacity of marine calcifiers to undergo metabolic adaptation is sparse. In Kiel Fjord, blue mussels thrive despite periodically high seawater PCO_2, making this population interesting for studying metabolic adaptation under OA. Consequently, we conducted a multi-generation experiment and compared physiological responses of F1 mussels from 'tolerant' and 'sensitive' families exposed to OA for 1 year. Family classifications were based on larval survival; tolerant families settled at all PCO_2 levels (700, 1120, 2400 µatm) while sensitive families did not settle at the highest PCO_2 (≥99.8% mortality). We found similar filtration rates between family types at the control and intermediate PCO_2 level. However, at 2400 µatm, filtration and metabolic scope of gill tissue decreased in tolerant families, indicating functional limitations at the tissue level. Routine metabolic rates (RMR) and summed tissue respiration (gill and outer mantle tissue) of tolerant families were increased at intermediate PCO_2, indicating elevated cellular homeostatic costs in various tissues. By contrast, OA did not affect tissue and routine metabolism of sensitive families. However, tolerant mussels were characterised by lower RMR at control PCO_2 than sensitive families, which had variable RMR. This might provide the energetic scope to cover increased energetic demands under OA, highlighting the importance of analysing intra-population variability. The mechanisms shaping such difference in RMR and scope, and thus species' adaptation potential, remain to be identified.

Keywords CO_2 · Multi-generation · Metabolic rate · Energy metabolism · Clearance rate · Protein biosynthesis

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Electronic supplementary material The online version of this article (doi:10.1007/s00360-016-1053-6) contains supplementary material, which is available to authorized users.

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Introduction

Ocean acidification resulting from increasing atmospheric CO_2 concentrations has been shown to adversely affect marine organisms (Wittmann and Pörtner 2013). Marine calcifiers, such as molluscs, appear to be particularly susceptible to ocean acidification (Kroeker et al. 2013). Numerous studies have shown that predicted shifts in seawater carbonate chemistry can directly impair calcification processes and the stability of carbonate structures (Orr et al. 2005; Kroeker et al. 2013; Gazeau et al. 2013). This impairment correlates with lowered seawater $CaCO_3$ saturation state (Ω), which can impair crystal formation (Waldbusser et al. 2015) and increases shell dissolution when seawater is undersaturated with calcium carbonate (Thomsen et al. 2010; Melzner et al. 2011). Alternatively,

calcification is impaired by the pH decrease, which affects the ability of calcifiers to excrete the excess H^+ produced during the calcification process (Thomsen et al. 2015; Bach 2015) or by a combination of both. In addition, elevated CO_2 levels are expected to alter energy budgets at the cellular and tissue and, consequently, whole organism level, due to increased homeostatic costs associated with energy demanding ion-regulatory processes to compensate for CO_2-induced acid–base disturbances in body fluids and calcification compartments (Pörtner et al. 2004; Melzner et al. 2009). Such energetic trade-offs can, in turn, affect biomineralisation in an indirect manner, as shell formation in many molluscs does not only include the precipitation of calcium carbonate polymorphs, but also the synthesis of an organic matrix which can take up a considerable amount of the organism's energy budget (Palmer 1992; Thomsen et al. 2013; Waldbusser et al. 2013; Thomsen et al. 2015).

One strategy to exploit existing capacity to compensate and meet an increased energy demand is increasing food uptake, if sufficient food is available. High food rations were shown to outweigh negative effects of ocean acidification on calcification, showing a clear link to the organisms' energy budget (Melzner et al. 2011; Hettinger et al. 2013; Thomsen et al. 2013; Waldbusser et al. 2013; Towle et al. 2015; Ramajo et al. 2016). Yet, there is growing evidence that exposure to high PCO_2 impairs digestion and ingestion rates in several calcifying invertebrates, possibly limiting the potential to maintain or increase food intake (Navarro et al. 2013; Vargas et al. 2013, 2015; Stumpp et al. 2013; Zhang et al. 2015; Clements 2016). If an increased energy assimilation cannot compensate for an increased energy demand, organisms may down-regulate their metabolism ('metabolic depression') as an energy preserving mechanism or, alternatively, available energy may be re-allocated among physiological functions and tissues (Stumpp et al. 2011; Sokolova et al. 2012; Dorey et al. 2013). A higher energy demand for homeostatic processes could thus lower energy availability for anabolic, fitness-related traits such as growth or reproduction (Sokolova et al. 2012). A decreased scope for growth at elevated CO_2 has been suggested for mussels, gastropods and sea urchins (Thomsen and Melzner 2010; Stumpp et al. 2011, 2012; Zhang et al. 2015). Knowledge about the patterns and limitations of organism's energy allocation to different physiological processes is thus crucial for a mechanistic understanding of a species sensitivity or tolerance towards elevated PCO_2, which highlights the need of studies that integrate tissue-specific into whole animal responses (Harvey et al. 2014; Pan et al. 2015).

Besides the need to deepen our mechanistic understanding of whether and how organisms respond to ocean acidification, long-term and multi-generation studies are required in order to realistically predict whether species possess the potential for acclimatisation or genetic adaptation (Kelly and Hofmann 2012). Due to the rapid rate of climate change, evolutionary adaptation of relatively long-living organisms such as bivalves or echinoderms is more likely to rely on existing genetic variation, rather than new mutations (Lande and Shannon 1996; Pespeni et al. 2013). Measuring the genetic variation of physiological and associated fitness-related traits of natural populations is thus a valuable tool for predicting a species' potential for evolutionary adaptation; especially if different genotypes show opposing reaction norms for such traits, potentially leading to overall unchanged mean population responses (Applebaum et al. 2014; Foo and Byrne 2016). However, due to the large effort that is required to perform experiments with multiple family lines of one population, few studies have been carried out in order to test this hypothesis and to investigate physiological mechanisms which correlate with increased or decreased fitness of different geno- and phenotypes. Species inhabiting naturally enriched CO_2 sites can serve as an interesting model to study long-term physiological acclimatisation or adaptation to elevated PCO_2 (Calosi et al. 2013). In Kiel Fjord, Western Baltic Sea, upwelling events of acidified bottom water during summer and autumn lead to strong seasonal and daily fluctuations in seawater PCO_2 level, with peak values of >2300 µatm far exceeding its present annual mean seawater PCO_2 of around 700 µatm (Thomsen et al. 2010; Saderne et al. 2013; Melzner et al. 2013). In spite of such unfavourable conditions, resilient marine calcifiers thrive and dominate in Kiel Fjord due to a high primary production and hence food availability (Thomsen et al. 2013; Pansch et al. 2014). Reproduction of blue mussels (*Mytilus edulis*) takes place in summer when the highest fluctuations in carbonate chemistry occur (Thomsen et al. 2010). Occasional high seawater PCO_2 levels during the larval phase might lead to selection for plastic or tolerant genotypes within the population (Thomsen et al., submitted). However, ongoing ocean acidification is expected to be amplified in coastal habitats and, in combination with eutrophication, PCO_2 values above 2000 µatm are likely to occur on a regular basis in Kiel Fjord by the end of this century (HELCOM 2009; Melzner et al. 2013), therefore the population may need to adapt in order to thrive in this habitat in the future.

We therefore conducted a long-term, multi-generation experiment with different generated family lines of blue mussels from Kiel Fjord to test for their adaptation potential under ocean acidification (see also Thomsen et al., submitted). Dams and sires of wild *M. edulis* were cross-bred and larvae from 16 generated families were exposed to different CO_2 levels. The CO_2 sensitivity of offspring (F1 generation) from these different family lines varied strongly, and thus families were classified as 'sensitive' and 'tolerant' (Thomsen et al., submitted). Our objective was to

compare adult F1 mussels of tolerant and sensitive families with regard to their physiological responses at tissue and organismal levels. By measuring filtration, routine metabolism and tissue respiration, we analysed energy allocation patterns and potential shifts in energy supply. Analyses of organismal performance and tissue-specific responses were linked by measurements of protein biosynthesis in calcifying mantle tissue and of metabolic scope of gill tissue. Using this multi-generation approach including studies across levels of biological organisation, we aimed to gain insight into potential intra-population variations in physiological responses to ocean acidification and the potential for metabolic adaption of *M. edulis*; a keystone species in the Baltic Sea.

Materials and methods

Animals and breeding design

In June 2012 mature individuals of *Mytilus edulis* (64.2 ± 5.5 mm) were collected from Kiel Fjord, Western Baltic Sea, and were kept at habitat temperature (18 °C) in flow-through aquaria supplied by natural seawater from the Fjord. After 24 h of recovery, animals were thermally induced to spawn by slowly increasing the water temperature (Δ 5–7 °C) in individual glass beakers containing filtered seawater (0.2 µm). Egg densities were determined and eggs subsequently fertilised at ambient conditions (pH_{NBS}: 8.22, 18.1 °C, 14.9 PSU). Gametes of 8 sires and 8 dams were crossbred in a reduced North Carolina I design resulting in a total of 16 full-sib families (Fig. 1). Fertilisation success was checked after 2–3 h and was high among all families (>90%).

Larval phase

Following fertilisation, the embryos of each family were randomly divided and transferred to the experimental units (5000 embryos per unit) to start the exposure to three different nominal PCO_2 levels [700 (control), 1120 (intermediate), 2400 (high) µatm] according to present and predicted PCO_2 levels in Kiel Fjord (Thomsen et al. 2013; Melzner et al. 2013) (Fig. 1). Each experimental unit (polyvinylchloride, KAUTEX, Germany) was filled with 500 ml filtered Kiel Fjord seawater (0.2 µm) at 18 °C and 15 PSU that was equilibrated to the respective PCO_2 level. For each of the 16 families the number of replication for each PCO_2 level was three (3 × 5000 eggs per family and CO_2 level), resulting in a total of 144 experimental units. Larvae were fed daily with fresh *Isochrysis* starting 2 days post-fertilisation. After 7 days, food was supplemented with fresh *Rhodomonas*. To account for an increasing food uptake with increasing larval age the total number of algae cells added per culture jar increased weekly (*Isochrysis*: week 1, 40,000; week 2, 60,000; week 3, 80,000; *Rhodomonas*: week 2, 10,000; week 3, 15,000). Algae were cultivated in F/2 or PES medium, respectively. Cell densities were monitored daily using a particle counter (Z2 Coulter® Particle count and size analyser, Beckman Coulter™, Germany). Water was exchanged weekly with filtered (0.2 µm) Kiel Fjord seawater set to 18 °C and equilibrated to the respective PCO_2 level. After approximately 21 days, larvae started to settle. At day 21, larval survival of all families was not significantly different between the control and intermediate PCO_2, but significantly reduced at the highest CO_2 treatment (Thomsen et al., submitted). In 11 of 16 families this translated into successful settlement (defined as >10 settled specimens per replicate) only at the control and intermediate CO_2 level (referred to as sensitive families in the following), while larvae of the remaining five families settled successfully at all three PCO_2 levels (referred to as tolerant families) (Fig. 1).

Juveniles and adults

After 9 weeks (September 2012) the settled spat of each family reached a size of about 1–2 mm and replicates were pooled and transferred into 20 l flow-through aquaria. Rearing of tolerant families continued at all three PCO_2 levels, whereas sensitive families were only reared at the control and intermediate CO_2 level since larvae did not settle at the highest PCO_2 (Fig. 1).

The flow-through system consisted of a large storage tank (300 l) that supplied seawater to a smaller reservoir tank, from which then each 20 l aquaria was supplied. The storage tank was constantly supplied with Kiel Fjord seawater that was filtered through a series of 50, 20, and 5 µm filters. Fresh *Rhodomonas* suspension was continually added to the reservoir tank. Each aquarium had an overflow equipped with mesh (3 mm mesh size) to prevent mussels from escaping and was individually bubbled with the respective gas composition.

After 2 months in the flow-through system, the number of mussels within each aquarium was randomly reduced down to ~20 in order to ensure standardised and optimised feeding conditions. Throughout the whole experiment *Rhodomonas* concentrations were monitored in the reservoir tank (mean ± SD; 2778 ± 936 cells ml^{-1}) and aquaria (mean ± SD; 1030 ± 417 cells ml^{-1}) using a particle counter (Z2 Coulter® Particle count and size analyser, Beckman CoulterTM, Germany).

After 12 months of acclimation (July 2013), all mussels of the F1 generation were induced to spawn, as described above, for a further breeding experiment with the F2 generation (Thomsen et al., submitted) and to minimise the

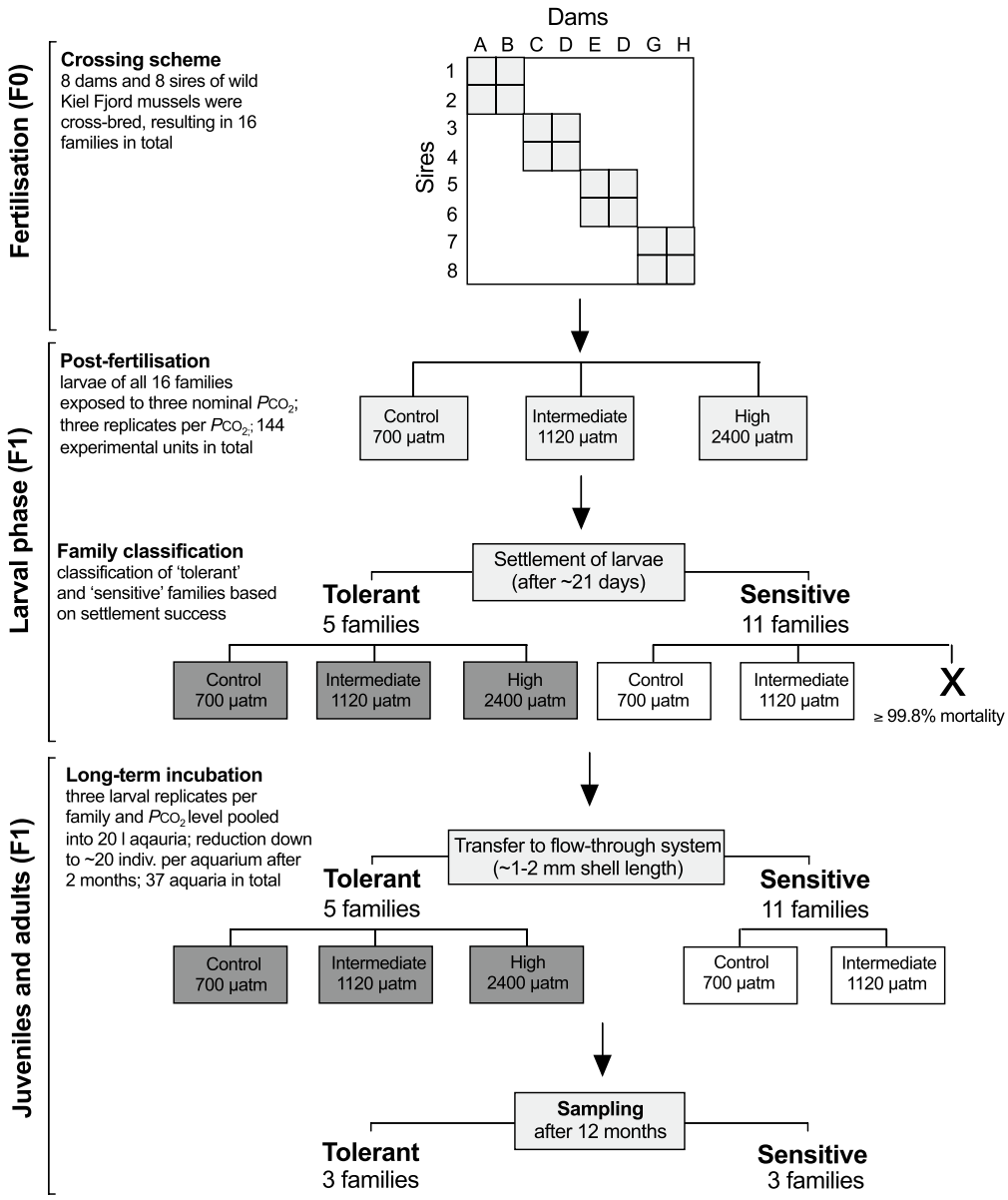

Fig. 1 Flow chart of the experimental design including the crossing scheme that was used for generating the 16 different families (F1 generation) of *Mytilus edulis*

effects of gonadal status on metabolic rates. After a 2-week recovery phase, three individuals per PCO_2 level from each of the three tolerant and three sensitive families were sampled for the physiological experiments (Fig. 1). To avoid allometric effects on physiological parameters, mussels of similar body size were picked from each family. Mean shell lengths ± SD were 27 ± 3.06 mm for tolerant and 26.4 ± 3.29 mm for sensitive mussels without significant differences between family types (2-way ANOVA, $F = 0.262$, $P = 0.613$) or CO_2 level, (2-way ANOVA, $F = 0.352$, $P = 0.558$; CO_2 within tolerant families, 1-way ANOVA, $F = 0.075$; $P = 0.928$).

Table 1 Mean seawater carbonate chemistry of the experimental units during the CO_2 exposure of F1 larvae and early juveniles (June to September 2012)

Treatment	pH_{NBS}	pH_{Total}	A_T (µmol kg^{-1})	DIC (µmol kg^{-1})	PCO_2 (µatm)	$\Omega_{calcite}$	$\Omega_{aragonite}$
Control	8.17 ± 0.07	7.97 ± 0.02	1884 ± 51	1803 ± 46	508 ± 14	2.08 ± 0.14	1.23 ± 0.62
Intermediate	7.75 ± 0.06	7.65 ± 0.01	1897 ± 76	1889 ± 79	1128 ± 82	1.05 ± 0.03	0.62 ± 0.02
High	7.46 ± 0.05	7.39 ± 0.03	1944 ± 51	1995 ± 50	2114 ± 108	0.61 ± 0.04	0.36 ± 0.02

Values for PCO_2, A_T, $\Omega_{calcite}$ and $\Omega_{aragonite}$ were calculated from salinity (15.5 ± 0.3 PSU), temperature (17.7 ± 0.1 °C), pH_{NBS} and DIC. Values are mean ± SE, $N = 48$

A_T total alkalinity, *DIC* dissolved inorganic carbon

Table 2 Mean seawater carbonate chemistry of the flow-through system during the CO_2 exposure of F1 juveniles and adults (September 2012 to September 2013)

Treatment	pH_{NBS}	pH_{Total}	A_T (µmol kg^{-1})	DIC (µmol kg^{-1})	PCO_2 (µatm)	$\Omega_{calcite}$	$\Omega_{aragonite}$
Control	8.01 ± 0.06	7.84 ± 0.07	2064 ± 122	2043 ± 125	734 ± 108	1.23 ± 0.32	0.71 ± 0.20
Intermediate	7.69 ± 0.07	7.57 ± 0.05	2068 ± 118	2108 ± 118	1381 ± 136	0.68 ± 0.18	0.40 ± 0.11
High	7.40 ± 0.10	7.33 ± 0.06	2146 ± 253	2258 ± 257	2515 ± 381	0.42 ± 0.12	0.24 ± 0.07

Values for PCO_2, A_T, $\Omega_{calcite}$ and $\Omega_{aragonite}$ were calculated from salinity (11.5 – 18.7 PSU), temperature (4.3 – 16.4 °C), pH_{NBS} and DIC. Values are mean ± SE, $N = 148$. Temperature and salinity varied throughout the year; given are minimum and maximum values

A_T total alkalinity, *DIC* dissolved inorganic carbon

Manipulation and monitoring of seawater carbonate chemistry

All seawater carbonate chemistry manipulations were conducted by direct bubbling of each experimental unit or aquarium with respective gas concentrations (PCO_2 of either 700, 1120 or 2400 µatm) using an automatic custom-made CO_2 mixing-facility (Linde Gas & HTK, Germany). During the larval phase pH_{NBS}, temperature and salinity were monitored daily in one-third of the culture jars (48 in total) using a WTW 330i pH meter equipped with a Sentix81 electrode and Cond 340i salinometer with a TetraCon 325 electrode. Weekly, water samples were analysed for dissolved inorganic carbon (DIC) using an AIRICA autoanalyser (Marianda GmbH, Kiel, Germany) and total scale pH by using a 626 Metrohm pH meter equipped with a glass electrode calibrated with Tris/HCl and 2-aminopyridine/HCl seawater buffers mixed for a salinity of 15 PSU according to Dickson et al. (2007). In the flow-through system DIC was determined once per week; pH_{NBS}, temperature and salinity twice a week. Seawater carbonate system specifications were calculated with CO2SYS (Lewis and Wallace 1998) using the dissociation constants for $KHSO_4$, K1 and K2 after Dickson et al. (2007) and Mehrbach et al. (1973) refitted by Dickson and Millero (1987), respectively. All data for the physicochemistry of the seawater are summarised in Tables 1 and 2.

Clearance rate

One day prior to clearance rate measurements mussels were placed in separate 800 ml glass beakers, which were submersed in the respective flow-through aquaria in order to allow byssal attachment and to reduce handling stress at the day of experimentation. The next day seawater in beakers was carefully exchanged with 700 ml filtered seawater (0.2 µm) equilibrated to the respective PCO_2 level at 18 °C. After 1 h, *Rhodomonas* cells were added (5000 cells ml^{-1}) and 12 ml water samples were taken every 5 min over a time course of 25 min. *Rhodomonas* concentration per sample was directly determined in duplicates using a Z2 Coulter® Particle count and size analyser (Beckman Coulter™, Krefeld, Germany). Clearance rates were standardised to shell length and calculated as the decrease of *Rhodomonas* concentration over time (verified as a straight line in a semi-log plot, $R^2 = 0.91$–0.99) according to the clearance formula of Coughlan (1969). Slight bubbling by air diffuser and respective gas mixtures prevented sedimentation of algae and ensured aerated conditions as well as constant PCO_2 level. A beaker without a mussel served as control. Final *Rhodomonas* concentration never fell below 1000 cells ml^{-1} to avoid cessation of filtration activity (Riisgård and Randløv 1981).

Table 3 Physicochemical conditions of the buffer used for oxygen consumption measurements of isolated gill and outer mantle tissue

Treatment	pH_{NBS}	PCO_2 (kPa)	PO_2 (kPa)	Osmolarity (mOsm/l)
Control	7.50	0.16	16	494
Intermediate	7.44	0.22	16	494
High	7.31	0.33	16	494

Whole animal oxygen consumption

Measurements of routine metabolic rates (RMR) were conducted in intermittent flow-through plexiglas chambers (~100 ml) placed in a 100 l tank containing 0.2 µm filtered seawater equilibrated to respective PCO_2 level. A tubing system connected to a peristaltic pump (ISMATEC, Switzerland) ensured a continuous water flow within each chamber. Prior to measurements mussels recovered for 1 h and chambers were constantly flushed with water from the tank using a small submersible aquarium pump. Subsequently, flushing stopped for 30 min and the oxygen consumption per mussel was measured as oxygen depletion (% air saturation) using oxygen micro-optodes (needle-type, Presens GmbH, Germany). Within a stop phase, the oxygen content never fell below 90% and was re-saturated to 100% air saturation during the subsequent flushing (15 min). Due to the relatively high variability of oxygen consumption measurements rates, respiration of each mussel was determined in triplicates (three stop phases). Opening status of mussel valves was monitored throughout the experiments and runs were excluded if closure of valves was observed. After measuring RMR, mussels were transferred back to respective aquaria and were kept individually in a mesh cage for a recovery phase (24–48 h) prior to the determination of tissue respiration rates.

Buffer composition

Metabolic rates of gill and outer mantle tissue were determined in buffer designed to closely mimic respective in vivo haemolymph conditions of *M. edulis* incubated at the different PCO_2 levels (700, 1120, 2400 µatm) at 15 PSU and 18 °C (Table 3, Table S1). Ionic buffer composition at a 15 PSU was calculated from Bayne (1976). Concentrations of free amino acids were taken from Mulvey and Feng (1981). Tyrosine, methionine and tryptophan levels were found to be below detection limits and, thus, were added at low concentration of 2 nmol l^{-1} (see supplementary Table S1). Glucose was added at a concentration of 2 mM (Bayne 1976). Data for haemolymph PCO_2 and pH (pH_e) were taken from Thomsen et al. (2010) and Heinemann et al. (2012). Haemolymph PCO_2 at the respective experimental seawater PCO_2 was calculated by linear regression ($R^2 = 0.96$) (Table 3). pH_e data available from the literature had been determined at various temperatures and were therefore temperature-corrected according to alphastat (Zittier et al. 2012). PO_2 was set according to ambient in vivo conditions at 18 °C (Kupprat 2014) (Table 3) . Buffers were equilibrated with the respective PCO_2 and PO_2 at 18 °C using custom-made gas mixtures and a water bath. pH_{NBS} was adjusted by adding the respective amount of fresh $NaHCO_3$ solution calculated from the Henderson–Hasselbalch equation by use of pK''' and solubility coefficient αCO_2 determined according to Heisler (1986). Buffer pH was checked before and after each run using a pH meter equipped with an InLab® semi-micro electrode (Mettler Toledo®) and an external temperature sensor.

Oxygen consumption of gill and outer mantle tissue

Mussels were dissected on ice. Gill and outer mantle (mantle margin with mantle folds) tissues were carefully chopped into similar-sized pieces using sterile scissors (mean dry weight ± SD; gill: 1 ± 0.3 mg, mantle: 1.5 ± 0.4 mg). Tissue pieces were transferred into petri dishes containing respective buffer and were allowed to recover from handling stress for at least 20 min. Subsequent measurements of tissue oxygen consumption rates were conducted in custom-made, closed, airtight water-jacketed glass respiration chambers in which temperature was set to 18 °C by an external thermostat (Haake or Lauda, Germany) (Strahl et al. 2011). The glass chambers were mounted on magnetic stirrers to ensure homogeneity during measurements. Self-made spacer consisting of coarse-meshed gauze (1 mm mesh size) attached to Tygon® tubing prevented tissues from damage by glass agitators. Chambers were closed airtight and oxygen micro-optodes (needle-type, Presens GmbH, Germany) were inserted through the plug to measure oxygen depletion within each chamber. Tissue respiration rates were determined for at least two pieces per tissue and mussel.

Metabolic scope of gill tissue and protein biosynthesis in outer mantle tissue

Directly after tissue respiration measurements, either cycloheximide (mantle) or serotonin solutions (gill) were injected into the chambers through the plug using a gas-tight microliter glass syringe (Hamilton, Switzerland). Response to each drug was measured for two tissue pieces per mussel. Cycloheximide is a specific inhibitor for protein biosynthesis and was applied at a final concentration of 100 µM (Cherkasov et al. 2006). Energy expense for protein biosynthesis was then calculated as the difference between respiration rates before and after the inhibitor had been applied. Values are given as fractional costs in per

cent of total mantle respiration. Serotonin acts as an excitatory nerve transmitter in gills and was applied at a concentration of 10 µM. This concentration is known to induce the maximal beat frequency of the lateral cilia which generate the water flow (Riisgård and Larsen 2007). Movement of lateral cilia is driven by the ATPase dynein, which accounts for up to 90% of the ATP demand in gills during the maximal beat frequency of cilia (Gibbons 1982; Clemmesen and Jørgensen 1987). This results in a tight correlation of the cilia beat frequency and oxygen consumption in isolated gill tissue of *M. edulis* (Clemmesen and Jørgensen 1987). Serotonin stimulated oxygen consumption of gill tissue was thus considered as the metabolic scope for filtration and will be termed 'metabolic scope' hereafter. Net metabolic scope (NMS) was calculated as the difference in oxygen consumption of stimulated and unstimulated gill tissue and factorial metabolic scope (FMS) as the ratio of stimulated to unstimulated gill oxygen consumption. Cyclohexiimide and serotonin were dissolved in DMSO. Final concentration did not exceed 0.2%, which showed only minor effects on tissue respiration (±0.2–5%). All chemicals were obtained from Sigma Aldrich (Germany).

Calculation of fractional oxygen consumption of gill and outer mantle tissue

In order to determine the contribution of tissue-specific to the whole organism metabolic rate, the fractional oxygen consumption of gill and outer mantel tissue in per cent to the total respiration (% $MO_{2\ Tissue}$) were calculated for each mussel using the following equation:

$$\%\, MO_{2\ Tissue} = (100/MO_2) \times (MO_{2\ Tissue}/WP_{Tissue}).$$

Oxygen consumption of the whole animal (MO_2) and tissue ($MO_{2\ Tissue}$) are in µmol O_2 g dry weight^{-1} h^{-1} and the weight proportion (WP_{Tissue}) of the respective tissue dry weight in per cent to the dry weight of the whole animal. To determine tissue weight proportions, 12 mussels were collected from Kiel Fjord, gill and outer mantle dissected and both tissues as well as the remaining tissue were dried (80 °C for 48 h) and then weighed. Mean dry weight proportion of the gill was 10.4 ± 0.8 and 15.3 ± 0.8% for the outer mantle.

Statistics

Data were analysed using Sigma Plot 12.0 (Systat Software Inc.). After data were checked for normality and equality of variances a full two-way ANOVA was performed for the control and intermediate CO_2 level to unravel the effects of family (sensitive or tolerant) and seawater PCO_2 followed by a Holm–Sidak post hoc test with pairwise comparisons of treatments. Additionally, a one-way ANOVA was performed to analyse the effects of PCO_2 level within the tolerant families followed by a Holm–Sidak post hoc test. $P < 0.05$ was accepted to indicate significant differences. All data are presented as mean ± SEM.

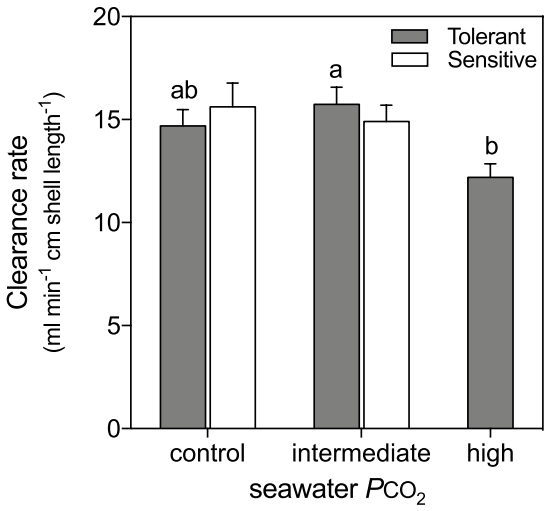

Fig. 2 Clearance rates of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, $N = 9$–12. *Different letters* indicate significantly different clearance rates within tolerant families at different seawater PCO_2

Results

Clearance rate

At the control and intermediate CO_2 clearance rates were not affected by 'family type' or 'PCO_2' (Fig. 2; Table 4); however, a significant CO_2 effect on clearance rates could be observed for tolerant families at the highest PCO_2 level (Table 5). Compared to the intermediate CO_2 level (1120 µatm), filtration decreased significantly by around 23% at 2400 µatm (15.7 ± 0.8 at 1120 µatm vs. 12.2 ± 0.7 ml min^{-1} cm shell length^{-1} at 2400 µatm, $P = 0.005$).

Whole animal oxygen consumption

Analysis of metabolic rates revealed a significant interaction between 'family type' and 'PCO_2' (Table 4). While oxygen consumption rates of tolerant families increased significantly with rising PCO_2 at intermediate compared to ambient CO_2 levels (Fig. 3a; Table 5, $P = 0.023$), metabolic rates of sensitive families were similar between

Table 4 Results of a two-way ANOVA performed to investigate effects of PCO_2 (control and intermediate level) and family type (tolerant, sensitive) on (a) whole animal oxygen consumption (MO_2) and filtration; (b) oxygen consumption, net (NMS) and factorial metabolic scope (FMS) of isolated gill tissue; (c) oxygen consumption and fractional costs of protein biosynthesis of outer mantle tissue; (d) summed oxygen consumption of gill and outer mantle and fractional contribution of gill and outer mantle to whole animal MO_2

Biological level		Parameter	PCO_2 effect			Family effect			Interaction		
			F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
(a)	Whole animal	Filtration	0.035	1	0.853	0.003	1	0.955	0.973	1	0.330
		MO_2	1.103	1	0.304	0.002	1	0.964	5.652	1	**0.026**
(b)	Gill	MO_2	2.056	1	0.161	0.370	1	0.547	0.896	1	0.351
		NMS	1.220	1	0.278	3.142	1	0.086	0.673	1	0.418
		FMS	1.741	1	0.196	0.008	1	0.929	0.488	1	0.490
(c)	Outer mantle	MO_2	0.310	1	0.582	0.239	1	0.132	4.475	1	**0.043**
		Protein biosynthesis	0.187	1	0.670	0.658	1	0.426	0.264	1	0.613
(d)	Gill + mantle	Summed MO2	2.503	1	0.124	1.275	1	0.268	2.928	1	0.097
		Fraction of total MO_2	0.253	1	0.620	0.096	1	0.760	3.559	1	0.072

Significant results are written in bold

Table 5 Results of a one-way ANOVA performed to investigate the PCO_2 effect (control, intermediate and high) within the tolerant families on (a) whole animal oxygen consumption (MO_2) and filtration; (b) oxygen consumption, net (NMS) and factorial metabolic scope (FMS) of isolated gill tissue; (c) oxygen consumption and fractional costs of protein biosynthesis of outer mantle tissue; (d) summed oxygen consumption of gill and outer mantle tissue

Biological level		Parameter	PCO_2 effect		
			F	*df*	*P*
(a)	Whole animal	Filtration	6.144	2	**0.006**
		MO_2	2.667	2	0.093
(b)	Gill	MO_2	2.734	2	0.082
		NMS	1.262	2	0.298
		FMS	3.865	2	**0.033**
(c)	Outer mantle	MO_2	2.137	2	0.136
		Protein biosynthesis	0.963	2	0.399
(d)	Gill + mantle	Summed MO_2	3.691	2	**0.038**

Significant results are written in bold

intermediate and control CO_2 levels. There was no significant difference in metabolic rates of tolerant compared to sensitive families; however, under control conditions, oxygen consumption rates of sensitive families tended to be elevated above those of tolerant families (43.6 ± 8.0 vs. 27.1 ± 3.6 µmol O_2 g dry weight^{-1} h^{-1}, $P = 0.088$).

Oxygen consumption of gill and outer mantle tissue

There was a significant interaction of 'family type' and 'PCO_2' on outer mantle respiration (Table 4). Control outer mantle respiration was similar for both family types (Fig. 3b). At the intermediate PCO_2 level mantle respiration of sensitive families decreased slightly by 14% while outer mantle respiration of tolerant families increased by around 25%, resulting in significantly higher outer mantle respiration than in sensitive families ($P = 0.013$). Although mantle respiration of tolerant families at different PCO_2 followed the same pattern as whole animal oxygen consumption rates no significant effect of PCO_2 was detected (Table 5).

We neither observed a significant effect of 'family type' or 'PCO_2', nor a significant interaction of these factors on metabolic rates of gill tissue isolated from both, sensitive and tolerant families (Fig. 3c; Table 4) but gill tissue of tolerant mussels respired around 18% more at 1120 and 2400 µatm than at ambient seawater PCO_2 (Table 5, $P = 0.082$).

When comparing the sum of gill and mantle tissue oxygen consumption rates for animals from the two family types, tolerant families were characterised by 22.8% higher respiration rates at intermediate than at control seawater PCO_2 (Fig. 4a; Table 5, $P = 0.038$).

Factorial and net metabolic scope of gill tissue

'Family type' and 'PCO_2' did not significantly affect the factorial metabolic scope (FMS) of isolated gill tissue at control and intermediate seawater PCO_2 (Table 4). However, FMS of gill tissue of tolerant mussels was significantly reduced by 21% at the highest CO_2 level compared to control conditions (3.8 ± 0.2 at control vs. 3.0 ± 0.2 at high seawater PCO_2, Fig. 5a; Table 5, $P = 0.003$). In contrast, net metabolic scope was neither affected by 'PCO_2', nor by 'family type' (Fig. 5b; Tables 4, 5).

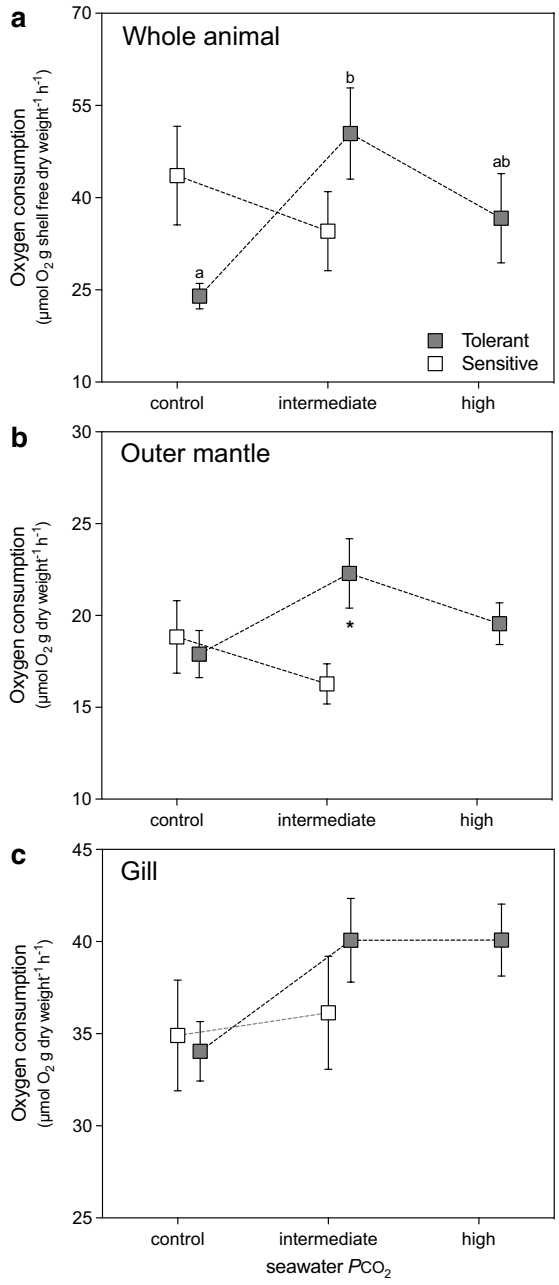

Fig. 3 **a** Oxygen consumption of the whole organism, **b** outer mantle tissue and **c** gill tissue of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE; $N = 7$–10 (whole animal), $N = 8$–12 (tissue). For better viewing clarity, the data points of tolerant families were shifted to the right. *Different letters* indicate significantly different oxygen consumption within tolerant families at different seawater PCO_2. *Hash* indicates a significantly different outer mantle oxygen consumption between tolerant and sensitive families

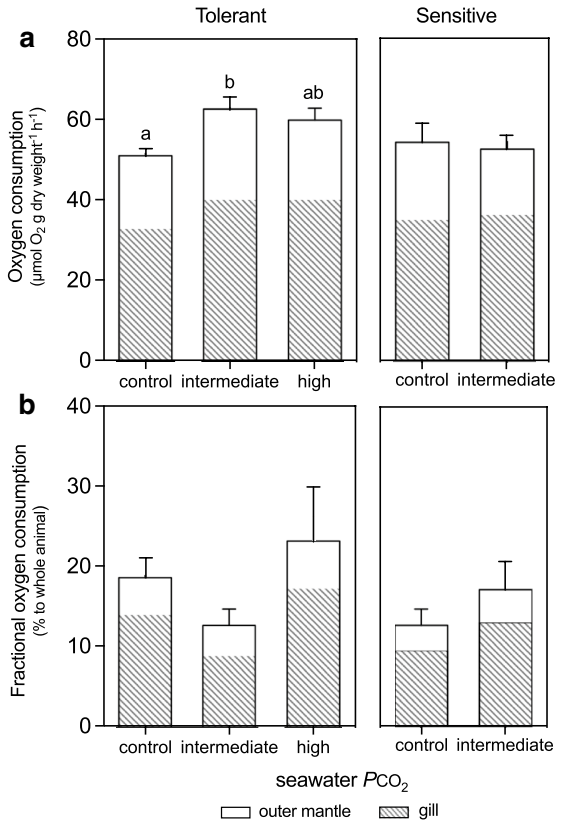

Fig. 4 Summed oxygen consumption of gill and outer mantle tissue (**a**) and fractional contribution of gill and outer mantle to whole animal respiration (**b**) of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, $N = 8$–12 (summed) and $N = 6$–11 (fractional). *Different letters* indicate significantly different summed tissue oxygen consumption within tolerant families at different seawater PCO_2

Energy allocation to protein biosynthesis in mantle tissue

Mean fractional energy allocated to protein biosynthesis in outer mantle tissue of both family types ranged between ~7 and 13%. Energy expenditure for protein biosynthesis in outer mantle tissue was neither affected in tolerant nor in sensitive mussels raised under elevated CO_2 (Fig. 6; Tables 4, 5).

Fractional tissue oxygen consumption

The fractional oxygen consumption of isolated gill and outer mantle tissue in relation to the whole animal oxygen consumption did not differ significantly between the tolerant and sensitive families as well as between

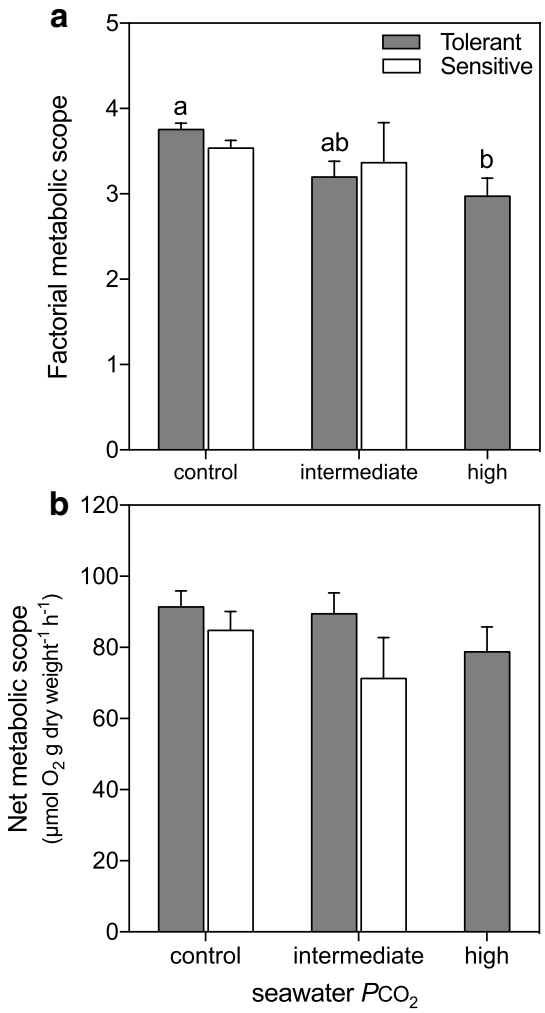

Fig. 5 Factorial (**a**) and net (**b**) metabolic scope of gill tissue isolated from tolerant and sensitive families of *Mytilus edulis* raised for 1 year at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, $N = 8$–12. *Different letters* indicate significantly different factorial metabolic scope within tolerant families at different seawater PCO_2

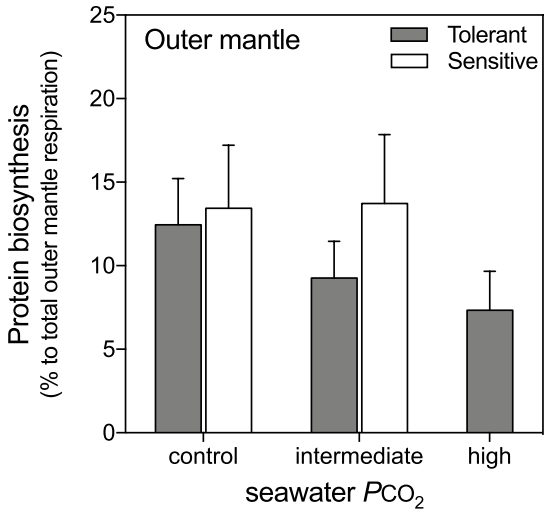

Fig. 6 Energy expenditure for protein biosynthesis in isolated outer mantle tissue of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, $N = 5$–10

CO_2 treatments (Fig. 4b; Table 4). Gill tissue contributed between 8.8 and 17.2% whilst the fraction of outer mantle tissue comprised between 3.5 and 5.9%.

Discussion

This study investigated family-specific metabolic responses of Baltic blue mussels (*Mytilus edulis*) selected and raised as larvae and juveniles under three different PCO_2 levels. We compared physiological traits of families whose larvae survived all PCO_2 levels with those whose larvae tolerated only moderately elevated CO_2 concentrations. After a 1-year acclimation period we observed contrasting metabolic responses between family types at the tissue as well as whole animal level. Thus, if data had been pooled, the observed metabolic variance between family types might have been obscured. In line with these observations, unchanged mean routine metabolic rates (RMR) but a large variability was observed between individual mussels from the same population, randomly collected as spat on settlement panels in the wild and acclimated for 1 year in the laboratory to PCO_2 levels similar to our study (Hüning 2014). However, in light of rapid climate change existing variation of metabolic responses may represent the physiological basis allowing a population to adapt to changing environmental conditions, stressing the importance of looking at trait variations within natural populations as a measure of their adaptive capacity (Applebaum et al. 2014; Foo and Byrne 2016).

Metabolic adaption to a changing environment is determined by an organism's ability to balance the energy costs of self-maintenance against those for life history traits, such as growth or reproduction. Mussels of tolerant families increased their whole animal RMR at intermediate CO_2, and, although alterations were more pronounced at the whole animal level, gill and mantle tissue followed the same pattern (Fig. 3). Summed gill and mantle respiration was significantly elevated at the intermediate PCO_2 levels (Fig. 4a) indicating that increased RMR of tolerant

families reflects a cumulative effect caused by slightly elevated metabolic rates in various tissues. Similarly, the fractional contribution of both tissues to the whole animal oxygen consumption did not change significantly between the treatments. Nevertheless, the contribution of gill and outer mantle to the whole animal respiration of tolerant families tended to decrease at the intermediate CO_2 level, potentially indicating that other tissues contributed relatively more to the observed increase of whole animal metabolic rate (Fig. 4b).

Ocean acidification is thought to increase cellular homeostatic costs through shifted acid–base equilibria that are challenging both pH regulation and calcification (Lannig et al. 2010; Thomsen and Melzner 2010; Beniash et al. 2010). Elevated CO_2 leads to a permanently decreased extracellular pH and increased PCO_2 in *Mytilus* spp. whilst intracellular pH is rapidly restored (Michaelidis et al. 2005; Thomsen et al. 2010; Heinemann et al. 2012; Gazeau et al. 2014; Zittier et al. 2015). This increases the proton flux into the intracellular space and requires additional energy for proton equivalent ion exchange (Boron 2004; Pörtner 2008; Melzner et al. 2009). Thus, a higher ability to regulate extracellular acid–base status during exposure to elevated CO_2 was proposed as a crucial trait promoting resilience towards ocean acidification (Pörtner 2008; Melzner et al. 2009). A recent transgenerational study on Sydney rock oysters (*Saccostrea glomerata*) showed that oysters exposed to ocean acidification responded with partial compensation of extracellular pH after one generation of exposure (Parker et al. 2015). In contrast to tolerant families, we found unchanged RMR and tissue metabolic rates of sensitive families at the intermediate elevated CO_2. In order to test whether different metabolic responses of sensitive and tolerant families correlated with different abilities of extracellular acid–base regulation we determined their extracellular pH (after Gazeau et al. 2014) in a subsequent experiment. However, there was no evidence for extracellular regulation of pH for neither tolerant nor sensitive families (see supplementary Fig. S1). This suggests that varying metabolic responses of adult mussels from different family types after long-term exposure to elevated CO_2 did not correlate with different abilities to regulate their extracellular pH.

Besides their varying metabolic responses to elevated CO_2, comparison of tolerant and sensitive families indicated different rates of routine metabolism under present-day (control) conditions. Although not statistically significant, due to high variability within sensitive families, individuals from tolerant families kept at control seawater PCO_2 had 1.6-fold lower mean RMR with a 2.6-fold decreased relative standard deviation than sensitive families. Low routine metabolic costs might thus be a beneficial trait when exposed to elevated CO_2, potentially providing the scope to cover elevated energetic costs of maintenance and calcification under elevated CO_2. Intra-specific variations of RMR is a ubiquitous phenomenon amongst most taxa and has been previously linked with organismal fitness (Burton et al. 2011). The so-called 'context dependence hypothesis' states that, while individuals with a higher relative RMR might have a fitness advantage under favourable conditions, low RMR is beneficial during adverse environmental conditions (e.g. low food availability) due to lower costs of self-maintenance (Burton et al. 2011). Although future studies need to confirm this hypothesis in the light of ocean acidification, our results for the highest experimental PCO_2 also suggest a selection for phenotypes with a reduced or more efficient routine metabolism. RMR of high CO_2-exposed tolerant mussels almost returned to control levels in contrast to a previous study with the same population were respiration rates peaked at this seawater PCO_2 (~2400 µatm)(Thomsen and Melzner 2010). Unlike the study of Thomsen and Melzner (2010), we acclimated mussels throughout their complete life cycle and, although tolerant families successfully settled in the high CO_2 treatment, we observed a significantly increased larval mortality at this PCO_2 level (Thomsen et al., submitted). Similarly, a transgenerational study with the calanoid copepod *Pseudocalanus acuspes* found that increased metabolic rates at an intermediate PCO_2 had returned to control levels at the highest PCO_2 level (Thor and Dupont 2015). This was associated with increased mortality at the high PCO_2 level and the authors interpreted this as a selection process (Thor and Dupont 2015), potentially for more efficient oxidative phosphorylation (De Wit et al. 2015).

For the genus *Mytilus,* intra-individual variations of RMR have been well studied in the context of intra-specific variation of growth rates. Specimens that grew faster had lower standard metabolic rates, partly stemming from an increased efficiency of protein deposition linked to different intensities of protein turnover (Diehl et al. 1986; Hawkins et al. 1986; Bayne and Hawkins 1997; Bayne 2004). Hawkins et al. (1986) hypothesised that this energy surplus might confer a fitness advantage for faster growers under adverse environmental conditions and, in fact, thermal tolerance of slow growing *Mytilus* was decreased compared to fast growers (Hawkins et al. 1987). Increasing growth efficiency could thus be a useful mechanism to counteract potential energy trade-offs under elevated PCO_2. Indeed, a study on the Sydney Rock oyster *Saccostrea glomerata* indicates a positive relationship between growth efficiency and CO_2 tolerance; oysters that were mass-selected for growth over seven generations showed a higher CO_2 tolerance compared to the wild population (Parker et al. 2010, 2012). We did not determine individual growth rates in this study and picked same-sized tolerant and sensitive mussels in order to avoid allometric effects. Mussels were of

the same age and hence had similar growth rates, however, potentially at different energetic costs as indicated by lower RMR of tolerant families at ambient PCO_2. Furthermore, Thomsen et al. (submitted) show that larvae from tolerant families had larger shell sizes at the highest CO_2 level after 2 days of development. At this larval stage, relative rates of somatic growth and calcification rates are highest, but larvae have to rely on their endogenous energy reserves (Rodriguez et al. 1990; Sánchez-Lazo and Martínez-Pita 2012). Egg sizes (i.e. proxy for energy content) did not differ between families, which might suggest a higher or more efficient energy allocation to (shell) growth in larvae of tolerant families (Thomsen et al., submitted).

In bivalves, the mantle is the tissue responsible for the secretion of the shells. At the intermediate PCO_2, outer mantle respiration was higher in tolerant compared to sensitive families. However, since data were derived from similar-sized mussels, this family-specific difference in outer mantle respiration was not correlated with a differential net calcification. This corresponds to earlier studies on this population which also found no effect on shell length after long-term exposure to a similar PCO_2 level (Thomsen et al. 2010, 2013). Apart from affecting calcification rates, elevated PCO_2 can also affect the mechanical properties and ultrastructure of mussel shells (Hüning et al., submitted; Hüning et al. 2013; Fitzer et al. 2014). Furthermore, a previous study with mussels from Kiel Fjord showed changes in the expression of genes involved in the processing of the organic shell matrix (Hüning et al. 2013). This proteinaceous shell matrix is mainly secreted by the outer mantle tissue (Beedham 1958; Hüning et al. 2016) and, although making up only a relatively small fraction of the total shell mass, the energetic costs of the synthesis can take up a significant amount of bivalves energy budget (Palmer 1992). Our data suggest, however, that family type-specific differences in outer mantle respiration were not linked with a differing energy allocation to protein biosynthesis. Relative costs for protein biosynthesis were highly variable between individuals but did not differ between family types or CO_2 levels; although, it should be considered that net protein deposition does also depend on degradation rates. Furthermore, the relatively low fraction of the cycloheximide-sensitive respiration (7–13% of total respiration) might have resulted in a relatively unfavourable signal-to-noise ratio between cycloheximide and its solvent DMSO, thereby contributing to the observed variability. Although matching the results of a previous study with a marine bivalve [*Crassostrea virginica*, (Cherkasov et al. 2006)], further experiments should confirm our results; for instance by use of a water-soluble inhibitor, such as emetine (Fenteany and Morse 1993), to minimise potential DMSO side effects.

At the highest PCO_2 level, RMRs of tolerant families were similar to those measured under control conditions, but were associated with decreased clearance rates and thus a decreased food (energy) uptake. This suggests a potentially unfavourable shift in energy demand and energy acquisition. Reduced feeding rates in response to ocean acidification were reported for larvae and adults of several marine invertebrates including *Mytilus* (Navarro et al. 2013; Vargas et al. 2013, 2015; Zhang et al. 2015; Xu et al. 2016). This could be related to a reduced catalytic efficiency of digestive enzymes (Stumpp et al. 2013), which could increase the gut passage time of food particles (Bayne et al. 1984), thereby reducing filtration in a secondary fashion. As an alternative explanation, the filtration mechanism itself may have been affected. Decreased filtration in tolerant families at the highest PCO_2 went hand in hand with a decreased factorial metabolic scope of gill tissue. In filter-feeding bivalves the gills retain food particles by generating a water flow through movement of lateral cilia that is driven by the ATPase dynein (Gibbons 1982). Cells that carry the lateral cilia make up only around 15% of the total gill mass, but account for up to 90% of the ATP demand in gills reflected in a tight correlation of ciliary beat frequency and oxygen consumption in isolated gill tissue (Clemmesen and Jørgensen 1987). The decreased metabolic scope of gill tissue may therefore be mechanistically linked to decreased clearance rates of *M. edulis* at the highest CO_2 level. The mechanism causing the observed reduction in functional capacities of ciliary activity remains to be explored. Upon acute hypoosmotic exposure ciliary activity in gills of *M. edulis* was decreased due to reallocation of ATP from ciliary activity towards vital functions—in this case osmoregulatory processes (Doeller et al. 1993 and references therein). Similar trade-offs may exist under elevated CO_2, when ATP may be reallocated to ion and acid–base regulation. Alternatively, CO_2-induced disturbances in acid–base equilibria may directly impact ciliary functioning. Acute changes in pH and PCO_2 were shown to decrease ciliary beat frequency in isolated *Mytilus* gills (Haywood 1925); however, tested pH levels (~pH 6.9) were below the range relevant in an ocean acidification context. Furthermore, mitochondrial capacities could become constrained under high CO_2 (Strobel et al. 2012). Since ciliated cells are densely packed with mitochondria to sufficiently fuel dynein ATPases (Paparo 1972), decreased mitochondrial capacities would possibly limit maximal ATP supply and thus metabolic scope of gills.

Irrespective of the underlying mechanism, food acquisition may be impaired and may decrease the fitness and productivity of this population under near-future conditions if no physiological adaptation or acclimatisation takes place. Presently blue mussels can thrive in the CO_2-enriched Kiel Fjord due to high food availability that seems to cover higher energetic demands under elevated CO_2 (Thomsen et al. 2010, 2013). The observed intra-population variability

of CO_2-related metabolic responses may suggest some capacity for metabolic adaptation within this population. A high variability of a given environmental driver likely increases the plasticity within populations (Valladares et al. 2014). Thomsen et al. (submitted) demonstrated that, due to patchy upwelling events the Kiel Fjords' carbonate chemistry can vary substantially between *Mytilus* larval cohorts from the same year with average mean PCO_2 levels during the planktonic phase ranging from ~800 to 1100 µatm, and peak values up to 2700 µatm. Thus, differing selective patterns during the larval phase are likely explaining the observed genotypic and/or phenotypic plasticity within this population (Thomsen et al., submitted). Besides selection processes at the larval stages CO_2 exposure of adults and larvae can lead to carryover and epigenetic effects as well as transgenerational plasticity (Ross et al. 2016). Near-future increases of PCO_2 fluctuations and mean PCO_2 level in Kiel Fjord will further increase selection pressure and likely favour CO_2-resistant larvae. However, such a unidirectional selective force may ultimately result in a reduced genetic variation and thus potentially limited capacity of this population to respond to other environmental drivers (Pistevos et al. 2011; Kelly and Hofmann 2012). As human-driven environmental change is not limited to ocean acidification, future studies should also address adaptive capacities to elevated CO_2 combined with multiple other environmental drivers.

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References

Applebaum SL, Pan TCF, Hedgecock D, Manahan DT (2014) Separating the nature and nurture of the allocation of energy in response to global change. Integr Comp Biol 54:284–295. doi:10.1093/icb/icu062

Bach LT (2015) Reconsidering the role of carbonate ion concentration in calcification by marine organisms. Biogeosciences 12:4939–4951. doi:10.5194/bg-12-4939-2015

Bayne BL (1976) Marine mussels: their ecology and physiology. Cambridge University Press, Cambridge

Bayne BL (2004) Phenotypic flexibility and physiological tradeoffs in the feeding and growth of marine bivalve molluscs. Integr Comp Biol 44:425–432. doi:10.1093/icb/44.6.425

Bayne BL, Hawkins AJS (1997) Protein metabolism, the costs of growth, and genomic heterozygosity: experiments with the mussel *Mytilus galloprovincialis* Lmk. Physiol Zool 70:391–402. doi:10.1086/515848

Bayne BL, Klumpp DW, Clarke KR (1984) Aspects of feeding, including estimates of gut residence time, in three mytilid species (Bivalvia, Mollusca) at two contrasting sites in the Cape Peninsula, South Africa. Oecologia 64:26–33. doi:10.1007/BF00377539

Beedham GE (1958) Observations on the mantle of Lamellibranchia. Q J Microsc Sci 99:181–197

Beniash E, Ivanina A, Lieb NS et al (2010) Elevated level of carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica*. Mar Ecol Prog Ser 419:95–108. doi:10.3354/meps08841

Boron WF (2004) Regulation of intracellular pH. AJP Adv Physiol Educ 28:160–179. doi:10.1152/advan.00045.2004

Burton T, Killen SS, Armstrong JD, Metcalfe NB (2011) What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? Proc R Soc B 278:3465–3473. doi:10.1098/rspb.2011.1778

Calosi P, Rastrick SPS, Lombardi C et al (2013) Adaptation and acclimatization to ocean acidification in marine ectotherms: an in situ transplant experiment with polychaetes at a shallow CO_2 vent system. Philos Trans R Soc B 368:20120444. doi:10.1098/rstb.2012.0444

Cherkasov AS, Biswas PK, Ridings DM et al (2006) Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. J Exp Biol 209:1274–1284. doi:10.1242/jeb.02093

Clements JC (2016) Meta-analysis reveals taxon- and life stage-dependent effects of ocean acidification on marine calcifier feeding performance. bioRxiv 066076. doi: 10.1101/066076

Clemmesen B, Jørgensen CB (1987) Energetic costs and efficiencies of ciliary filter feeding. Mar Biol 94:445–449. doi:10.1007/BF00428251

Coughlan J (1969) The estimation of filtering rate from the clearance of suspensions. Mar Biol 2:356–358. doi:10.1007/BF00355716

De Wit P, Dupont S, Thor P (2015) Selection on oxidative phosphorylation and ribosomal structure as a multigenerational response to ocean acidification in the common copepod *Pseudocalanus acuspes*. Evol Appl 9:1112–1123. doi:10.1111/eva.12335

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res Pt I 34:1733–1743. doi:10.1016/0198-0149(87)90021-5

Dickson AG, Sabine CL, Christian JR (2007) Guide to Best Practices for Ocean CO_2 Measurements. PICES Special Publications 3

Diehl WJ, Gaffney PM, Koehn RK (1986) Physiological and Genetic Aspects of Growth in the Mussel *Mytilus edulis*. I. Oxygen Consumption, Growth, and Weight Loss. Physiol Zool 59:201–211. doi:10.2307/30156034

Doeller JE, Kraus DW, Shick JM, Gnaiger E (1993) Heat flux, oxygen flux, and mitochondrial redox state as a function of oxygen availability and ciliary activity in excised gills of *Mytilus edulis*. J Exp Zool 265:1–8. doi:10.1002/jez.1402650102

Dorey N, Lançon P, Thorndyke M, Dupont S (2013) Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. Glob Change Biol 19:3355–3367. doi:10.1111/gcb.12276

Fenteany G, Morse DE (1993) Specific inhibitors of protein synthesis do not block RNA synthesis or settlement in larvae of a marine

gastropod mollusk (*Haliotis rufescens*). Biol Bull 184:6–14. doi:10.2307/1542375

Fitzer SC, Cusack M, Phoenix VR, Kamenos NA (2014) Ocean acidification reduces the crystallographic control in juvenile mussel shells. J Struct Biol 188:39–45. doi:10.1016/j.jsb.2014.08.007

Foo SA, Byrne M (2016) Acclimatization and adaptive capacity of marine species in a changing ocean. Adv Mar Biol 74:69–116. doi:10.1016/bs.amb.2016.06.001

Gazeau F, Parker LM, Comeau S et al (2013) Impacts of ocean acidification on marine shelled molluscs. Mar Biol 160:2207–2245. doi:10.1007/s00227-013-2219-3

Gazeau F, Alliouane S, Bock C et al (2014) Impact of ocean acidification and warming on the Mediterranean mussel (*Mytilus galloprovincialis*). Front Mar Sci 1:1–12. doi:10.3389/fmars.2014.00062

Gibbons IR (1982) Introduction: dynein ATPases. Cell Motil 2:87–93. doi:10.1002/cm.970020718

Harvey BP, Al-Janabi B, Broszeit S et al (2014) Evolution of marine organisms under climate change at different levels of biological organisation. Water 6:3545–3574. doi:10.3390/w6113545

Hawkins AJS, Bayne BL, Day AJ (1986) Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: the basis of variable age-specific growth. Proc R Soc B 229:161–176. doi:10.1098/rspb.1986.0080

Hawkins AJS, Wilson IA, Bayne BL (1987) Thermal responses reflect protein turnover in *Mytilus edulis* L. Funct Ecol 1:339–351. doi:10.2307/2389790

Haywood C (1925) The relative importance of pH and carbon dioxide tension in determining the cessation of ciliary movement in acidified sea water. J Gen Physiol 7:693–697. doi:10.1085/jgp.7.6.693

Heinemann A, Fietzke J, Melzner F et al (2012) Conditions of *Mytilus edulis* extracellular body fluids and shell composition in a pH-treatment experiment: acid–base status, trace elements and δ^{11}B. Geochem Geophys Geosyst 13:Q01005. doi:10.1029/2011GC003790

Heisler N (1986) Buffering and transmembrane ion transfer processes. In: Heisler N (ed) Acid–Base regulation in animals. Elsevier, Amsterdam, pp 3–47

HELCOM (2009) Eutrophication in the Baltic Sea—an integrated thematic assessment of the effects of nutrient enrichment and eutrophication in the Baltic Sea region. Balt Sea Environ Proc No 115B:148. ISSN 0357–2994

Hettinger A, Sanford E, Hill TM et al (2013) The influence of food supply on the response of Olympia oyster larvae to ocean acidification. Biogeosciences 10:6629–6638. doi:10.5194/bg-10-6629-2013

Hüning (2014) Responsiveness of *Mytilus edulis* towards mechanical stress and elevated pCO_2—combined transcriptomic, proteomic and physiological analyses. Dissertation, University of Bremen

Hüning AK, Melzner F, Thomsen J et al (2013) Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: implications for shell formation and energy metabolism. Mar Biol 160:1845–1861. doi:10.1007/s00227-012-1930-9

Hüning AK, Lange SM, Ramesh K et al (2016) A shell regeneration assay to identify biomineralization candidate genes in mytilid mussels. Mar Genom 27:57–67. doi:10.1016/j.margen.2016.03.011

Kelly MW, Hofmann GE (2012) Adaptation and the physiology of ocean acidification. Funct Ecol 27:980–990. doi:10.1111/j.1365-2435.2012.02061.x

Kroeker KJ, Kordas RL, Crim R et al (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Glob Change Biol 19:1884–1896. doi:10.1111/gcb.12179

Kupprat F (2014) Determination of acute protein biosynthesis rates in the blue mussel *Mytilus edulis:* The role of hemolymph parameters in temperature-dependent growth. Master's Thesis, University of Bremen

Lande R, Shannon S (1996) The role of genetic variation in adaptation and population persistence in a changing environment. Evolution 50:434–437. doi:10.2307/2410812

Lannig G, Eilers S, Pörtner HO et al (2010) Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. Mar Drugs 8:2318–2339. doi:10.3390/md8082318

Lewis E, Wallace D (1998) Program Developed for CO_2 System Calculations. ORNL/CDIAC-105. Carbon dioxide Information Analysis Center, Oak Ridge National Laboratory. US Dep. of Energy, Oak Ridge, TN

Mehrbach C, Culberso CH, Hawley JE, Pytkowic RM (1973) Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18:897–907

Melzner F, Gutowska MA, Langenbuch M et al (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6:2313–2331. doi:10.5194/bg-6-2313-2009

Melzner F, Stange P, Trübenbach K et al (2011) Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. PLoS One 6:e24223. doi:10.1371/journal.pone.0024223.t003

Melzner F, Thomsen J, Koeve W et al (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar Biol 160:1875–1888. doi:10.1007/s00227-012-1954-1

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293:109–118. doi:10.3354/meps293109

Mulvey M, Feng SY (1981) Hemolymph constituents of normal and *Proctoeces maculatus* infected *Mytilus edulis*. Comp Biochem Physiol A 70A:119–125. doi:10.1016/0300-9629(81)90407-2

Navarro JM, Torres R, Acuña K et al (2013) Impact of medium-term exposure to elevated pCO_2 levels on the physiological energetics of the mussel *Mytilus chilensis*. Chemosphere 90:1242–1248. doi:10.1016/j.chemosphere.2012.09.063

Orr JC, Fabry VJ, Aumont O et al (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681–686. doi:10.1038/nature04095

Palmer AR (1992) Calcification in marine molluscs: how costly is it? Proc Natl Acad Sci USA 89:1379–1382. doi:10.1073/pnas.89.4.1379

Pan TCF, Applebaum SL, Manahan DT (2015) Experimental ocean acidification alters the allocation of metabolic energy. Proc Natl Acad Sci USA 112:4696–4701. doi:10.1073/pnas.1416967112

Pansch C, Schaub I, Havenhand J, Wahl M (2014) Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. Glob Change Biol 20:765–777. doi:10.1111/gcb.12478

Paparo A (1972) Innervation of the lateral cilia in the mussel *Mytilus Edulis* L. Biol Bull 143:592–604

Parker LM, Ross PM, O'Connor WA (2010) Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. Mar Biol 158:689–697. doi:10.1007/s00227-010-1592-4

Parker LM, Ross PM, O'Connor WA et al (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18:82–92. doi:10.1111/j.1365-2486.2011.02520.x

Parker LM, O'Connor WA, Raftos DA et al (2015) Persistence of positive carryover effects in the oyster, *Saccostrea glomerata*, following transgenerational exposure to ocean acidification. PLoS One 10:e0132276. doi:10.1371/journal.pone.0132276.t001

Pespeni MH, Sanford E, Gaylord B et al (2013) Evolutionary change during experimental ocean acidification. Proc Natl Acad Sci USA 110:6937–6942. doi:10.1073/pnas.1220673110

Pistevos JCA, Calosi P, Widdicombe S, Bishop JDD (2011) Will variation among genetic individuals influence species responses to global climate change? Oikos 120:675–689. doi:10.1111/j.1600-0706.2010.19470.x

Pörtner H (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373:203–217. doi:10.3354/meps07768

Pörtner HO, Langenbuch M, Reipschläger A (2004) Biological impact of elevated ocean CO_2 Concentrations: lessons from animal physiology and earth history. J Oceanogr 60:705–718. doi:10.1007/s10872-004-5763-0

Ramajo L, Pérez-Léon E, Hendriks IE et al (2016) Food supply confers calcifiers resistance to ocean acidification. Sci Rep 6:19374. doi:10.1038/srep19374

Riisgård HU, Larsen PS (2007) Viscosity of seawater controls beat frequency of water-pumping cilia and filtration rate of mussels *Mytilus edulis*. Mar Ecol Prog Ser 343:141–150. doi:10.3354/meps06930

Riisgård HU, Randløv A (1981) Energy budget, growth and filtration rates in *Mytilus edulis* at different algal concentrations. Mar Biol 61:227–234. doi:10.1007/BF00386664

Rodriguez JL, Sedano FJ, García-Martín LO et al (1990) Energy metabolism of newly settled *Ostrea edulis* spat during metamorphosis. Mar Biol 106:109–111. doi:10.1007/BF02114680

Ross PM, Parker L, Byrne M (2016) Transgenerational responses of molluscs and echinoderms to changing ocean conditions. ICES J Mar Sci fsv254. doi: 10.1093/icesjms/fsv254

Saderne V, Fietzek P, Herman PMJ (2013) Extreme variations of pCO_2 and pH in a macrophyte meadow of the Baltic Sea in summer: evidence of the effect of photosynthesis and local upwelling. PLoS One 8:e62689. doi:10.1371/journal.pone.0062689

Sánchez-Lazo C, Martínez-Pita I (2012) Biochemical and energy dynamics during larval development of the mussel *Mytilus galloprovincialis* (Lamarck, 1819). Aquaculture 358–359:71–78. doi:10.1016/j.aquaculture.2012.06.021

Sokolova IM, Frederich M, Bagwe R et al (2012) Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 79:1–15. doi:10.1016/j.marenvres.2012.04.003

Strahl J, Dringen R, Schmidt MM et al (2011) Metabolic and physiological responses in tissues of the long-lived bivalve *Arctica islandica* to oxygen deficiency. Comp Biochem Physiol A 158:513–519. doi:10.1016/j.cbpa.2010.12.015

Strobel A, Bennecke S, Leo E et al (2012) Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front Zool 9:1. doi:10.1186/1742-9994-9-28

Stumpp M, Wren J, Melzner F et al (2011) CO_2 induced seawater acidification impacts sea urchin larval development I: elevated metabolic rates decrease scope for growth and induce developmental delay. Comp Biochem Physiol A 160:331–340. doi:10.1016/j.cbpa.2011.06.022

Stumpp M, Trübenbach K, Brennecke D et al (2012) Resource allocation and extracellular acid–base status in the sea urchin *Strongylocentrotus droebachiensis* in response to CO_2 induced seawater acidification. Aquat Toxicol 110–111:194–207. doi:10.1016/j.aquatox.2011.12.020

Stumpp M, Hu M, Casties I et al (2013) Digestion in sea urchin larvae impaired under ocean acidification. Nat Clim Change 3:1044–1049. doi:10.1038/nclimate2028

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157:2667–2676. doi:10.1007/s00227-010-1527-0

Thomsen J, Gutowska MA, Saphörster J et al (2010) Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7:3879–3891. doi:10.5194/bg-7-3879-2010

Thomsen J, Casties I, Pansch C et al (2013) Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. Glob Change Biol 19:1017–1027. doi:10.1111/gcb.12109

Thomsen J, Haynert K, Wegner KM, Melzner F (2015) Impact of seawater carbonate chemistry on the calcification of marine bivalves. Biogeosciences 12:4209–4220. doi:10.5194/bg-12-4209-2015

Thor P, Dupont S (2015) Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. Glob Change Biol 21:2261–2271. doi:10.1111/gcb.12815

Towle EK, Enochs IC, Langdon C (2015) Threatened caribbean coral is able to mitigate the adverse effects of ocean acidification on calcification by increasing feeding rate. PLoS One 10:e0123394. doi:10.1371/journal.pone.0123394

Valladares F, Matesanz S, Guilhaumon F et al (2014) The effects of phenotypic plasticity and local adaptation on forecasts of species range shifts under climate change. Ecol Lett 17:1351–1364. doi:10.1111/ele.12348

Vargas CA, de al Hoz M, Aguilera V et al (2013) CO_2-driven ocean acidification reduces larval feeding efficiency and changes food selectivity in the mollusk *Concholepas concholepas*. J Plankton Res 35:1059–1068. doi:10.1093/plankt/fbt045

Vargas CA, Aguilera VM, Martín VS et al (2015) CO_2-driven ocean acidification disrupts the filter feeding behavior in chilean gastropod and bivalve species from different geographic localities. Estuaries Coasts 38:1163–1177. doi:10.1007/s12237-014-9873-7

Waldbusser GG, Brunner EL, Haley BA et al (2013) A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. Geophys Res Lett 40:2171–2176. doi:10.1002/grl.50449

Waldbusser GG, Hales B, Langdon CJ et al (2015) Saturation-state sensitivity of marine bivalve larvae to ocean acidification. Nat Clim Change 5:273–280. doi:10.1038/nclimate2479

Wittmann AC, Pörtner HO (2013) Sensitivities of extant animal taxa to ocean acidification. Nat Clim Change 3:995–1001. doi:10.1038/nclimate1982

Xu X, Yang F, Zhao L, Yan X (2016) Seawater acidification affects the physiological energetics and spawning capacity of the Manila clam *Ruditapes philippinarum* during gonadal maturation. Comp Biochem Physiol A 196:20–29. doi:10.1016/j.cbpa.2016.02.014

Zhang H, Shin PKS, Cheung SG (2015) Physiological responses and scope for growth upon medium-term exposure to the combined effects of ocean acidification and temperature in a subtidal scavenger *Nassarius conoidalis*. Mar Environ Res 106:51–60. doi:10.1016/j.marenvres.2015.03.001

Zittier Z, Bock C, Pörtner HO (2012) Impact of ocean acidification on the thermal tolerance and acid–base regulation capacity of *Mytilus edulis* from the White Sea, The Ocean in a High-CO_2 World—Third Symposium, Monterey, California, 24 September 2012—27 September 2012, hdl:10013/epic.40120

Zittier Z, Bock C, Lannig G, Pörtner HO (2015) Impact of ocean acidification on thermal tolerance and acid–base regulation of *Mytilus edulis* (L.) from the North Sea. J Exp Mar Bio Ecol 473:16–25. doi:10.1016/j.jembe.2015.08.001

Publication II

CO_2-dependent adjustments of metabolic pathways differ within a population of blue mussels (*Mytilus edulis*)

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CO_2-dependent adjustments of metabolic pathways differ within a population of blue mussels (*Mytilus edulis*)

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Abstract

Exploring intra-population variation in physiological responses to ocean acidification is crucial to assess species' adaptation potential. A previous study on *Mytilus edulis* from CO_2 enriched Kiel Fjord revealed family-specific survival of F1 larval at the highest experimental seawater PCO_2, which translated into differing physiological responses between adult mussels of 'tolerant' and 'sensitive' families after raising the F1 larvae for over 1 year under the same PCO_2 level (700 (control), 1120, 2400 μatm). Here, we explore the underlying biochemical mechanisms by measuring capacities of key metabolic enzymes in gill and outer mantle tissues originating from adult mussels of the same experiment. We show that, under control conditions, tolerant families had a 30% higher gill aerobic capacity (citrate synthase (CS), cytochrome-C-oxidase (COX)) than sensitive families but similar metabolic rates (reported previously). This indicates a higher gill mitochondrial scope in tolerant mussels, supporting slightly elevated tissue metabolic costs under elevated PCO_2. By contrast, elevated PCO_2 did not depress gill aerobic enzyme capacities in sensitive families but increased capacities for lipid (3-hydroxyacyl-CoA dehydrogenase) and carbohydrate metabolism (pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK)). At the highest PCO_2, tolerant families had decreased mantle COX/CS ratio and gill PK/PEPCK. This indicates constraints of aerobic metabolism, potentially explaining the decreased gill metabolic scope and filtration rates reported in our previous study. In conclusion, present study suggests that a high gill mitochondrial scope may promote CO_2 resilience in mussels and highlights the need to study tissue specific biochemical mechanisms underlying intra-population variations of physiological traits under environmental change.

Introduction

Ever-increasing human activity is leading to rapid global environmental changes that are largely unprecedented in evolutionary history (Pörtner et al. 2014). As a consequence, the ability to understand and predict the effects of global change on the biosphere in order to estimate its resilience is of central importance.

The distribution patterns of species are shaped by organisms' physiological limits under the prevailing abiotic environmental conditions and biotic interactions (Pörtner and Farrell 2008; Somero 2012). If environmental changes exceed those limits, species have to either shift their distribution range (if possible) or they have to undergo physiological acclimatisation and/or genetic adaptation in order to ultimately avoid extinction (Sunday et al. 2014; Calosi et al. 2016). Predicting the potential for physiological acclimatisation and/or genetic adaptation of populations to a given environmental driver requires an understanding of affected biochemical mechanisms that set physiological limits as well as the capacity and rate at which they are able to adjust to new environmental conditions.

In the marine realm, ocean acidification (OA) is one of the main threats associated with global change, alongside warming and pollution (Doney et al. 2009; Pörtner et al. 2014). The world's oceans are a major sink for anthropogenic CO_2 emissions (Sabine et al. 2004). This oceanic CO_2 uptake causes a decrease in seawater pH and changes its carbonate chemistry (Caldeira and Wickett 2003), which has particularly negative effects on marine ectothermic species with large calcified body structures such as corals, echinoderms and bivalves (Kroeker et al. 2013; Wittmann and Pörtner 2013).

The constantly growing body of literature dealing with the effects of OA on marine calcifying invertebrates shows that projected levels of the associated physiochemical changes have the potential to affect a range of fundamental physiological processes (Parker et al. 2013; Gazeau et al. 2013). Besides direct negative effects on the shell and skeleton precipitation (e.g. Orr et al. 2005) and increased energetic costs for shell conservation (e.g. Melzner et al. 2011), ocean acidification has the potential to impact vital processes such as immune (e.g. Brothers et al. 2016; Leite Figueiredo et al. 2016) or antioxidant defence (e.g. Tomanek 2012) and can also alter feeding and metabolic rates (e.g. Thomsen and Melzner 2010; Dorey et al. 2013). Alterations in basic energetic costs of maintenance and homeostatic processes such as ion- and acid-base regulation (e.g. Stumpp et al. 2012a), may divert energy away from fitness-related traits such as growth and reproduction (Stumpp et al. 2012b; e.g. Pan et al. 2015), which may translate into population- and ultimately ecosystem-level consequences (Pörtner

2008). The ability to balance the allocation of available energy (ATP) between physiological maintenance and fitness-related traits is crucial for an organism to thrive under changing environmental conditions (Applebaum et al. 2014) and may include adjustments of ATP-producing pathways as well as ATP-consuming mechanisms (Lannig et al. 2010; Sokolova et al. 2012). Understanding impacts of ocean acidification on an organisms' energy metabolism may provide access to a more mechanism based understanding of vulnerability and potential for physiological adaptation among species (Sokolova et al. 2012; Sokolova 2013).

Although most studies on marine calcifiers show overall negative effects of ocean acidification, there is a growing body of literature indicating that CO_2 sensitivity can differ at an inter-specific (Ries et al. 2009; e.g. Griffith and Gobler 2017), but also intra-specific level (Wood et al. 2016; Calosi et al. 2017; Thomsen et al. 2017), even including intra-population levels (Pansch et al. 2014; Stapp et al. 2017). Spatial and temporal variability in carbonate chemistry as well as other abiotic factors seem to be a major environmental force in shaping the variation in OA sensitivity within and among species and natural populations (Kelly et al. 2013; Kroeker et al. 2016; Calosi et al. 2017). In general, heterogeneous environments are believed to increase genetic polymorphism within natural populations (Hedrick 2006). Due to the rapid rate of on-going and projected CO_2-induced changes to seawater carbonate chemistry, acclimatisation or genetic adaptation of relatively long-lived species is likely to depend strongly on the standing genetic variation within the existing gene pool (Pespeni et al. 2013). Investigating existing variation in OA responses within and between species and populations may allow elucidation of physiological mechanisms that underlie their vulnerability or resilience while also assessing their ability to respond to ocean acidification via natural selection.

The Kiel Fjord in the western Baltic Sea is a naturally CO_2-enriched habitat characterised by strong seasonal and daily seawater PCO_2 fluctuations with peak values exceeding 2300 μatm and an annual average seawater PCO_2 of around 700 μatm (Thomsen et al. 2010; Saderne et al. 2013; Melzner et al. 2013). The fluctuation of the carbonate chemistry is caused by patchy upwelling events which occur mainly in summer and autumn and coincide with the reproductive time of the local blue mussel (*Mytilus edulis*) population (Thomsen et al. 2010). In order to assess whether this translates into a high variability of OA sensitivity within this population, we recently investigated the OA response of different families during a long-term multi-generational CO_2 acclimation experiment (Thomsen et al. 2017; Stapp et al. 2017). CO_2 sensitivity of F1-larvae varied substantially between family lines. While larvae of some families showed successful recruitment under all three applied experimental PCO_2 levels (700, 1120, 2400 μatm), larvae of the majority of family lines (11 out of 16) did not survive at the

highest PCO_2 (2400 µatm) and only settled successfully at control and intermediate PCO_2 levels (700 and 1120 µatm) (Thomsen et al. 2017; Stapp et al. 2017). Therefore, in order to simplify the terminology, former families were referred as 'tolerant' and the latter as 'sensitive'. To elucidate the underlying physiology, F1-larvae were raised for one year at different PCO_2 levels followed by measurements of physiological traits at tissue and whole animal levels (Stapp et al. 2017). In sensitive families long-term CO_2 exposure did not affect tissue and whole-animal metabolism, whereas tolerant families had increased whole-animal metabolic rates at intermediate PCO_2, in line with an increased summed tissue respiration of gill and outer mantle tissue. However, tolerant mussels were characterised by lower whole-animal metabolic rates at control PCO_2 than sensitive families, which had variable metabolic rates. Furthermore, tolerant mussels raised under the highest PCO_2 level showed decreased filtration rates that were associated with a reduced factorial metabolic scope of the gills; however, the underlying biochemical mechanisms remained unclear.

Here, using gill and outer mantle tissue samples originating from tolerant and sensitive families of our previous study, we aimed to further characterise the biochemical regulation and metabolic framework/pathways underlying the variation in physiological OA responses. To do so, we investigated capacities of key metabolic enzymes representing major pathways of energy metabolism (cytochrome-c-oxidase (COX), citrate synthase (CS), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), 3-hydroxyacyl-CoA dehydrogenase (HADH), and glutamate dehydrogenase (GDH)). COX and CS were chosen as proxies for mitochondrial functioning and aerobic capacity. PK and PEPCK represent the phosphoenolpyruvate (PEP) branchpoint, which controls the flux of glycolytic substrates for aerobic or anaerobic energy production in *Mytilus* spp. (Kluytmans et al. 1980; Greenway and Storey 1999). HADH plays a central role in fatty acid oxidation (Kluytmans et al. 1985) whereas GDH is involved in amino acid metabolism (Moyes et al. 1985).

Material and Methods

Animals and experimental design

Tissue samples of *M. edulis* originated from a long-term, multigenerational CO_2 acclimation experiment previously described in Stapp et al. (2017) and Thomsen et al. (2017). Briefly, in June 2012 adult *M. edulis* (64.2 ± 5.5 mm) were collected in Kiel Fjord and thermally induced to spawn at control conditions following a one-night recovery in a flow-through seawater setup. Gametes of 8 sires and 8 dams (F_0 generation) were crossbred in a reduced North Carolina I design, resulting in a total of 16 full-sib families within four half-sib groups. Embryos of each family (F_1 generation) were subsequently randomly divided and transferred into experimental units (5000 embryos per unit, three replicates) that contained filtered Kiel Fjord seawater (0.2 µm) at 18°C with a nominal PCO_2 level of either 700 (control), 1120 (intermediate) or 2400 (high) µatm which were selected according to present and predicted PCO_2 levels in Kiel Fjord (Thomsen et al. 2010; Melzner et al. 2013). Starting two days post fertilisation, larvae were fed with fresh *Isochrysis* and *Rhodomonas* suspension and water exchanged weekly. After around three weeks, larvae started to settle. Families with successful larval settlement (defined as >10 settled specimens per replicate) at all three PCO_2 levels were termed 'tolerant' (5 of 16 families), while families whose larvae only settled at the control and intermediate PCO_2 were termed 'sensitive'. At a size of about 1-2 mm, larvae of the three replicates of each family and PCO_2 level were pooled and 20 individuals randomly picked and transferred into a flow-through setup where they were cultured at their respective PCO_2. The flow-through setup consisted of a header tank that was constantly supplied with filtered (5 µm) Kiel Fjord seawater as well as fresh *Rhodomonas* suspension. The header tank itself constantly supplied each experimental aquarium by gravity feed. Experimental aquaria were equipped with a mesh-covered overflow and were individually aerated with pressurized air containing the respective PCO_2. Throughout the experiment, *Rhodomonas* density was monitored in the header tank (mean ± SD; 2778 ± 936 cells ml^{-1}) and in experimental aquaria (mean ± SD; 1030 ± 417 cells ml^{-1}) in order to assure adequate feeding of mussels. During the larval phase pH_{NBS}, temperature and salinity were measured daily in one third of the experimental units and twice a week in the flow-through setup. Dissolved inorganic carbon (DIC) was measured weekly throughout the whole experiment. For a more detailed description of experimental procedures as well as measurements, calculations and respective seawater conditions see Stapp et al. (2017).

Tissue sampling

After 15-months of CO_2 exposure (October 2013), gill and outer mantle tissue (mantle margin with mantle folds) of three individuals per PCO_2 level of the F_1 generation of three tolerant and three sensitive families (n = 9 per family type) were sampled for enzyme assays. Mussels were shucked quickly and gill and outer mantle tissue dissected and immediately snap frozen in liquid nitrogen. Afterwards samples were stored at -80°C until day of experimentation. Mean shell length was 24.6 ± 2.1 mm for tolerant and 25.7 ± 2.4 mm for sensitive families, with no statistical difference between family types (2-way ANOVA, $F = 0.624$, $p = 0.435$) and PCO_2 level (2-way ANOVA, $F = 0.017$, $p = 0.896$; PCO_2 within tolerant families, 1-way ANOVA, $F = 1.245$; $p = 0.306$).

Maximum enzyme activities

Frozen samples of gill and outer mantle tissue were transferred into pre-cooled Precellys tubes (Precellys soft tissue homogenizing kit CK14, 1.4 mm ceramic beads) that contained extraction buffer (20 mM Tris-HCl buffer, pH 7.5 at 4°C, supplemented with 1mM EDTA and 0.1% Triton X-100) at a ratio of 1 ml buffer per 0.1 g tissue. Subsequently, tissues were homogenised at 4°C and 6500 rpm (2 times for 8 sec., 30 sec. pause in between) using a Precellys tissue homogenizer (Precellys24, Bertin Technology, France). Following homogenisation, cell debris was removed from samples by centrifugation (11 min, 1000 g, 4°C) and the supernatant used as crude extract. Maximal enzyme activities of citrate synthase (CS), cytochrome-C-oxidase (COX), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), glutamate dehydrogenase (GDH) and 3-hydroxyacyl-CoA-dehydrogenase (HADH) were determined by means of a microplate reader (PowerWave HT, Biotek, Germany). All assays were performed at the experimental temperature of 18°C (temperature in the flow-through system at the time of tissue sampling). Temperature was controlled by incubating the microplate before and in between measurements on a custom-made, temperature controlled aluminium block set to 18°C (for technical details see Weiss et al. 2012). Per tissue sample, enzyme activities were determined in duplicates for two amounts of crude extract (single and double amount, four measurements in total) in order to check for reproducibility of measured enzyme activities. Enzyme activities were measured in a final volume of 200 µl. All assay components except for the start reagent were mixed in the microplate and a few readings performed in order to assure a stable absorbance signal. Subsequently, adding the respective start reagent started the reaction. Assay components, start substrate and wavelength (λ) varied between enzymes and are outlined in the following:

Citrate synthase (CS) catalyses the first reaction of the tricarboxylic acid (TCA) cycle. CS activity was determined after Sidell et al. (1987) using Tris-HCl buffer (75 mM, pH 8.0 at 18°C) that contained 0.25 mM DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]) and 0.4 mM acetyl-CoA. The reaction was started with 0.5 mM oxaloacetate and CS activity measured as the increase in absorbance at λ = 412 nm.

Cytochrome-C-oxidase (COX) is the last enzyme in the respiratory chain located in the mitochondrial membrane. Its activity was measured after a modified protocol of Moyes et al. (1997) using Tris-HCl buffer (20 mM, pH 7.80 at 18°C) supplemented with 0.5 % Tween 20. Start reagent was 0.05 mM of reduced cytochrome C. COX activity was measured as the decrease in absorbance at λ = 550 nm.

Pyruvate kinase (PK) catalyses the final step of glycolysis. PK was measured after a modified protocol of Driedzic and De Almeida-Val. (1996) using Tris-HCl buffer (80 mM, pH 7.5 at 18°C) that contained 80 mM KCl, 5 mM $MgSO_4$, 5 mM ADP, 0.2 mM NADH and 5 µg/ml lactate dehydrogenase (LDH). The reaction was started with 1 mM of phospoenolpyruvate (PEP) and PK activity was measured as the decrease in absorbance at λ = 340 nm.

Phosphoenolpyruvate carboxykinase (PEPCK) converts PEP to oxaloacetate or vice versa as part of anaerobic or gluconeogenic pathways, respectively. PEPCK activity was determined following a protocol of Lockwood and Somero (2012) based on Harlocker et al. (1991). The assay was performed in Imidazol-HCl buffer (65 mM, pH 6.6 at 18°C) containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM PEP, 1.5 mM IDP, 0.15 mM NADH and 6 U/ml malate dehydrogenase (MDH) that was degassed with N_2 prior to the measurement. The reaction was started with 20 mM $NaHCO_3$ and PEPCK activity measured as the decrease in absorbance at λ = 340 nm.

3-Hydroxyacyl-CoA-Dehydrogenase (HADH) catalyses the third step of ß-oxidation, the oxidation of L-3-hydroxyacyl CoA into 3-ketoacyl-CoA. The assay was performed after a modified protocol of McClelland et al. (2005) using imidazole buffer (50 mM, pH 7.5 at 18°C) supplemented with 0.15 mM NADH and 2.5 µg/ml Antimycin A. Start reagent was 0.1 mM of aceto-acetyl-CoA and HADH activity was measured as the decrease in absorbance at λ = 340 nm.

Glutamate dehydrogenase (GDH) converts glutamate to α-ketoglutarate, and vice versa. GDH activity was determined following a modified protocol of Sanchez-Muros et al. (1998). Phosphate buffer (40 mM, pH 7.4 at 18°C) was supplemented with 100 mM ammonium

acetate, 1 mM ADP and 0.2 mM NADH and the reaction initiated by adding 5 mM of α-ketoglutarate. GDH activity was then measured as the decrease in absorbance at λ = 340 nm.

In order to standardise data analysis, the maximum slope of change in absorbance for each enzyme measurement was detected using a Python script (Python Software Foundation, https://www.python.org). Maximum enzyme activities were then referred to g tissue fresh weight (U/g FW) as well as per mg tissue protein (U/mg protein) using the respective extinction coefficient (NADH ε (340 nm) = 6.31 mM^{-1} cm^{-1}; thionitrobenzoic acid ε (412 nm) = 13.6 mM^{-1} cm^{-1}; ferricytochrome c ε (550 nm) = 19.1 mM^{-1} cm^{-1}). Protein content of the tissue crude extracts (mg per g FW) was determined according to Bradford (1976), using lyophilized bovine serum albumin as protein standard. All samples were measured randomised with respect to family type and PCO_2 level.

Statistics

Data were analysed using the R software version 3.2.3 (R Development Core Team 2015). All data were checked for normality of residuals (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Subsequently, a full two-way ANOVA was performed for data collected from individuals at the control and intermediate CO_2 level to test for the effects of 'family type' (sensitive or tolerant) and 'seawater PCO_2' on shell length, maximum enzyme activities, enzyme activity ratios as well as total protein content in gill and outer mantle. Additionally, a one-way ANOVA was performed to analyse the effects of PCO_2 level for the same parameters within the tolerant families. If significant family or CO_2 effects were detected by ANOVA, a TukeyHSD post hoc test was performed for pairwise comparisons of treatments. $p < 0.05$ was accepted to indicate significant differences. All data are presented as mean ± SEM.

Results

Tissue protein content

Total protein content of gill and mantle tissue differed significantly between tolerant and sensitive families (2-way ANOVA: gill, $F_{1,32} = 11.980$, $p = 0.002$; mantle, $F_{1,32} = 8.827$, $p = 0.006$) (Fig. 1). In both tissues, protein content at control seawater PCO_2 was around 40% higher in tolerant than in sensitive families (Tukey's HSD: gill, $p = 0.019$; mantle, $p = 0.011$). In addition, seawater PCO_2 affected the protein content of mantle tissue from tolerant families (1-way ANOVA: $F_{1,24} = 8.29$, $p = 0.002$) reflected in a significant increase from around 164 mg/g FW at the control and intermediate PCO_2 to 203 mg/g FW at the highest seawater PCO_2 (Tukey's HSD: $p < 0.05$).

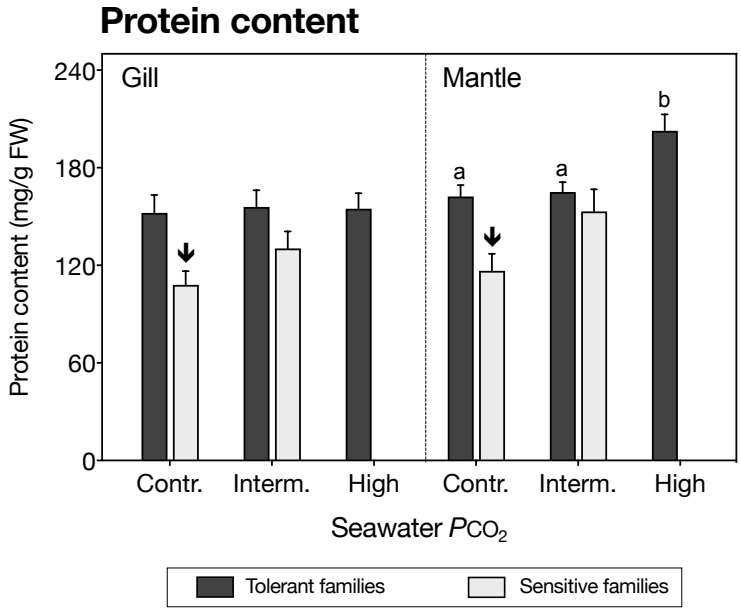

Fig. 1 Total protein content in gill (left panel) and mantle (right panel) tissue of tolerant and sensitive families of *Mytilus edulis* raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Different lower case letters indicate significant difference in protein content between PCO_2 levels within tolerant families detected by TukeyHSD post hoc analysis ($p < 0.05$); arrows indicate significant difference ($p < 0.05$) in protein content between tolerant and sensitive families at a given PCO_2 (TukeyHSD post hoc; $p < 0.05$). Values are given as mean ± SE, $n = 9$.

Maximal enzyme activities

Maximal enzyme activities related to tissue fresh weight (FW) are depicted in Fig. 2-5 whereas enzyme activities referred to total tissue protein content (PC) are summarised in Table 1. Furthermore, we calculated the ratio of activities of several enzymes in order to detect poten-

tial shifts in metabolic fuel usage and pathways. For the sake of clarity and for the ease of the reader, a simplified overview of FW-related enzyme activities is provided in Table 2.

Table 1 Maximal enzyme activities per mg tissue protein in gill and mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Lower case letters indicate significant differences ($p < 0.05$) in maximal enzyme activities between tolerant and sensitive families at the control and intermediate seawater PCO_2; upper case letters indicate significant PCO_2 effects on maximal enzyme activities within tolerant families. Values are given as mean ± SE (n =7-9 for citrate synthase (CS), cytochrome-C-oxidase (COX), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), glutamate dehydrogenase (GDH) and 3-hydroxyacyl-CoA-dehydrogenase (HADH).

Tissue	Enzyme	Control PCO_2		Intermediate PCO_2		High PCO_2
		Tolerant	Sensitive	Tolerant	Sensitive	Tolerant
Gill	CS	0.017 ± 0.002^{aA}	0.016 ± 0.003^{ab}	0.014 ± 0.004^{abAB}	0.012 ± 0.003^{b}	0.012 ± 0.003^{B}
	COX	0.021 ± 0.012^{A}	0.022 ± 0.018	0.013 ± 0.004^{AB}	0.019 ± 0.008	0.012 ± 0.004^{B}
	GDH	0.002 ± 0.001^{a}	0.002 ± 0.000^{ab}	0.001 ± 0.0003^{ab}	0.001 ± 0.0002^{b}	0.001 ± 0.0001
	HADH	0.005 ± 0.001	0.004 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.004 ± 0.002
	PK	0.009 ± 0.004	0.007 ± 0.002	0.009 ± 0.002	0.010 ± 0.003	0.006 ± 0.005
	PEPCK	0.003 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
Mantle	CS	0.009 ± 0.002	0.010 ± 0.003	0.009 ± 0.003	0.009 ± 0.003	0.010 ± 0.004
	COX	0.019 ± 0.006^{A}	0.021 ± 0.009	0.022 ± 0.006^{A}	0.020 ± 0.006	0.012 ± 0.003^{B}
	GDH	0.001 ± 0.0003	0.002 ± 0.001	0.001 ± 0.0003	0.001 ± 0.0003	0.001 ± 0.0003
	HADH	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.002 ± 0.0004
	PK	0.011 ± 0.003	0.014 ± 0.003	0.015 ± 0.001	0.012 ± 0.003	0.012 ± 0.002
	PEPCK	0.003 ± 0.001	0.004 ± 0.001	0.003 ± 0.002	0.004 ± 0.001	0.003 ± 0.001

Aerobic capacity (COX and CS)

In gills, the effect of PCO_2 on FW-related COX and CS activities was family-dependent as shown by a significant interaction between both factors (2-way ANOVA: COX, $F_{1,28} = 6.816$, $p = 0.014$; CS, $F_{1,32} = 9.574$, $p = 0.004$) (Fig. 2). Whereas FW-related COX and CS activities remained at control levels in gills of sensitive families at the intermediate PCO_2 level (Fig. 2 a), FW-related gill COX and CS activity in tolerant families were significantly depressed by 39% and 24% at the intermediate and by 45% and 29% at high PCO_2 level, respectively (1-way ANOVA: COX, $F_{1,21} = 6.225$, $p = 0.008$; CS, $F_{1,24} = 5.871$, $p = 0.008$; Tukey's HSD: $p < 0.05$) (Fig. 2a). Furthermore, FW-related gill CS capacities differed significantly between

family types (2-way ANOVA: $F_{1,32} = 21.612$, $p < 0.001$). At control PCO_2, CS capacity of tolerant families was around 65% higher than for sensitive families (Tukey's HSD: $p < 0.0001$), which exhibited CS capacities similar to those of tolerant families at elevated PCO_2 (Fig. 2a). Due to the similar responses of gill COX and CS activities, the COX/CS ratio was constant in both family types across the different CO_2 treatments (Fig. 2a).

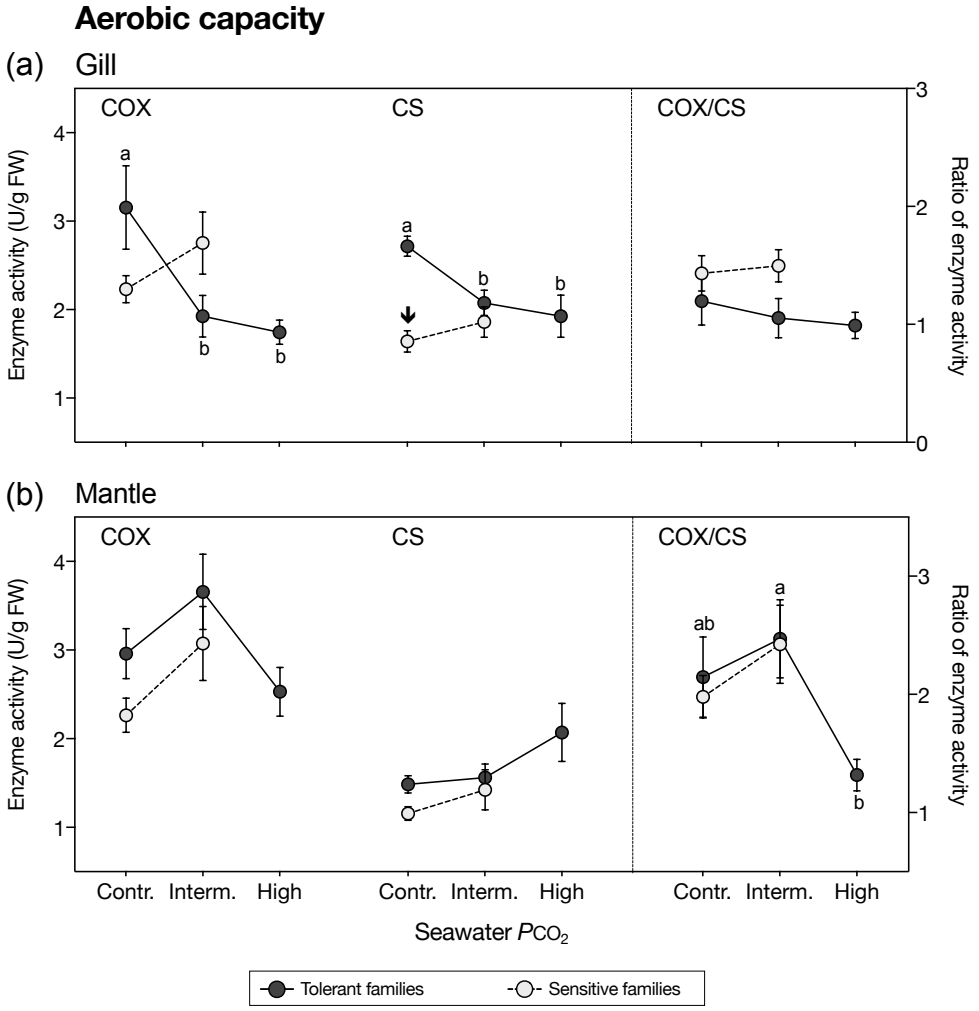

Fig. 2 Maximal enzyme activities per gram fresh weight (FW) of cytochrome-C-oxidase (COX, left panel), citrate synthase (CS, middle) as well as the ratio of COX to CS (right panel) in (a) gill and (b) mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Different lower case letters indicate significant difference in maximal enzyme activities and enzyme ratios between PCO_2 levels within tolerant families detected by TukeyHSD post hoc analysis ($p < 0.05$); arrows indicate significant difference ($p < 0.05$) in maximal enzyme activities between tolerant and sensitive families at a given PCO_2 (TukeyHSD post hoc; $p < 0.05$). Values are given as mean ± SE, n = 7-9.

When related to protein content (PC), gill COX activity did not differ between family types but was significantly affected by 'seawater PCO_2' in tolerant families (1-way ANOVA:

$F_{1,21} = 3.517$, $p = 0.048$), which was visible as a lower COX capacity at the high compared to the control seawater PCO_2 level (Tukey's HSD: $p = 0.05$) (Table 1). PC-related CS activity was affected by 'seawater PCO_2' in gills of both family types (2-way ANOVA: $F_{1,32} = 7.318$, $p = 0.011$; 1-way ANOVA: $F_{1,24} = 4.504$, $p = 0.022$) (Table 1). In tolerant families, PC-related CS capacity in gills decreased progressively with rising seawater PCO_2 and was significantly lower at the high compared to the control PCO_2 level (Tukey's HSD: $p = 0.017$) (Table 1). Similar, PC-related CS capacity was depressed by 21% in gills of sensitive mussels at the intermediate compared to control PCO_2.

In mantle, there was no effect on FW- as well as PC-related CS capacities (Fig. 2b, Table 1) but an overall significant effect of 'seawater PCO_2' on FW-related COX activity in both family types (2-way ANOVA: $F_{1,32} = 4.835$, $p = 0.035$) (Fig. 2b). Acclimation to the intermediate PCO_2 level resulted in a 24% increase of mantle COX-activity in tolerant and a 35% increase in sensitive families (Fig. 2b). At the highest PCO_2, FW-related COX activity returned to control levels in tolerant mussels, which resulted in a COX/CS ratio that was significantly lower compared to the intermediate seawater PCO_2 (1-way ANOVA: 'seawater PCO_2'-effect, $F_{1,24} = 4.381$, $p = 0.024$; Tukey's HSD: $p = 0.022$) (Fig. 2b). PC-related mantle COX activities were similar between families and between the control and intermediate PCO_2, but significantly depressed in tolerant families acclimated to the high seawater PCO_2 (1-way ANOVA: 'seawater PCO_2'-effect, $F_{1,24} = 7.8$, $p = 0.002$; Tukey's HSD: $p < 0.05$).

Carbohydrate metabolism (PK and PEPCK)

PK activities related to FW were affected in a tissue- and family-specific manner (Fig. 3). In gills, the effect of the seawater PCO_2 level was family dependent as shown by a significant interaction between both factors (2-way ANOVA: $F_{1,28} = 5.283$, $p = 0.029$) (Fig. 3a). Whereas FW-related PK activity remained constant in gills of tolerant families among the different PCO_2 treatments, sensitive families possessed significantly increased PK capacity at the intermediate PCO_2 level (Tukey's HSD: $p = 0.042$) (Fig. 3a).

In mantle, PK capacity (per FW) was affected by both, 'family type' (2-way ANOVA: $F_{1,29} = 9.530$, $p = 0.004$) and 'seawater PCO_2' (2-way ANOVA: $F_{1,29} = 7.041$, $p = 0.013$) (Fig. 3b). Mussels of both families showed similar FW-related PK capacities at the control PCO_2 and, while PK activity under intermediate elevated seawater PCO_2 remained at this level in sensitive families, it increased significantly in tolerant families (Tukey's HSD: $p < 0.05$).

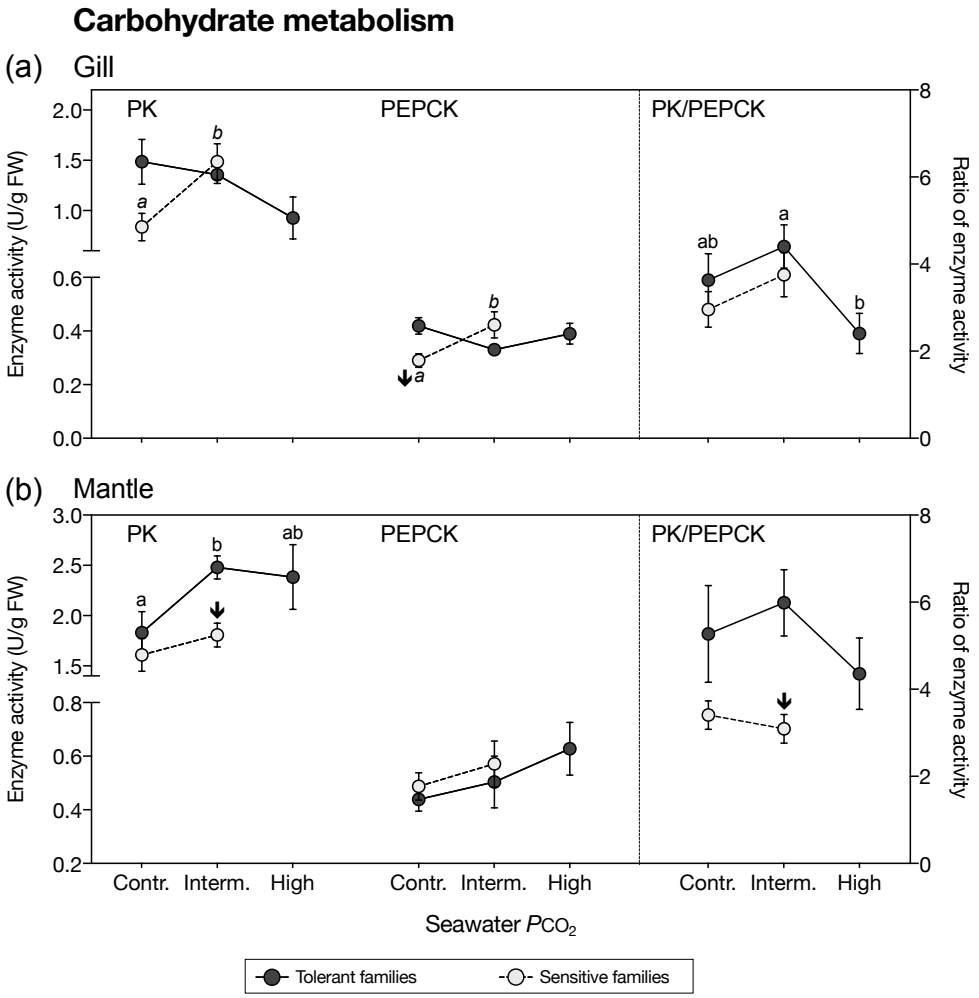

Fig. 3 Maximal enzyme activities per gram fresh weight (FW) of pyruvate kinase (PK, left panel), phosphoenolpyruvate carboxykinase (PEPCK, middle) and the ratio of PK to CS (right panel) in gill and mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Different lower case letters indicate significant difference in maximal enzyme activities and enzyme ratios between PCO_2 levels within tolerant (non-italic) and sensitive (*italic*) families detected by TukeyHSD post hoc analysis ($p < 0.05$); arrows indicate significant difference ($p < 0.05$) in maximal enzyme activities between tolerant and sensitive families at a given PCO_2 (TukeyHSD post hoc; $p < 0.05$) Values are given as mean ± SE, n = 7-9.

When related to PC, there was no effect on gill PK capacity, but a significant interaction between 'family type' and 'seawater PCO_2' in mantle tissue (2-way ANOVA: $F_{1,29} = 7.565$, $p = 0.010$) (Table 1). While PC-related mantle PK activity tended to decrease under intermediate PCO_2 in sensitive families, it increased in tolerant families but the change was marginally non-significant (Tukey's HSD: tolerant control vs. tolerant intermediate seawater PCO_2, $p = 0.058$).

While gill PEPCK activity remained unaffected when referred to PC, FW-related PEPCK changed in response to PCO_2 dependent on the family type (2-way ANOVA: 'seawater PCO_2' × 'family type', $F_{1,32} = 11.349$, $p = 0.002$) (Fig. 3a). In tolerant families, gill PEPCK capacity remained constant across all seawater PCO_2 level. At the control PCO_2, sensitive families showed significantly lower FW-related PEPCK activity in comparison to tolerant families (Tukey's HSD: $p = 0.043$). When exposed to elevated seawater PCO_2, PEPCK capacity rose significantly in gills of sensitive families (Tukey's HSD: $p = 0.034$). PEPCK capacity related to FW as well as protein did neither differ between families nor between PCO_2 levels in mantle tissue (Fig. 3b).

In gills, there was no family specific difference in the PK/PEPCK ratio but a significant 'PCO_2' effect within tolerant families (1-way ANOVA: $F_{1,21} = 3.740$, $p = 0.041$) (Fig. 3a). Compared to control PCO_2, the PK/PEPCK ratio increased slightly at the intermediate and dropped at the highest PCO_2 resulting in a significant difference of means between the elevated PCO_2 treatments (Tukey's HSD: $p = 0.036$). In mantle, PK/PEPCK ratios were not affected by PCO_2 but varied significantly between families (2-way ANOVA: $F_{1,29} = 11.838$, $p = 0.002$) (Fig. 3b). In tolerant families, mantle PK/PEPCK ratios were overall higher than in sensitive families leading to statistically significant difference between families at the intermediate PCO_2 (Tukey's HSD: $p = 0.039$).

Lipid metabolism (HADH)

PCO_2 effects on HADH were tissue specific (Fig. 4, Table 1). In mantle, FW- and PC-related HADH activities as well as the HADH/CS ratio were similar among family types and seawater PCO_2 level (Fig. 4b; Table1). In contrast, while gill HADH activities remained unaffected when expressed per PC, there was a significant effect of 'family type' (2-way ANOVA: $F_{1,32} = 9.184$, $p = 0.005$) as well as a significant interaction between 'seawater PCO_2' and 'family type' (2-way ANOVA: $F_{1,32} = 8.574$, $p = 0.006$) on FW-related capacities (Fig. 4a, Table 1). At the control PCO_2, tolerant families had significantly higher gill HADH capacity than sensitive families (Tukey's HSD: $p = 0.001$). At the intermediate seawater PCO_2, gill HADH activity in tolerant families remained at control level whereas it significantly increased in sensitive mussels by around 67% (Tukey's HSD: $p = 0.01$) leading to activity levels similar to those found in tolerant mussels. As FW-related gill CS capacity remained constant in sensitive families irrespective of PCO_2, ANOVA detected a significant effect of 'seawater PCO_2' on the HADH/CS ratio which was higher in gills of sensitive families at the intermediate compared to control PCO_2 level (2-way ANOVA: $F_{1,32} = 8.023$, $p = 0.008$; Tukey's HSD:

p = 0.036) (Fig. 4a). Furthermore, while gill HADH capacities in tolerant families remained unaffected at the intermediate seawater PCO_2, exposure to the highest PCO_2 significantly depressed gill HADH activity by around 33 % (1-way ANOVA: $F_{1,324}$ = 3.722, p = 0.039; Tukey's HSD: p = 0.037).

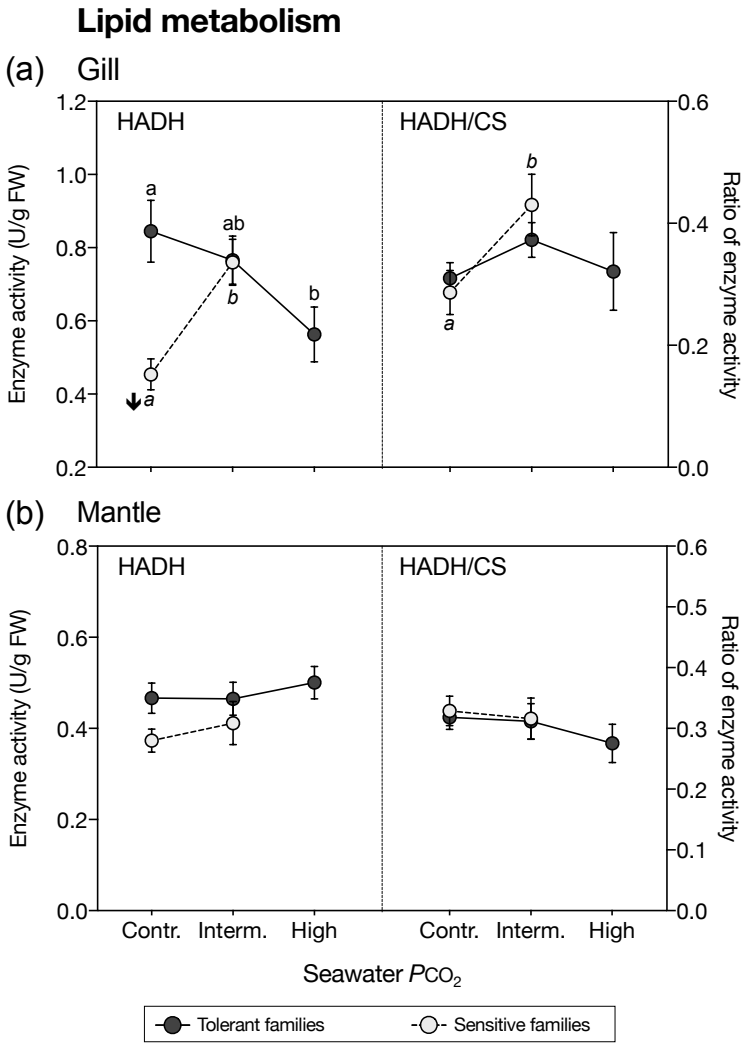

Fig. 4 Maximal enzyme activities per gram fresh weight (FW) of hydroxyacyl-CoA-dehydrogenase (HADH, left panel) and the ratio of HADH to CS (right panel) in gill and mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Different lower case letters indicate significant difference in maximal enzyme activities and enzyme ratios between PCO_2 levels within tolerant (non-italic) and sensitive (*italic*) families detected by TukeyHSD post hoc analysis ($p < 0.05$); arrows indicate significant difference ($p < 0.05$) in maximal enzyme activities between tolerant and sensitive families at a given PCO_2 (TukeyHSD post hoc; $p < 0.05$). Values are given as mean ± SE, n = 9

Amino acid metabolism (GDH)

GDH showed a similar level of activity in both tissues (Fig. 5; Table 1). When related to FW, overall GDH activities in gill and mantle tissue were affected by 'family type' but not by 'seawater PCO_2' (2-way ANOVA: gill, $F_{1,31} = 11.299$, $p = 0.002$; mantle, $F_{1,32} = 7.068$, $p = 0.012$). In gill tissue, the highest FW-related GDH activity was detected in tolerant mussels from the control treatment resulting in significantly higher activity of around 40% when compared to sensitive families at the control and intermediate PCO_2 treatment (Tukey's HSD: $p = 0.011$ and 0.007) (Fig. 5a), respectively. Similarly, at the control PCO_2, FW-related mantle GDH activity of tolerant families was 32% higher than in sensitive mussels. However, this difference was only marginally significant (Tukey's HSD: $p = 0.052$) (Fig. 5b).

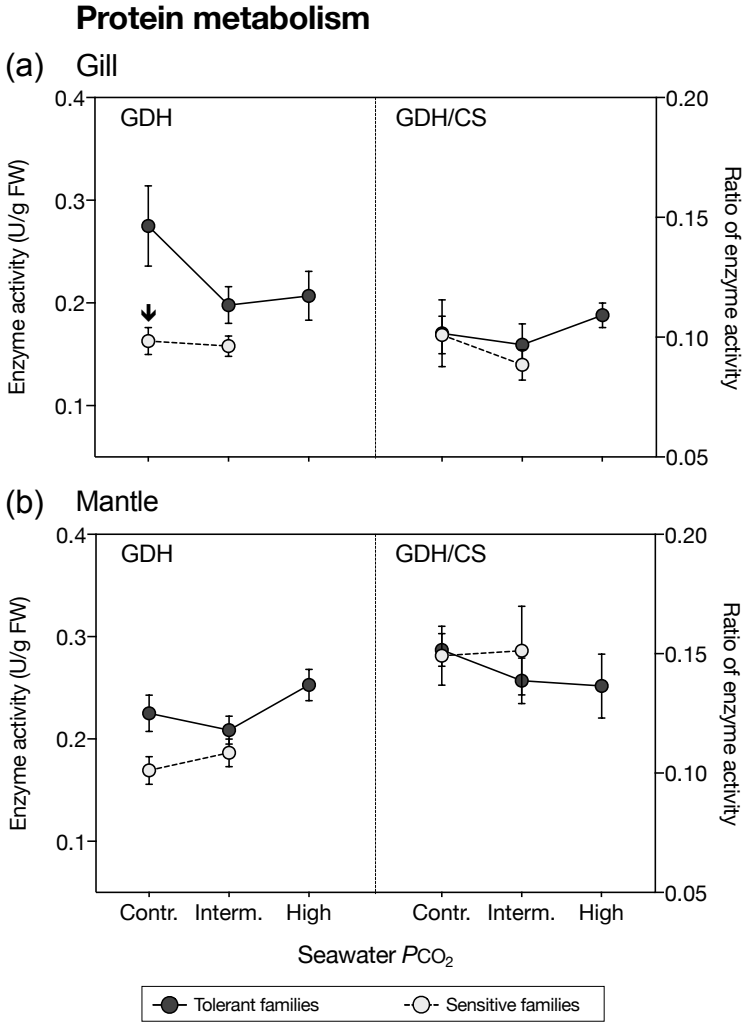

Fig. 5 Maximal enzyme activities per gram fresh weight (FW) of glutamate dehydrogenase (GDH, left panel) and the ratio of GDH to CS (right panel) in gill and mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Arrows indicate significant difference in maximal enzyme activities between tolerant and sensitive families at a given PCO_2 (TukeyHSD post hoc; $p < 0.05$). Values are given as mean ± SE, $n = 9$

When the activities of gill and mantle GDH were expressed relative to PC, family specific differences were no longer visible. However, while mantle PC-related GDH activities were similar for family types and PCO_2 level, 'seawater PCO_2' significantly affected PC-related GDH activity in gill tissue (2-way ANOVA: $F_{1,31} = 8.026$, $p = 0.008$). An increase in seawater PCO_2 reduced the GDH capacity by 27% in tolerant and by 43% in sensitive families. The ratio of GDH to CS was constant in both tissues between family types as well as PCO_2 level (Fig. 5a, b).

Table 2 Simplified overview of fresh weight-related enzyme activities in gill and mantle tissue of tolerant and sensitive families of *Mytilus edulis* that were raised for 15 months at control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. ■ and □ indicate enzyme activities at control level in tolerant and sensitive families, respectively. Black arrows indicate significantly decreased (🡻) or elevated (🡹) or slight trend of increasing (🡽) or decreasing (🡾) enzyme activities within each family type compared to control. White arrows indicate significantly decreased (⇩) or elevated (⇧) or slight trend of increasing (⇗) or decreasing (⇘) enzyme activities within in sensitive compared to tolerant families at the respective PCO_2 level.

Metabolic pathway	Enzyme	Tissue	Tolerant			Sensitive	
			Control	Intermed.	High	Control	Intermed.
Aerobic capacity	COX	Gill	■	🡻	🡻	⇘	□
		Mantle	■	🡽	■	□	🡽
	CS	Gill	■	🡻	🡻	⇩	□
		Mantle	■	■	🡽	□	□
	COX/ CS	Gill	■	■	■	□	□
		Mantle	■	■	🡻	□	□
Carbohydrate metabolism	PK	Gill	■	■	🡾	⇘	🡹
		Mantle	■	🡹	🡽	□	⇩
	PEPCK	Gill	■	■	■	⇩	🡹
		Mantle	■	■	■	□	□
	PK/ PEPCK	Gill	■	🡽	🡾	□	□
		Mantle	■	■	■	⇘	⇩
Lipid metabolism	HADH	Gill	■	🡾	🡻	⇩	🡹
		Mantle	■	■	■	□	□
	HADH/ CS	Gill	■	■	■	□	🡹
		Mantle	■	■	■	□	□
Protein metabolism	GDH	Gill	■	🡾	🡾	⇩	□
		Mantle	■	■	■	□	□
	GDH/ CS	Gill	■	■	■	□	□
		Mantle	■	■	■	□	□

Discussion

Knowledge about the existing variation in metabolic and biochemical responses to rapidly changing environmental drivers is a prerequisite in order to assess the adaptive capacity of natural populations (Applebaum et al. 2014). Here we show that the capacity of key metabolic enzymes varied substantially in both gill and outer mantle tissue between CO_2-sensitive and -tolerant families within a population of Baltic blue mussel (*Mytilus edulis*) raised for over one year under elevated PCO_2. These results provide insight into the biochemical background of whole animal responses contrasting between family types of *M. edulis* (CO_2 -sensitive and -tolerant families) with reference to our previous study (Stapp et al. 2017).

Impact of elevated PCO_2 on aerobic capacity

Citrate synthase (CS) is a marker for mitochondrial density and commonly used, together with cytochrome-C-oxidase (COX), as a proxy for the aerobic capacity of cells and tissues (Moyes et al. 1997; Dalziel et al. 2005). In gills of tolerant families, fresh weight (FW) and protein content (PC) related CS and COX capacities showed a simultaneous reduction at elevated seawater PCO_2 level. At unchanged total gill tissue PC, this suggests a reduced aerobic capacity in gills of tolerant families under elevated seawater PCO_2. In contrast, aerobic capacity in gills from sensitive families did not differ between control and intermediate PCO_2 levels. However, at control conditions, FW-related COX and CS capacities of sensitive families amounted to only around 60% of aerobic capacity of tolerant families. In fact, all measured gill mitochondrial enzymes (GDH, HADH, COX, CS) showed the same differences between tolerant and intolerant families. Under control conditions, FW-related capacities in sensitive families were between 30-45% lower than capacities in tolerant families. When related to PC, these differences were no longer visible. However, gill PC being 30% lower in sensitive than in tolerant mussels suggests that the significantly lower aerobic capacity was due to a lower mitochondrial density in sensitive mussels.

Differences in gill mitochondrial enzyme capacities/densities between sensitive and tolerant mussels at control PCO_2 were, however, not reflected in different rates of gill tissue respiration. Our companion study showed that respiration of isolated gills (per gram of tissue) did not differ between tolerant and sensitive mussels under control PCO_2 (Stapp et al. 2017). A higher mitochondrial capacity and/or density at the same oxygen consumption rate in tolerant and sensitive mussels indicates a higher mitochondrial scope in tolerant mussels, i.e. a lower fraction of gill mitochondrial capacity is used under baseline (control) conditions. Under elevated PCO_2, this greater gill baseline mitochondrial scope of tolerant families may

allow to cover a higher energy demand in spite of decreased aerobic capacity. Our previous study showed that gill and also mantle tissue respiration was slightly elevated in tolerant mussels at intermediate compared to control PCO_2 levels, which resulted in a significantly increased total tissue respiration rate, and, consequently, higher whole animal routine metabolic rates (RMR) (Stapp et al. 2017). Sensitive families, in contrast, showed unchanged gill and RMRs and slightly decreased mantle respiration at the intermediate PCO_2 level, which resulted in a significant difference of mantle respiration and RMR between sensitive and tolerant families at this PCO_2 level (Stapp et al. 2017). This may indicate that sensitive families rely on energy reallocation, and hence trade-offs, rather than an increase of aerobic metabolism to cover an altered energy demands imposed by elevated seawater PCO_2. Interestingly, baseline (control) RMRs of sensitive families were in the range of those of tolerant families under elevated PCO_2 (Stapp et al. 2017). Under control conditions, although not statistically significant due to a high variability within sensitive families (p=0.08), individuals from sensitive families even had 1.6-fold higher mean RMR with a 2.6-fold increased relative standard deviation than tolerant families. A lower baseline (control) RMR at whole animal level in tolerant versus sensitive families would support a larger aerobic scope but were associated with similar clearance rates (i.e. food intake) (Stapp et al. 2017). This may suggest an overall higher metabolic efficiency (i.e. lower maintenance costs at similar energy intake) in tolerant than sensitive families, an efficiency reflected in their greater gill mitochondrial scope and ability to increase their RMR at the intermediate PCO_2 compared to sensitive families.

The higher aerobic scope in gills of tolerant mussels may also allow for a fraction of aerobic capacity that can be depressed as an energy saving strategy under elevated PCO_2. This is indicated by decreased aerobic enzyme capacities in tolerant but not in sensitive families. A reduction in aerobic, and therefore functional scope, as a mechanism to reduce costs of metabolic maintenance in response to elevated seawater PCO_2 was previously suggested for fish (Strobel et al. 2012); 5 weeks of CO_2 exposure (~2000 µatm CO_2) led to reduced state III respiration (maximal mitochondrial respiration and phosphorylating activity) and reduced COX capacities but did not affect state IV respiration (non-phosphorylating respiration) in liver tissue of Antarctic rock cod, *Notothenia rossii* (Strobel et al. 2012). In the present study, the CO_2 induced reductions of aerobic enzyme capacities in gills of tolerant families occurred in a concentration dependent manner; i.e. PC-related CS and COX capacities were lowest at the highest PCO_2 level in spite of a constant PC in gills. In filter-feeding bivalves, the gills are densely packed with mitochondria (Paparo 1972) to meet the high ATP demand of the dynein motor ATPase of the water current producing lateral cilia (Gibbons 1982). The gill

cells that carry those cilia therefore account for up to 90% of gill respiration (Clemmesen and Jørgensen 1987). In isolated gill tissue, beating of the lateral cilia can be induced in a concentration dependent manner using the excitatory nerve transmitter serotonin (Aiello 1960). In our companion study (Stapp et al. 2017), we showed that gills of tolerant mussels responded to the highest seawater PCO_2 level with a significantly reduced factorial metabolic scope (i.e. factorial difference in respiration of serotonin-unstimulated gills vs. respiration of gill tissue during maximal beat frequency of lateral cilia). This reduction of gill metabolic scope was accompanied by a significant reduction of filtration rates of tolerant mussels at the same PCO_2 level. Thus, the level of reduction of aerobic scope at the highest PCO_2 level seems to translate into a measurable reduction in tissue functional scope (i.e. lower factorial metabolic scope) that may translate into functional constraints that are also visible at the whole animal level (i.e. lower filtration rates). Thereby, the CO_2 induced reduction in CS and COX capacities may limit the ATP supply necessary to fuel the large ciliary machinery in gills. This hypothesis is supported by results of an earlier proteomic study on the same population which showed a PCO_2 induced decrease in abundance of NAD-dependent isocitrate dehydrogenase (IDH) in gill tissue after long-term (1 year) CO_2 exposure (at 1300 and 2400 µatm CO_2). This effect was also associated with a concentration dependent reduction of filtration rates (1300 and 2400 µatm; Hüning 2014). IDH catalyses the conversion of isocitrate to α-ketoglutarate in the TCA cycle and marks, together with CS and α-ketoglutarate dehydrogenase, one of the three irreversible steps of the TCA cycle.

Decreased filtration rates of tolerant families at the highest PCO_2 level were associated with RMRs that were at similar levels as under control conditions (Stapp et al. 2017). This indicated a shift in the balance of energy availability for filtration and energy supply and, therefore, some degree of metabolic down-regulation and/or reorganisation at the highest compared to the intermediate PCO_2 level. Besides the decrease in gill aerobic capacity, aerobic enzyme capacities in mantle of tolerant families also indicate a shift of mitochondrial metabolism between intermediate and high PCO_2 level. At the intermediate PCO_2 level, FW-related COX activity increased slightly, indicating a higher activity of the electron transport chain and increased ATP demand, matching the above mentioned slight increase of mantle respiration in tolerant families at the intermediate PCO_2 level. By contrast, at the highest PCO_2 level, FW-related COX activity as well as tissue respiration (Stapp et al. 2017) returned to control levels in mantle tissue of tolerant families and COX activity referred to PC was significantly reduced compared to the control and intermediate PCO_2 level. This was associated with slightly increased FW- and PC-related CS capacities and significantly higher PC in mantle of toler-

ant families at the highest PCO_2, indicating an unchanged mitochondrial density but a relative increase in matrix (CS) over membrane functions (COX), reflected in the significantly lower COX/CS ratio. In line with this, a previous study on the same population showed that gene expression of F-ATPase subunits from both the F_O and the F_1 complex were down-regulated in outer mantle following a two month exposure to a similar elevated PCO_2 (Hüning et al. 2013). Taken together, this may indicate a reduced capacity of the electron transport chain and for aerobic ATP synthesis in mantle tissue of tolerant mussels at the highest PCO_2 level.

The changed ratio of COX to CS in mantle of tolerant families at the highest PCO_2 requires explanation. Reduced COX at unchanged CS capacities, and the resulting lowered COX/CS ratio, were also found in liver of Antarctic rock cod, *Notothenia rossii*, after 5 weeks of CO_2 exposure (Strobel et al. 2013). The authors interpreted this as a shunting away of TCA intermediates from the electron transport chain towards cataplerotic processes, i.e. the removal of TCA intermediates for biosynthesis, potentially to support other oxidative tissues during decreased energy assimilation (i.e. lower filtration rates). In *M. edulis*, mantle tissue is known to be both gluconeogenic and lipogenic as it serves as a major glycogen storage and is also involved in gametogenesis (Bayne 1976). A shift to gluconeogenesis seems, however, unlikely, as mantle PEPCK did not show a substantial increase at the highest PCO_2 level. We did not measure any lipogenic enzymes, but lipogenesis from endogenous glycogen stores is known to occur within mantle tissue of *M. edulis* (Zaba and Davies 1979), mainly during gametogenesis (Bayne 1976). Speculatively, lipogenesis may have occurred in mantle of tolerant mussels at the highest PCO_2 level in order to support other tissues such as the digestive gland (hepatopancreas), which uses lipids as a preferred mitochondrial substrate (Ballantyne and Moon 1985). The increase in PC per gram mantle tissue in tolerant families at the highest PCO_2 could be indicative for a relative decrease of other tissue components such as glycogen or lipids.

Impact of elevated PCO_2 on carbohydrate metabolism

In mussels, the phosphoenpolpyruvate (PEP) branchpoint controls the flux of glycolytic substrates to either aerobic (formation of pyruvate via pyruvate kinase (PK)) or anaerobic energy production (formation of oxaloacetate, and subsequently fumarate and succinate) via phosphoenolpyruvate carboxykinase (PEPCK)) (De Zwaan and Holwerda 1972; Holwerda and De Zwaan 1973; De Vooys 1987). Thus, a decrease in PK/PEPCK ratio reflects an activation of anaerobic pathways.

While the PK/PEPCK ratio as well as FW and PC related enzyme activities remained unaffected in gills of tolerant mussels at the intermediate PCO_2, the PK/PEPCK ratio dropped significantly at the highest PCO_2. The lower PK/PEPCK suggests an increased contribution of anaerobic pathways and potentially lowered rates of pyruvate supply to aerobic metabolism at the highest PCO_2, which matches the decreased aerobic capacity and metabolic scope in gills of tolerant mussels (Stapp et al. 2017). Similarly, CO_2 exposure was shown to induce partial anaerobiosis (measured as succinate accumulation) in gill tissue of *Crassostrea gigas,* potentially due to slightly hypoxemic conditions as indicated by a drop in hemolymph oxygenation at a constant RMR (Lannig et al. 2010). In filter-feeding bivalves, filtration and ventilation are coupled processes. The lower filtration capacity observed in tolerant families at the highest PCO_2 could initiate a slight decrease in tissue oxygenation, which may trigger the activation of anaerobic metabolic pathways.

In contrast to tolerant families, sensitive mussels exhibited a simultaneous increase in FW-related PK and PEPCK capacities, and thus a stable PK/PEPCK ratio in gill tissue at the intermediate PCO_2. Besides its role in anaerobic energy production, PEPCK in the carboxylating direction can serve in an anaplerotic function by channelling oxaloacetate (via malate) into the TCA cycle (Hochachka and Somero, 1984). Higher FW-related PEPCK capacities in sensitive families level were associated with higher HADH capacities, indicating increased ß-oxidation (see discussion below) and therefore an increased demand of oxaloacetate due to the channelling of acetyl-CoA into the TCA cycle. The potentially higher ß-oxidation in concert with higher PK capacities may indicate an increased reliance on glucose as well as lipid-based substrates in gills of sensitive mussels at the intermediate PCO_2. Similarly, a study on *Crassostrea virginica* showed that during osmotic stress a mix of fatty acids as well as pyruvate was oxidised to meet the energy demand in isolated gills (Ballantyne and Moyes, 1987). The relevance of these findings for CO_2 tolerance needs to be investigated.

In mantle tissue of tolerant families, the long-term exposure to elevated PCO_2 resulted in an increased activity of FW-related PK activity, which peaked at the intermediate PCO_2 level. This indicates an increase of the glycolytic pathway, which is supported by data showing that hexokinase expression – which, besides PK, is one of the three regulatory enzymes in glycolysis – was up-regulated in outer mantle tissue of *M. edulis* of the same population after an eight-week exposure to elevated PCO_2 (Hüning et al. 2013). A potential increase of the glycolytic pathway in tolerant families correlates with the slightly elevated FW-related COX capacities, which may fuel the above-mentioned higher mantle respiration at the intermediate PCO_2.

In contrast, sensitive families, showed unchanged PK and PEPCK capacities in mantle tissue under elevated CO_2. Although we observed no CO_2 effect on PK and PEPCK capacities in sensitive families, they were characterised by an overall lower PK/PEPCK ratio compared to tolerant families. This suggests an increased anaerobic and/or gluconeogenic component in mantle tissue of sensitive mussels independent of CO_2 exposure. The underlying reason for this difference of glycolytic enzyme ratios and how this related to the different levels of CO_2 vulnerability remains to be explored.

Impact of elevated PCO_2 on lipid metabolism

Mitochondrial 3-Hydroxyacyl-CoA-Dehydrogenase (HADH) is involved in lipid oxidation, which has been shown to play an important role in the energy metabolism of certain tissues in mollusks. Lipids are the preferred substrate in hepatopancreas mitochondria of *M. edulis* (Ballantyne and Moon 1985) and a recent study on *Sepia officinalis* shows that oxidation of fatty acids and ketone bodies play an important role in gill catabolism during starvation (Speers-Roesch et al. 2016). In line with these findings, our results and those of an earlier study (Kluytmans et al. 1985) show overall higher HADH activity in gill than in mantle tissue of *M. edulis*. Furthermore, we found family-specific PCO_2 effects on FW-related HADH capacities in gill tissue. While FW-related HADH activities decreased in line with the decreased aerobic capacity in gill tissue of tolerant families, sensitive families exhibited higher gill HADH activities under elevated PCO_2. The latter suggests an increased reliance on lipids as an energy source in sensitive families under elevated PCO_2.

Lipids are the major energy sources during early shell formation and metamorphosis when larvae rely solely on endogenous energy reserves (Bayne 1976). Thus, an increased lipid oxidation at unchanged larval growth rates (see Thomsen et al. 2017; Stapp et al. 2017) could result in a depletion of lipid reserves before metamorphosis is completed, potentially explaining the higher larval mortality at the highest PCO_2 treatment in sensitive compared to tolerant mussels. Yet, further measurements of larval enzyme capacities and lipid contents are required to test this hypothesis.

Impact of elevated PCO_2 on protein metabolism

Glutamate dehydrogenase (GDH) is located in the mitochondria and is involved in amino acid catabolism and synthesis; it catalyses the reversible formation of alpha ketoglutarate and ammonia (NH_4^+) out of glutamate. Earlier short-term studies on blue mussels from Kiel Fjord showed an increased ammonia excretion following an eight-week exposure to elevated

PCO_2 (Thomsen and Melzner 2010). This indicates increased protein catabolism which might aid in acid base regulation under hypercapnic conditions due to the excretion of positively charged ions (Lindinger et al. 1984) as well as the formation of bicarbonate ions as part of protein breakdown process (Langenbuch and Pörtner 2002; Michaelidis et al. 2005). However, longer-term exposure (1 year) to similar PCO_2 diminished this effect, as ammonia excretion of *M. edulis* did not differ between CO_2 treatments (Hüning 2014). Instead, proteomics revealed a lower abundance of gill proteins involved in protein degradation at elevated PCO_2 (Hüning 2014). A decrease in protein turnover over time could be an energy saving strategy to offset increased energy demands under elevated PCO_2 as well as a compromised energy assimilation at the highest PCO_2 level (i.e. decreased clearance rates, Stapp et al. 2017; Hüning 2014). In fact, a stable and increased gill PC in tolerant and sensitive mussels, respectively, at decreased PC-related GDH capacities supports this idea. Furthermore, mantle PC increased at the intermediate PCO_2 in sensitive and at the highest PCO_2 level in tolerant families. Our previous study did not show differences in oxygen demand of protein biosynthesis between families and PCO_2 level (Stapp et al. 2017), thus, higher mantle PC at similar costs of protein synthesis might also indicate a lowered protein degradation in mantle tissue at elevated PCO_2.

Conclusions

This study showed that 15 months exposure of different family lines of Baltic blue mussel (*M. edulis*) to a control (700 µatm) and elevated seawater PCO_2 levels (1120 and 2400 µatm) resulted in gill and outer mantle specific adjustment of capacities of key metabolic enzymes that differed between CO_2 tolerant compared to CO_2 sensitive family lines. The most striking difference between family types appeared to be a greater gill mitochondrial density and thus gill aerobic capacity (CS, COX) and scope under control conditions in tolerant compared to sensitive families. Under elevated PCO_2, this higher gill aerobic scope of tolerant families seems to cover slightly elevated homeostatic costs; our previous study revealed increased summed tissue and whole animal metabolic rates in tolerant but not in sensitive families at the intermediate PCO_2 level (see Stapp et al. 2017). In addition, it seems to allow for a 'reserve' that can be depressed, potentially as an energy saving mechanisms in response to CO_2; aerobic capacity and concomitantly lipid metabolism decreased in gill tissue of tolerant families under elevated PCO_2. Sensitive mussels, in contrast, may not be able to follow this 'strategy' of a decreased aerobic capacity due to their *per se* lower gill aerobic capacity compared to tolerant families.

Although tolerant families survived the highest PCO_2 level, our data for gill and mantle show that this appears to be associated with metabolic constraints. Depression of gill aerobic capacity was highest at the highest PCO_2 level and was accompanied by an increased contribution of anaerobic energy production (lower PK/PEPCK ratio). Decreased aerobic mitochondrial capacities and lower ATP supply at the highest PCO_2 level may explain the decrease in gill metabolic scope and filtration rate observed in our previous study (Stapp et al. 2017).

In mantle, COX capacities and associated mantle respiration of tolerant families were increased at the intermediate PCO_2 level and likely fuelled by the simultaneous increase of the glycolytic metabolic pathway (higher PK capacity). This seems, however, not sustainable at the highest PCO_2 level; survivors of tolerant families at the highest CO_2 level were characterised by COX capacity and mantle as well as whole animal respiration similar to those under control conditions. Furthermore, a decreased ratio of COX/CS in mantle at the highest PCO_2 suggests shunting away of TCA intermediates from the electron transport chain towards cataplerotic processes. We hypothesise that lipogenesis may have occurred, potentially to support the energy demand of other tissues.

Taken together, this suggests that CO_2 resilience in mussels correlated with an enhanced metabolic efficiency (i.e. lower RMR at similar energy intake (Stapp et al. 2017); greater gill mitochondrial scope (this study)), which seems to be selected for in a high CO_2 environment. Although a significant number of mussels from tolerant families survived at the highest PCO_2, larval mortality was higher compared to the control and intermediate PCO_2 (Thomsen et al. 2017). Furthermore, while our results suggest metabolic constraints at the highest PCO_2, our previous studies showed that tolerant mussels at the highest PCO_2 grew to a similar size as mussels at the control and intermediate PCO_2 and were able to reproduce after one year of CO_2 exposure; yet, larval mortality rates and fitness were not improved in the F_2 generation (Thomsen et al. 2017). However, the observed intra-population variation in physiological traits at the biochemical (this study) and also whole animal level (Stapp et al. 2017) suggests some capacity for physiological adaptation to ocean acidification within this population. As global change is not limited to ocean acidification, future studies have to reassess these results in light of multiple environmental drivers. For example, the observed decrease in gill aerobic scope may result in a narrowing of their thermal performance window (Pörtner, 2002) making mussels more vulnerable to ocean warming.

Overall, these results warrant more research on understanding biochemical mechanisms that underlie intra-population, inter-individual and inter-tissue responses driven by CO_2 induced changes to seawater carbonate chemistry. Such knowledge is crucial in order to make accurate projections regarding the consequences of ocean acidification and species adaptive capacities.

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References

Aiello EL (1960) Factors affecting ciliary activity on the gill of the mussel *Mytilus edulis*. Biol Bull 33:120–135. https://doi.org/10.1086/physzool.33.2.30152299

Applebaum SL, Pan TCF, Hedgecock D, Manahan DT (2014) Separating the Nature and Nurture of the Allocation of Energy in Response to Global Change. Integr Comp Biol 54:284–295. doi: 10.1093/icb/icu062

Ballantyne JS, Moon TW (1985) Hepatopancreas mitochondria from *Mytilus edulis*: substrate preferences and effects of pH and osmolarity. Mar Biol 87:239–244. doi: 10.1007/BF00397800

Ballantyne J.S. and C.D. Moyes. 1987. Osmotic Effects on Fatty Acid, Pyruvate, and Ketone Body Oxidation in Oyster Gill Mitochondria. Physiol Zool 60:713–729. https://doi.org/10.1086/physzool.60.6.30159987

Bayne BL (1976) Marine Mussels: Their Ecology and Physiology. Cambridge University Press, Cambridge

Bradford M (1976) A rapid and sensitive method for quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brothers CJ, Harianto J, McClintock JB, Byrne M (2016) Sea urchins in a high-CO_2 world: the influence of acclimation on the immune response to ocean warming and acidification. Proc R Soc B 283:20161501. doi: 10.1098/rspb.2016.1501

Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. Nature 425:365–365. doi: 10.1038/425365a

Calosi P, De Wit P, Thor P, Dupont S (2016) Will life find a way? Evolution of marine species under global change. Evol Appl 9:1035–1042. doi: 10.1111/eva.12418

Calosi P, Melatunan S, Turner LM, et al (2017) Regional adaptation defines sensitivity to future ocean acidification. Nature Communications 8:13994. doi: 10.1038/ncomms13994

Clemmesen B, Jørgensen CB (1987) Energetic Costs and Efficiencies of Ciliary Filter Feeding. Mar Biol 94:445–449. doi: 10.1007/BF00428251

Dalziel AC, Moore SE, Moyes CD (2005) Mitochondrial enzyme content in the muscles of high-performance fish: evolution and variation among fiber types. Am J Physiol Regul Integr Comp Physiol 288:R163–R172. doi: 10.1152/ajpregu.00152.2004

De Vooys CGN (1987) Adaptation to anaerobic metabolism in two mussel species, *Mytilus edulis* and *Mytilus galloprovincialis*, from the tidal zone at Arcachon Bay, France. Neth J Sea Res 21:17–23. doi: 10.1016/0077-7579(87)90019-6

De Zwaan A, Holwerda DA (1972) The effect of phosphoenolpyruvate, fructose 1, 6-diphosphate and pH on allosteric pyruvate kinase in muscle tissue of the bivalve *Mytilus edulis* L. J Proteom 276:430–433. doi: 10.1016/0005-2744(72)91004-2

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean Acidification: The Other CO_2 Problem. Annu Rev Mar Sci 1:169–192. doi: 10.1146/annurev.marine.010908.163834

Dorey N, Lançon P, Thorndyke M, Dupont S (2013) Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. Glob Change Biol 19:3355–3367. doi: 10.1111/gcb.12276

Driedzic WR, Fonseca de Almeida Val VM (1996) Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). J Exp Zool 274:327–333. doi: 10.1002/(SICI)1097-010X(19960415)274:6<327::AID-JEZ1>3.0.CO;2-Q

Gazeau F, Parker LM, Comeau S, et al (2013) Impacts of ocean acidification on marine shelled molluscs. Mar Biol 160:2207–2245. doi: 10.1007/s00227-013-2219-3

Gibbons IR (1982) Introduction: dynein ATPases. Cell Motil 2:87–93. doi: 10.1002/cm.970020718

Greenway SC, Storey KB (1999) The effect of prolonged anoxia on enzyme activities in oysters (*Crassostrea virginica*) at different seasons. J Exp Mar Bio Ecol 242:259–272. doi: 10.1016/S0022-0981(99)00103-3

Griffith AW, Gobler CJ (2017) Transgenerational exposure of North Atlantic bivalves to ocean acidification renders offspring more vulnerable to low pH and additional stressors. Sci Rep 7:11394. doi: 10.1038/s41598-017-11442-3

Harlocker SL, Kapper MA, Greenwalt DE, Bishop SH (1991) Phosphoenolpyruvate carboxykinase from ribbed mussel gill tissue: Reactivity with metal ions, kinetics, and action of 3-mercaptopicolinic acid. J Exp Zool 257:285–298. doi: 10.1002/jez.1402570302

Hedrick PW (2006) Genetic Polymorphism in Heterogeneous Environments: The Age of Genomics. Annu Rev Ecol Evol Syst 37:67–93. doi: 10.1146/annurev.ecolsys.37.091305.110132

Hochachka P. W. and G. N. Somero. 1984. Biochemical adaptation: mechanism and process in physiological evolution. Princeton University Press, Princeton

Holwerda DA, De Zwaan A (1973) Kinetic and molecular characteristics of allosteric py-

ruvate kinase from muscle tissue of the sea mussel *Mytilus edulis* L. J Proteom 309:296–306. doi: 10.1016/0005-2744(73)90028-4

Hüning (2014) Responsiveness of *Mytilus edulis* towards mechanical stress and elevated pCO_2—combined transcriptomic, proteomic and physiological analyses. Dissertation, University of Bremen

Hüning AK, Melzner F, Thomsen J, et al (2013) Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: Implications for shell formation and energy metabolism. Mar Biol 160:1845–1861. doi: 10.1007/s00227-012-1930-9

Kelly MW, Padilla-Gamiño JL, Hofmann GE (2013) Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus purpuratus*. Glob Change Biol 19:2536–2546. doi: 10.1111/gcb.12251

Kluytmans JH, Boot JH, Oudejans RCHM, Zandee DI (1985) Fatty acid synthesis in relation to gametogenesis in the mussel *Mytilus edulis* L. J Proteom 81:959–963. doi: 10.1016/0305-0491(85)90097-5

Kluytmans JH, Zandee DI, Zurburg W, Pieters H (1980) The influence of seasonal changes on energy metabolism in *Mytilus edulis* (L.).—III. Anaerobic energy metabolism. Comp Biochem Physiol B Biochem Mol Biol 67:307–315. doi: 10.1016/0305-0491(80)90149-2

Kroeker KJ, Kordas RL, Crim R, et al (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Glob Change Biol 19:1884–1896. doi: 10.1111/gcb.12179

Kroeker KJ, Sanford E, Rose JM, et al (2016) Interacting environmental mosaics drive geographic variation in mussel performance and predation vulnerability. Ecol Lett 19:771–779. doi: 10.1111/ele.12613

Langenbuch M, Pörtner HO (2002) Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid-base variables. J Exp Biol 205:1153–1160.

Lannig G, Eilers S, Pörtner HO, et al (2010) Impact of Ocean Acidification on Energy Metabolism of Oyster, *Crassostrea gigas*—Changes in Metabolic Pathways and Thermal Response. Mar Drugs 8:2318–2339. doi: 10.3390/md8082318

Leite Figueiredo DA, Branco PC, Santos dos DA, et al (2016) Ocean acidification affects parameters of immune response and extracellular pH in tropical sea urchins *Lytechinus variegatus* and *Echinometra luccunter*. Aquat Toxicol 180:84–94. doi: 10.1016/j.aquatox.2016.09.010

Lindinger MI, Lauren DJ, McDonald DG (1984) Acid-base balance in the sea mussel, *Mytilus edulis*. III: Effects of environmental hypercapnia on intra-and extracellular acid-base balance. J Proteom 5:371–381.

Lockwood BL, Somero GN (2012) Functional Determinants of Temperature Adaptation in Enzymes of Cold- versus Warm-Adapted Mussels (Genus *Mytilus*). Molecular Biology and Evolution 29:3061–3070. doi: 10.1093/molbev/mss111

McClelland GB, Dalziel AC, Fragoso NM, Moyes CD (2005) Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. J Exp Biol 208:515–522. doi: 10.1242/jeb.01423

Melzner F, Stange P, Trübenbach K, et al (2011) Food Supply and Seawater pCO_2 Impact Calcification and Internal Shell Dissolution in the Blue Mussel *Mytilus edulis*. PLoS ONE 6:e24223. doi: 10.1371/journal.pone.0024223.t003

Melzner F, Thomsen J, Koeve W, et al (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. Mar Biol 160:1875–1888. doi: 10.1007/s00227-012-1954-1

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293:109–118. doi: 10.3354/meps293109

Moyes CD, Mathieu-Costello OA, Tsuchiya N, et al (1997) Mitochondrial biogenesis during cellular differentiation. Am J Physiol Cell Physiol 272:C1345–C1351. doi: 10.1152/ajpcell.1997.272.4.C1345

Moyes CD, Moon TW, Ballantyne JS (1985) Glutamate catabolism in mitochondria from *Mya arenaria* mantle: Effects of pH on the role of glutamate dehydrogenase. J Exp Zool 236:293–301. doi: 10.1002/jez.1402360306

Orr JC, Fabry VJ, Aumont O, et al (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437:681–686. doi: 10.1038/nature04095

Pan TCF, Applebaum SL, Manahan DT (2015) Experimental ocean acidification alters the allocation of metabolic energy. Proc Natl Acad Sci USA 112:4696–4701. doi: 10.1073/pnas.1416967112

Pansch C, Schaub I, Havenhand J, Wahl M (2014) Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. Glob Change Biol 20:765–777. doi: 10.1111/gcb.12478

Paparo A (1972) Innervation of the Lateral Cilia in the Mussel *Mytilus Edulis* L. Biol Bull 143:592–604. https://doi.org/10.2307/1540185

Parker L, Ross P, O'Connor W, et al (2013) Predicting the Response of Molluscs to the Impact of Ocean Acidification. Biology 2:651–692. doi: 10.3390/biology2020651

Pespeni MH, Sanford E, Gaylord B, et al (2013) Evolutionary change during experimental ocean acidification. Proc Natl Acad Sci USA 110:6937–6942. doi: 10.1073/pnas.1220673110

H.O Pörtner (2002) Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp Biochem Physiol A Mol Integr Physiol 132: 739-761. https://doi.org/10.1016/S1095-6433(02)00045-4.

Pörtner H (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar Ecol Prog Ser 373:203–217. doi: 10.3354/meps07768

Pörtner H-O, Karl DM, Boyd PW, et al (2014) Ocean Systems. In: Field CB, Barros VR, Dokken DJ, et al. (eds) Climate Change 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, NY, pp 411–484

Pörtner HO, Farrell AP (2008) Physiology and Climate Change. Science 322:690–692. doi: 10.1126/science.1163156

Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. Geology 37:1131–1134. doi: 10.1130/G30210A.1

Sabine CL, Feely RA, Gruber N, et al (2004) The oceanic sink for anthropogenic CO_2. Science 305:367–371. doi: 10.1126/science.1097403

Saderne V, Fietzek P, Herman PMJ (2013) Extreme Variations of pCO_2 And pH in a Macrophyte Meadow of the Baltic Sea in Summer: Evidence of the Effect of Photosynthesis and Local upwelling. PLoS ONE 8:e62689. doi: 10.1371/journal.pone.0062689

Sánchez-Muros MJ, García-Rejón L, García-Salguero L, et al (1998) Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diet on glutamate dehydrogenase and alanine aminotransferase kinetics. Int J Biochem Cell Bio 30:55–63. doi: 10.1016/S1357-2725(97)00100-3

Sidell BD, Driedzic WR, Stowe DB, Johnston IA (1987) Biochemical Correlations of Power Development and Metabolic Fuel Preferenda in Fish Hearts. Physiol Zool 60:221–232. doi: 10.1086/physzool.60.2.30158646

Sokolova IM (2013) Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr Comp Biol 53:597–608. doi: 10.1093/icb/ict028

Sokolova IM, Frederich M, Bagwe R, et al (2012) Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar Environ Res 79:1–15. doi: 10.1016/j.marenvres.2012.04.003

Somero GN (2012) The physiology of global change: linking patterns to mechanisms. Ann Rev Mar Sci 4:39–61. doi: 10.1146/annurev-marine-120710-100935

Speers-Roesch B, Callaghan NI, MacCormack TJ, et al (2016) Enzymatic capacities of metabolic fuel use in cuttlefish (*Sepia officinalis*) and responses to food deprivation: insight into the metabolic organization and starvation survival strategy of cephalopods. J Comp Physiol B 186:711–725. doi: 10.1007/s00360-016-0991-3

Stapp LS, Thomsen J, Schade H, et al (2017) Intra-population variability of ocean acidification impacts on the physiology of Baltic blue mussels (*Mytilus edulis*): integrating tissue and organism response. J Comp Physiol B 187:529–543. doi: 10.1007/s00360-016-1053-6

Strobel A., E. Leo, H.O. Pörtner, and F.C. Mark. 2013. Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*. Comp Biochem Physiol B Biochem Mol Biol 166:48–57. doi: 10.1016/j.cbpb.2013.06.006

Strobel A., S. Bennecke, E. Leo, K. Mintenbeck, H.O. Pörtner, and F.C. Mark. 2012. Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front Zool 9:1. doi: 10.1186/1742-9994-9-28

Stumpp M, Hu MY, Melzner F, et al (2012a) Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. Proc Natl Acad Sci USA 109:18192–18197. doi: 10.1073/pnas.1209174109

Stumpp M, Trübenbach K, Brennecke D, et al (2012b) Resource allocation and extracellular acid–base status in the sea urchin *Strongylocentrotus droebachiensis* in response to CO_2 induced seawater acidification. Aquat Toxicol 110-111:194–207. doi: 10.1016/j.aquatox.2011.12.020

Sunday JM, Calosi P, Dupont S, et al (2014) Evolution in an acidifying ocean. Trends Ecol Evol 29:117–125. doi: 10.1016/j.tree.2013.11.001

Thomsen J, Gutowska MA, Saphörster J, et al (2010) Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7:3879–3891. doi: 10.5194/bg-7-3879-2010

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar Biol 157:2667–2676. doi: 10.1007/s00227-010-1527-0

Thomsen J, Stapp LS, Haynert K, et al (2017) Naturally acidified habitat selects for ocean acidification–tolerant mussels. Sci Adv 3:e1602411. doi: 10.1126/sciadv.1602411

Tomanek L (2012) Environmental Proteomics of the Mussel *Mytilus*: Implications for Tolerance to Stress and Change in Limits of Biogeographic Ranges in Response to Climate Change. Integr Comp Biol 52:648–664. doi: 10.1093/icb/ics114

Weiss M, Heilmayer O, Brey T, et al (2012) Physiological capacity of *Cancer setosus* larvae — Adaptation to El Niño Southern Oscillation conditions. J Exp Mar Bio Ecol 413:100–105. doi: 10.1016/j.jembe.2011.11.023

Wittmann AC, Pörtner HO (2013) Sensitivities of extant animal taxa to ocean acidification. Nature Clim Change 3:995–1001. doi: doi:10.1038/nclimate1982

Wood HL, Sundell K, Almroth BC, et al (2016) Population-dependent effects of ocean acidification. Proc R Soc B 283:20160163. doi: 10.1098/rspb.2016.0163

Zaba B.N. and I. Davies. 1979. The Contribution of the Pentose Phosphate Cycle to the Central Pathways of Metabolism in the Marine Mussel, *Mytilus edulis* L. Biochem Soc Trans. 7:900-902. doi: 10.1042/bst0070900

Publication III

Sensitivity to ocean acidification differs between populations of the Sydney rock oyster: Role of filtration and ion-regulatory capacities

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Sensitivity to ocean acidification differs between populations of the Sydney rock oyster: Role of filtration and ion-regulatory capacities

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ABSTRACT

Understanding mechanisms of intraspecific variation in resilience to environmental drivers is key to predict species' adaptive potential. Recent studies show a higher CO_2 resilience of Sydney rock oysters selectively bred for increased growth and disease resistance ('selected oysters') compared to the wild population. We tested whether the higher resilience of selected oysters correlates with an increased ability to compensate for CO_2-induced acid-base disturbances. After 7 weeks of exposure to elevated seawater PCO_2 (1100 µatm), wild oysters had a lower extracellular pH (pH_e = 7.54 ± 0.02 (control) vs. 7.40 ± 0.03 (elevated PCO_2)) and increased hemolymph PCO_2 whereas extracellular acid-base status of selected oysters remained unaffected. However, differing pH_e values between oyster types were not linked to altered metabolic costs of major ion regulators (Na^+/K^+-ATPase, H^+-ATPase and Na^+/H^+-exchanger) in gill and mantle tissues. Our findings suggest that selected oysters possess an increased systemic capacity to eliminate metabolic CO_2, possibly through higher and energetically more efficient filtration rates and associated gas exchange. Thus, effective filtration and CO_2 resilience might be positively correlated traits in oysters.

1. Introduction

The combustion of fossil fuels over the last 250 years has led to an increase in atmospheric CO_2 from pre-industrial concentrations of 280 ppm to the present level of 400 ppm (Collins et al., 2013), a rate unprecedented during the past 66 million years (Zeebe et al., 2016). The world's oceans absorb around one third of these CO_2 emissions (Sabine et al., 2004), resulting in increased seawater PCO_2 which causes a shift in the oceans carbonate system towards lower pH values and carbonate ion concentrations (Cao and Caldeira, 2008), a process called ocean acidification (OA). If mitigation strategies are not implemented, a drop in pH by 0.42 units below the pre-industrial average pH 8.17 and a corresponding PCO_2 level of around 930 µatm are predicted for open ocean surface waters by the end of this century (RCP 8.5; Pörtner et al., 2014).

The biological impacts of ocean acidification have been extensively studied in recent years. Impacts on marine fauna are generally broad and species-specific (Harvey et al., 2013; Kroeker et al., 2013) and often dependent on various factors such as the studied population (e.g. Wood et al., 2016; Wright et al., 2014); the life-history stage (e.g. Baumann et al., 2012; Scanes et al., 2014); the duration of exposure (e.g. Dupont et al., 2012; Form and Riebesell, 2012); natural habitat variability (e.g. Eriander et al., 2016; Pansch et al., 2014); transgenerational exposure (e.g. Miller et al., 2012; Thor and Dupont, 2015), and; interactions with other environmental drivers (e.g. Dahlke et al., 2016; Dickinson et al., 2012). Nonetheless, a general consensus is that marine calcifying organisms are amongst the taxa most sensitive to ocean acidification (Kroeker et al., 2013; Wittmann and Pörtner, 2013). The formation and stability of their calcified skeletons and structures will likely be impaired by projected changes in ocean carbonate chemistry (Orr et al., 2005; Waldbusser et al., 2015). In addition, numerous studies indicate that ocean acidification causes energy allocation trade-offs in marine calcifiers (e.g. Stumpp et al., 2012; Dorey et al., 2013) which may compromise biomineralisation in a secondary fashion (e.g. Ivanina et al., 2013; Thomsen et al., 2013; Waldbusser et al., 2013). The energetic basis of ocean acidification effects is further underpinned by recent studies showing that high food (i.e. energy) availability has the potential to outweigh adverse CO_2 effects (Hettinger et al., 2013;

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Ramajo et al., 2016; Thomsen et al., 2013). The underlying physiological processes causing these energy trade-offs are, however, not yet fully understood.

Disturbances in acid-base equilibria and potentially associated altered maintenance costs caused by excess CO_2 are suggested as a primary cause for energetic trade-offs under OA (Melzner et al., 2009; Pörtner, 2008). Marine ectothermic organisms eliminate metabolic CO_2 via diffusive gradients, thus, elevated seawater PCO_2 also leads to an elevated P_eCO_2 in their body fluids (Melzner et al., 2009; Pörtner et al., 2004). This results in a drop of pH in the extra- (pH_e) and intracellular space (pH_i) (Larsen et al., 1997; Michaelidis et al., 2005). Owing to high levels of non-bicarbonate buffer, the buffering capacity of the intracellular space is usually high in all marine ectotherms (Heisler, 1986), while a high capacity to buffer pH_e seems to correlate with high metabolic rates and an active mode of life (Melzner et al., 2009) and can be mainly found in marine vertebrates such as fish (Esbaugh et al., 2012; Larsen et al., 1997) but also in cephalopods (Gutowska et al., 2010; Hu et al., 2014) and some crustaceans (Pane and Barry, 2007; Rastrick et al., 2014). In contrast, most studied sessile calcifying species show an incomplete compensation of pH_e in response to elevated PCO_2 (e.g. Lannig et al., 2010; Miles et al., 2007; Scanes et al., 2017), potentially mirroring their increased sensitivity towards ocean acidification (Melzner et al., 2009; Pörtner, 2008; Widdicombe and Spicer, 2008). Uncompensated reductions of pH_e can lead to metabolic depression (Michaelidis et al., 2005; Reipschläger and Pörtner, 1996) or, alternatively, increased energetic costs for acid-base and ion-regulatory processes because defending pH_i at uncompensated pH_e requires the up-regulation of proton equivalent ion exchange across body epithelia which is driven by energetically expensive ion-pumps (Melzner et al., 2009). As a result, energy budgets of cells, tissues and consequently whole animals may be challenged (e.g. Pan et al., 2015; Stapp et al., 2016; Stumpp et al., 2012). Knowledge of physiological mechanisms that underlie whole animal effects and performance limitations is, thus, key to ultimately predicting ecological consequences (Pörtner, 2008; Sokolova et al., 2012; Somero, 2012).

Comparing sensitive with more resilient phenotypes or species can serve as a powerful tool to determine key functional traits promoting resilience to ocean acidification (Applebaum et al., 2014). For example, the uneven distribution of two sea urchin species along a natural CO_2 gradient of a shallow CO_2 vent was recently correlated with their species-specific acid–base and ion-regulatory capacities (Calosi et al., 2013). The species which occurred in closer proximity to the vent had a relatively higher capacity to compensate for acid base disturbances (Calosi et al., 2013), supporting the hypothesis that a high capacity of the acid-base regulatory apparatus may be a key physiological trait for resilience towards ocean acidification (Melzner et al., 2009; Pörtner, 2008). Furthermore, the existence of marine calcifiers in naturally CO_2 enriched habitats highlights their potential capability for physiological acclimatisation or adaptation towards increased seawater PCO_2.

The rapid rate of anthropogenic ocean acidification is forcing species to undergo rapid acclimatisation or adaptation in order to survive (Sunday et al., 2014). Recent studies suggest that marine invertebrates may possess some potential for rapid adaptation towards ocean acidification through mechanisms already existing within the standing genetic variation (Kelly et al., 2013; Pespeni et al., 2013; Thomsen et al., 2017), transgenerational plasticity (Parker et al., 2012; Thor and Dupont, 2015) or exaptation (i.e. fitness mediated by a coopted trait that was selected for a different purpose) (Cole et al., 2016; Waldbusser et al., 2016). One example is the Sydney rock oyster, *Saccostrea glomerata*, an ecologically and economically important species inhabiting intertidal and shallow subtidal habitats along the southeast coast of Australia. It is a key species of Australia's edible oyster industry and, despite declines in production, accounts for about 40% of Australia's total oyster industry production value (Schrobback et al., 2014). The declining productivity of the industry led to the initiation of a breeding program in 1990 where oysters were initially mass selected for faster growth and, later, for increased disease resistance (hereafter termed 'selected oysters') (for details see Nell et al., 2000; O'Connor and Dove, 2009). Interestingly, when compared to the wild population, this selection for growth and immune competence also resulted in a significantly higher resilience towards ocean acidification through lower reductions in shell growth and reduced larval sensitivity (Parker et al., 2012, 2010). The selected oysters also had a higher standard metabolic rate at control conditions than wild-type oysters, which was further increased at elevated PCO_2 (Parker et al., 2012). In addition, negative CO_2 effects on early life stages were alleviated when their parents were previously exposed to elevated CO_2 (Parker et al., 2012). Similar positive carry-over effects were also observed for wild-type oysters, however, offspring of selected oysters still performed better (Parker et al., 2012). A subsequent study on the effects of transgenerationally CO_2 exposed wild-type oysters showed that adult F1 oysters had an improved capability to compensate for extracellular acidosis under elevated CO_2 (Parker et al., 2015). However, the physiological mechanisms promoting the higher resilience of selected compared to wild-type oysters are not yet understood.

The aim of this study was to investigate and compare the physiology of selected and wild-type oysters in response to elevated CO_2. Adults of both oyster types were incubated for 7 weeks at a control and elevated PCO_2 level before determining their extracellular acid-base status, their metabolic rates of isolated gill and mantle tissues and their respective energy demands of major ion-regulators (Na^+/K^+-ATPase, H^+-ATPase, Na^+/H^+-exchanger). We hypothesised that (i) the higher resilience of selected oysters is due to a higher capacity to compensate for CO_2 induced extracellular acid-base disturbances, and (ii) that this capacity is driven by metabolic and ion-regulatory costs at the cellular/tissue level.

2. Material and methods

2.1. Animals and experimental set-up

Two different populations of the Sydney rock oyster, *Saccostrea glomerata* (Gould, 1850) a wild and a selected population were used for the experiments. The selected line (line B2, 7th generation) was selectively bred for faster growth and increased immune competence and originates from a breeding program, which was initiated by NSW Fisheries in 1990. Oysters were initially mass selected for enhanced growth and later also for resistance against diseases (winter mortality, QX disease). For detailed information see O'Connor and Dove (2009) and Parker et al. (2012, 2010).

Selected and wild oysters experienced the same treatments throughout their complete life history. In 2012, broodstocks of both populations were collected at Cromarty Bay in Port Stephens (152°03′E, 32°42′S) and transferred to the Department of Primary Industries (DPI), Port Stephens Fisheries Institute (PSFI), Taylors Beach, NSW, Australia. Subsequently, 750 oysters of each population were mass spawned by thermal and salinity manipulation (for detailed procedure see O'Connor et al., 2008) and resulting larvae were reared at the PSFI in filtered seawater (FSW, 1 µm) at 23 °C. Water was changed daily and larvae were fed twice daily with fresh algae suspension consisting of 50% *Chaetoceros calcitrans,* 25% *Pavlova lutheri* and 25% *Tisochrysis lutea* (O'Connor et al., 2008). Post-settlement, larvae remained in the hatchery until they were retained on a 3 mm screen (~5 weeks). Subsequently, the spat of both populations was transferred to cultivation trays in Cromarty Bay where they developed into adults at the same lease. Around three years later (March 2015), oyster trays were brought back to the PSFI and 100 individuals (70–110 mm shell length) per population randomly chosen. Fouling organisms were scrubbed off and oysters were distributed between two re-circulating acclimation tanks and maintained at 24 °C and a salinity of 35. Each acclimation tank consisted of a 750 L reservoir tank and two 40 l tubs that were installed on top of the reservoir tank. Tubs contained baskets with either selected- or wild-type oysters and were constantly supplied with FSW

from the reservoir tank via spray bars connected to a small submersible aquarium pump (after Parker et al., 2012). After two days, all oysters were exposed to spawning-inducing conditions (see above) to induce gamete release of oysters that were gravid in order to achieve comparable gonadal status of all oysters. Following this procedure, all oysters were acclimated to laboratory conditions for another three weeks. Subsequently, the CO_2 incubation started. As the physiological experiments on isolated tissues were time-consuming, oysters were successively transferred from the acclimation into the incubation tanks over a period of 2.5 weeks to ensure a standardised period of 7 weeks in the incubation tanks for each oyster. Control animals were maintained in aerated local FSW (PCO_2 level of ~500 µatm (~0.05 kPa)), while CO_2 exposed specimens were incubated in aerated local FSW with a PCO_2 level of ~1000 µatm (~0.1 kPa) as predicted for 2100 according to the RCP 8.5 of the latest report of the Intergovernmental Panel on Climate Change (Pörtner et al., 2014). The number of replicate tanks per PCO_2 level was three (6 individual tanks in total). The experimental set-up was identical during incubation and acclimation except for the elevated CO_2 tanks, which were additionally equipped with computerised pH feedback systems (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia; accuracy ± 0.01) in order to maintain elevated PCO_2 level. The system regulates pH (NIST, accuracy ± 0.01 units) by adding pure gaseous CO_2 directly into the seawater. CO_2 was injected via CO_2 reactors to ensure proper mixing within the tank.

Throughout the whole experiment, oysters were fed twice daily with a mixture of fresh algae (50% *Chaetoceros calcitrans,* 25% *Pavlova lutheri* and 25% *Tisochrysis lutea;* O'Connor et al., 2008) at a concentration of approximately 2×10^9 cells oyster^{-1} d^{-1}. A complete water change using pre-equilibrated FSW was done every second or third day. The baskets that contained the oysters were rinsed with fresh water and the reservoir tanks and tubs cleaned using Virkon S solution (Antec Corp, North Bend, WA, USA). All seawater used during the experiment was locally collected at either Little Beach (152°07′E, 32°72′S) or Shoal Bay Beach (152°10′E, 32°43′S). Mortality did not exceed 5% in all tanks.

2.2. Monitoring of seawater carbonate chemistry

At each water change, the pH probes of the feedback systems were calibrated (NIST buffer) and alkalinity determined in triplicate by Gran titration. Additionally, pH (NIST), temperature and salinity were checked twice daily in each tank using a daily-calibrated pH probe (InLab Routine Pt1000, Mettler Toledo GmbH, Germany) connected to a pH meter (WTW 3310, Weilheim, Germany) and a salinometer (WTW LF 197, Germany), respectively. Corresponding seawater carbonate system speciations were calculated with CO2SYS (Lewis and Wallace, 1998) using the dissociation constants for $KHSO_4$, K1 and K2 after Dickson et al. (2007) and Mehrbach et al. (1973) refitted by Dickson and Millero (1987), respectively. Data for the physicochemistry of the seawater are summarised in Table 1.

2.3. Condition index

The condition index (CI) of wild and selected oysters was determined before (9 oysters per population) and after the 7 weeks of acclimation to different seawater PCO_2 (4 oysters per replicate tank; i.e. 12 oysters per treatment and oyster type). Body tissues and shells were dried for 48 h at 80 °C and the condition index calculated after Lawrence and Scott (1982):

$$CI = \left(\frac{body_{DW}}{shell_{DW}}\right) \times 100 \quad (1)$$

with DW = dry weight in gram.

2.4. Extracellular acid-base status

The extracellular acid-base status of oysters from all treatments was determined on the same day following 7 weeks of acclimation. Oysters showing filtration activity (i.e. shells open) were randomly sampled (3–4 oysters from each replicate tank), quickly shucked and hemolymph extracted by pericardial puncture. Extracellular pH (NIST scale) was immediately measured at acclimation temperature (24 °C) using a micro electrode (InLab Ultra-Micro, Mettler Toledo GmbH, Germany) connected to a pH meter (WTW 3310, Germany), that was calibrated with NIST buffers at 24 °C. Total extracellular CO_2 (C_eCO_2) was measured in a 100 µL subsample using a Ciba-Corning 965 CO_2 analyser (Olympic Analytical Service, UK) that was operated in a linear range from 0.71 to 11.36 mM and calibrated daily using a fresh $NaHCO_3$ standard (1 g l^{-1}). To account for potential instrument drift, distilled water was measured prior to, and standard solution after, each hemolymph sample. Extracellular carbonate chemistry (P_eCO_2, $[HCO_3^-]_e$) was calculated using the modified Henderson-Hasselbalch equations:

$$P_eCO_2 = C_eCO_2 \times (10^{pH\text{-}pK'''} \times \alpha CO_2 + \alpha CO_2)^{-1} \quad (2)$$

$$[HCO_3^-]_e = C_eCO_2 - \alpha CO_2 \times P_eCO_2 \quad (3)$$

The CO_2 solubility coefficient α (0.0346 mmol/l × mmHg) and the negative logarithm of the dissociation constant pK''' at 24 °C were calculated after Heisler (1986). Ionic strength (*I*) and the molarity of dissolved species (*M*) were taken from seawater (salinity 35) to represent these values in the hemolymph (Hammer et al., 2011). Hemolymph protein concentration (0.05 g l^{-1}) was adopted from Peters and Raftos (2003). Sodium concentration (468 mM) was measured using a blood gas analyser (ABL80, Radiometer Medical, Brønshøj, Denmark).

2.5. Tissue metabolic rates and ion-regulatory energy budget

Oysters (3–4 oysters from each replicate tank) were shucked, gill and mantle tissue carefully dissected and immediately transferred into buffer-filled Petri dishes in which they were cut into equal sized pieces (mean dry weight ± SD; gill: 2.65 ± 0.9 mg, mantle: 2.9 ± 1.1 mg, N = 173) using sterile scissors and forceps. Buffer for respiration experiments was designed to mimic respective *in vivo* hemolymph conditions and contained 500 mM NaCl, 9.1 mM KCl, 9.3 mM $CaCl_2 \cdot 2H_2O$, 22.9 mM $MgCl_2 \cdot 6H_2O$, 25.5 mM $MgSO_4 \cdot 7H_2O$, 5 mM glucose, 20 mM HEPES (after Cherkasov et al., 2006). Osmolarity was adjusted to 1100 mOsm, to match hemolmyph of *Saccostrea glomerata* at seawater

Table 1
Seawater physiochemical conditions for each tank during the 7-week exposure. Values are given as mean ± SD, *n* = 29.

Tank	Salinity	Temp. (°C)	pH_{NIST}	A_T (µmol kg^{1-})	C_T (µmol kg^{-1})	PCO_2 (µatm)	PCO_2 (kPa)	$\Omega_{calcite}$	$\Omega_{aragonite}$
Contr. 1	34.5 ± 0.8	23.8 ± 0.5	8.09 ± 0.02	2399 ± 73	2156 ± 73	540 ± 34	0.055 ± 0.003	4.35 ± 0.16	2.86 ± 0.11
Contr. 2	34.6 ± 0.8	23.7 ± 0.6	8.09 ± 0.01	2399 ± 73	2156 ± 72	537 ± 32	0.054 ± 0.003	4.35 ± 0.15	2.86 ± 0.10
Contr. 3	34.5 ± 0.8	23.9 ± 0.6	8.09 ± 0.02	2399 ± 73	2156 ± 74	540 ± 36	0.055 ± 0.004	4.36 ± 0.13	2.86 ± 0.09
CO_2 1	34.5 ± 0.8	23.9 ± 0.4	7.81 ± 0.02	2399 ± 73	2284 ± 72	1129 ± 81	0.114 ± 0.008	2.51 ± 0.14	1.64 ± 0.09
CO_2 2	34.6 ± 0.8	23.9 ± 0.4	7.80 ± 0.03	2399 ± 73	2288 ± 77	1162 ± 101	0.118 ± 0.010	2.45 ± 0.13	1.61 ± 0.09
CO_2 3	34.7 ± 0.9	24.0 ± 0.5	7.80 ± 0.02	2399 ± 73	2287 ± 74	1162 ± 74	0.118 ± 0.010	2.46 ± 0.09	1.61 ± 0.06

Values for PCO_2, C_T, $\Omega_{aragonite}$ and $\Omega_{calcite}$ calculated from salinity, temperature, pH_{NIST} and total alkalinity (A_T).

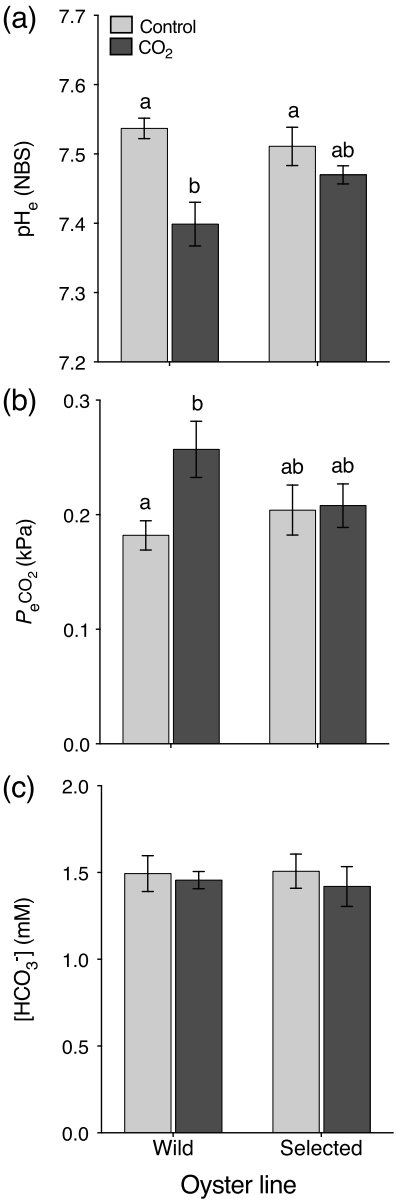

Fig. 1. Extracellular pH (a), P_eCO_2 (b) and $[HCO_3^-]$ (c) of wild and selected oysters (*Saccostrea glomerata*) exposed for 7 weeks to control or elevated seawater PCO_2. Different letters indicate significant differences between groups detected by *post hoc* analysis. Values are given as mean ± SEM, n = 9–12.

salinity of 35 (Nell and Dunkley, 1984). In order to mimic respective hemolymph gas composition buffer was gassed using pre-mixed gases (BOC gas & gear, Australia) with either a PO_2 of 18 kPa and PCO_2 of 0.15 kPa for the control oysters, or with a PO_2 of 18 kPa and PCO_2 of 0.3 kPa for CO_2-exposed oysters (Lannig et al., 2010). Buffer pH was set to respective pH_e values of wild (pH at 24 °C: 7.53 (control); 7.39 (elevated PCO_2)) and selected oysters (pH at 24 °C: 7.51 (control); 7.47 (elevated PCO_2)) that were measured after the 7 weeks of experimental exposure (see above and Fig. 1a) and adjusted by adding the respective amount of fresh $NaHCO_3$ solution calculated from the Henderson-Hasselbalch equation by use of pK‴ solubility coefficient αCO_2 determined according to Heisler (1986). Buffer pH was checked before and after each respiration experiment using an InLab® ultra-micro (Mettler Toledo®) electrode connected to pH-meter (WTW 3310, Germany) and was found to be stable.

After a recovery phase of around 20 min post-dissection, oxygen consumption of tissue pieces was measured in custom made closed, airtight and water-jacketed glass respiration chambers maintained at 24 °C by a connected external thermostat (Lauda Ecoline, Germany). Chambers were equipped with glass agitators and placed on magnetic stirrers to ensure homogeneity during measurements. Tissue pieces were placed on spacers consisting of coarse meshed gauze (1 mm mesh size) attached to a piece of Tygon® tubing to avoid mechanical damage by glass agitators. Oxygen content within each chamber was monitored using oxygen micro-optodes (needle-type, Presens GmbH, Germany) that were previously 2-point calibrated at 24 °C using oversaturated sodium sulfite solution for 0% and aerated buffer for 100% air saturation. For both tissues, total respiration was measured in quadruplicate. After uninhibited respiration rates had been measured for around 30 min, drugs that were either inhibiting Na^+/K^+-ATPase (ouabain, 1 mM), or H^+-ATPase (bafilomycin A1, 100 nM), or the Na^+/H^+-exchanger (EIPA (5-(*N*-ethyl-*N*-isopropyl) amiloride), 100 µM), were injected into the chambers using gas tight syringes (Hamilton, Switzerland) in order to determine metabolic cost of respective ion regulators. All drugs were dissolved in DMSO and applied in the lowest effective concentration to avoid unspecific inhibition (Wieser and Krumschnabel, 2001). Final DMSO concentration did not exceed 0.2% (v/v) to avoid impact on tissue respiration (Stapp et al., 2016). After each run, tissue pieces were dried (80 °C, 24h) and weighed on a fine scale and oxygen consumption rates calculated as nmol O_2 per mg tissue dry weight per min (nmol O_2 mg dw^{-1} min^{-1}). Metabolic costs of Na^+/K^+-ATPase, H^+-ATPase and Na^+/H^+-exchanger are presented as net and fractional oxygen demand. The latter was calculated as the fraction of total tissue oxygen consumption inhibited by the respective inhibitor, and net oxygen by subtracting the inhibited oxygen consumption from total oxygen consumption rates of the respective tissue piece.

2.6. Statistics

All statistical analyses were performed using the R software version 3.2.3 (R Development Core Team 2015). At first, linear mixed effect models (LMMs) with 'oyster type' and 'seawater PCO_2' as fixed factors and 'tank' as random factor nested within fixed factors was used to determine significant effects on condition index, extracellular acid-base variables, metabolic rates of gill and mantle tissue as well as energy demand of ion-transporters using the LMERTEST package. As no significant 'tank' effect ($P > 0.1$) was detected for any measured parameter (RAND function of LMERTEST), the random factor was removed from analysis and data analysed using linear models (CAR package) with the fixed factors 'oyster type' and 'seawater PCO_2'. For multiple comparisons of means, *post hoc* analysis was performed after Tukey. All data were checked for normality of residuals (Shapiro-Wilk test) and homogeneity of variances (Levene's test). $P \leq 0.05$ was accepted to indicate significant differences. If not stated otherwise, all data are presented as mean ± SEM.

3. Results

3.1. Condition index

The condition indices of oysters were not significantly affected by the 7 weeks of experimental exposure. Furthermore, there were no significant differences between the condition indices of wild and selected oysters as well as between the control and elevated seawater PCO_2 treatments (Table 2).

Table 2
Condition index of wild and selected oysters (*Saccostrea glomerata*) before the start of the experiment and after the 7-week exposure to control or elevated seawater PCO_2. Values are given as mean ± SEM, n = 9–12.

Oyster type	Condition index		
	Pre-exposure	Control	CO_2
Wild	4.37 ± 0.23	4.01 ± 0.26	4.88 ± 0.40
Selected	4.03 ± 0.22	3.80 ± 0.27	4.10 ± 0.37

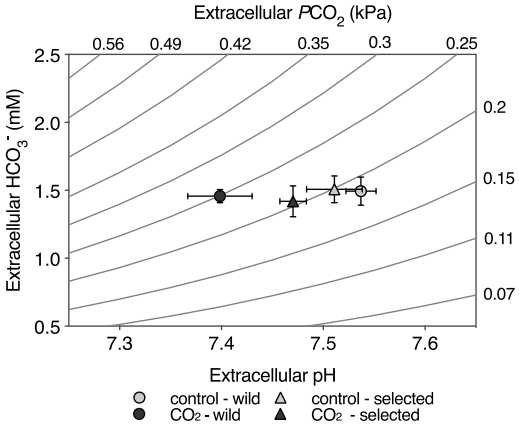

Fig. 2. Davenport diagram showing the relationship of extracellular pH, PCO_2 and $[HCO_3^-]$ of wild and selected oysters (*Saccostrea glomerata*) after 7-week exposure to control or elevated seawater PCO_2. Curved lines represent the PCO_2 isopleths calculated for the different pH and $[HCO_3^-]$. Values are given as mean ± SEM, n = 9–12.

3.2. Extracellular acid-base status

The extracellular acid-base status of wild and selected oysters after the 7-week exposure to control or elevated seawater PCO_2 is presented in Figs. 1 and 2. There was a significant effect of 'seawater PCO_2' (Fig. 1a, ANOVA: $F_{1,39} = 15.825$, $P < 0.001$) on extracellular pH as well as a significant interaction between 'seawater PCO_2' and 'oyster type' (Fig. 1a, ANOVA: $F_{1,39} = 4.110$, $P < 0.05$). The pH_e of wild and selected oysters was similar in the control treatment. The pH_e of wild oysters, however, was significantly reduced (Tukey's HSD: $P < 0.001$) under elevated seawater CO_2, while selected oysters showed no significant reduction of their pH_e (Fig. 1a). Similarly, there was a significant 'seawater PCO_2' effect on the extracellular P_eCO_2 (Fig. 1b, ANOVA: $F_{1,39} = 4.691$, $P < 0.05$), however, the effect differed between oyster populations. Hemolymph P_eCO_2 of wild oysters rose from 0.18 kPa under control (seawater PCO_2 of ~0.055 kPa) to 0.26 kPa under high CO_2 conditions (seawater PCO_2 of ~0.117 kPa) (Fig. 1b, Tukey's HSD: $P < 0.05$), but it remained unaffected in selected oysters. Irrespective of seawater PCO_2, extracellular bicarbonate concentrations were similar between oyster populations and mean values ranged between 1.41 and 1.51 mM (Fig. 1c). When displayed in a Davenport diagram (Fig. 2), it becomes more clear that the differing pH_e values of wild and selected oysters after high CO_2 exposure are not related to an active accumulation of $[HCO_3^-]$ in selected oysters, but due to an increased P_eCO_2 in wild oysters under elevated seawater PCO_2.

3.3. Metabolic rates of isolated gill and mantle tissue

'Seawater PCO_2' significantly affected oxygen consumption of mantle tissue (Fig. 3a, ANOVA: $F_{1,40} = 7.106$, $P < 0.05$). At control conditions, mantle respiration was similar between wild and selected oysters, but the response to elevated CO_2 differed between populations. Under high CO_2, wild oysters showed a significant 30% increase in mantle respiration (Tukey's HSD: $P < 0.05$), whereas mantle respiration of selected oysters was only slightly (11%) but non-significantly increased.

In contrast to mantle respiration, there was no effect of 'seawater PCO_2' on gill respiration but a significant 'oyster type' effect (ANOVA: $F_{1,42} = 6.591$, $P < 0.05$). Irrespective of seawater PCO_2, gill respiration of selected oysters was around 20% lower compared to wild oysters (Fig. 3b).

3.4. Energy demand of major ion-regulators in isolated gill and mantle tissue

Net O_2 demand of Na^+/K^+-ATPase and H^+-ATPase in gill and mantle tissue (Table 3) and their fractional respiratory contribution to the respective total tissue respiration (Fig. 4) was neither affected by 'oyster type' nor by 'seawater PCO_2'. Fractional oxygen consumption of the two ATPases was similar for both tissues and oyster types ranging between 12 and 18% in gill and 13–18% in mantle for Na^+/K^+-ATPase, and between 10 and 15% in gill and 9–14% in mantle for H^+-ATPase. Similarly, in mantle tissue the net and fractional O_2 demand of Na^+/H^+-exchanger was not affected by 'oyster type' or 'seawater PCO_2' (Table 3, Fig. 3). By contrast, there was an overall significant effect of 'seawater PCO_2' on the fractional contribution of Na^+/H^+-exchanger to total gill respiration (ANOVA: $F_{1,31} = 5.266$, $P < 0.05$). In both oyster types, the fractional contribution of Na^+/H^+-exchanger to total gill respiration increased by around 12% (Fig. 3b). However, only marginally significant differences were detected in pairwise comparison (Tukey's HSD: $P = 0.054$). In contrast, net oxygen demand for Na^+/H^+-exchanger was affected by both 'seawater PCO_2' (Table 3, ANOVA: $F_{1,31} = 4.528$, $P < 0.05$) and 'oyster type' (Table 3, ANOVA: $F_{1,31} = 5.683$, $P < 0.05$). Acclimation to elevated CO_2 increased net oxygen demand for Na^+/H^+-exchanger by 57% in wild and by 81% in selected oysters (Table 3). Overall, the mean net oxygen demand for Na^+/H^+-exchanger was higher in wild than selected oysters (Table 3).

4. Discussion

This study aimed to shed light on the physiological mechanisms that contribute to the higher CO_2 resilience of Sydney rock oysters (*Saccostrea glomerata*) that were selectively bred for increased growth and disease resistance over 7 generations in comparison to the more CO_2 sensitive wild population.

4.1. Effect of elevated CO_2 on extracellular acid base variables of wild vs. selected S. glomerata

The 7-week exposure to elevated CO_2 led to a significant extracellular acidosis in the wild but not in selected oysters. This supports our first hypothesis that selected oysters have a higher capacity to regulate their extracellular acid-base status and the proposed concept that a high capacity to buffer extracellular acid base parameters against environmental hypercapnia positively correlates with CO_2 resilience in marine ectotherms (Melzner et al., 2009; Pörtner, 2008; Widdicombe and Spicer, 2008).

In marine ectotherms, the release rate of metabolic CO_2 is directly proportional to the outward PCO_2 gradient between the extracellular fluid and the surrounding seawater; thus, an increased P_eCO_2 in body fluids is required if diffusive gradients are to be maintained in an elevated PCO_2 environment ($\Delta P_eCO_2/PCO_2$). Hemolymph P_eCO_2 of wild oysters increased under elevated seawater PCO_2 thereby maintaining a $\Delta P_eCO_2/PCO_2$ similar to control conditions ($\Delta P_eCO_2/PCO_2$: 0.13 kPa (control PCO_2) vs. 0.14 kPa (high PCO_2)). The resulting extracellular acidosis remained chronically uncompensated, which is in line with most previous studies addressing the effects of OA on the acid base physiology of marine bivalves (Lannig et al., 2010; Schalkhausser et al.,

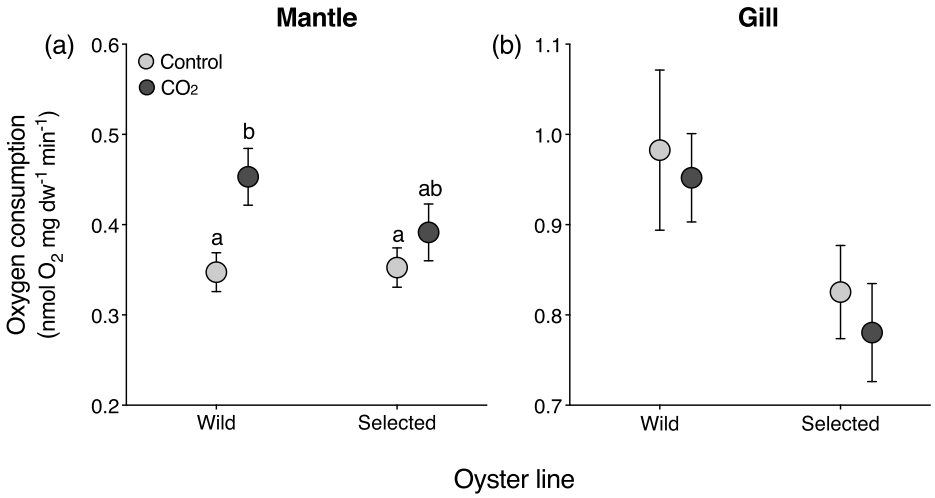

Fig. 3. Oxygen consumption of isolated mantle (a) and gill (b) tissue of wild and selected oysters (*Saccostrea glomerata*) after 7-week exposure to control or elevated seawater PCO_2. Different *letters* indicate significant differences between groups detected by *post hoc* analysis. Values are given as mean ± SEM, n = 11–12.

Table 3
Net O_2 demand of Na^+/K^+-ATPase, Na^+/H^+-exchanger and H^+-ATPase in mantle and gill tissue of wild and selected oysters (*Saccostrea glomerata*) exposed for 7 weeks to control or elevated seawater PCO_2. Different letters indicate significant differences between groups detected by *post hoc* analysis. Values are given as mean ± SEM, n = 6–12.

Tissue	Oyster type	Treatment	Net O_2 demand (nmol O_2 mg dw^{-1} min^{-1})		
			Na^+/K^+-ATPase	Na^+/H^+-exchanger	H^+-ATPase
Mantle	Wild	Control	0.082 ± 0.02	0.100 ± 0.07	0.056 ± 0.04
		CO_2	0.092 ± 0.03	0.070 ± 0.02	0.060 ± 0.01
	Selected	Control	0.045 ± 0.01	0.055 ± 0.01	0.047 ± 0.01
		CO_2	0.063 ± 0.01	0.065 ± 0.02	0.043 ± 0.01
Gill	Wild	Control	0.190 ± 0.06	0.228 ± 0.06 ^{ab}	0.149 ± 0.05
		CO_2	0.139 ± 0.03	0.360 ± 0.07 ^{b}	0.101 ± 0.03
	Selected	Control	0.167 ± 0.03	0.106 ± 0.03 ^{a}	0.147 ± 0.04
		CO_2	0.116 ± 0.03	0.192 ± 0.04 ^{ab}	0.131 ± 0.05

2013; Thomsen et al., 2010), including wild *Saccostrea glomerata* (Parker et al., 2015; Scanes et al., 2017). Interestingly, in selected oysters hemolymph P_eCO_2 was unaffected by elevated PCO_2 resulting in a 40% reduction in the outward PCO_2 gradient compared to control conditions (Δ P_eCO_2 0.15 kPa (control) vs. 0.09 kPa (high PCO_2)). As hemolymph samples were measured at random on the same day, with the same instrument, methodological flaws can be excluded. Our data, thus, indicate that selected oysters seem to possess a higher or more efficient machinery to eliminate metabolic CO_2, thereby circumventing CO_2 induced extracellular acidosis and potential negative downstream effects.

In water breathers, ventilatory CO_2 release is thought to play a minor role due to a relatively small PCO_2 diffusion gradient compared to air breathers. Indeed, active bicarbonate buffering is the prevailing mechanism to compensate for hypercapnia related extracellular acid-base disturbance in most higher marine ectotherms, such as fish or crustaceans (for review see Melzner et al., 2009). In bivalves, ventilation is achieved by the water pumping activity of the gills. The same water current is utilised for filter-feeding; thus, ventilation and filtration are coupled processes in bivalves. Bayne and co-workers (Bayne, 2000; Bayne et al., 1999) investigated the physiological basis for the faster growth of the selected compared to the wild oyster line from the Port Stephens breeding programme. They showed that their faster growth is associated with higher filtration rates found in selected compared to wild oysters (Bayne, 2000; Bayne et al., 1999). It is well known that filtration rates in marine bivalve scale with gill surface area (Honkoop et al., 2003; Meyhöfer, 1985; Pouvreau et al., 1999; Riisgård, 1988). Surface area in turn is proportional to diffusion rates (Fick, 1855), potentially explaining the increased capacity of selected oysters to diffusively eliminate metabolic CO_2. Furthermore, an elevated filtration rate increases ventilation of the large mantle surface, which is known to contribute significantly to gas exchange (Shumway, 1982).

Maintaining a large ciliary gill pump is, however, associated with considerable energetic costs (Honkoop et al., 2003). Gill respiration was around twice as high as those of mantle tissue (see Fig. 3) and recent studies in blue mussel (*Mytilus edulis*) show that gill respiration can take up around 20% of the whole animal oxygen demand (Riisgård et al., 2015; Stapp et al., 2016). Due to this high energetic demand oysters are known to adjust their gill and palp size in response to variations of food quality and availability (Barille et al., 2000; Honkoop et al., 2003). Selected oysters seem to be able to lower the energetic trade off of sustaining large gills. Bayne et al. (1999) showed that filtration rates were not only higher in selected oysters, but also more metabolically efficient than in wild oysters; they respired only around 0.24 J for every joule ingested compared to 0.45 J in wild oysters. Our data suggest that this metabolic efficiency of filtration may be based on decreased metabolic maintenance costs of the gill tissue as gill oxygen consumption of selected oysters was around 20% lower compared to wild oysters.

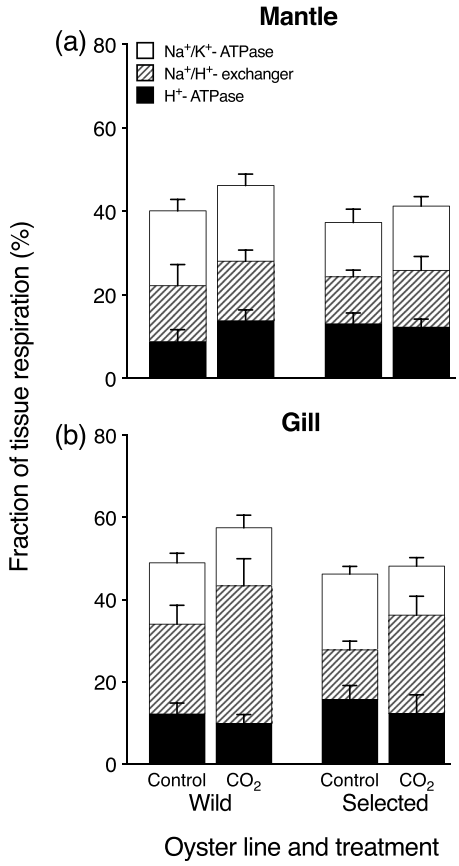

Fig. 4. Fractional contribution of Na^+/K^+-ATPase, Na^+/H^+-exchanger and H^+-ATPase to total tissue respiration of isolated mantle (a) and gill (b) tissue of wild and selected oysters (*Saccostrea glomerata*) after 7-week exposure to control or elevated seawater PCO_2. Values are given as mean ± SEM, n = 6–12.

Higher filtration rates in combination with lower metabolic maintenance costs have been also identified as the physiological basis for different growth rates between fast and slow growing spat of the Pacific oyster (*Crassostrea gigas*) (Tamayo et al., 2014).

The mechanistic basis of reduced metabolic maintenance costs in gills of selected versus wild *S. glomerata* remains unclear but could be related to an increased efficiency in protein metabolism; Bayne (2000) showed that selected Sydney rock oysters have a reduced protein turnover lowering their metabolic costs for growth. Differences related to protein metabolism were also found between fast and slow growers of larvae of *C. gigas* (Hedgecock et al., 2007; Meyer and Manahan, 2010) as well as blue mussels (Hawkins et al., 1986). Protein synthesis was shown to account for up to 23% of total ATP turn over in isolated gill cells of the eastern oyster (*Crassostrea virginica*) (Cherkasov et al., 2006), thus, energy savings related to protein deposition are likely to be reflected in reduced tissue metabolic rates.

Although further studies are necessary to unravel the mechanistic basis for lower metabolic rates in gills of selected oysters, the findings of this and the earlier studies of Bayne et al. (Bayne, 2000; Bayne et al., 1999) clearly show that selected oysters are able to filter at a higher rate and energetic efficiency than wild oysters. When exposed to elevated CO_2, the filtration apparatus seems to enable selected oysters to maintain their extracellular P_eCO_2, and, thus pH_e, by means of ventilatory adjustments.

4.2. Effect of elevated CO_2 on metabolic and ion-regulatory cost in isolated tissue of wild vs. selected S. glomerata

The findings do not support our second hypothesis that the higher pH_e-regulatory capacity of selected oysters is based on increased epithelial rates of active proton equivalent ion exchange. At both seawater PCO_2 levels, selected and wild oysters showed unchanged levels of hemolymph bicarbonate ions of around 1.5 mM (see Davenport diagram, Fig. 2), which agrees with values previously reported for Sydney rock oysters (Scanes et al., 2017). Increased rates and costs of active proton equivalent ion exchange in marine bivalves under high PCO_2 are suggested to be caused by an increased proton flux into the intracellular space caused by the uncompensated extracellular acidosis (Boron, 2004; Melzner et al., 2009; Pörtner, 2008). Stable pH_e in selected oysters thus prevented a CO_2 induced intracellular acid-load. In line with this, fractional and net metabolic costs of two major ion regulatory and ATP consuming pumps, the Na^+/K^+-ATPase and V-type H^+-ATPase remained constant in gill and mantle tissue of selected oysters at both PCO_2 level. Similarly, we observed no altered overall oxygen demand in both tissues (Fig. 3). In selected oysters, only the net and fractional O_2 demand for Na^+/H^+ exchange in gills responded with an increased O_2 demand under elevated CO_2. This might be due to a lowered seawater pH, potentially resulting in upregulation of apical acid extruding mechanisms in order to maintain intracellular pH in the gill epithelium. The Na^+/H^+-exchanger is a secondary active ion transporter that is energetically coupled to the Na^+-gradient maintained by the Na^+/K^+-ATPase. Nevertheless, oxygen demand for Na^+/K^+-ATPase remained unchanged suggesting that the higher energy demand of Na^+/H^+-exchange did not translate into a measurable increase in metabolic costs of gill tissue in selected oysters.

Despite their extracellular acidosis, ion-regulatory costs in gill and mantle tissue of wild oysters were similar to that of selected oysters when exposed to elevated seawater PCO_2 (Fig. 4). Again, only the branchial metabolic costs for Na^+/H^+ antiporter increased under elevated seawater PCO_2. The higher PCO_2 initiated a 12% rise in the fractional contribution of Na^+/H^+-exchange to gill metabolic in both oyster types. However, the overall net oxygen demand for Na^+/H^+ antiport was around 2-times higher in gills of wild oysters matching their overall higher oxygen demand per gram gill tissue compared to selected oysters. This finding might reflect the need for a higher proton excretion due to their lower ventilatory capacities for CO_2 release in wild than in selected *S. glomerata*. Nevertheless, as for the selected oysters, the increased net and fractional demand of Na^+/H^+ exchange at elevated CO_2 did not translate into a significant rise in branchial Na^+/K^+-ATPase and total oxygen demand above control levels. Thus, oysters might be able to modulate ion transporters in response to hypercapnia without significantly affecting overall ATP turnover.

Unlike in gill tissue, CO_2 exposure led to a 30% rise in oxygen demand of mantle tissue of wild oysters, which correlates with their extracellular acidosis during CO_2 exposure. However, as outlined above, this increased energy demand seems to be unrelated to an increased energy demand of major ion-transporters investigated in the present study. The oyster mantle is a multifunctional tissue involved in various physiological processes such as gas exchange (Shumway, 1982) storage metabolism (Berthelin et al., 2000), immune defence (Ertl et al., 2016) or feeding; however, its main function lies in the formation of the shell (Gosling, 2003). Although the exact mechanisms of biomineralisation in bivalves are not yet fully understood and may also involve hemocytes (Mount et al., 2004), it is obvious that CO_2-driven changes in carbonate chemistry challenge calcification processes (Orr et al., 2005). In adult oysters and mussels, the shell consists of a mix of calcite and aragonite and is covered by an organic proteinaceous layer, the periostracum. This uppermost organic layer has been demonstrated to be fairly chemical resistant (Waite, 1983) and may be the reason why calcifiers are able to thrive and protect their shells from external dissolution during corrosive seawater conditions (Tambutté et al., 2015; Thomsen et al.,

2010; Tunnicliffe et al., 2009). While the periostracum might aid to protect against external shell dissolution under elevated seawater PCO_2, it has been shown that the even higher hemolymph P_eCO_2 levels and associated lower pH_e and carbonate concentrations that occur in during CO_2 exposure led to an increased inner shell dissolution in *M. edulis* (Melzner et al., 2011). The extent of the internal shell dissolution was thereby dependent on the animals' food ration (i.e. low food = more shell corrosion) showing that mussels are able to dynamically allocate energy to shell conservation depending on their energetic status (Melzner et al., 2011). Throughout our experiment, all oysters were fed twice a day, showed new shell growth at the fringe (personal observation) and no reduction in their condition index among all treatments (Table 2). Thus, the higher mantle respiration of wild compared to selected oysters could be the result of an increased energy allocation to shell conservation as a result of the extracellular acidosis that wild, but not selected oysters, experienced during CO_2 exposure.

Increased mantle metabolic rates correlate with an increased whole animal metabolic rate of wild oysters under elevated CO_2 found in the previous studies of Parker et al. (2015, 2012). However, acclimation to high CO_2 resulted in an even stronger elevation of whole animal metabolic rate in selected oysters (Parker et al., 2015, 2012), which contrasts with unchanged tissue respiration for selected oysters in this study. This could be due to a CO_2-driven increased energy demand of an organ (e.g. hepatopancreas, muscle tissue) or organ system (e.g. circulatory system) other than gills or the pallial mantle. In the study by Parker et al. (2012), metabolic rates of selected oysters under present day PCO_2 level were also found to be higher than those of wild oysters. This is in contrast to results by Bayne (2000), who found that oxygen consumption rates of selected oysters were lower than of wild oysters across a range of three different food rations, which correlates with our results for gill tissue (see discussion above). When comparing these results, it should be noted that, although oysters were supplied from the same breeding programme and had approximately the same age, Bayne (2000) used selected oysters of the fourth generation, whereas Parker et al. (2012) used oysters selected that were selected for 7 generations. The ongoing selection could have acted on metabolic rates. Furthermore, it should be noted that Parker et al. (2012) measured metabolic rates of gravid oysters, as their oysters had been conditioned for 5 weeks for a subsequent larval experiment. Honkoop (2003) quantified the average metabolic costs of producing and maintaining reproductive versus somatic tissue in Sydney rock oysters. The study (Honkoop, 2003), showed that the relative oxygen consumption (per gram tissue) for maintaining gonadal tissue is on average 16% less than for somatic tissue due to the fact that gonads mainly consist of storage materials (i.e. lipids), which have very low maintenance costs (Kooijman, 2010). Interestingly, it is known that the growth selection of the Port Stephens Sydney rock oysters led to a reduced gonadal area in selected compared to wild oysters (Dove and O'Connor, 2012), caused by the increased protein growth in selected oysters at the expense of lipid storage (i.e. gametogenesis) (see Bayne, 2000; Bayne, 2004). Thus, the higher metabolic rates per gram tissue dry mass in selected oysters found by Parker et al. (2012) at control conditions might be due to a lower proportion of gonadal to somatic tissue in selected oysters, a proportion which may potentially be even lower under elevated CO_2. Strip spawning did not allow for a determination of fecundity in the study of Parker et al. (2012). In wild oysters, egg size and lipid content were unaffected when adults were exposed to CO_2 during gametogenesis (Parker et al., 2017). Whether this is the case for total gonad size as well as for the selected oysters remains to be explored.

5. Conclusion

The 7-week exposure to elevated PCO_2 led to an increased hemolymph P_eCO_2 and associated drop in pH_e in wild Sydney rock oysters, *S. glomerata* but not in oysters selectively bred for faster growth and disease resistance over 7 generations. Thus, the physiological basis of the higher CO_2 resilience of the selected compared to the wild oysters' population reported in previous studies (Parker et al., 2010, 2012) seems to be correlated with an increased ability for systemic CO_2 release likely brought about by higher and energetically more efficient filtration rates found in selected oysters. This supports the concept that a high capacity to defend extracellular acid-base variables against CO_2-induced perturbations plays a pivotal role in an organisms' resilience to ocean acidification. Furthermore, the results support earlier findings that sessile marine bivalves seem to be unable to compensate for CO_2-induced extracellular respiratory acidosis through active extracellular bicarbonate accumulation. Systemic (i.e. ventilatory) CO_2 release may be, however, an alternative mechanism to cope with CO_2 induced acid-base disturbances in putatively sensitive sessile bivalves; although future studies have to reveal whether this mechanism is effective at seawater PCO_2 surpassing the PCO_2 level applied in the present study. Nevertheless, the higher CO_2 resilience of selected oysters was achieved within the – in evolutionary terms – rapid time span of only a few generations. Due to the rapid rate of atmospheric CO_2 increase, adaptation to climate change is likely to rely on selection of pre-existing standing genetic variation within natural populations (Barrett and Schluter, 2008). Given the fact that along the North American Pacific coast oyster hatcheries already experience higher larval mortalities primarily caused by upwelling of deeper acidified water masses (Barton et al., 2012; Feely et al., 2008), insights into physiological traits of CO_2 resilience could inform human-assisted selection of adaptive traits for ocean acidification resilience. However, as global change is not limited to ocean acidification, potential resilience traits to a single environmental driver must be reassessed in the light of multiple environmental drivers (see Parker et al., 2017).

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References

Applebaum, S.L., Pan, T.C.F., Hedgecock, D., Manahan, D.T., 2014. Separating the nature and nurture of the allocation of energy in response to global change. Integr. Comp. Biol. 54, 284–295. http://dx.doi.org/10.1093/icb/icu062.

Barille, L., Haure, J., Cognie, B., Leroy, A., 2000. Variations in pallial organs and eulatero-frontal cirri in response to high particulate matter concentrations in the oyster *Crassostrea gigas*. Can. J. Fish. Aquat. Sci. 57, 837–843. http://dx.doi.org/10.1139/cjfas-57-4-837.

Barrett, R., Schluter, D., 2008. Adaptation from standing genetic variation. Trends Ecol. Evol. 23, 38–44. http://dx.doi.org/10.1016/j.tree.2007.09.008.

Barton, A., Hales, B., Waldbusser, G.G., Langdon, C., Feely, R.A., 2012. The Pacific oyster, *Crassostrea gigas*, shows negative correlation to naturally elevated carbon dioxide levels: implications for near-term ocean acidification effects. Limnol. Oceanogr. 57, 698–710. http://dx.doi.org/10.4319/lo.2012.57.3.0698.

Baumann, H., Talmage, S.C., Gobler, C.J., 2012. Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. Nat. Clim. Change 2, 38–41.

http://dx.doi.org/10.1038/nclimate1291.
Bayne, B.L., Svensson, S., Nell, J.A., 1999. The physiological basis for faster growth in the Sydney rock oyster, *Saccostrea commercialis*. Biol. Bull. 197, 377–387. http://dx.doi.org/10.2307/1542792.
Bayne, B., 2000. Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). J. Exp. Mar. Biol. Ecol. 251, 185–203. http://dx.doi.org/10.1016/S0022-0981(00)00211-2.
Bayne, B.L., 2004. Phenotypic flexibility and physiological tradeoffs in the feeding and growth of marine bivalve molluscs. Integr. Comp. Biol. 44, 425–432. http://dx.doi.org/10.1093/icb/44.6.425.
Berthelin, C., Kellner, K., Mathieu, M., 2000. Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France). Comp. Biochem. Physiol. B. 125, 359–369. http://dx.doi.org/10.1016/S0305-0491(99)00187-X.
Boron, W.F., 2004. Regulation of intracellular pH. Adv. Physiol. Educ. 28, 160–179. http://dx.doi.org/10.1152/advan.00045.2004.
Calosi, P., Rastrick, S.P.S., Graziano, M., Thomas, S.C., Baggini, C., Carter, H.A., Hall-Spencer, J.M., Milazzo, M., Spicer, J.I., 2013. Distribution of sea urchins living near shallow water CO_2 vents is dependent upon species acid-base and ion-regulatory abilities. Mar. Pollut. Bull. 73, 470–484. http://dx.doi.org/10.1016/j.marpolbul.2012.11.040.
Cao, L., Caldeira, K., 2008. Atmospheric CO_2 stabilization and ocean acidification. Geophys. Res. Lett. 35, L19609. http://dx.doi.org/10.1029/2008GL035072.
Cherkasov, A.S., Biswas, P.K., Ridings, D.M., Ringwood, A.H., Sokolova, I.M., 2006. Effects of acclimation temperature and cadmium exposure on cellular energy budgets in the marine mollusk *Crassostrea virginica*: linking cellular and mitochondrial responses. J. Exp. Biol. 209, 1274–1284. http://dx.doi.org/10.1242/jeb.02093.
Cole, V.J., Parker, L.M., O'Connor, S.J., O'Connor, W.A., Scanes, E., Byrne, M., Ross, P.M., 2016. Effects of multiple climate change stressors: ocean acidification interacts with warming, hyposalinity, and low food supply on the larvae of the brooding flat oyster *Ostrea angasi*. Mar. Biol. 163, 125. http://dx.doi.org/10.1007/s00227-016-2880-4.
Collins, M., Knutti, R., Arblaster, J., Dufresne, J.-L., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W.J., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A.J., Wehner, M., 2013. Long-term climate change: projections, commitments and irreversibility. In: Stocker, T.F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.), 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, United Kingdom and New York, NY, USA.
Dahlke, F.T., Leo, E., Mark, F.C., Pörtner, H.-O., Bickmeyer, U., Frickenhaus, S., Storch, D., 2016. Effects of ocean acidification increase embryonic sensitivity to thermal extremes in Atlantic cod, *Gadus morhua*. Global Change Biol. 23, 1499–1510. http://dx.doi.org/10.1111/gcb.13527.
Dickinson, G.H., Ivanina, A.V., Matoo, O.B., Pörtner, H.O., Lannig, G., Bock, C., Beniash, E., Sokolova, I.M., 2012. Interactive effects of salinity and elevated CO_2 levels on juvenile eastern oysters, *Crassostrea virginica*. J. Exp. Biol. 215, 29–43. http://dx.doi.org/10.1242/jeb.061481.
Dickson, A.G., Millero, F.J., 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep-Sea Res. Pt. I 34, 1733–1743. http://dx.doi.org/10.1016/0198-0149(87)90021-5.
Dickson, A.G., Sabine, C.L., Christian, J.R., 2007. Guide to Best Practices for Ocean CO_2 Measurements. PICES Special Publications 3http://dx.doi.org/10.1007/978-1-4302-6491-0_26.
Dorey, N., Lançon, P., Thorndyke, M., Dupont, S., 2013. Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. Global Change Biol. 19, 3355–3367. http://dx.doi.org/10.1111/gcb.12276.
Dove, M.C., O'Connor, W.A., 2012. Reproductive cycle of Sydney rock oysters, *Saccostrea glomerata* (Gould 1850) selectively bred for faster growth. Aquaculture 324–325, 218–225. http://dx.doi.org/10.1016/j.aquaculture.2011.10.022.
Dupont, S., Dorey, N., Stumpp, M., Melzner, F., Thorndyke, M., 2012. Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar. Biol. 160, 1835–1843. http://dx.doi.org/10.1007/s00227-012-1921-x.
Eriander, L., Wrange, A.L., Havenhand, J.N., 2016. Simulated diurnal pH fluctuations radically increase variance in—but not the mean of—growth in the barnacle *Balanus improvisus*. ICES J. Mar. Sci. 73, 596–603. http://dx.doi.org/10.1093/icesjms/fsv214.
Ertl, N.G., O'Connor, W.A., Wiegand, A.N., Elizur, A., 2016. Molecular analysis of the Sydney rock oyster (*Saccostrea glomerata*) CO_2 stress response. Climate Change Responses 3, 6. http://dx.doi.org/10.1186/s40665-016-0019-y.
Esbaugh, A.J., Heuer, R., Grosell, M., 2012. Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. J. Comp. Physiol. B 182, 921–934. http://dx.doi.org/10.1007/s00360-012-0668-5.
Feely, R.A., Sabine, C.L., Hernandez-Ayon, J.M., Ianson, D., Hales, B., 2008. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science 320, 1490–1492. http://dx.doi.org/10.1126/science.1155676.
Fick, A., 1855. Ueber diffusion. Ann. Phys. 170, 59–86. http://dx.doi.org/10.1002/andp.18551700105.
Form, A.U., Riebesell, U., 2012. Acclimation to ocean acidification during long-term CO_2 exposure in the cold-water coral *Lophelia pertusa*. Global Change Biol. 18, 843–853. http://dx.doi.org/10.1111/j.1365-2486.2011.02583.x.
Gosling, E., 2003. Bivalve Molluscs: Biology, Ecology and Culture. Blackwell Publishing Ltd, Oxford.
Gutowska, M.A., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G., Pörtner, H.O., 2010. Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. J. Comp. Physiol. B 180, 323–335. http://dx.doi.org/10.1007/s00360-009-0412-y.
Hammer, K.M., Kristiansen, E., Zachariassen, K.E., 2011. Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae). Mar. Environ. Res. 72, 135–142. http://dx.doi.org/10.1016/j.marenvres.2011.07.002.
Harvey, B.P., Gwynn Jones, D., Moore, P.J., 2013. Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. Ecol. Evol. 3, 1016–1030. http://dx.doi.org/10.1002/ece3.516.
Hawkins, A.J.S., Bayne, B.L., Day, A.J., 1986. Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: the basis of variable age-specific growth. Proc. R. Soc. B 229, 161–176. http://dx.doi.org/10.1098/rspb.1986.008.
Hedgecock, D., Lin, J.-Z., DeCola, S., Haudenschild, C.D., Meyer, E., Manahan, D.T., Bowen, Ben, 2007. Transcriptomic analysis of growth heterosis in larval Pacific oysters (*Crassostrea gigas*). Proc. Natl. Acad. Sci. USA 104, 2313–2318. http://dx.doi.org/10.1073/pnas.0610880104.
Heisler, N., 1986. Buffering and transmembrane ion transfer processes. In: Heisler, N. (Ed.), Acid-base Regulation in Animals. Elsevier, Amsterdam, pp. 3–47.
Hettinger, A., Sanford, E., Hill, T.M., Hosfelt, J.D., Russell, A.D., Gaylord, B., 2013. The influence of food supply on the response of Olympia oyster larvae to ocean acidification. Biogeosciences 10, 6629–6638. http://dx.doi.org/10.5194/bg-10-6629-2013.
Honkoop, P.J.C., 2003. Physiological costs of reproduction in the Sydney rock oyster *Saccostrea glomerata*. How expensive is reproduction? Oecologia 135, 176–183. http://dx.doi.org/10.1007/s00442-002-1172-5.
Honkoop, P.J.C., Bayne, B.L., Drent, J., 2003. Flexibility of size of gills and palps in the Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) and the Pacific oyster *Crassostrea gigas* (Thunberg, 1793). J. Exp. Mar. Biol. Ecol. 282, 113–133. http://dx.doi.org/10.1016/S0022-0981(02)00463-X.
Hu, M.Y., Guh, Y.-J., Stumpp, M., Lee, J.-R., Chen, R.-D., Sung, P.-H., Chen, Y.-C., Hwang, P.-P., Tseng, Y.-C., 2014. Branchial NH_4^+-dependent acid–base transport mechanisms and energy metabolism of squid (*Sepioteuthis lessoniana*) affected by seawater acidification. Front. Zool. 11, 55. http://dx.doi.org/10.1186/s12983-014-0055-z.
Ivanina, A.V., Dickinson, G.H., Matoo, O.B., Bagwe, R., Dickinson, A., Beniash, E., Sokolova, I.M., 2013. Interactive effects of elevated temperature and CO_2 levels on energy metabolism and biomineralization of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. Comp. Biochem. Physiol. 166, 101–111. http://dx.doi.org/10.1016/j.cbpa.2013.05.016.
Kelly, M.W., Padilla-Gamiño, J.L., Hofmann, G.E., 2013. Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus purpuratus*. Global Change Biol. 19, 2536–2546. http://dx.doi.org/10.1111/gcb.12251.
Kooijman, S.A.L.M., 2010. Dynamic Energy Budgets in Biological Systems, first ed. Cambridge University Press, New York.
Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M., Gattuso, J.-P., 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. Global Change Biol. 19, 1884–1896. http://dx.doi.org/10.1111/gcb.12179.
Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M., Bock, C., 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. Mar. Drugs 8, 2318–2339. http://dx.doi.org/10.3390/md8082318.
Larsen, B.K., Pörtner, H.O., Jensen, F.B., 1997. Extra-and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. Mar. Biol. 128, 337–346.
Lawrence, D.R., Scott, G.I., 1982. The determination and use of condition index of oysters. Estuaries 5, 23–27. http://dx.doi.org/10.2307/1352213.
Lewis, E., Wallace, D., 1998. Program Developed for CO_2 System Calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory. US Dep. of Energy, Oak Ridge, TN.
Mehrbach, C., Culberso, C.H., Hawley, J.E., Pytkowic, R.M., 1973. Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol. Oceanogr. 18, 897–907.
Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313–2331. http://dx.doi.org/10.5194/bg-6-2313-2009.
Melzner, F., Stange, P., Trübenbach, K., Thomsen, J., Casties, I., Panknin, U., Gorb, S.N., Gutowska, M.A., 2011. Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. PLos One 6, e24223. http://dx.doi.org/10.1371/journal.pone.0024223.t003.
Meyer, E., Manahan, D.T., 2010. Gene expression profiling of genetically determined growth variation in bivalve larvae (*Crassostrea gigas*). J. Exp. Biol. 213, 749–758. http://dx.doi.org/10.1242/jeb.037242.
Meyhöfer, E., 1985. Comparative pumping rates in suspension-feeding bivalves. Mar. Biol. 85, 137–142. http://dx.doi.org/10.1007/BF00397432.
Michaelidis, B., Ouzounis, C., Paleras, A., Pörtner, H.O., 2005. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 293, 109–118. http://dx.doi.org/10.3354/meps293109.
Miles, H., Widdicombe, S., Spicer, J.I., Hall-Spencer, J., 2007. Effects of anthropogenic seawater acidification on acid–base balance in the sea urchin *Psammechinus miliaris*. Mar. Pollut. Bull. 54, 89–96. http://dx.doi.org/10.1016/j.marpolbul.2006.09.021.
Miller, G.M., Watson, S.-A., Donelson, J.M., McCormick, M.I., Munday, P.L., 2012. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nature Clim. Change 2, 858–861. http://dx.doi.org/10.1038/nclimate1599.
Mount, A.S., Wheeler, A.P., Paradkar, R.P., Snider, D., 2004. Hemocyte-mediated shell mineralization in the eastern oyster. Science 304, 297–300. http://dx.doi.org/10.

1126/science.1090506.
Nell, J.A., Dunkley, P.R., 1984. Effects of temperature, nutritional factors and salinity on the uptake of L-methionine by the Sydney rock oyster *Saccostrea commercialis*. Mar. Biol. 80, 335–339. http://dx.doi.org/10.1007/BF00392829.
Nell, J.A., Smith, I.R., McPhee, C.C., 2000. The Sydney rock oyster *Saccostrea glomerata* (Gould 1850) breeding programme: progress and goals. Aquacult. Res. 31, 45–49.
O'Connor, W.A., Dove, M.C., Finn, B., O'Connor, S.J., 2008. Manual for Hatchery Production of Sydney Rock Oysters (*Saccostrea Glomerata*). Final Report to Fisheries Research and Development Corporation, Deakin, ACT, Australia, vol. 20. New South Wales Department of Primary Industries – Fisheries Research Report Series, pp. 55.
O'Connor, W.A., Dove, M.C., 2009. The changing face of oyster culture in New South Wales, Australia. J. Shellfish Res. 28, 803–811. http://dx.doi.org/10.2983/035.028.0409.
Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.-F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437, 681–686. http://dx.doi.org/10.1038/nature04095.
Pan, T.C.F., Applebaum, S.L., Manahan, D.T., 2015. Experimental ocean acidification alters the allocation of metabolic energy. Proc. Natl. Acad. Sci. USA 112, 4696–4701. http://dx.doi.org/10.1073/pnas.1416967112.
Pane, E.F., Barry, J.P., 2007. Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Mar. Ecol. Prog. Ser. 334, 1–9. http://dx.doi.org/10.3354/meps334001.
Pansch, C., Schaub, I., Havenhand, J., Wahl, M., 2014. Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. Global Change Biol. 20, 765–777. http://dx.doi.org/10.1111/gcb.12478.
Parker, L.M., Ross, P.M., O'Connor, W.A., 2010. Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. Mar. Biol. 158, 689–697. http://dx.doi.org/10.1007/s00227-010-1592-4.
Parker, L.M., Ross, P.M., O'Connor, W.A., Borysko, L., Raftos, D.A., Pörtner, H.-O., 2012. Adult exposure influences offspring response to ocean acidification in oysters. Global Change Biol. 18, 82–92. http://dx.doi.org/10.1111/j.1365-2486.2011.02520.x.
Parker, L.M., O'Connor, W.A., Raftos, D.A., Pörtner, H.-O., Ross, P.M., 2015. Persistence of positive carryover effects in the oyster, *Saccostrea glomerata*, following transgenerational exposure to ocean acidification. PLos One 10, e0132276. http://dx.doi.org/10.1371/journal.pone.0132276.t001.
Parker, L.M., O'Connor, W.A., Byrne, M., Coleman, R.A., Virtue, P., Dove, M., Gibbs, M., Spohr, L., Scanes, E., Ross, P.M., 2017. Adult exposure to ocean acidification is maladaptive for larvae of the Sydney rock oyster *Saccostrea glomerata* in the presence of multiple stressors. Biol. Lett. 13, 20160798. http://dx.doi.org/10.1098/rsbl.2016.0798.
Pespeni, M.H., Sanford, E., Gaylord, B., Hill, T.M., Hosfelt, J.D., Jaris, H.K., LaVigne, M., Lenz, E.A., Russell, A.D., Young, M.K., Palumbi, S.R., 2013. Evolutionary change during experimental ocean acidification. Proc. Natl. Acad. Sci. USA 110, 6937–6942. http://dx.doi.org/10.1073/pnas.1220673110.
Peters, R., Raftos, D.A., 2003. The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). Aquaculture 223, 29–39. http://dx.doi.org/10.1016/S0044-8486(03)00169-8.
Pouvreau, S., Jonquières, G., Buestel, D., 1999. Filtration by the pearl oyster, *Pinctada margaritifera*, under conditions of low seston load and small particle size in a tropical lagoon habitat. Aquaculture 176, 295–314. http://dx.doi.org/10.1016/S0044-8486(99)00102-7.
Pörtner, H.O., Langenbuch, M., Reipschläger, A., 2004. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. J. Oceanogr. 60, 705–718. http://dx.doi.org/10.1007/s10872-004-5763-0.
Pörtner, H., 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. Mar. Ecol. Prog. Ser. 373, 203–217. http://dx.doi.org/10.3354/meps07768.
Pörtner, H.-O., Karl, D.M., Boyd, P.W., Cheung, W., Lluch-Cota, S.E., Nojiri, Y., Schmidt, D.N., Zavialov, P.O., 2014. Ocean systems. In: Field, C.B., Barros, V.R., Dokken, D.J., Mach, K.J., Mastrandrea, M.D., Bilir, T.E., Chatterjee, M., Ebi, K.L., Estrada, Y.O., Genova, R.C., Girma, B., Kissel, E.S., Levy, A.N., MacCracken, S., Mastrandrea, P.R., White, L.L. (Eds.), Climate Change 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, NY, pp. 411–484.
R Core Team, 2015. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
Ramajo, L., Pérez-Léon, E., Hendriks, I.E., Marbà, N., Krause-Jensen, D., Sejr, M.K., Blicher, M.E., Lagos, N.A., Olsen, Y.S., Duarte, C.M., 2016. Food supply confers calcifiers resistance to ocean acidification. Sci. Rep. 6, 19374. http://dx.doi.org/10.1038/srep19374.
Rastrick, S.P.S., Calosi, P., Calder-Potts, R., Foggo, A., Nightingale, G., Widdicombe, S., Spicer, J.I., 2014. Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. J. Exp. Biol. 217, 2499–2508. http://dx.doi.org/10.1242/jeb.089011.
Reipschläger, A., Pörtner, H.O., 1996. Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J. Exp. Biol. 199, 1801–1807.
Riisgård, H.U., 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Mar. Ecol. Prog. Ser. 45, 217–223. http://dx.doi.org/10.3354/meps045217.
Riisgård, H.U., Funch, P., Larsen, P.S., 2015. The mussel filter–pump – present understanding, with a re-examination of gill preparations. Acta Zool. 96, 273–282. http://dx.doi.org/10.1111/azo.12110.
Sabine, C.L., Feely, R.A., Gruber, N., Key, R.M., Lee, K., Bullister, J.L., Wanninkhof, R., Wong, C.S., Wallace, D.W.R., Tilbrook, B., Millero, F.J., Peng, T.-H., Kozyr, A., Ono, T., Rios, A.F., 2004. The oceanic sink for anthropogenic CO_2. Science 305, 367–371. http://dx.doi.org/10.1126/science.1097403.
Scanes, E., Parker, L.M., O'Connor, W.A., Ross, P.M., 2014. Mixed effects of elevated pCO_2 on fertilisation, larval and juvenile development and adult responses in the mobile subtidal scallop *Mimachlamys asperrima* (lamarck, 1819). PLos One 9. http://dx.doi.org/10.1371/journal.pone.0093649.
Scanes, E., Parker, L.M., O'Connor, W.A., Stapp, L.S., Ross, P.M., 2017. Intertidal oysters reach their physiological limit in a future high-CO_2 world. J. Exp. Biol. 220, 765–774. http://dx.doi.org/10.1242/jeb.151365.
Schalkhausser, B., Bock, C., Stemmer, K., Brey, T., Pörtner, H.O., Lannig, G., 2013. Impact of ocean acidification on escape performance of the king scallop, *Pecten maximus*, from Norway. Mar. Biol. 160, 1995–2006. http://dx.doi.org/10.1007/s00227-012-2057-8.
Schrobback, P., Pascoe, S., Coglan, L., 2014. History, status and future of Australia's native Sydney rock oyster industry. Aquat. Living Resour. 27, 153–165. http://dx.doi.org/10.1051/alr/2014011.
Shumway, S.E., 1982. Oxygen consumption in oysters: an overview. Mar. Biol. Lett. 3, l–23.
Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. http://dx.doi.org/10.1016/j.marenvres.2012.04.003.
Somero, G.N., 2012. The physiology of global change: linking patterns to mechanisms. Annu. Rev. Mater. Sci. 4, 39–61. http://dx.doi.org/10.1146/annurev-marine-120710-100935.
Stapp, L.S., Thomsen, J., Schade, H., Bock, C., Melzner, F., Pörtner, H.O., Lannig, G., 2016. Intra-population variability of ocean acidification impacts on the physiology of Baltic blue mussels (*Mytilus edulis*): integrating tissue and organism response. J. Comp. Physiol. B 1–15. http://dx.doi.org/10.1007/s00360-016-1053-6.
Stumpp, M., Trübenbach, K., Brennecke, D., Hu, M.Y., Melzner, F., 2012. Resource allocation and extracellular acid–base status in the sea urchin *Strongylocentrotus droebachiensis* in response to CO_2 induced seawater acidification. Aquat. Toxicol. 110–111, 194–207. http://dx.doi.org/10.1016/j.aquatox.2011.12.020.
Sunday, J.M., Calosi, P., Dupont, S., Munday, P.L., Stillman, J.H., Reusch, T.B.H., 2014. Evolution in an acidifying ocean. Trends Ecol. Evol. 29, 117–125. http://dx.doi.org/10.1016/j.tree.2013.11.001.
Tamayo, D., Ibarrola, I., Urrutxurtu, I., Navarro, E., 2014. Physiological basis of extreme growth rate differences in the spat of oyster (*Crassostrea gigas*). Mar. Biol. 161, 1627–1637. http://dx.doi.org/10.1007/s00227-014-2447-1.
Tambutté, E., Venn, A.A., Holcomb, M., Segonds, N., Techer, N., Zoccola, D., Allemand, D., Tambutté, S., 2015. Morphological plasticity of the coral skeleton under CO_2-driven seawater acidification. Nat. Commun. 6, 7368. http://dx.doi.org/10.1038/ncomms8368.
Thomsen, J., Gutowska, M.A., Saphörster, J., Heinemann, A., Trübenbach, K., Fietzke, J., Hiebenthal, C., Eisenhauer, A., Körtzinger, A., Wahl, M., Melzner, F., 2010. Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7, 3879–3891. http://dx.doi.org/10.5194/bg-7-3879-2010.
Thomsen, J., Casties, I., Pansch, C., Körtzinger, A., Melzner, F., 2013. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. Global Change Biol. 19, 1017–1027. http://dx.doi.org/10.1111/gcb.12109.
Thomsen, J., Stapp, L.S., Haynert, K., Schade, H., Danelli, M., Lannig, G., Wegner, K.M., Melzner, F., 2017. Naturally acidified habitat selects for ocean acidification–tolerant mussels. Sci. Adv 3, e1602411. http://dx.doi.org/10.1126/sciadv.1602411.
Thor, P., Dupont, S., 2015. Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. Global Change Biol. 21, 2261–2271. http://dx.doi.org/10.1111/gcb.12815.
Tunnicliffe, V., Davies, K.T.A., Butterfield, D.A., Embley, R.W., Rose, J.M., Chadwick, W.W., 2009. Survival of mussels in extremely acidic waters on a submarine volcano. Nat. Geosci. 2, 344–348. http://dx.doi.org/10.1038/ngeo500.
Waite, J.H., 1983. Quinone-tanned scleroproteins. In: Metabolic Biochemistry and Molecular Biomechanics. Elsevier, pp. 467–504. http://dx.doi.org/10.1016/B978-0-12-751401-7.50018-1.
Waldbusser, G.G., Brunner, E.L., Haley, B.A., Hales, B., Langdon, C.J., Prahl, F.G., 2013. A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. Geophys. Res. Lett. 40, 2171–2176. http://dx.doi.org/10.1002/grl.50449.
Waldbusser, G.G., Gray, M.W., Hales, B., Langdon, C.J., Haley, B.A., Gimenez, I., Smith, S.R., Brunner, E.L., Hutchinson, G., 2016. Slow shell building, a possible trait for resistance to the effects of acute ocean acidification. Limnol. Oceanogr. 61, 1969–1983. http://dx.doi.org/10.1002/lno.10348.
Waldbusser, G.G., Hales, B., Langdon, C.J., Haley, B.A., Schrader, P., Brunner, E.L., Gray, M.W., Miller, C.A., Gimenez, I., 2015. Saturation-state sensitivity of marine bivalve larvae to ocean acidification. Nat. Clim. Change 5, 273–280. http://dx.doi.org/10.1038/nclimate2479.
Widdicombe, S., Spicer, J.I., 2008. Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us? J. Exp. Mar. Biol. Ecol. 366, 187–197. http://dx.doi.org/10.1016/j.jembe.2008.07.024.
Wieser, W., Krumschnabel, G., 2001. Hierarchies of ATP-consuming processes: direct compared with indirect measurements, and comparative aspects. Biochem. J. 355, 389–395. http://dx.doi.org/10.1042/bj3550389.

Wittmann, A.C., Pörtner, H.O., 2013. Sensitivities of extant animal taxa to ocean acidification. Nature Clim. Change 3, 995–1001. http://dx.doi.org/10.1038/nclimate1982.

Wood, H.L., Sundell, K., Almroth, B.C., Sköld, H.N., Eriksson, S.P., 2016. Population-dependent effects of ocean acidification. P. Roy. Soc. B Biol. Sci. 283, 20160163. http://dx.doi.org/10.1098/rspb.2016.0163.

Wright, J.M., Parker, L.M., O'Connor, W.A., Williams, M., Kube, P., Ross, P.M., 2014. Populations of pacific oysters *Crassostrea gigas* respond variably to elevated CO_2 and predation by *Morula marginalba*. Biol. Bull. 226, 269–281.

Zeebe, R.E., Ridgwell, A., Zachos, J.C., 2016. Anthropogenic carbon release rate unprecedented during the past 66 million years. Nat. Geosci. 9, 325–329. http://dx.doi.org/10.1038/NGEO2681.

4 Discussion

According to the fifth IPCC report "Ocean acidification (OA) will exert negative effects on species and whole ecosystems and their services, especially those relying on carbonate structures" (Pörtner et al. 2014, p. 464). Against this backdrop, this thesis seeks to contribute to the understanding of physiological mechanisms and their intra-specific variance that underpin and define the OA vulnerability of ecologically and economically important blue mussels (*Mytilus edulis*) and Sydney rock oysters (*Saccostrea glomerata*).

This chapter will discuss the main findings of the three core publications (Publication I-III) while also considering the results of peer-reviewed literature and the additional publications (Publication A1-A4), where appropriate. The first part (Section 4.1) addresses the key results of the long-term multi-generation experiment with Baltic *M. edulis*, which revealed varying CO_2 sensitivities between different family lines ('tolerant' and 'sensitive' families) (Publication I and II), while the second part (Section 4.2) discusses physiological mechanism that underlie the different CO_2 sensitivity of wild versus selectively-bred *S. glomerata* (Publication III). Based on the individual results of Publications I-III, Section 4.3 attempts to identify potential physiological traits that may be correlated with CO_2 resilience in marine mussels and oysters. Lastly, overall conclusions will be drawn from the results presented in this thesis accompanied by a brief discussion of potential evolutionary and ecosystem implications (Section 4.4).

4.1 Physiological responses of tolerant and sensitive families of *M. edulis*

The following section discusses the main results of the long-term multi-generation experiment performed with *M. edulis* from Kiel Fjord (Experiment 1). During Experiment 1, 16 family lines were created using wild specimens from Kiel Fjord and the resulting offspring were exposed to three nominal PCO_2 levels (700 (control), 1120 (intermediate) and 2400 (high) µatm). This resulted in large variation in final larval survival and, consequently, settlement success between family lines at the highest PCO_2 treatment. Based on these differences in settlement success, family lines were classified as either tolerant (i.e. successful settlement at all three PCO_2 level) or sensitive (i.e. successful settlement only at control and intermediate PCO_2 level). After raising these offspring for over one year at the respective seawater PCO_2, physiological responses to elevated PCO_2 and their potential variation between tolerant and sensitive families were determined at the whole animal, tissue (Publication I) and biochemical level (Publication II).

4.1.1 Clearance rates

In physiological terms, maintaining the balance of energy uptake and expenditure is key to survival in heterotrophic organisms. Assuming that food abundance is high, increasing energy (food) intake and/or uptake efficiency, if possible, could be a viable strategy to meet an increased energy demand for self-maintenance, which may be evoked by altered environmental conditions. In fact, previous work on *M. edulis* from Kiel Fjord showed that, up to a seawater PCO_2 level of around 3000 µatm, shell growth (i.e. calcification) is much more strongly determined by food abundance than by seawater PCO_2 (Thomsen et al. 2013), showing that high food availability can ameliorate negative PCO_2 effects. Thus, to see whether potential differences in food uptake may have contributed to the different physiological responses and associated CO_2 sensitivities between family types, clearance rates (CR) (i.e. volume of water completely cleared of particles per unit of time) were assessed for tolerant and sensitive families (Publication I). Whilst there were no differences in CRs between sensitive and tolerant family lines and between the control and intermediate PCO_2 levels, clearance rates of tolerant families at the highest PCO_2 level were reduced by around 20%, indicating a decreased energy assimilation. As routine metabolic rates (RMR) of tolerant mussels were at control levels at the highest PCO_2 level, a potentially lower food uptake at an unchanged RMR points towards an unfavourable shift in the balance of energy uptake and expenditure (Publication I, see Section 4.1.2). This result confirms those of an earlier study on mussels from the same population. After a one year acclimation to similar (600, 1300, 2400 µatm) and a very high seawater PCO_2 (4000 µatm), CRs decreased linearly with increasing seawater PCO_2 (Hüning 2014). In concert with unchanged metabolic and ammonia excretion rates this resulted in a linear decrease in scope for growth with increasing PCO_2 level (Hüning 2014). Taken together, these results likely explain the abovementioned observation of Thomsen et al. (2013) that the ameliorating effect of food abundance on shell growth diminished at high PCO_2 levels that are likely to be realised in Kiel Fjord by the end of this century (Melzner et al. 2013, Reusch et al. 2018).

However, when interpreting these results, it should be noted that energy uptake in filter-feeding bivalves is not only determined by CR but also by absorption efficiency (AE) which, in turn, is a function of gut capacity, the residence time of food in the gut and the actual ingestion rate (i.e. the seston cleared from suspension minus seston rejected as pseudofaeces) (Gosling 2003). It is known that AE is plastic in mytilid mussels and can change, for instance, with food abundance (Thompson & Bayne 1974) mediated by alterations of gut residence

time (Bayne et al. 1988) or changes of digestive enzyme activities (Connor et al. 2016). Therefore, it cannot be ruled out that different AE, and consequently different rates of energy uptake between tolerant and sensitive families, contributed to the differing levels of CO_2 sensitivity and whether AE increased in tolerant mussels at the highest PCO_2 in an attempt to compensate for the CO_2 induced decrease in CR. While further studies are necessary to sufficiently resolve these questions, the results for *Mytilus* species from other studies argue against the latter hypothesis; *M. chilensis* and *M. edulis* both responded with a reduced AE in concert with a reduced and unchanged CR, respectively to chronic CO_2 exposure (*M. chilensis*, 1200 µatm, Navarro et al. 2013, *M. edulis,* 1000 µatm, Rastrick et al. 2018).

Besides the abovementioned results, a recent meta-analysis suggests depressed filtration rates as a general trend for suspension-feeding molluscs in response to elevated PCO_2 (Clements and Darrow 2018). This raises the question of what is the underlying physiological cause? Direct and indirect causes (or a combination of both) seem plausible. For example, a depressing effect of CO_2 on the digestive capacity would likely affect food uptake indirectly. Such indirect CO_2 effects on food ingestion have been reported for sea urchin larvae, where a reduced efficiency of digestive enzymes evoked, in this case, compensatory feeding (Stumpp et al. 2013). Alternatively, CO_2 could directly inhibit the feeding mechanisms itself. As outlined in Section 2.5, filtration in *Mytilus* and other suspension-feeding bivalves is achieved via ciliary movement within the gills; a process that is neuronally controlled via the excitatory neurotransmitter serotonin and its antagonist dopamine (Paparo and Aiello 1970). It has been suggested that CO_2-induced effects on GABA-A receptors may be the underlying cause for a reduced filtration activity of suspension feeding molluscs (Clements and Darrow 2018); GABA has been shown to inhibit serotonergic neurons in the oyster *Crassostrea viginica* (Mathieu et al. 2014). Whilst this mechanism seems plausible, particularly when considering that CO_2-induced behavioural impairments have been linked to altered GABA-A receptor activity in molluscs (Watson et al. 2014), the results presented in this thesis suggest an additional or alternative mechanism which causes the decrease in filtration activity observed for tolerant families at the observed highest seawater PCO_2. It is hypothesised that CO_2-induced constraints of gill aerobic metabolism (Publication II) impair gill functional scope and consequently filtration activity (Publication I). This hypothesis will be further elaborated in Section 4.1.3.1.

4.1.2 Routine metabolic rates

Routine metabolic rates (RMR) represent the energy costs of self-maintenance (here measured as oxygen consumption) when allowing for low levels of spontaneous activity. From a life-history theory perspective, self-maintenance forms one component that competes for an organism's finite energy budget alongside fitness-related functions such as growth and reproduction (Stearns 1992). Due to the compulsory trade-offs that exist among these functions, environmental conditions that provoke a higher energy allocation to self-maintenance may impose implications for organismal fitness. Consequently, the ability of an organism to adjust its (routine) metabolic rate and energy allocation in such a way that allows for the maintenance of positive life-history traits is a determining factor for its resilience against changing environmental conditions.

Following one year of acclimation to the different seawater PCO_2, RMRs varied substantially between tolerant and sensitive families (Publication I). While tolerant families displayed a bell-shaped response (i.e. elevated RMR at the intermediate and unchanged RMR at high PCO_2), mean RMR of sensitive families were similar at the control and intermediate PCO_2 levels (Publication I). These results clearly show the importance of investigating physiological responses to rapidly changing environmental drivers at a higher resolution than mean population responses. In fact, if RMR data of tolerant and sensitive had been pooled, no CO_2 effect would have been observed, which may explain an earlier finding of no CO_2 effect on RMR data for the same population (Hüning 2014). After acclimating Kiel Fjord blue mussels that were collected as spat on settlement panels and subsequently raised and acclimated in the laboratory to similar PCO_2 levels (600, 1300, 2400 or 4200 µatm), Hüning (2014) found no difference in mean RMRs but large variation between replicates. However, in the face of rapidly occurring ocean change, quantifying this pre-existing variation is essential as it provides the raw material for natural selection, and thus the potential for adaptation, to occur within a population (further discussed in Section 4.4).

But, what drives changes of RMR and the observed intra-population variation in response to elevated seawater PCO_2? As outlined in the introduction (see Section 1.2.1), OA-induced changes of seawater carbonate chemistry are predicted to challenge the RMRs of sessile calcifying invertebrates through an increased energy demand for homeostatic processes. In tolerant mussels, the increase in RMR was accompanied by a simultaneous increase of metabolic rates at the tissue level (summed gill and outer mantle MO_2). RMR represent a 'sum signal' of energy demands and allocation patterns at lower organisational levels, i.e. tissues and cells

(Pörtner and Lannig 2009). As the fractional contribution of gill and mantle to RMR at the intermediate PCO_2 level did not change, this suggested that elevated costs for cellular maintenance costs in various tissues (i.e. more than just gill and mantle) integrated into elevated RMRs in tolerant families at intermediate PCO_2.

The increase in RMR of tolerant mussels was not associated with a similarly strong increase in CR, which may indicate an unfavourable shift in energy demand and supply, given the assumptions that AE (see Section 4.1.1, Figure 4.1) and energy loss by ammonia excretion were lower and similar, respectively, between the different CO_2 treatments (see Hüning 2014). This unfavourable shift also becomes evident when comparing the balance of mean CR to mean RMR of tolerant mussels between the control and intermediate PCO_2 levels (CR/RMR = 0.61 (control) vs. 0.31 (intermediate PCO_2 level)). At the highest PCO_2, RMR returned to control levels but were associated with decreased CRs, which interestingly resulted in a similar ratio of CR/RMR (0.33) than at the intermediate PCO_2 (Figure 4.1). This indicates that elevated PCO_2 induces the onset of energy-limitation in tolerant mussels (*sensu* Sokolova et al. 2012, Sokolova 2013). Thereby, the cause may differ between the intermediate and high seawater PCO_2 treatments (i.e. higher RMR at unchanged CR vs. unchanged RMR at lower CR), potentially driven by decreasing aerobic capacity with increasing PCO_2 (Publication II, see discussion Section 4.1.3).

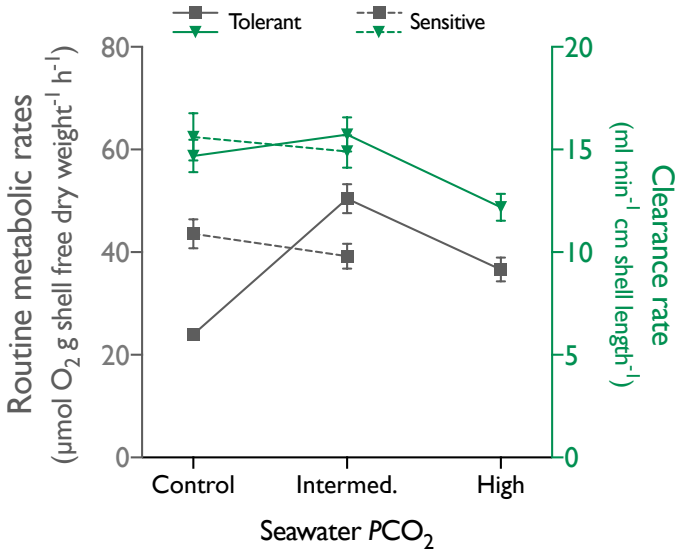

Figure 4.1: Routine metabolic rates and clearance rates of tolerant and sensitive families. Routine metabolic rates and clearance rates of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, n = 5–13.

Therefore, elevated PCO_2 may lead to a reduction of the aerobic power budget of tolerant mussels, which is defined as the proportion of the energy flux (and the corresponding metabolic power (i.e. mitochondria) that supports this flux) that is available to an organism after the costs of self-maintenance are met (Guderley & Pörtner 2010, Sokolova 2013, Pörtner et al. 2017). Although it should be stressed that further research (i.e. ideally measurements of whole animal aerobic scope) is necessary to confirm this hypothesis, CO_2-induced impairments of whole animal aerobic scope have been reported for active bivalves, such as the swimming King scallop, *Pecten maximus* (Schalkhausser et al. 2013) and also fish (Munday et al. 2009).

In contrast, sensitive families were characterised by unchanged RMRs and tissue respiration rates at the intermediate compared to the control PCO_2. However, when interpreting these results, it should be noted that at control PCO_2, although not statistically significant due to high variability within sensitive families (p=0.08), individuals from sensitive families had a 1.6-fold higher mean RMR (2.6-fold increase with regards to relative standard deviation) compared to tolerant families. Thus, RMRs of sensitive families at control PCO_2 were in the range of those of tolerant mussels at the intermediate PCO_2 (see Publication I, Figure 3A). At similar CRs, this translated into a CR/RMR ratio of around 0.37 which, interestingly, is similar to the balance of CR to RMR of tolerant families found at elevated PCO_2 (Figure 4.1). This may indicate a CO_2-independent lower metabolic efficiency (i.e. higher maintenance costs at similar energy intake) and potentially lower aerobic power budget in sensitive compared to tolerant families, reflected in their inability to increase RMRs above control levels and lowered gill aerobic capacities at the intermediate PCO_2 (see Section 4.1.3). Therefore, sensitive families may have to rely on energy reallocation, and thus trade-offs, to cover an altered energy demand imposed by elevated seawater PCO_2 (see discussion Publication II). In fact, while shell length did not differ between tolerant and sensitive mussels, soft tissue dry weight of sensitive mussels tended to decrease at the intermediate PCO_2, which may indicate that sensitive mussels were not able to build up as much body tissue within one year compared to tolerant families (Figure 4.2). In marine calcifying invertebrates, the correlation between skeletal and tissue growth can be weakened by unfavourable environmental conditions with tissue growth being more responsive to resource limitations than skeletal growth (Anthony et al. 2002).

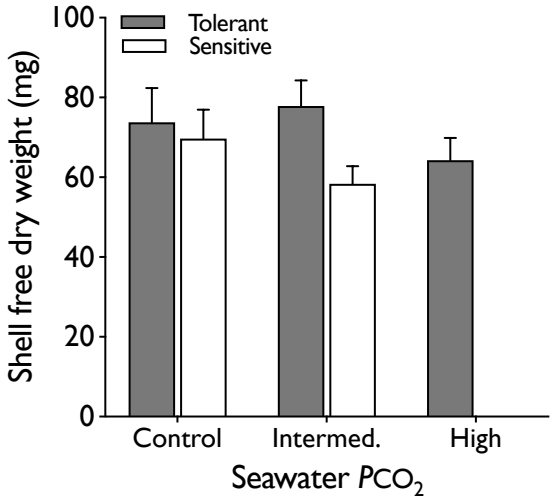

Figure 4.2: Shell free dry weight of tolerant and sensitive families. Body tissue dry weight of tolerant and sensitive families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and for tolerant families at high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, n = 8–13.

4.1.3 Key metabolic enzymes and tissue level responses

Whilst whole organism effects (e.g. RMR) and resource allocation are indisputably most relevant in an ecological context, they may only provide a relatively coarse measure of causative physiological mechanisms shaping those responses (Pörtner 2012, Publication A1). Therefore, biochemical strategies (i.e. metabolic pathways) which accommodate potential shifts in tissue level energy demands and associated tissue metabolic rates were determined in order to gain a mechanism-based understanding of whole animal metabolic consequences.

4.1.3.1 Gill tissue

In filter-feedings bivalves the water current produced by gills is created through mechanical work of the cytoskeletal motor protein dynein, which results in the beating of lateral cilia. This process of transforming chemically stored energy (in form of ATP) into mechanical work is fuelled by high mitochondrial densities within the gill filaments (Paparo 1972, Rivera-Ingraham et al. 2016). Mitochondria are the primary site for oxygen consumption and associated aerobic energy production at the cellular level. Accordingly, aerobic capacities of tissues and consequently whole animal aerobic scope are governed by aerobic capacities of individual mitochondria (Pörtner 2001).

Citrate synthase (CS), the first enzyme of the citric acid cycle (TCA-cycle), together with cytochrome c oxidase (COX), the final enzyme of the electron transport chain (ETC), are commonly used to assess mitochondrial aerobic capacity within cells and tissues (e.g. Moyes

et al. 1997, Lannig et al. 2003, Strobel et al. 2013b). Maximal capacities of both enzymes (expressed on a fresh weight (FW) basis) decreased in gills of tolerant families at elevated PCO_2 (Publication II). When normalised to protein content (PC), there was a progressive reduction of enzyme capacities with increasing PCO_2. In combination with a stable tissue PC, these results point towards a decreased gill aerobic capacity, which was lowest at the highest PCO_2 level. Decreased aerobic capacities were associated with slightly elevated tissue respiration (albeit this effect was not statistically significant (p= 0.082)); gill tissue of tolerant families respired around 18% more at elevated than at ambient seawater PCO_2 (Publication I). Consequently, gill factorial metabolic scope (i.e. factorial difference in respiration of serotonin-unstimulated gills vs. respiration of gill tissue during maximal beat frequency of lateral cilia) was reduced at the highest PCO_2 level. The observed CO_2-induced constraint of tissue aerobic capacity is therefore proposed to be the mechanistic underpinning for the decreased clearance rates of tolerant mussels at the highest PCO_2 level (Figure 4.3); although further research is necessary to verify this hypothesis (see discussion above and Publication I, II).

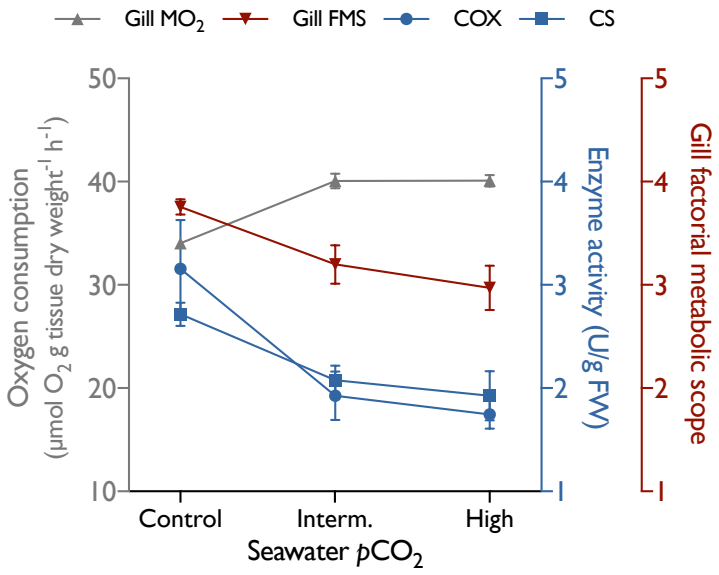

Figure 4.3: Overview of gill parameters of tolerant families of *Mytilus edulis*. Oxygen consumption, maximal enzyme activities of citrate synthase (CS) and cytochrome-c-oxidase (COX) (proxy for tissue aerobic capacity) and factorial gill metabolic scope of tolerant families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater PCO_2. Values are given as mean ± SE, n = 9–13.

The phosphoenpolpyruvate (PEP) branchpoint controls the flux of glycolytic substrates to either aerobic (formation of pyruvate) or anaerobic energy production (formation of fumarate and succinate via oxaloacetate) via pyruvate kinase (PK) and phosphoenolpyruvate

carboxykinase (PEPCK), respectively (De Zwaan & Holwerda 1972, Holwerda & De Zwaan 1973, De Vooys 1987) (Figure 4.4). Hence, a decrease in PK/PEPCK ratio reflects an activation of anaerobic pathways. Accordingly, the lower ratio of PK to PEPCK in gills of tolerant families at the highest PCO_2 supports the idea of reduced aerobic energy capacity as it implies a higher flux of PEP into anaerobic energy production.

The underlying mechanisms causing a CO_2-induced decrease in gill mitochondrial capacities remain to be explored. Recent studies on bivalves and fish suggest intracellular bicarbonate (HCO_3^-) concentrations as a modulator for mitochondrial capacity during environmental hypercapnia (Strobel et al. 2012, Haider et al. 2016). Elevated intracellular HCO_3^- concentrations inhibited state III respiration (maximal mitochondrial respiration and phosphorylating activity) in a concentration dependent manner (0-10 mM HCO_3^-) in gill mitochondria of the hard clam (*Mercenaria mercenaria*) and bay scallop (*Agropecten irradians*) (Haider et al. 2016). Similarly, elevated intracellular HCO_3^- concentrations were suggested to cause reduced state III respiration and reduced COX activities in liver tissue of Antarctic fish, *Notothenia rossii*, after ~1 month acclimation to elevated PCO_2 (~2000 μatm) (Strobel et al. 2012). While exposure to elevated CO_2 leads to a permanently decreased extracellular pH and increased PCO_2 in Baltic *M. edulis* (Publication I, Thomsen et al. 2010), intracellular pH is rapidly restored, which includes a rise in intracellular HCO_3^- (Lindinger et al. 1984). However, the fact that CS and COX activities (normalised to FW and PC) remained more or less unaffected in gills of sensitive families under elevated PCO_2 in spite of similar levels of extracellular acidosis (hence, presumably similar levels of intracellular HCO_3^-) speaks against a ubiquitous depressing effect of elevated intracellular HCO_3^- concentrations on gill mitochondrial capacities in this study. Instead, reduced CS and COX activities in gills of tolerant mussels may be a plastic response to elevated PCO_2, brought about by a higher gill aerobic scope in tolerant compared to sensitive families. This higher gill aerobic scope is reflected in the fact that, under control PCO_2, FW-related capacities of all measured gill mitochondrial enzymes, namely CS, COX, glutamate dehydrogenase (GDH) and 3-hydroxyacyl-CoA dehydrogenase (HADH) were around 30-45% lower in sensitive than in tolerant families (Publication II). In combination with similar gill respiration rates at control PCO_2, this suggest that tolerant mussels use a smaller fraction of their gill mitochondrial capacity under control conditions than sensitive ones. Under elevated PCO_2, this higher gill aerobic scope of tolerant families may allow for both, coverage of slightly elevated homeostatic costs at the tissue level, and also reduced aerobic capacity as an energy preserving mechanism in response to elevated CO_2. However, a slightly higher energy demand for gill maintenance, in concert with a decreased aerobic ca-

pacity, implies that tolerant mussels reach a 'ceiling' of physiological capacity. Beyond this capacity a mismatch of tissue aerobic capacity and basic energy demand may occur, which requires an increased anaerobic energy production at the expense of functional scope, such as a lower gill factorial metabolic scope.

In gills of sensitive mussels this 'ceiling' may be reached earlier, potentially not only related to their apparent lower gill mitochondrial scope, but also due to different biochemical strategies at elevated PCO_2. HADH is a key enzyme of the ß oxidation pathway (i.e. lipid oxidation, Figure 4.4) and while tolerant families showed progressively decreasing HADH capacities with an increasing seawater PCO_2 (in line with their decreasing aerobic capacity), gills of sensitive families displayed an almost 70% increase of HADH capacities (normalised to FW) compared to control conditions. The resulting increased HADH/CS ratio suggested an increased reliance on lipids as a fuel for aerobic energy production at the intermediate PCO_2 level. Higher HADH capacities were accompanied by a simultaneous increase of FW-related PK and PEPCK capacities, hence a stable PK/PEPCK ratio. It was therefore proposed (Publication II) that higher PEPCK capacities may serve an anaplerotic function by supplying oxaloacetate (via malate) to the TCA cycle (Hochachka & Somero 1984) to support increased lipid oxidation. In concert with an increased glucose oxidation, this may then fuel the gill energy demand under elevated PCO_2. Besides supporting aerobic lipid oxidation, the simultaneous increase of PEPCK and HADH could also be indicative of anaerobic lipid consumption in the gills of sensitive families at elevated PCO_2. The ß-oxidation cycle delivers acetyl-CoA, which can enter the TCA cycle through condensation via oxaloacetate, but in facultative anaerobes, such as *Mytilus*, it can also be converted into acetate, thereby yielding energy via substrate level phosphorylation (De Zwaan & Wijsman 1976). For every mole of acetyl-CoA, this requires the reduction of two moles of fumarate into succinate in order to replenish the reducing equivalents ($FADH_2$ and NADH). Thereby, fumarate is produced from glucose (via PEPCK, Figure 4.4), thus conversion of fatty acids during anaerobiosis requires a simultaneous mobilisation of carbohydrates (DeZwann & Wijsman 1976). Therefore, anaerobic lipid oxidation (increased HADH and PEPCK capacities) in concert with aerobic glucose oxidation (increased PK capacity) could be a mechanism to increase ATP production beyond aerobic capacities. Nevertheless, independent of whether lipid metabolism may have occurred aerobically or anaerobically, the data indicate an increased reliance on lipids as a metabolic fuel under elevated PCO_2 in sensitive compared to tolerant families. As discussed in Publication II, if an increased lipid consumption in sensitive families was already prevalent during the larval phase, this could have contributed to the observed larval

mortality of sensitive families at the highest seawater PCO_2 due to a potential depletion of egg lipid reserves before metamorphosis was completed. Indeed, decreasing lipid contents with increasing PCO_2 level have been reported for larvae of other bivalves, such as hard clams (*M. mercenaria*) and bay scallops (*A. irradians*) (Talmage & Gobler 2010).

Glutamate dehydrogenase (GDH), which catalyses the formation of α-ketoglutarate and ammonia out of glutamate is an indicator for protein metabolism. When related to PC, elevated PCO_2 reduced GDH capacities in tolerant (by 27%) and sensitive families (by 43%). In combination with a stable and increased gill PC in tolerant and sensitive families, respectively, this was discussed as a potential indication for a lowered protein metabolism and degradation as an energy saving strategy at elevated PCO_2 (Publication II). These results support earlier findings of Hüning (2014) who, after one year exposure to elevated PCO_2, showed unchanged ammonia excretion but a lower abundance of proteins involved in protein degradation (proteasome subunits) in the gill proteome of *M. edulis* of the same population. However, this is contrary to the results of Thomsen and Melzner (2010), who found ammonia excretion, and hence protein breakdown, increased linearly with increasing seawater PCO_2 (400-4000 μatm) during an 8-week acclimation experiment. In the course of Experiment 1, ammonia samples were taken, but unfortunately, due to small-sized mussels and different measurement modi (individual (this study) vs. pooled (10 animals per measurement, Thomsen and Melzner 2010)) ammonia excretion rates were below or just above the detection limit and, therefore, data were not reliable. However, in the 8-week study of Thomsen and Melzner (2010) increased protein metabolism was associated with a decrease in shell length at elevated PCO_2, whereas unchanged ammonia excretion in the one year study by Hüning (2014) was associated with similar shell lengths up to a PCO_2 level of 2400 μatm, matching present findings of unchanged shell length growth (Publication I, Publication A4). Thus, adjustments of protein metabolism might differ depending on acclimation time, which highlights the importance of long-term studies in order to address climate change effects. Compared to the oxidation of carbohydrates and lipids, protein oxidation is energetically less efficient due to steady energy loss through ammonia excretion (Tedengren & Kautsky 1986). Therefore, a relative decrease of protein as metabolic fuel in favour of lipid and carbohydrate seems a viable strategy under PCO_2 in the long run. In addition, elevated PCO_2 could potentially constrain protein catabolism via decreasing filtration rates which may in turn affect ammonia excretion, as it has recently been found that *Mytilus* excrete ammonia across their respiratory epithelia; a process facilitated by the water current produced for filter feeding (Thomsen et al. 2016).

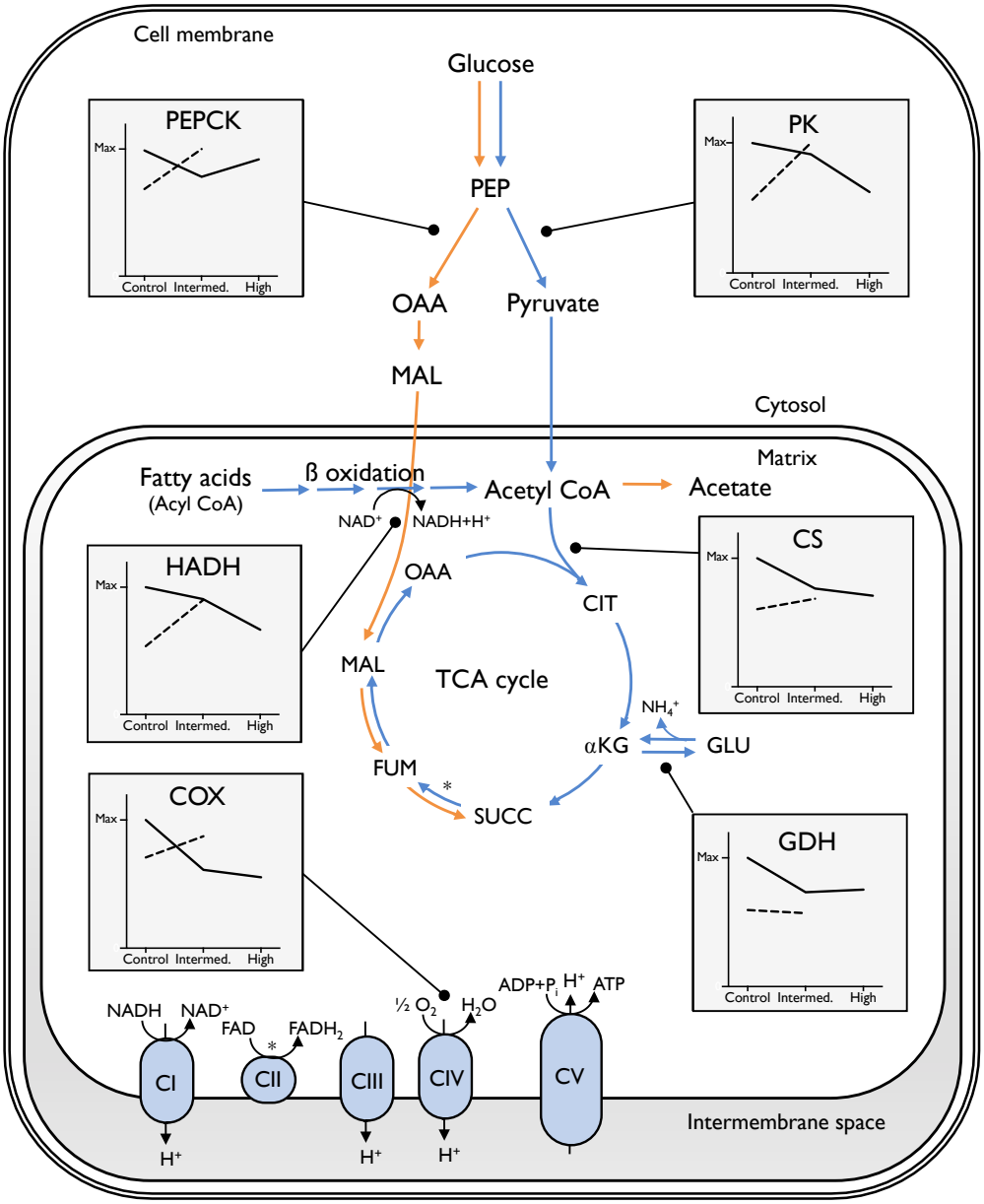

Figure 4.4: Overview of metabolic pathways and responses of key metabolic enzymes to elevated seawater *P*CO_2 in gills of tolerant and sensitive mussel families. The overview shows major aerobic (blue arrows) and anaerobic (orange arrows) metabolic pathways in *Mytilus edulis* (after Müller et al. 2012, Bayne 1976). Grey boxes show maximal enzyme capacities in gills of tolerant (solid line) and sensitive (dashed line) families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater *P*CO_2. Black lines extending from grey boxes indicate the reaction catalysed by the respective enzyme. For the sake of clarity, maximal enzyme capacities are scaled to the respective maximum value within each data set. Please note, the overview is not comprehensive in terms of metabolic pathways and redox reactions. Abbreviations: TCA: tricarboxylic acid cycle, PEPCK: phosphoenolpyruvate carboxykinase, PK: pyruvate kinase, HADH: 3-hydroxyacyl-CoA dehydrogenase, CS: citrate synthase, COX: cytochrome-c-oxidase, GDH: glutamate dehydrogenase, PEP: phosphoenolpyruvate, OAA: oxaloacetate, MAL: malate, FUM: fumarate, SUCC: succinate, αKG: α-ketoglutarate, CIT: citrate, GLU: glutamate, CI: Complex I (NADH dehydrogenase), CII: Complex II (succinate dehydrogenase), CIII: Complex III (cytochrome c oxidoreductase), CIV: Complex IV (COX), CV: Complex V (F_OF_1 ATP synthase). The * indicates that CII catalyses this reaction.

4.1.3.2 Outer mantle tissue

The outer mantle tissue plays a central role in the excretion of the shell (Gosling 2003). As outlined in the introduction, OA is expected to challenge bio-mineralisation, either through direct interference by altering available seawater carbonate species for the calcification processes or in an indirect manner, through whole animal energetic trade-offs. This can be primarily attributed to the fact that shell formation not only involves the precipitation of inorganic shell components but also the energetically costly formation of an organic matrix (Palmer 1992). A recent study showed that in the brackish Kiel Fjord, shell formation in the local *Mytilus* population can demand between 30-60% of the total assimilated energy (Sanders et al. 2018). Thus, changes in mantle energy metabolism may be crucial in mediating whole animal responses.

In tolerant families, outer mantle respiration displayed a similar PCO_2-dependent shape as the respective RMR (Figure 3B in Publication I). At the intermediate PCO_2, this was associated with a slight increase in FW specific COX capacities and increased PK capacities at unchanged CS, GDH and HADH capacities (Figure 4.5). COX (Complex IV) is the last enzyme of the ETC and reduces molecular oxygen to water molecules. In tolerant families, elevated COX capacities matched their increased mantle tissue oxygen demand at the intermediate PCO_2. Furthermore, the data indicated (Publication II) that this increased oxygen demand appears to be mainly fuelled by an increased activity of the glycolytic pathway, as FW related PK capacity increased at unchanged HADH and GDH capacities. This matches previous observations from Hüning et al. (2013), who found a CO_2-induced increased gene expression of hexokinase in outer mantle of *M. edulis*.

By contrast, results from the highest PCO_2 treatment indicated a shift in mitochondrial pathways in the mantle of tolerant families, which was reflected in a lower COX/CS ratio evoked by decreasing COX at slightly increased CS capacities (Publication II). This indicates enhanced citrate synthesis over ETC capacities in mantle mitochondria at the highest PCO_2 and, therefore, a potential reduced capacity of the ETC and, hence, aerobic ATP synthesis. This assumption is further supported by a previous study on this population which reported a down-regulation of F-ATPase subunits at a similar elevated PCO_2 (Hüning et al. 2013). As discussed in Publication II, a relative increase of mantle CS over COX capacities in tolerant families at high PCO_2 could indicate an increase of cataplerotic reactions with CS providing excess citrate for lipogenesis, which may support oxidative metabolism in other tissues (Strobel et al. 2013b).

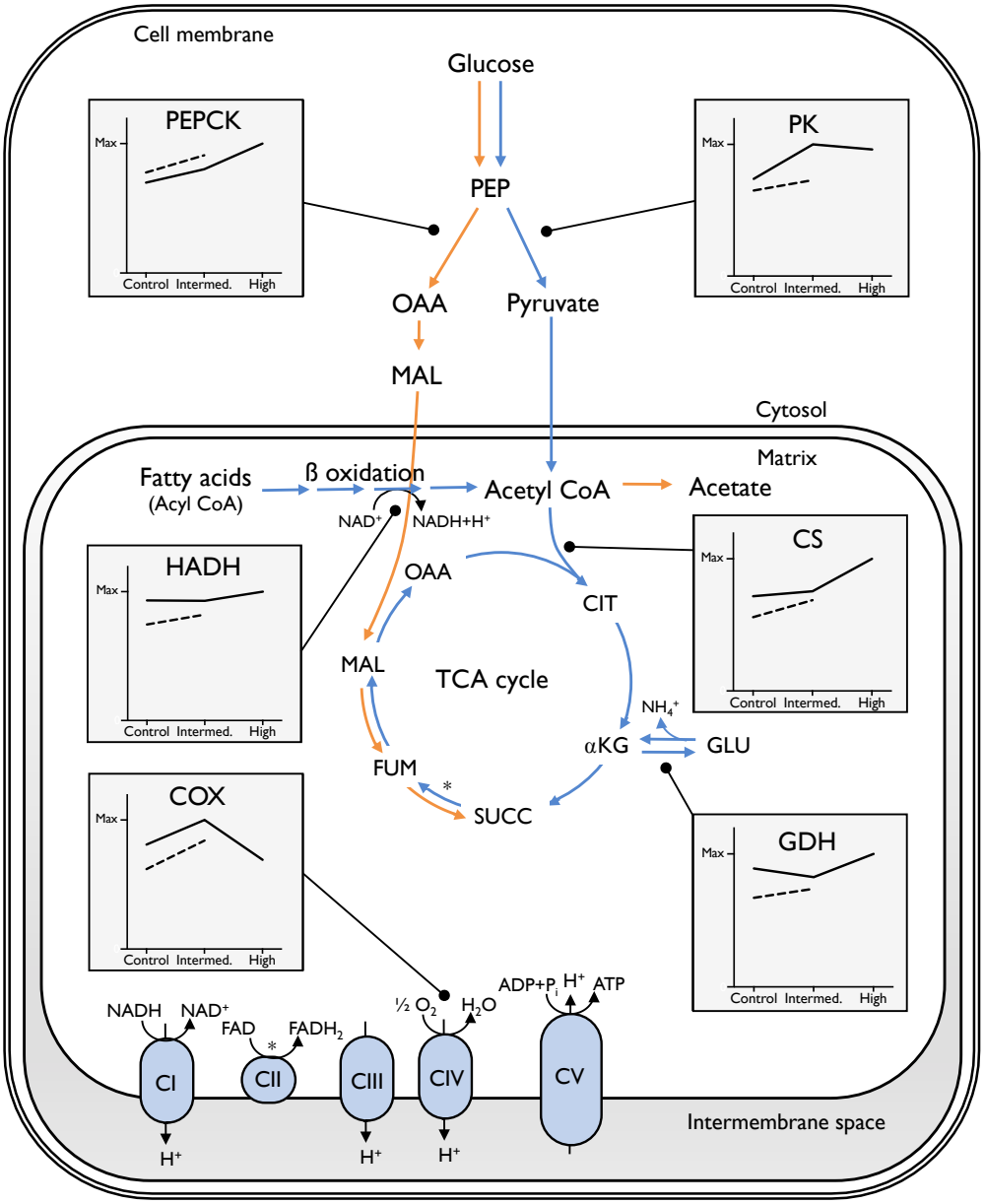

Figure 4.5: Overview of metabolic pathways and responses of key metabolic enzymes to elevated seawater *P*CO_2 in outer mantle tissue of tolerant and sensitive mussel families. The overview shows major aerobic (blue arrows) and anaerobic (orange arrows) metabolic pathways in *Mytilus edulis* (after Müller et al. 2012, Bayne 1976). Grey boxes show maximal enzyme capacities in outer mantle tissue of tolerant (solid line) and sensitive (dashed line) families of *Mytilus edulis* raised for 1 year at nominal control (700 µatm), intermediate (1120 µatm) and high (2400 µatm) seawater *P*CO_2. Black lines extending from grey boxes indicate the reaction catalysed by the respective enzyme. For the sake of clarity, maximal enzyme capacities are scaled to the respective maximum value within each data set. Please note, the overview is not comprehensive in terms of metabolic pathways and redox reactions. Abbreviations: TCA: tricarboxylic acid cycle, PEPCK: phosphoenolpyruvate carboxykinase, PK: pyruvate kinase, HADH: 3-hydroxyacyl-CoA dehydrogenase, CS: citrate synthase, COX: cytochrome-c-oxidase, GDH: glutamate dehydrogenase, PEP: phosphoenolpyruvate, OAA: oxaloacetate, MAL: malate, FUM: fumarate, SUCC: succinate, αKG: α-ketoglutarate, CIT: citrate, GLU: glutamate, CI: Complex I (NADH dehydrogenase), CII: Complex II (succinate dehydrogenase), CIII: Complex III (cytochrome c oxidoreductase), CIV: Complex IV (COX), CV: Complex V (F_OF_1 ATP synthase). The * indicates that CII catalyses this reaction.

In spite of increased maintenance costs at the intermediate PCO_2 and potentially decreased ETC capacities at the highest PCO_2, tolerant mussels were able to maintain calcification rates as shell length did not differ between mussels of the different PCO_2 treatments or between family types (Publication I, Publication A4). As outlined earlier, shell growth includes the energetically costly synthesis of an organic matrix (Palmer 1992, Sanders et al. 2018). Under corrosive seawater conditions (i.e. $\Omega < 1$), organic shell components and particularly the uppermost organic layer (periostracum) may protect the shell form dissolution (Tunnicliffe et al. 2009, Thomsen et al. 2010, Tambutté et al. 2015, Telesca et al. 2019). Oxygen demand for protein synthesis did not differ between mussels exposed to different PCO_2 levels and family types, but mantle PC increased at the intermediate PCO_2 in sensitive and at the highest PCO_2 level in tolerant families. A higher mantle PC at similar costs of protein synthesis and unchanged protein catabolism (i.e. stable GDH capacities between CO_2 treatments, Figure 4.5) was therefore suggested to be an indication of lowered protein degradation in mantle tissue of sensitive and tolerant mussels at elevated PCO_2. Thus, it appears that mussels prioritise maintenance of protein anabolism in mantle and also gill tissue (see Section 4.1.3.1) under elevated PCO_2.

Similar to tolerant mussels, COX capacities were slightly elevated at the intermediate PCO_2 and at a similar level as in tolerant families. However, mantle respiration of sensitive families was significantly lower than in tolerant families. Given that complex IV capacity is usually in excess in order to sustain high oxygen affinity of mitochondria (Gnaiger et al. 1998, Dudognon et al. 2013), increasing COX capacities at unchanged tissue respiration therefore seems counterintuitive. One explanation could be that respiration rates of isolated tissues may not mirror actual *in vivo* respiration, although it should be noted that experimental conditions for enzyme assays and tissues respiration experiments were identical for sensitive and tolerant families. Furthermore, mantle respiration correlated with RMR in both family types. Another explanation could be that exposure to elevated CO_2 induced an inhibitory effect on COX activity *in vivo*, which may be compensated for by an increase in total enzyme capacity. Under elevated PCO_2, COX (i.e. Complex IV) could be, for example, inhibited via nitric oxide (NO). NO is a reactive nitrogen species and plays an important role in marine invertebrates as a cellular mediator for various functions in various tissues (for review see Palumbo 2005). Intracellularly NO is almost exclusively catalysed by NO synthases (Palumbo 2005) and it is known that NO decreases the oxygen affinity of COX (Abele et al. 2007, Taylor & Moncada 2010). A recent study on blood clams (*Tegillarca granosa*) showed that CO_2 exposure upregulated genes involved in NO synthesis and increased hemocyte NO production in a

concentration dependent manner (Su et al. 2018). Furthermore, experimental addition of a NO donor was shown to strongly inhibit complex IV activities of isolated *M. edulis* gill mitochondria and consequently gill respiration in a concentration dependent manner (González et al. 2019). Thereby, the increase in NO may be related to an oxidative stress under elevated PCO_2 (see discussion Su et al. 2018 for potential mechanisms). Oxidative stress markers were not determined during Experiment 1, but an earlier proteomic study on the same population showed a higher abundance of proteins associated with oxidative stress in gill and mantle tissue at elevated PCO_2 when compared to control conditions (Hüning 2014). In addition, CO_2 exposure was shown to increase reactive oxygen species (ROS) level in hemocytes of *M. edulis* in a concentration-dependent manner (Sun et al. 2017) and several other studies also suggest that elevated seawater PCO_2 induces oxidative stress in other marine bivalves (Tomanek et al. 2011, Timmins-Schiffman et al. 2014).

While the abovementioned potential role of NO and/or ROS as a mechanistic underpinning for OA effects on mitochondrial functioning lies in the realm of speculation, the observed variation of CS and COX capacities clearly shows that elevated PCO_2 affects key mitochondrial enzymes in a tissue- and family type-specific manner. Future research is required to fully characterise mitochondrial properties and functioning under elevated PCO_2. Thereby, with respect to the widely observed increase of CO_2 induced oxidative stress, it would be worthwhile to investigate whether difference in antioxidant capacities between tolerant and sensitive families may have contributed to the observed variation in metabolic responses. This seems likely, as antioxidant capacity in *Mytilus* can be highly plastic within a population and in response to environmental conditions (Jimenez et al. 2015). Moreover, high constitutive antioxidant efficiency was correlated with higher CO_2 tolerance in a polychaete (*Platynereis dumerilii*) at a natural CO_2 vent system (Ricevuto et al. 2015).

4.2 Different CO_2 sensitivity of selectively-bred vs. wild *S. glomerata*

Previous studies on Sydney rock oysters established a higher CO_2 resilience in an aquaculture line selectively bred for increased growth and disease resistance ('selected oyster') compared to the wild population ('wild oysters') (Section 1.4.2; Parker et al. 2010, 2012). The following section discusses the main result of Publication III, which investigated whether the higher CO_2 resilience of selected oysters correlates with an increased capacity to compensate for CO_2-induced extracellular acid-base disturbances (Section 4.2.1) and whether this capacity is driven by metabolic and ion-regulatory costs at the cellular/tissue level (Section 4.2.2).

4.2.1 Extracellular acid-base regulation

Following 7-weeks of acclimation to an elevated PCO_2, wild oysters displayed a drop in pH_e, while selectively-bred oysters maintained a pH_e similar to control conditions (Publication III). These results support the concept that the ability of an organism to regulate its extracellular pH is positively correlated with CO_2 resilience (Pörtner 2008, Widdicombe and Spicer 2008, Melzner et al. 2009).

As outlined in the introduction, previous studies on marine ectotherms have found that the ability to compensate for a CO_2 induced extracellular acidosis is associated with the active accumulation and/or retention of HCO_3^- via energy consuming ion-regulatory processes (reviewed by Melzner et al. 2009). Thereby, the initial extracellular acidosis is brought about by a rise in extracellular PCO_2 in order to maintain diffusive gradients for elimination of metabolic CO_2. Unexpectedly, this was not the case for selected oysters. Extracellular PCO_2 levels remained at control levels in selected oysters at elevated PCO_2. Accordingly, HCO_3^- remained at control levels and selected oysters experienced a 40% reduction in the diffusive gradient between their extracellular space and the environment (Figure 1 and 2 in Publication III). By contrast, wild oysters experienced an increase in extracellular PCO_2 under elevated PCO_2, and, thus, maintained an outwards PCO_2 gradient similar to control conditions.

The enhanced ability of selected oysters to eliminate metabolic CO_2 was proposed to be related to higher and energetically more efficient feeding (i.e. filtration) rates (see discussion below, Publication III), which is known to be a physiological basis for their faster growth (Bayne et al. 1999, Bayne 2000). As filtration and ventilation is a coupled process in filter-feeders, an increased water pumping activity increases the ventilation of the body cavity which facilitates diffusive gas exchange.

Higher ventilation rates may also be of benefit in light of the intertidal habitat of Sydney rock oysters. Oysters close their valves when emersed, which restricts gas exchange and results in a strong increase of hemoylmph PCO_2 and a concomitant drop of extracellular pH (Burnett 1988, Publication A3) and associated metabolic depression (Guppy & Withers 1999). Thus, a larger water pumping capacity may allow selected oysters a faster recovery from respiratory acidosis once immersed. A faster recovery from a depressed metabolic state is likely to maximise energy assimilation, an aspect that putatively promotes growth within a feeding-time constrained habitat such as the intertidal zone. Furthermore, it is known that a significant drop in pH_e can have a depressing effect on protein synthesis in marine invertebrates (Δ pH 1.2 units; Langenbuch et al. 2006), which may translate into reduced somatic growth. And

lastly, a faster recovery from a respiratory acidosis may also be correlated with the increased disease resistance of the selected oysters; low pH_e during intertidal conditions was previously shown to be directly related to a depressed immune response in *Crassostrea virginica* (Boyd & Burnett 1999).

Given these interrelations, the question arises why natural selection does not favour faster growth and a larger filtration capacity in the wild population? It may be explained from a life-history trade-off perspective (Stearns 1992). Bayne (2000, 2004) showed that the increased growth in selected oysters is correlated with a higher deposition of available energy to protein growth at the expense of lipid deposition compared to the wild population, which in turn translates into a reduced gonadal area in selected compared to wild oysters (Dove & O'Connor 2012). In simple terms, enhanced growth in the selected line seems to come at the expense of reproductive output and hence Darwinian fitness. Similarly, a recent study in which three different populations of Olympia oysters (*Ostrea lurida*) were raised under common garden conditions (until F2 generation) reported the lowest reproductive capacity in the fastest growing population and the authors suggested a potential adaptive trade-off (Silliman et al. 2018).

4.2.2 Tissue respiration and ion-regulatory costs

The larger ventilatory (i.e. filtration) capacity in selected than wild oysters is likely to be attributed to a larger gill surface area, as filtration rates scale with gill surface area (Meyhöfer 1985, Tamayo et al. 2014). Compared to other tissue, a relatively high mitochondrial density is necessary in gills to support the ciliary machinery (Paparo 1972), and therefore, maintaining large gills is energetically costly and represents an energetic trade-off in oysters (Honkoop et al. 2003). In line with this, mean gill respiration (per g tissue DW) of both oyster types (and also for *M. edulis*, Publication I) was at least twice as high as the mantle respiration when compared at control conditions (Figure 3 in Publication III). Furthermore, the results showed that while PCO_2 had no effect on the gill respiration of both oyster types, oxygen consumption in gills of selected oysters was overall around 20% lower than in wild oysters. Thus, lower gill maintenance costs may enhance the scope for organ size and were discussed (Publication III) as a potential mechanistic underpinning for the observations of Bayne et al. (1999) that filtration in selected oysters was not only higher, but also energetically more efficient as reflected in a lower ratio of energy respired per joule of energy ingested.

The underlying cause for lower gill maintenance costs remains unknown but several mechanisms seem plausible. As discussed in Publication III, lowered gill maintenance costs in se-

lected oysters could be related to an increased efficiency of protein deposition. This hypothesis was based on earlier findings by Bayne (2000), who showed that selected oysters have a reduced protein turnover compared to the wild type. Furthermore, Ertl et al. (2016) showed that wild Sydney rock oysters exposed to four weeks of increased PCO_2 (1100 μatm) had an upregulation of genes that are involved in protein synthesis. Thus, if elevated PCO_2 also evokes an increase in protein synthesis in selected Sydney rock oysters, lower costs for protein deposition would have an additional benefit during CO_2 exposure. Furthermore, if also applicable for the larval stage, a presumably higher efficiency in protein deposition could also contribute to the observed increased larval growth in selected and wild oysters (Parker et al. 2010, 2012) (further discussed in Section 4.3). Alternatively, or additionally, growth selection of oysters might have led to an enhanced energetic efficiency of the lateral cilia, which would depend on optimising cilia number as well as synchronisation of beating between adjacent cilia (Gueron & Levit-Gurevich 1999, Osterman & Vilfan 2011). A higher ciliary energy efficiency may allow for an increased beat frequency and increased pumping rate at unchanged energetic costs while potentially also minimising boundary layer effects, which would facilitate diffusive gas exchange (i.e. increased capacity to eliminate metabolic CO_2).

In contrast to gill tissue, mantle respiration at control PCO_2 did not differ between wild and selected oyster. However, while mantle respiration remained at control levels in selected oysters at elevated PCO_2, it increased significantly in wild oysters (Publication III). Thus, similar to the *Mytilus* results presented in Publication I and II, PCO_2 effects were tissue specific. Tissue specific PCO_2 effects have been observed in whole range of other studies (e.g. Publication A2, Lannig et al. 2010, Strobel et al. 2013b, Nardi et al. 2018), stressing the importance of studying PCO_2 effects at different levels of biological organisation in order to gain a holistic effect of OA responses (see also Publication A1).

Increased mantle respiration in wild, but not in selected oysters, indicates that the PCO_2 induced shift of extracellular acid-base variables evoked a higher mantle energy demand. Unexpectedly, this increase in mantle respiration was not correlated with an increased energy demand for ion regulation, as the fractional energy demand of the measured ion transporters (Na^+/K^+-ATPase, H^+-ATPase, Na^+/H^+-exchanger) did not differ between the control and elevated PCO_2 treatment. As outlined in the introduction, a decrease in extracellular pH increases the proton flux into the intracellular space (Boron 2004) and requires the active removal of excess proton equivalents via proton equivalent ion exchange (Pörtner 2008, Melzner et al. 2009). This mechanism has potential implications for cellular/tissue and consequently whole animal energy demand. Only in gill tissue did net and fractional O_2 demand

for Na^+/H^+ exchange increase in wild oysters in response to elevated PCO_2. However, this was not correlated with a higher gill oxygen demand under elevated PCO_2. Furthermore, the same response (i.e. increased net and fractional energy demand of Na^+/H^+ exchange) was found in selected oysters in spite of their stable extracellular pH. This was discussed as being related to an upregulation of apical acid extruding mechanisms in order to defend intracellular pH in the gill epithelium against the lower seawater pH in the OA treatment (Publication III). Na^+/H^+ exchangers are thought to be thermodynamically favoured over H^+-ATPases in marine taxa due to the strong Na^+ gradient between cytosol (low [Na^+]) and seawater (high [Na^+]) (Claiborne et al. 1999) and thereby suggested to represent a common pathway for proton excretion in most marine species, including invertebrates (Hu et al. 2015). For example, Na^+/H^+ exchange was also upregulated in nauplii of the copepod, *Calanus glacialis* under elevated PCO_2 (Bailey et al. 2017). The Na^+/H^+-antiport is a secondary active ion transporter that is energetically coupled to the Na^+-gradient maintained by the Na^+/K^+-ATPase. However, oxygen demand for Na^+/K^+-ATPase remained unchanged in gills of wild and selected oysters. Overall, these results led to the conclusion that Sydney rock oysters may be able to modulate ion transporters in response to elevated PCO_2 without significant effects on overall ATP turnover, at least in gill and mantle tissue.

However, some considerations have to be made regarding the methodology that was applied. While the indirect determination of energy demands for cellular processes, such as ion transport, via specific inhibitors (measured as difference in tissue oxygen consumption before and after inhibition; see Section 2.5.3 for methodological details) allows for a direct link to cellular/tissue and consequently whole animal energy budget and has been applied in various studies (e.g. Smith & Houlihan 1995, Mark et al. 2005, Lannig et al. 2010), data should be seen as qualitative, rather than quantitative. This is because inhibitors may have non-specific side effects (e.g. Wieser & Krumschnabel 2001), which may lead to an overestimation of energy demands due to the simultaneous inhibition of other cellular processes. Conversely, energy demands could be underestimated if inhibition of one transporter may result in stimulation of another in order to compensate a potential intracellular ion imbalance elicited by the inhibition (Pörtner et al. 2000). Furthermore, measured *in vivo* energy demands are a snapshot and do not necessarily reflect actual maximal capacities, as shown for the gill Na^+/K^+-ATPase in Atlantic cod (*Gadus morhua*), where results suggested that maximal enzyme capacities (measured *in vitro*) are not exploited *in vivo* (Kreiss et al. 2014). Thus, further studies would be useful to underpin the abovementioned results. Ideally, a combination of gene-expression and protein expression studies, measurements of *in vitro* maximal enzyme capaci-

ties and also the determination of actual *in vivo* functioning rates (e.g. via rubidium uptake for Na^+/K^+-ATPase) would complement measured *in vivo* energy demands such that a comprehensive picture regarding ion-regulation and regulatory responses and associated metabolic costs on all functional levels would emerge.

Nonetheless, the data presented in Publication III should allow for detection of relative shifts in ion-regulatory energy demand between oyster types and PCO_2 level. Except for gill Na^+/H^+-exchange, no CO_2 shift in fractional and net O_2 demand of ion transporters could be observed in wild and selected oysters. However, when comparing the summed net O_2 demands of all three ion transporters measured under control conditions in gill and mantle tissues of both oyster types, it appears as if wild oysters have a *per se* higher energy demand for ion regulation than selected oysters (Figure 4.6). Such a constitutively higher energy demand of the ion-transport in wild oysters may be related to the higher relative fluctuations of acid-base parameters that they may experience in their intertidal environment, which selected oysters seem to avoid (as discussed above). Likewise, reduced inherent costs for ion-regulation in selected oysters could be a mechanism to reduce tissue maintenance costs (such as observed in gill tissue) and would likely increase the energetic scope for anabolic processes such as growth. Clearly, future research is necessary to investigate this speculative hypothesis. Furthermore, an in-depth analysis of transepithelial ion transporters as well as their isoforms and associated energetic costs is required to advance our general understanding of acid-base and ion-regulatory mechanisms in oysters and other marine bivalves, and their potentially changing role under ocean acidification scenarios.

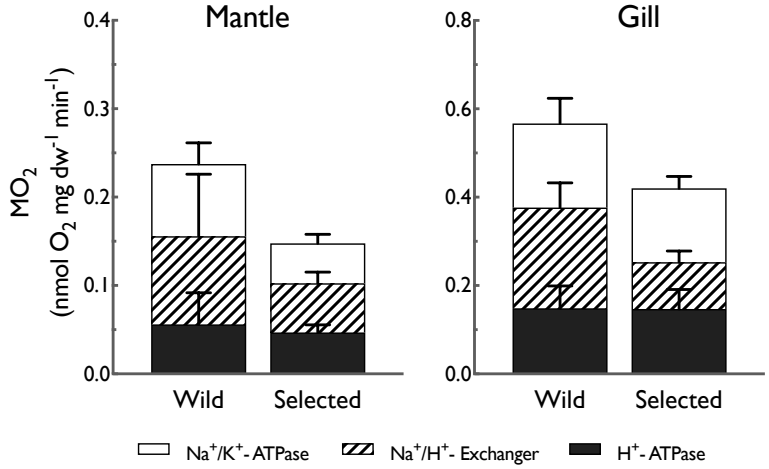

Figure 4.6 Summed net oxygen demand of major ion transporters in mantle (*left panel*) and gill (*right panel*) tissue of wild and selected *Saccostrea glomerata* measured at control PCO_2. Net O_2 demand of Na^+/K^+-ATPase, Na^+/H^+-exchanger and H^+-ATPase in mantle and gill tissue of wild and selected oysters (*Saccostrea glomerata*) exposed for 7 weeks to control seawater PCO_2. Values are given as mean ± SEM, n=6–12.

4.3 Potential resilience traits in mussels and oysters

Comparative studies that link whole-organism responses with cellular mechanisms can be a powerful tool in advancing our understanding of traits that are associated with OA tolerance in marine ectotherms (Stillman & Paganini 2015). With this in mind, the aim of this section is to identify physiological traits that may be associated with CO_2 resilience, derived from the comparative studies presented in Publication I-III and the corresponding literature.

Routine metabolic rate

Compared to the majority of previous OA studies on marine bivalves, RMR in Publication I was determined after mussels had been acclimated to the different PCO_2 levels for over one year and, most importantly, throughout their complete life cycle. This is crucial as species' persistence under environmental change is inevitably dictated by the sensitivity of the 'weakest link', which, for broadcast spawning marine invertebrates, can be usually found in the larval stages (Pechenik 1999, Byrne 2012).

In Experiment 1 (Publication I and II), exposure to the highest PCO_2 level resulted in drastic larval mortality. Only offspring from 5 out of 16 families were able to metamorphose into juveniles at the highest PCO_2 level, a result on which the classification into tolerant and sensitive families was based (Publication I, A4). Although tolerant families successfully settled in the high PCO_2 treatment, larval mortality was, however, still significantly higher compared to the control and intermediate PCO_2 levels, thus, a selection process occurred at the highest PCO_2 level. The results suggest that this selection favoured phenotypes with RMRs that remained at control level. This larval selection process prior to metamorphosis may also explain why results for RMRs differed from those of previous studies on the same population. Thomsen and Melzner (2010) reported a peak of RMR at PCO_2 levels similar to the high PCO_2 treatment in Experiment 1 (around 2400 μatm), while Hüning (2014) reported unchanged RMRs over wide range of PCO_2 levels (600, 1300, 2400 or 4200 μatm). Thomsen and Melzner (2010) used mussels collected in Kiel Fjord with a mean shell length 15 ± 0.2 mm, while Hüning (2014) incubated mussels that were collected as spat on settlement panels. Thus, unlike in Experiment 1, CO_2 acclimation in both these studies commenced post metamorphosis. Metamorphosis represents a bottleneck in mussels' ontogeny, due to a massive re-organisation of body parts while solely relying on internally stored energy resources (Gosling 2003, Byrne 2012), and is sensitive to PCO_2, as indicated by Publication I, Publication A4.

A selection process favouring phenotypes with RMRs similar to control levels has also been reported for a transgenerational experiment with the calanoid copepod *Pseudocalanus acuspes*, where it was found that metabolic rates increased at an intermediate PCO_2, while they returned to control levels at the highest PCO_2 level and were associated with increased mortality (Thor and Dupont 2015). Reduced metabolic rates are also the adaptive mechanism that enables some marine polychaete species to inhabit CO_2 vents (Calosi et al. 2013). Based on these results, one could hypothesis that CO_2 exposure during early ontogeny selects for low rather than elevated metabolic rates. Such a relationship may be explained in light of the so-called 'context-dependence hypothesis', which proposes that "high-RMR individuals are likely to have relatively high fitness when environmental conditions are favourable (…) while low-RMR individuals may be somewhat buffered against the environment owing to their lower costs of maintenance" (Burton et al. 2011, p. 3468). The fact that mean RMRs of tolerant families at control PCO_2 were lower than in sensitive families supports this idea. Likewise, Bayne (2000) showed that whole animal oxygen consumption of selected oysters was lower than in wild oysters. In marine mussels and oysters, relatively lower RMRs can be associated with enhanced growth efficiencies (Hawkins et al. 1986, Bayne 1999, 2000), as discussed below.

Costs of growth

It is well known that growth rates within natural populations of marine bivalves, and particularly their larval stages, vary significantly (e.g. Mallet & Haley 2011), with this growth variation being persistent within larval cohorts reared under identical controlled laboratory conditions (e.g. Pan et al. 2018). This persistent variability indicates that growth is highly plastic, potentially due to underlying adaptive trade-offs (see discussion above, Section 4.2.1.).

As mentioned before, increased costs for self-maintenance and/or compromised energy acquisition provoked by elevated seawater PCO_2 may leave less energy for other, mainly anabolic, fitness-related traits such as growth and reproduction. Consequently, reducing costs for growth is likely to help facilitate these energetic trade-offs, particularly during the larval phase, when relative growth rates are highest. In selected Sydney rock oysters, higher growth compared to the wild population is achieved through an increased energetic efficiency of protein deposition (Bayne 2000) fuelled by higher and energetically more efficient filtration rates (Bayne 2000, Bayne et al. 1999, Publication III). Frieder et al. (2018) recently showed that reduced shell length of *C. gigas* larvae under elevated PCO_2 was directly related to an impeded larval ability to deposit shell and whole-body protein content caused by a CO_2 in-

duced increase of protein degradation. Thus, the increased energetic efficiency of protein deposition in selected oysters may explain the decreased CO_2 effect on larval growth in selected compared to wild oysters (Parker et al. 2010, 2012). This is further emphasised by the fact that elevated PCO_2 has no impact on maternal energy provision (measured as egg size and lipid content) in wild oysters (Parker et al. 2018).

Differences in larval shell growth were also evident between tolerant and sensitive mussel families during Experiment 1; prodissoconch I (PD I) shell length declined in both family types with increasing PCO_2 but, two days post fertilisation, PDI lengths of larvae of tolerant families were larger than those of sensitive families (Publication A4). The difference in shell size between family types did not correlate with different egg sizes, which is a proxy for energy content (Publication A4). This suggests that differences in larval shell length may not be related to differences in maternal energy provision, but instead may be due to an increased efficiency of protein deposition in tolerant compared to sensitive families, as seen for Sydney rock oysters.

Unfortunately, costs for whole animal growth were not determined for tolerant and sensitive mussel families, but instead same-sized mussels were picked for physiological measurements to avoid allometric effects. Since tolerant and sensitive mussels were of the same age (i.e. fertilisation occurred at the same day), shell length growth was similar between family types. The fact that this was associated with a lower control RMR in tolerant mussels and similar costs for protein biosynthesis between family types (at least in mantle tissue) may suggest that growth efficiency is higher in tolerant than sensitive families. Indeed, earlier studies on intra-individual growth variation in *Mytilus* revealed that fast growers have lower metabolic rates than slow growers, partly stemming from an increased efficiency of protein deposition linked to different intensities of protein turnover (Diehl et al. 1986, Hawkins et al. 1986, Bayne & Hawkins 1997).

Taken together, a relative decrease in protein turnover may be a favourable trait under elevated seawater PCO_2, as is also indicated by the gill and mantle enzyme data (see discussion Section 4.1.3). Lower protein turnover may, however, come at the cost of a reduced metabolic flexibility (e.g. mobilisation and selective distribution of amino acids), which is associated with high rates of protein turnover (for review see Hawkins 1991). Furthermore, as discussed above (Section 4.2.1), and at least for Sydney rock oysters, there seems to be an adaptive trade-off between growth efficiency and reproductive output (Dove and O'Connor 2012).

Extracellular pH

As outlined in the introduction (Section 1.2.1), the ability of an organism to regulate its extracellular pH is proposed to be positively correlated with CO_2 resilience. While there was no difference in extracellular pH between tolerant and sensitive families of *M. edulis* (Publication I), the higher CO_2 resilience of selected oysters was correlated with the ability to maintain their extracellular pH under elevated PCO_2 (Publication III). However, as discussed in Section 4.2.1 and Publication III, this ability is not brought about by the ability to actively elevate extracellular HCO_3^- but is rather a 'by-product' of the increased filtration activity and associated larger gill surface area which allows for the increased ventilatory elimination of metabolic CO_2. This said, future studies will have to establish how extracellular acid base variables of selected oysters are affected by elevated seawater PCO_2 under varying ration levels and across seasons, as gill size in Sydney rock oysters can vary with season, likely mediated by differences in food availability (Honkoop et al. 2003). However, these results also show that gill size is plastic which may provide an adaptive capacity. Parker et al. (2015) demonstrated that wild Sydney rock oysters, who originated from CO_2 exposed parents, and who were reared at elevated PCO_2 post metamorphosis, had an improved ability to regulate their pH_e when exposed to elevated PCO_2 level after they had been grown out in the field for 14 months. The underlying mechanism remains unknown, but the results presented in Publication III suggest that the improved ability to regulate pH_e is not related to an active accumulation of extracellular HCO_3^- ions. Whether this effect was related to an increased gill size in response to elevated PCO_2 remains to be explored, but the results of Publication III suggest existing gill size plasticity in oysters may become a beneficial trait in an acidifying ocean and could be an example of exaptation (*sensu* Gould & Vrba 1982).

Gill metabolism and mitochondria

The gill is a central organ in bivalves' due to its role in feeding and gas exchange. The large surface area that this function brings about means that gills are largely exposed to environmental perturbations, which may imply that a high gill metabolic plasticity may be beneficial during environmentally challenging conditions.

Both tolerant mussel families and selected oysters showed some type of increased metabolic efficiency in gill tissue compared to sensitive families and wild oysters, respectively, which may provide an advantage under elevated PCO_2. In tolerant mussel families, this increased metabolic efficiency in gills was reflected in a higher aerobic capacity at similar gill respiration when compared to sensitive families, whereas selected oysters had overall lower gill respira-

tion rates than wild oysters. Unfortunately, no gill mitochondrial markers were determined for selected and wild oysters in the present thesis. However, a recent study on isolated hemocytes of wild and selectively-bred Sydney rock oysters using fluorescent dyes showed that after 15 days of CO_2 exposure, selected oysters showed unchanged mitochondrial fluorescence but reduced production of ROS, while the opposite was observed for the wild oysters (Goncalves et al. 2018). This suggests that hemocyte mitochondrial responses to CO_2 differ between selected and wild oysters, however, future research is necessary to test whether such differences in CO_2 responses are also prevalent in gill mitochondria.

The fact that survivors of tolerant mussel families at the highest PCO_2 level also showed mitochondrial changes in mantle tissue (i.e. reduced COX/CS ratio) suggests that natural selection under elevated CO_2 targets mitochondrial properties. Similarly, Gibbin et al. (2017) reported an increase in the ratio of CS to ETS (electron transport system) in the polychaete *Ophryotrocha labronica* in the fifth generation raised under elevated PCO_2. Selection on mitochondrial genes involved in oxidative phosphorylation (i.e. mitochondrial functioning) have also been observed in *Pseudocalanus acuspes* after two generations of CO_2 exposure (De Wit et al. 2016). Taken together, these results suggest that the scope of organisms to modify mitochondrial properties could be a key trait that underpins their ability to adapt to ocean acidification.

4.4 Conclusions and eco-evolutionary perspectives

By comparing physiological traits between families and populations of marine mussels (*Mytilus edulis*) and oysters (*Saccostrea glomerata*), respectively, this thesis demonstrates that physiological responses to OA can vary significantly at intra- and inter-population levels. This finding is in line with a series of other studies which show variation in CO_2 sensitivity at an inter-population (e.g. Pansch et al. 2014, Wood et al. 2016, Calosi et al. 2017), intra-population (e.g. Vihtakari et al. 2016, Frieder et al. 2018) and even inter-individual level (e.g. Pistevos et al. 2011, Guscelli et al. 2019). These findings emphasise that caution is advised when attempting to extrapolate species vulnerability to environmental drivers from single-population experiments. Furthermore, they suggest that some populations or individuals may already be pre-adapted or acclimatised to CO_2 induced changes of carbonate chemistry (Hofmann et al. 2014, Publication A4). In fact, Publication A4 demonstrates that, under common garden conditions, mussel larvae of CO_2 enriched Kiel Fjord were less affected by elevated PCO_2 when compared to a North Sea population (higher survival and smaller shell length reduction

in Kiel Fjord mussels), which experiences more stable and lower variation in carbonate chemistry than mussels in Kiel Fjord.

In Kiel Fjord, mussel reproduction occurs during the summer months, which also marks the time of the highest fluctuations of carbonate chemistry, with peak seawater PCO_2 levels of >2300 µatm (see Figure 1.4; Figure 1 in Publication A4); hence, natural selection of mussel larvae in Kiel Fjord can occur in a high PCO_2 environment. Thereby, the level of CO_2 exposure can vary significantly between larval cohorts of the same year (Figure 1 in Publication A4). This heterogeneity of selective pressure during the larval phase is reflected in the large intra-population variability of physiological responses of larvae and adult *M. edulis* reported in Publication I, II and A4. Future research is necessary to assess whether the observed differences in physiological performance between tolerant and sensitive families are related to genetic differences or phenotypic plasticity. In particular, effects of transgenerational phenotypic plasticity may have contributed to the differential responses between mussel families. Modulation of responses to ocean acidification through transgenerational phenotypic plasticity have been reported for several molluscs and other marine invertebrates (reviewed by Ross et al. 2011). Furthermore, differences in mitochondrial capacities (as observed in Publication II) can be mediated via transgenerational phenotypic plasticity (Shama et al. 2016). Phenotypic plasticity can provide a temporal buffer for genetic adaptation to 'catch-up' (Sunday et al. 2014). Thus, regardless of the origin of the observed difference of responses between sensitive and tolerant families (i.e. genotypic or phenotypic), the intra-population variability and increased CO_2 tolerance compared to the North Sea mussels suggests some capacity within this population to adapt to OA and indicates some degree of pre-existing adaptation to elevated PCO_2 in Kiel Fjord mussels.

The results for Sydney rock oysters also suggest some capacity for rapid adaptation to ocean OA within this species; the higher CO_2 tolerance of selectively-bred compared to wild oysters occurred within the - in evolutionary terms - rapid time scale of just a few generations. Furthermore, the higher capacity of selected oysters, and also in transgenerational CO_2 exposed wild oysters (Parker et al. 2015), to withstand CO_2 induced acidification of the extracellular fluid appears to be mediated by an already existing trait (i.e. gill size plasticity) suggesting that exaptation could be an additional mechanism that aids rapid adaptation to OA in marine bivalves.

Whilst these results exhibit capacities for a rapid evolutionary response to OA within the studied bivalve populations, future studies will have to reassess potential adaptive capacities

to OA in the light of other environmental drivers (e.g. temperature and hypoxia). This is because adaptation to one environmental driver may be constrained by evolutionary trade-offs mediated by negative genetic correlations of traits (Kelly & Hoffman 2012, Sunday et al. 2014). For example, a trait might be beneficial under OA scenarios but maladaptive under ocean warming. In a combined scenario (e.g. ocean warming & OA), such a negative genetic correlation will slow down an adaptive response to both drivers. In fact, the positive effect that transgenerational CO_2 exposure had on larval performance of wild Sydney rock oysters under an OA scenario, diminished dramatically when larvae were exposed to a multi-stressor scenario (elevated CO_2, elevated temperature and reduced food availability) (Parker et al. 2017).

In physiological terms, adaptation to changing environmental conditions requires an organisms' ability to adjust its energy assimilation, energy allocation and associated trade-offs in such a way that additional energetic costs incurred by the environmental driver are met while positive life-history traits (e.g. growth and reproduction) are preserved (Sokolova et al. 2013). In selected Sydney rock oysters, the higher CO_2 resilience seems mediated by their increased growth efficiency, however, this may be associated with a decreased fecundity. Likewise, although tolerant and sensitive families of Kiel Fjord mussels showed comparable shell growth rates between control and elevated PCO_2 levels and were able to reproduce after long-term CO_2 exposure (Publication A4), the results revealed that this seems to come with energetic trade-offs particularly at the highest, yet for the habitat realistic, PCO_2 level. RMRs and tissue metabolic rates were increased in tolerant families at the intermediate PCO_2, indicating CO_2 induced increases to maintenance costs which were, however, not compensated for by an increased energy assimilation. This incipient imbalance in energy demand and supply is reflected in the fact that survival at the highest PCO_2 level required significant metabolic adjustments. These adjustments translated into low RMR and reduced feeding rates that correlated with reduced mitochondrial capacities and associated increased anaerobic energy production as well as shifted mitochondrial pathways in gill and mantle tissue, respectively. Thereby, the ability of sensitive mussel families for such metabolic adjustments at the high PCO_2 level may have been constrained by a potential inherent lower metabolic efficiency (higher RMR at similar energy intake) associated with a lower gill mitochondrial scope and increased reliance on lipids as metabolic fuel in gills. As outlined above, future studies will have to assess whether the metabolic adjustments that allowed survival of tolerant mussels at the high PCO_2 level are sustainable in combination with other environmental drivers and, for example, variable levels of food availability across seasons (Wasmund & Uhlig 2003).

The physiology of marine bivalves is tightly linked with their functions within ecosystems. The fact that blue mussels can contribute up to 90% of the Baltic benthic invertebrate biomass (Kautsky et al. 1990) suggests that OA induced physiological impairments could have far reaching consequences at an ecosystem scale. For example, the finding that *M. edulis* responds to elevated seawater PCO_2 with reduced clearance rates (Publication I) could be particularly deleterious in eutrophic systems, such as Kiel Fjord. High abundance of suspension-feeders can directly reduce the amount of excess particulate organic matter (Newell 2004). As nutrient pollution is predicted to further increase in the Baltic Sea (Reusch et al. 2018), decreased filtration activities of the local mussel community could amplify negative consequences such as the expansion of hypoxic water masses. Moreover, the observed CO_2 induced shifts in metabolic pathways of mussels could result in a lower nutritional value of mussels. For example, increased tissue protein content observed in mantle tissue under elevated PCO_2 suggests a relative decrease of other components such as lipids. Furthermore, sensitive mussels had an overall lower whole body dry weight. A lower nutritional content per mussel could translate into food web consequences (Kroeker et al. 2014) and a reduction of their value in an aquaculture context (Martin et al. 2019).

In conclusion, the findings of this thesis contribute to the growing evidence that future OA will likely impair the physiology of marine mussels and oysters with potential associated ecosystem consequences. The observed intra-specific variation of OA responses suggests standing genetic variation, which is likely to be key for the persistence of populations under rapidly occurring OA (Pespeni et al. 2013). Future studies will have to incorporate other environmental drivers in order to be able to precisely determine the fate of marine mussels and oysters in the face of anthropogenic global change.

References

Abele D, Philip E, Gonzalez PM, Puntarulo S (2007) Marine invertebrate mitochondria and oxidative stress. *Frontiers in Bioscience* 12: 933–946.

Acin-Perez R, Salazar E, Kamenetsky M, Buck J, Levin LR, Manfredi G (2009) Cyclic AMP Produced inside Mitochondria Regulates Oxidative Phosphorylation. *Cell Metabolism* 9: 265–276.

Anthony KRN, Connolly SR, Willis BL (2002) Comparative analysis of energy allocation to tissue and skeletal growth in corals. *Limnology and Oceanography* 47: 1417–1429.

Applebaum SL, Pan TCF, Hedgecock D, Manahan DT (2014) Separating the Nature and Nurture of the Allocation of Energy in Response to Global Change. *Integrative and Comparative Biology* 54: 284–295.

Bailey A, De Wit P, Thor P, Browman HI, Bjelland R, Shema S et al. (2017) Regulation of gene expression is associated with tolerance of the Arctic copepod *Calanus glacialis* to CO_2-acidified sea water. *Ecology and Evolution* 7: 7145-7160.

Barton A, Hales B, Waldbusser GG, Langdon C, Feely RA (2012) The Pacific oyster, *Crassostrea gigas,* shows negative correlation to naturally elevated carbon dioxide levels: Implications for near-term ocean acidification effects. *Limnology and Oceanography* 57: 698–710.

Baumann H, Smith EM (2017) Quantifying Metabolically Driven pH and Oxygen Fluctuations in US Nearshore Habitats at Diel to Interannual Time Scales. *Estuaries and Coasts* 41: 1102–1117.

Baumann H, Talmage SC, Gobler CJ (2012) Reduced early life growth and survival in a fish in direct response to increased carbon dioxide. *Nature Climate Change* 2: 38–41.

Bax N, Williamson A, Aguero M, Gonzalez E, Geeves W (2003) Marine invasive alien species: a threat to global biodiversity. *Marine Policy* 27: 313–323.

Bayne B (2000) Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). *Journal of Experimental Marine Biology and Ecology* 251: 185–203.

Bayne BL (1976) *Marine Mussels: Their Ecology and Physiology*. Cambridge University Press, Cambridge.

Bayne BL (1999) Physiological components of growth differences between individual oysters (*Crassostrea gigas*) and a comparison with *Saccostrea commercialis*. *Physiological and biochemical zoology* 72: 705–713.

Bayne BL (2004) Phenotypic Flexibility and Physiological Tradeoffs in the Feeding and Growth of Marine Bivalve Molluscs. *Integrative and Comparative Biology* 44: 425–432.

Bayne BL, Hawkins AJS (1997) Protein Metabolism, the Costs of Growth, and Genomic Heterozygosity: Experiments with the Mussel *Mytilus galloprovincialis* Lmk. *Physiological Zoology* 70: 391–402.

Bayne BL, Hawkins AJS, Navarro E (1988) Feeding and Digestion in Suspension-Feeding Bivalve Molluscs: The Relevance of Physiological Compensations. *Integrative and Comparative Biology* 28: 147–159.

Bayne BL, Svensson S, Nell JA (1999) The physiological basis for faster growth in the Sydney rock oyster, *Saccostrea commercialis*. *The Biological bulletin* 197: 377–387.

Behrenfeld MJ, Randerson JT, McClain CR, Feldman GC, Los SO, Tucker CJ et al. (2001) Biospheric Primary Production During an ENSO Transition. *Science* 291: 2594–2597.

Bibby R, Widdicombe S, Parry H, Spicer J, Pipe R (2008). Effects of ocean acidification on the immune response of the blue mussel *Mytilus edulis*. Aquatic Biology 2: 62–74.

Boron WF (2004) Regulation of intracellular pH. *Advances in Physiology Education* 28: 160–179.

Bouchet P (2006) The Magnitude of Marine Biodiversity. In: Duarte CM (ed) *The Exploration of Marine Biodiversity:* Scientific and Technological Challenges, pp. 33–64. Fundación BBVA, Bilbao.

Boutilier RG, Heming TA, Iwama GK (1984). Appendix: Physicochemical parameters for use in fish respiratory physiology. *Fish Physiol* XA, 403-430.

Boyd J, Burnett L (1999) Reactive oxygen intermediate production by oyster hemocytes exposed to hypoxia. *The Journal of Experimental Biology* 22: 3135–3143.

Boyd PW, Brown CJ (2015) Modes of interactions between environmental drivers and marine biota. *Frontiers in Marine Science* 2: 1-7.

Boyd PW, Collins S, Dupont S, Fabricius K, Gattuso J-P, Havenhand J et al. (2018) Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change—A review. *Global Change Biology* 24: 2239–2261.

Bozinovic F, Pörtner HO (2018) Physiological ecology meets climate change. *Ecology and Evolution* 5: 1025-1030.

Bradford M (1976) A rapid and sensitive method for quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248–254.

Burnett LE (1988) Physiological Responses to Air Exposure: Acid-Base Balance and the Role of Branchial Water Stores. *Integrative and Comparative Biology* 28: 125–135.

Burton T, Killen SS, Armstrong JD, Metcalfe NB (2011) What causes intraspecific variation in resting metabolic rate and what are its ecological consequences? *Proceedings of the Royal Society B: Biological Sciences* 278: 3465–3473.

Byrne M (2012) Global change ecotoxicology: Identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. *Marine Environmental Research* 76: 3–15.

Cai WJ, Hu X, Huang WJ, Murrell MC, Lehrter JC, Lohrenz SE et al. (2011) Acidification of subsurface coastal waters enhanced by eutrophication. *Nature Geoscience* 4: 766–770.

Caldeira K, Wickett ME (2003) Anthropogenic carbon and ocean pH. *Nature* 425: 365–365.

Calosi P, De Wit P, Thor P, Dupont S (2016) Will life find a way? Evolution of marine species under global change. *Evolutionary Applications* 9: 1035–1042.

Calosi P, Melatunan S, Turner LM, Artioli Y, Davidson RL, Byrne JJ, Viant MR, Widdicombe S, Rundle SD (2017) Regional adaptation defines sensitivity to future ocean acidification. *Nature Communications* 8: 13994.

Calosi P, Rastrick SPS, Lombardi C, de Guzman HJ, Davidson L, Jahnke M et al. (2013) Adaptation and acclimatization to ocean acidification in marine ectotherms: an *in situ* transplant experiment with polychaetes at a shallow CO_2 vent system. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368: 20120444.

Chan KYK, Grünbaum D, O'Donnell MJ (2011) Effects of ocean-acidification-induced morphological changes on larval swimming and feeding. *Journal of Experimental Biology* 214: 3857–3867.

Chevin LM, Lande R, Mace GM (2010) Adaptation, Plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory. *PLOS Biology* 8: e1000357.

Claiborne JB, Blackston CR, Choe KP, Dawson DC, Harris SP, Mackenzie LA, Morrison-Shetlar AI (1999) A mechanism for branchial acid excretion in marine fish: Identification of multiple Na^+/H^+ antiporter (NHE) isoforms in gills of two seawater teleosts. *Journal of Experimental Biology* 202: 315–324.

Clements JC, Darrow ES (2018) Eating in an acidifying ocean: a quantitative review of elevated CO_2 effects on the feeding rates of calcifying marine invertebrates. *Hydrobiologia*: 820: 1–21.

Clements JC, Hunt HL (2015) Marine animal behaviour in a high CO_2 ocean. *Marine Ecology Progress Series* 536: 259–279.

Clemmesen B, Jørgensen CB (1987) Energetic Costs and Efficiencies of Ciliary Filter Feeding. *Marine Biology* 94: 445–449.

Connor KM, Sung A, Garcia NS, Gracey AY, German DP (2016) Modulation of digestive physiology and biochemistry in *Mytilus californianus* in response to feeding level acclimation and microhabitat. *Biology Open* 5: bio.019430–1210.

Coughlan J (1969) The estimation of filtering rate from the clearance of suspensions. *Marine Biology* 2: 356–358.

Cubasch U, Wuebbles D, Chen D, Facchini MC, Frame D, Mahowald N, Winther JG (2013). Introduction. In Climate Change 2013: *The Physical Science Basis.Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (eds. TF Stocker, D Qin, G-K Plattner, M Tignor, SK Allen, JBoschung, A Nauels, Y Xia, V Bex and PM Midgley), pp. 119-158. Cambridge, UK and New York, NY, USA: Cambridge University Press.

Dahlke FT, Leo E, Mark FC, Pörtner H-O, Bickmeyer U, Frickenhaus S, Storch D (2017) Effects of ocean acidification increase embryonic sensitivity to thermal extremes in Atlantic cod, *Gadus morhua*. *Global Change Biology* 23: 1499–1510.

Dam HG (2013) Evolutionary adaptation of marine zooplankton to global change. *Annual Review of Marine Science* 5: 349–370.

Daskalov GM, Grishin AN, Rodionov S, Mihneva V (2007) Trophic cascades triggered by overfishing reveal possible mechanisms of ecosystem regime shifts. *Proceedings of the National Academy of Sciences* 104: 10518–10523.

De Vooys CGN (1987) Adaptation to anaerobic metabolism in two mussel species, *Mytilus edulis* and *Mytilus galloprovincialis*, from the tidal zone at Arcachon Bay, France. *Netherlands Journal of Sea Research* 21: 17–23.

De Wit P, Dupont S, Thor P (2016) Selection on oxidative phosphorylation and ribosomal structure as a multigenerational response to ocean acidification in the common copepod *Pseudocalanus acuspes*. *Evolutionary Applications* 9: 1112–1123.

De Zwaan A, Holwerda DA (1972) The effect of phosphoenolpyruvate, fructose 1, 6-diphosphate and pH on allosteric pyruvate kinase in muscle tissue of the bivalve *Mytilus edulis* L. *Biochmica et Biophysica Acta* 276: 430–433.

De Zwaan A, Wijsman TCM (1976) Anaerobic metabolism in bivalvia (Mollusca) Characteristics of anaerobic metabolism. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 54: 313–323.

De Vries T, Holzer M, Primeau F (2017) Recent increase in oceanic carbon uptake driven by weaker upper-ocean overturning. *Nature* 542: 215–218.

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Research Part A - Oceanographic Research Papers* 34: 1733–1743.

Dickson AG, Sabine CL, Christian JR (2007) *Guide to Best Practices for Ocean CO_2 Measurements*. PICES Special Publications 3.

Diehl WJ, Gaffney PM, Koehn RK (1986) Physiological and Genetic Aspects of Growth in the Mussel *Mytilus edulis*. I. Oxygen Consumption, Growth, and Weight Loss. *Physiological Zoology* 59: 201–211.

Dorey N, Lançon P, Thorndyke M, Dupont S (2013) Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. *Global Change Biology* 19: 3355–3367.

Dove MC, O'Connor WA (2012) Reproductive cycle of Sydney rock oysters, *Saccostrea glomerata* (Gould 1850) selectively bred for faster growth. *Aquaculture* 324-325: 218–225.

Driedzic WR, Fonseca de Almeida Val VM (1996) Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). *Journal of Experimental Zoology* 274: 327–333.

Dudognon T, Soudant P, Seguineau C, Quéré C, Auffret M, Kraffe E (2013) Functional capacities of gill mitochondria in oyster *Crassostrea gigas* during an emersion/immersion tidal cycle. *Aquatic Living Resources* 26: 249–256.

Dupont S, Dorey N, Stumpp M, Melzner F, Thorndyke M (2012) Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. *Marine Biology* 160: 1835–1843.

Enderlein P, Wahl M (2004) Dominance of blue mussels versus consumer-mediated enhancement of benthic diversity. *Journal of Sea Research* 51: 145–155.

Ertl NG, O'Connor WA, Wiegand AN, Elizur A (2016) Molecular analysis of the Sydney rock oyster (*Saccostrea glomerata*) CO_2 stress response. *Climate Change Responses* 3: 6.

Esbaugh AJ, Heuer R, Grosell M (2012) Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 182: 921–934.

Etheridge DM, Steele LP, Langenfelds RL, Francey RJ, Barnola JM, Morgan VI (1996) Natural and anthropogenic changes in atmospheric CO_2 over the last 1000 years from air in Antarctic ice and firn. *Journal of Geophysical Research: Atmospheres* 101: 4115–4128.

Fabricius KE, De'ath G, Noonan S, Uthicke S (2013) Ecological effects of ocean acidification and habitat complexity on reef-associated macroinvertebrate communities. *Proceedings of the Royal Society B: Biological Sciences* 281: 20132479.

Fabricius KE, Langdon C, Uthicke S, Humphrey C, Noonan S, De'ath G et al. (2011) Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nature Climate Change* 1: 165–169.

FAO (2016) The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

FAO (2018) The State of World Fisheries and Aquaculture 2018- Meeting the sustainable development goals. Rome. 210 pp.

Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, Fabry VJ, Millero FJ (2004) Impact of Anthropogenic CO_2 on the $CaCO_3$ System in the Oceans. *Science* 305: 362–366.

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science* 320: 1490–1492.

Feely R, Doney S, Cooley S (2009) Ocean Acidification: Present Conditions and Future Changes in a High-CO_2 World. *Oceanography* 22: 36–47.

Form AU, Riebesell U (2012) Acclimation to ocean acidification during long-term CO_2 exposure in the cold-water coral *Lophelia pertusa*. *Global Change Biology* 18: 843–853.

Frieder CA, Applebaum SL, Pan F, Manahan DT (2018) Shifting Balance of Protein Synthesis and Degradation Sets a Threshold for Larval Growth Under Environmental Stress. *Biological Bulletin* 234: 45–57.

Gattuso JP, Frankignoulle M, Wollast R (1998) Carbon And Carbonate Metabolism In Coastal Aquatic Ecosystems. *Annual Review of Ecology and Systematics* 29: 405–434.

Gattuso JP, Magnan A, Bille R, Cheung WWL, Howes EL, Joos F et al. (2015) Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. *Science* 349: aac4722.

Gaylord B, Kroeker KJ, Sunday JM, Anderson KM, Barry JP, Brown NE et al. (2015) Ocean acidification through the lens of ecological theory. *Ecology* 96: 3–15.

Génio L, Kiel S, Cunha MR, Grahame J, Little CTS (2012) Shell microstructures of mussels (Bivalvia: Mytilidae: Bathymodiolinae) from deep-sea chemosynthetic sites: Do they have a phylogenetic significance? *Deep Sea Research Part I: Oceanographic Research Papers* 64: 86–103.

Ghalambor CK, McKay JK, Carroll S, Reznick DN (2007) Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology* 21: 394–407.

Gibbin EM, Chakravarti LJ, Jarrold MD, Christen F, Turpin V, N'Siala GM, Blier PU, Calosi P (2017) Can multi-generational exposure to ocean warming and acidification lead to the adaptation of life history and physiology in a marine metazoan? *Journal of Experimental Biology* 220: 551–563.

Gnaiger E, Lassnig B, Kuznetsov A, Rieger G, Margreiter R (1998) Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *Journal of Experimental Biology* 201: 1129–1139.

Goncalves P, Anderson K, Raftos DA, Thompson EL (2018) The capacity of oysters to regulate energy metabolism-related processes may be key to their resilience against ocean acidification. *Aquaculture Research* 49: 2059–2071.

González PM, Rocchetta I, Abele D, Rivera-Ingraham GA (2019) Hypoxically Induced Nitric Oxide: Potential Role as a Vasodilator in *Mytilus edulis* Gills. *Frontiers in Physiology* 9: 1709.

Gosling E (2003) *Bivalve molluscs: biology, ecology and culture*. Blackwell Publishing Ltd, Oxford.

Gould SJ, Vrba ES (1982) Exaptation—a Missing Term in the Science of Form. *Paleobiology* 8: 4–15.

Gran G (1952). Determination of the equivalence point in potentiometric titrations. Part II. *Analyst* 77, 661-671.

Gray JS, Wu RS-S, Or YY (2002) Effects of hypoxia and organic enrichment on the coastal marine environment. *Marine Ecology Progress Series* 238: 249–279.

Gruber N, Clement D, Carter BR, Feely RA,van Heuven S, Hoppema M et al. (2019) The oceanic sink for anthropogenic CO_2 from 1994 to 2007. *Science* 363: 1193-1199.

Gruber N (2011) Warming up, turning sour, losing breath: ocean biogeochemistry under global change. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Science* 369: 1980-1996.

Guderley HG, Pörtner HO (2010) Metabolic power budgeting and adaptive strategies in zoology: examples from scallops and fish. *Canadian Journal of Zoology* 88: 753–763.

Gueron S, Levit-Gurevich K (1999) Energetic considerations of ciliary beating and the advantage of metachronal coordination. *Proceedings of the National Academy of Sciences* 96: 12240–12245.

Guppy M, Withers P (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews of the Cambridge Philosophical Society* 74: 1–40.

Guscelli E, Spicer JI, Calosi P (2019) The importance of inter-individual variation in predicting species' responses to global change drivers. *Ecology and Evolution* 9: 4327–4339.

Gutiérrez JL, Jones CG, Strayer DL, Iribarne OO (2003) Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos* 101: 79–90.

Gutowska MA, Melzner F, Langenbuch M, Bock C, Claireaux G, Pörtner HO (2010) Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 180: 323–335.

Haider F, Falfushynska H, Ivanina AV, Sokolova IM (2016) Effects of pH and bicarbonate on mitochondrial functions of marine bivalves. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 198: 41–50.

Hall-Spencer JM, Rodolfo-Metalpa R, Martin S, Ransome E, Fine M, Turner SM, Rowley SJ, Tedesco D, Buia M-C (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature* 454: 96–99.

Hammer KM, Kristiansen E, Zachariassen KE (2011) Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae). *Marine Environmental Research* 72: 135–142.

Harley CDG (2011) Climate Change, Keystone Predation, and Biodiversity Loss. *Science* 334: 1124–1127.

Harlocker SL, Kapper MA, Greenwalt DE, Bishop SH (1991) Phosphoenolpyruvate carboxykinase from ribbed mussel gill tissue: Reactivity with metal ions, kinetics, and action of 3-mercaptopicolinic acid. *Journal of Experimental Zoology* 257: 285–298.

Hawkins AJS (1991) Protein Turnover: A Functional Appraisal. *Functional Ecology* 5: 222.

Hawkins AJS, Bayne BL, Day AJ (1986) Protein Turnover, Physiological Energetics and Heterozygosity in the Blue Mussel, *Mytilus edulis*: The Basis of Variable Age-Specific Growth. *Proceedings of the Royal Society Part B* 229: 161–176.

Heisler N (1986) Buffering and transmembrane ion transfer processes. In: Heisler N (ed) *Acid-Base Regulation in Animals*, 3–47. Elsevier, Amsterdam.

Heisler N (1989) Interactions between gas exchange, metabolism, and ion transport in animals: an overview. *Canadian Journal of Zoology* 67: 2923–2935.

Hernroth B, Baden S, Thorndyke M, Dupont S (2011) Immune suppression of the echinoderm *Asterias rubens* (L.) following long-term ocean acidification. *Aquatic Toxicology* 103: 222–224.

Heuer RM, Grosell M (2016) Elevated CO_2 increases energetic cost and ion movement in the marine fish intestine. *Scientific Reports* 6: 34480.

Hochachka PW, Somero GN (1984) *Biochemical Adaptation*. Princeton University Press, Princeton.

Hofmann GE, Evans TG, Kelly MW, Padilla-Gamiño JL, Blanchette CA, Washburn L et al. (2014) Exploring local adaptation and the ocean acidification seascape; studies in the California Current Large Marine Ecosystem. *Biogeosciences* 11: 1053–1064.

Holwerda DA, De Zwaan A (1973) Kinetic and molecular characteristics of allosteric pyruvate kinase from muscle tissue of the sea mussel *Mytilus edulis* L. *Biochimica et Biophysica Acta - Enzymology* 309: 296–306.

Honkoop PJC, Bayne BL, Drent J (2003) Flexibility of size of gills and palps in the Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) and the Pacific oyster *Crassostrea gigas* (Thunberg, 1793). *Journal of Experimental Marine Biology and Ecology* 282: 113–133.

Hu MY, Hwang P-P, Tseng Y-C (2015) Recent advances in understanding trans-epithelial acid-base regulation and excretion mechanisms in cephalopods. *Tissue Barriers* 3: 1–12.

Hüning (2014) Kompensationsvermögen von *Mytilus edulis* gegenüber mechanischem Stress und erhöhtem pCO_2 - kombinierte transkriptomische, proteomische und physiologische Analysen. PhD thesis, University of Bremen, Germany

Hüning AK, Melzner F, Thomsen J, Gutowska MA, Krämer L, Frickenhaus S et al. (2013) Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: Implications for shell formation and energy metabolism. *Marine Biology* 160: 1845–1861.

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 [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

Islam MS, Tanaka M (2004) Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Pollution Bulletin* 48: 624–649.

Jackson JBC, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ et al. (2001) Historical Overfishing and the Recent Collapse of Coastal Ecosystems. *Science* 293: 629–637.

Jimenez AG, Jayawardene S, Alves S, Dallmer J, Dowd WW (2015) Micro-scale environmental variation amplifies physiological variation among individual mussels. *Proceedings of the Royal Society B: Biological Sciences* 282: 20152273.

Kapsenberg L, Kelley AL, Shaw EC, Martz TR, Hofmann GE (2015) Near-shore Antarctic pH variability has implications for the design of ocean acidification experiments. *Scientific Reports* 5: 9638.

Kautsky N, Johannesson K, Tedengren M (1990) Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplants. I. Growth and morphology. *Marine Ecology Progress Series* 59: 203–210.

Kelly MW, Hofmann GE (2013) Adaptation and the physiology of ocean acidification. *Functional Ecology* 27: 980-990.

Kelly MW, Padilla-Gamiño JL, Hofmann GE (2013) Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus purpuratus*. *Global Change Biology* 19: 2536–2546.

Kreiss CM, Michael K, Bock C, Lucassen M, Pörtner HO (2014) Impact of long-term moderate hypercapnia and elevated temperature on the energy budget of isolated gills of Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 182C: 102–112.

Kreiss CM, Michael K, Lucassen M, Jutfelt F, Motyka R, Dupont S, Pörtner HO (2015) Ocean warming and acidification modulate energy budget and gill ion regulatory mechanisms in Atlantic cod (*Gadus morhua*). *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 185: 767–781.

Kroeker KJ, Kordas RL, Crim R, Hendriks IE, Ramajo L, Singh GS, Duarte CM, Gattuso J-P (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Global Change Biology* 19: 1884–1896.

Kroeker KJ, Sanford E, Jellison BM, Gaylord B (2014) Predicting the effects of ocean acidification on predator-prey interactions: a conceptual framework based on coastal molluscs. *The Biological Bulletin* 226: 211–222.

Lande R, Shannon S (1996) The Role of Genetic Variation in Adaptation and Population Persistence in a Changing Environment. *Evolution* 50: 434–437.

Langenbuch M, Bock C, Leibfritz D, Pörtner HO (2006) Effects of environmental hypercapnia on animal physiology: a ^{13}C NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 144: 479–484.

Lannig G, Eckerle L, Serendero I, Sartoris FJ, Fischer T, Knust R, Johansen T, Pörtner HO (2003) Temperature adaptation in eurythermal cod (*Gadus morhua*): a comparison of mitochondrial enzyme capacities in boreal and Arctic populations. *Marine Biology* 142: 589–599.

Lannig G, Eilers S, Pörtner HO, Sokolova IM, Bock C (2010) Impact of Ocean Acidification on Energy Metabolism of Oyster, *Crassostrea gigas*—Changes in Metabolic Pathways and Thermal Response. *Marine Drugs* 8: 2318–2339.

Lawrence DR, Scott GI (1982) The determination and use of condition index of oysters. *Estuaries* 5: 23–27.

Le Quéré C, Raupach MR, Canadell JG, Marland G, Bopp L, Ciais P et al. (2009) Trends in the sources and sinks of carbon dioxide. *Nature Geoscience* 2: 831–836.

Lee K, Tong LT, Millero FJ, Sabine CL, Dickson AG, Goyet C et al. (2006) Global relationships of total alkalinity with salinity and temperature in surface waters of the world's oceans. *Geophysical Research Letters* 33: L196505.

Lewis E, Wallace D (1998) Program Developed for CO_2 System Calculations. ORNL/ CDIAC-105. Carbon dioxide Information Analysis Center, Oak Ridge National Laboratory. US Department of Energy, Oak Ridge, TN.

Lindinger MI, Lauren DJ, McDonald DG (1984) Acid-base balance in the sea mussel, *Mytilus edulis*. III: Effects of environmental hypercapnia on intra-and extracellular acid-base balance. *Marine Biology Letters* 5: 371–381.

Lockwood BL, Somero GN (2012) Functional Determinants of Temperature Adaptation in Enzymes of Cold- versus Warm-Adapted Mussels (Genus *Mytilus*). *Molecular Biology and Evolution* 29:3061–3070.

Maggi E, Bertocci I, Vaselli S, Benedetti-Cecchi L (2009) Effects of changes in number, identity and abundance of habitat-forming species on assemblages of rocky seashores. *Marine Ecology Progress Series* 381: 39–49.

Mallet AL, Haley LE (2011) Growth Rate and Survival in Pure Population Matings and Crosses of the Oyster *Crassostrea virginica*. *Canadian Journal of Fisheries and Aquatic Sciences* 40: 948–954.

Mark FC, Hirse T, Pörtner HO (2005) Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. *Polar Biology* 28: 805–814.

Martin VAS, Gelcich S, Lavín FV, Oliva RDP, Hernández JI, Lagos NA, Birchenough SNR, Vargas CA (2019) Linking social preferences and ocean acidification impacts in mussel aquaculture. *Scientific Reports* 9: 1–9.

Mathieu S, Sylvain D, Walden F, Catapane E, Carroll M (2014) GABA is an inhibitory neurotransmitter in ganglia of the bivalve mollusc, *Crassostrea virginica*. *The FASEB Journal* 28: Supplement 1059.4.

Mathis JT, Pickart RS, Byrne RH, McNeil CL, Moore GWK, Juranek LW et al. (2012) Storm-induced upwelling of high pCO_2 waters onto the continental shelf of the western Arctic Ocean and implications for carbonate mineral saturation states. *Geophysical Research Letters* 39: L07606

Maus B, Bock C, Pörtner HO (2018) Water bicarbonate modulates the response of the shore crab *Carcinus maenas* to ocean acidification. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 459: 1–16.

McClelland GB, Dalziel AC, Fragoso NM, Moyes CD (2005) Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. *Journal of Experimental Biology* 208: 515–522.

McNeil BI, Sasse TP (2016) Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. *Nature* 529: 383–386.

Mehrbach C, Culberso CH, Hawley JE, Pytkowic RM (1973) Measurement of Apparent Dissociation Constants of Carbonic Acid in Seawater at Atmospheric Pressure. *Limnology and Oceanography* 18: 897–907.

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner HO (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* 6: 2313–2331.

Melzner F, Stange P, Trübenbach K, Thomsen J, Casties I, Panknin U, Gorb SN, Gutowska MA (2011) Food Supply and Seawater pCO_2 Impact Calcification and Internal Shell Dissolution in the Blue Mussel *Mytilus edulis*. *PLoS ONE* 6: e24223.

Melzner F, Thomsen J, Koeve W, Oschlies A, Gutowska MA, Bange HW, Hansen HP, Körtzinger A (2013) Future ocean acidification will be amplified by hypoxia in coastal habitats. *Marine Biology* 160: 1875–1888.

Meure CM, Etheridge D, Trudinger C, Steele P, Langenfelds R, van Ommen T, Smith A, Elkins J (2006) Law Dome CO_2, CH_4 and N_2O ice core records extended to 2000 years BP. *Geophysical Research Letters* 33: L14810.

Meyhöfer E (1985) Comparative pumping rates in suspension-feeding bivalves. *Marine Biology* 85: 137–142.

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Marine Ecology Progress Series* 293: 109–118.

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B (2011) How Many Species Are There on Earth and in the Ocean? *PLOS Biology* 9: e1001127.

Moyes CD, Mathieu-Costello OA, Tsuchiya N, Filburn C, Hansford RG (1997) Mitochondrial biogenesis during cellular differentiation. *American Journal of Physiology-Cell Physiology* 272: C1345–C1351.

Müller M, Mentel M, van Hellemond JJ, Henze K, Woehle C et al. (2012) Biochemistry and Evolution of Anaerobic Energy Metabolism in Eukaryotes. *Microbiology and Molecular Biology Reviews* 76: 444-495.

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

Munday PL, Crawley NE, Nilsson GE (2009) Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Marine Ecology Progress Series* 388: 235–242.

Munday PL, Warner RR, Monro K, Pandolfi JM, Marshall DJ (2013) Predicting evolutionary responses to climate change in the sea. *Ecology Letters* 16: 1488–1500.

Nardi A, Benedetti M, Fattorini D, Regoli F (2018) Oxidative and interactive challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*. *Aquatic Toxicology* 196: 53–60.

Narita D, Rehdanz K, Tol RSJ (2012) Economic costs of ocean acidification: a look into the impacts on global shellfish production. *Climatic Change* 113: 1049–1063.

Navarro JM, Torres R, Acuña K, Duarte C, Manríquez PH, Lardies M, Lagos NA, Vargas C, Aguilera V (2013) Impact of medium-term exposure to elevated pCO_2 levels on the physiological energetics of the mussel *Mytilus chilensis*. *Chemosphere* 90: 1242–1248.

Nell JA, Smith IR, McPhee CC (2000) The Sydney rock oyster *Saccostrea glomerata* (Gould 1850) breeding programme: progress and goals. *Aquaculture Research* 31: 45-49.

Newell RIE (2004) Ecosystem influences of natural and cultivated populations of suspension-feeding bivalve molluscs: a review. *Journal of Shellfish Research* 23: 51–62.

Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sørensen C, Watson S-A, Munday PL (2012) Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change* 2: 201–204.

O'Connor WA, Dove MC (2009) The Changing Face of Oyster Culture in New South Wales, Australia. *Journal of Shellfish Research* 28: 803–811.

O'Connor WA, Dove MC, Finn B, O'Connor SJ (2008) *Manual for hatchery production of Sydney rock oysters (*Saccostrea glomerata*)*. NSW Department of Primary Industries.

Orr JC, Fabry VJ, Aumont O, Bopp L, Doney SC, Feely RA et al. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* 437: 681–686.

Osterman N, Vilfan A (2011) Finding the ciliary beating pattern with optimal efficiency. *Proceedings of the National Academy of Sciences* 108: 15727–15732.

Palmer AR (1992) Calcification in marine molluscs: how costly is it? *Proceedings of the National Academy of Sciences* 89: 1379–1382.

Palumbo A (2005) Nitric oxide in marine invertebrates: A comparative perspective. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 142: 241–248.

Pan TCF, Applebaum SL, Manahan DT (2015) Experimental ocean acidification alters the allocation of metabolic energy. *Proceedings of the National Academy of Sciences* 112: 4696–4701.

Pan TCF, Applebaum SL, Frieder CA, Manahan DT (2018) Biochemical bases of growth variation during development: a study of protein turnover in pedigreed families of bivalve larvae (*Crassostrea gigas*). *Journal of Experimental Biology* 221: jeb171967.

Pansch C, Schaub I, Havenhand J, Wahl M (2014) Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. *Global Change Biology* 20: 765–777.

Paparo A (1972) Innervation of the Lateral Cilia in the Mussel *Mytilus Edulis* L. *The Biological bulletin* 143: 592–604.

Paparo A, Aiello E (1970) Cilio-inhibitory effects of branchial nerve stimulation in the mussel, *Mytilus edulis*. *Comparative and General Pharmacology* 1: 241–250.

Parker LM, O'Connor WA, Byrne M, Coleman RA, Virtue P, Dove M et al. (2017) Adult exposure to ocean acidification is maladaptive for larvae of the Sydney rock oyster *Saccostrea glomerata* in the presence of multiple stressors. *Biology Letters* 13: 20160798–5.

Parker LM, O'Connor WA, Raftos A, Pörtner HO, Ross PM (2015) Persistence of Positive Carryover Effects in the Oyster, *Saccostrea glomerata,* following Transgenerational Exposure to Ocean Acidification. *PLoS ONE* 10: e0132276.

Parker LM, O'Connor WA, Byrne M, Dove M, Coleman RA, Pörtner HO et al. (2018) Ocean acidification but not warming alters sex determination in the Sydney rock oyster, *Saccostrea glomerata*. *Proceedings of the Royal Society B: Biological Sciences* 285: 20172869–9.

Parker LM, Ross PM, O'Connor WA (2010) Populations of the Sydney rock oyster, *Saccostrea glomerata*, vary in response to ocean acidification. *Marine Biology* 158: 689–697.

Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Pörtner H-O (2012) Adult exposure influences offspring response to ocean acidification in oysters. *Global Change Biology* 18: 82–92.

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. A*nnual Review of Ecology, Evolution, and Systematics* 37: 637–669.

Pechenik JA (1999) On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series* 177: 269–297.

Perkins FO, Wolf PH (1976) Fine Structure of *Marteilia sydneyi* sp. n.: Haplosporidan Pathogen of Australian Oysters. *The Journal of Parasitology* 62: 528–538.

Pespeni MH, Sanford E, Gaylord B, Hill TM, Hosfelt JD, Jaris HK et al. (2013) Evolutionary change during experimental ocean acidification. *Proceedings of the National Academy of Sciences* 110: 6937–6942.

Peters R, Raftos DA (2003) The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). *Aquaculture* 223: 29–39.

Pistevos JCA, Calosi P, Widdicombe S, Bishop JDD (2011) Will variation among genetic individuals influence species responses to global climate change? *Oikos* 120: 675–689.

Pörtner HO (2008) Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series* 373: 203–217.

Pörtner HO (2012) Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Marine Ecology Progress Series* 470: 273–290.

Pörtner HO, Farrell AP (2008) Physiology and Climate Change. *Science* 322: 690–692.

Pörtner HO, Lannig G (2009). Oxygen and capacity limited thermal tolerance. In: Richards JG, Farrell AP, Brauner CJ (eds) *Fish Physiology:* Hypoxia (vol 27), Academic Press, pp.143–191.

Pörtner HO, Bock C, Mark FC (2017) Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. *Journal of Experimental Biology* 220: 2685–2696.

Pörtner HO, Bock C, Reipschläger A (2000) Modulation of the cost of pH_i regulation during metabolic depression: a ^{31}P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. *Journal of Experimental Biology* 203: 2417–2428.

Pörtner HO, Langenbuch M, Reipschläger A (2004) Biological Impact of Elevated Ocean CO_2 Concentrations: Lessons from Animal Physiology and Earth History. *Journal of Oceanography* 60: 705–718.

Pörtner HO (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* 88, 137-146.

Pörtner HO, Karl DM, Boyd PW, Cheung W, Lluch-Cota SE, Nojiri Y, Schmidt DN, Zavialov PO (2014) Ocean Systems. In: Field CB, Barros VR, Dokken DJ, Mach KJ, Mastrandrea MD, Bilir TE et al. (eds) *Climate Change 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*, 411–484. Cambridge University Press, NY.

Rahmstorf S (2002) Ocean circulation and climate during the past 120,000 years. *Nature* 419: 207–214.

Ramajo L, Pérez-Léon E, Hendriks IE, Marbà N, Krause-Jensen D, Sejr MK et al. (2016) Food supply confers calcifiers resistance to ocean acidification. *Scientific Reports* 6: 19374.

Rastrick SPS, Calosi P, Calder-Potts R, Foggo A, Nightingale G, Widdicombe S, Spicer JI (2014) Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *The Journal of Experimental Biology* 217: 2499–2508.

Rastrick SPS, Collier V, Graham H, Strohmeier T, Whiteley NM, Strand Ø (2018) Feeding plasticity more than metabolic rate drives the productivity of economically important filter feeders in response to elevated CO_2 and reduced salinity. *ICES Journal of Marine Science* 75: 2117–2128.

Reusch TBH (2013) Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. *Evolutionary Applications* 7: 104–122.

Reusch TBH, Dierking J, Andersson HC, Bonsdorff E, Carstensen J, Casini M et al. (2018) The Baltic Sea as a time machine for the future coastal ocean. *Science Advances* 4: eaar8195.

Riahi K, Grübler A, Nakicenovic N (2007) Scenarios of long-term socio-economic and environmental development under climate stabilization. *Technological Forecasting and Social Change* 74: 887–935.

Ricevuto E, Benedetti M, Regoli F, Spicer JI, Gambi MC (2015) Antioxidant capacity of polychaetes occurring at a natural CO_2 vent system: Results of an *in situ* reciprocal transplant experiment. *Marine Environmental Research* 112: 44–51.

Ridgwell A, Zeebe RE (2005) The role of the global carbonate cycle in the regulation and evolution of the Earth system. *Earth and Planetary Science Letters* 234: 299–315.

Riebesell U, Fabry VJ, Hansson L, Gattuso JP (Eds) (2010) *Guide to Best Practices for Ocean Acidification Research and Data Reporting*. Publications Office of the European Union, Luxembourg.

Riebesell U, Gattuso J-P (2015) Lessons learned from ocean acidification research. *Nature Climate Change* 5: 12-14.

Riisgård HU, Larsen PS (2007) Viscosity of seawater controls beat frequency of water-pumping cilia and filtration rate of mussels *Mytilus edulis*. *Marine Ecology Progress Series* 343: 141–150.

Riisgård HU, Randløv A (1981) Energy budgest, growth and filtration rates in *Mytilus edulis* at different algal concentrations. *Marine Biology* 61: 227–234.

Rivera-Ingraham GA, Rocchetta I, Bickmeyer U, Meyer S, Abele D (2016) Spatial compartmentalization of free radical formation and mitochondrial heterogeneity in bivalve gills revealed by live-imaging techniques. *Frontiers in Zoology* 13: 4.

Ross PM, Parker L, O'Connor WA, Bailey EA (2011) The Impact of Ocean Acidification on Reproduction, Early Development and Settlement of Marine Organisms. *Water* 3: 1005–1030.

Rossoll D, Bermúdez R, Hauss H, Schulz KG, Riebesell U, Sommer U, Winder M (2012) Ocean Acidification-Induced Food Quality Deterioration Constrains Trophic Transfer. *PLoS ONE* 7: e34737.

Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL et al. (2004) The oceanic sink for anthropogenic CO_2. *Science* 305: 367–371.

Salin K, Auer SK, Rey B, Selman C, Metcalfe NB (2015) Variation in the link between oxygen consumption and ATP production, and its relevance for animal performance. *Proceedings of the Royal Society B: Biological Sciences* 282: 20151028.

Sanders T, Schmittmann L, Nascimento-Schulze JC, Melzner F (2018) High Calcification Costs Limit Mussel Growth at Low Salinity. *Frontiers in Marine Science* 5: 1417.

Sánchez-Muros MJ, García-Rejón L, García-Salguero L, de laHiguera M, Lupiáñez JA (1998) Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diet on glutamate dehydrogenase and alanine aminotransferase kinetics. *The International Journal of Biochemistry & Cell Biology* 30: 55–63.

Scanes E, Parker LM, O'Connor WA, Ross PM (2014) Mixed Effects of Elevated pCO_2 on Fertilisation, Larval and Juvenile Development and Adult Responses in the Mobile Subtidal Scallop *Mimachlamys asperrima* (Lamarck, 1819). *PLoS ONE* 9.

Schalkhausser B, Bock C, Stemmer K, Brey T, Pörtner HO, Lannig G (2013) Impact of ocean acidification on escape performance of the king scallop, *Pecten maximus*, from Norway. *Marine Biology* 160: 1995–2006.

Schrobback P, Pascoe S, Coglan L (2014) History, status and future of Australia's native Sydney rock oyster industry. *Aquatic Living Resources* 27: 153–165.

Shama LNS, Mark FC, Strobel A, Lokmer A, John U, Mathias Wegner K (2016) Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. *Evolutionary Applications* 9: 1096–1111.

Sidell BD, Driedzic WR, Stowe DB, Johnston IA (1987) Biochemical Correlations of Power Development and Metabolic Fuel Preferenda in Fish Hearts. *Physiological Zoology* 60: 221–232.

Silliman KE, Bowyer TK, Roberts SB (2018) Consistent differences in fitness traits across multiple generations of Olympia oysters. *Scientific Reports* 8: 1–8.

Smith RW, Houlihan DF (1995) Protein synthesis and oxygen consumption in fish cells. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology* 165: 93–101.

Sokolova IM (2018) Mitochondrial Adaptations to Variable Environments and Their Role in Animals' Stress Tolerance. *Integrative and Comparative Biology* 58: 519-531.

Sokolova IM (2013) Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology* 53: 597–608.

Sokolova IM, Frederich M, Bagwe R, Lannig G, Sukhotin AA (2012) Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research* 79: 1–15.

Spiers ZB, Gabor M, Fell SA, Carnegie RB, Dove M, Connor WO et al. (2014) Longitudinal study of winter mortality disease in Sydney rock oysters *Saccostrea glomerata*. *Diseases of Aquatic Organisms* 110: 151–164.

Sswat M, Stiasny MH, Taucher J, Algueró-Muñiz M, Bach LT, Jutfelt F, Riebesell U, Clemmesen C (2018) Food web changes under ocean acidification promote herring larvae survival. *Nature Ecology & Evolution* 2: 836–840.

Stearns S (1992) *The Evolution of Life History*. Oxford University Press, Oxford, United Kingdom.

Stillman JH, Paganini AW (2015) Biochemical adaptation to ocean acidification. *Journal of Experimental Biology* 218: 1946–1955.

Stocker TF, Qin D, Plattner GK, Alexander LV, Allen SK, Bindoff NL et al. (2013) Technical Summary. In: Climate Change 2013: *The Physical Science Basis. Contribution of Working Group I to the Fifth Assess- ment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Strobel A, Bennecke S, Leo E, Mintenbeck K, Pörtner HO, Mark FC (2012) Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. *Frontiers in Zoology* 9: 28.

Strobel A, Graeve M, Pörtner HO, Mark FC (2013a) Mitochondrial acclimation capacities to ocean warming and acidification are limited in the antarctic Nototheniid Fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. *PLoS ONE* 8: e68865.

Strobel A, Leo E, Pörtner HO, Mark FC (2013b) Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 166: 48–57.

Stuckas H, Stoof K, Quesada H, Tiedemann R (2009) Evolutionary implications of discordant clines across the Baltic Mytilus hybrid zone (M*ytilus edulis* and *Mytilus trossulus*). *Heredity* 103: 146–156.

Stumpp M, Hu MY, Casties I, Saborowski R, Bleich M, Melzner F, Dupont S (2013) Digestion in sea urchin larvae impaired under ocean acidification. *Nature Climate Change* 3: 1044-1049.

Su W, Rong J, Zha S, Yan M, Fang J, Liu G (2018) Ocean Acidification Affects the Cytoskeleton, Lysozymes, and Nitric Oxide of Hemocytes: A Possible Explanation for the Hampered Phagocytosis in Blood Clams, *Tegillarca granosa*. *Frontiers in Physiology* 9: 182.

Sun T, Tang X, Jiang Y, Wang Y (2017) Seawater acidification induced immune function changes of haemocytes in *Mytilus edulis*: a comparative study of CO_2 and HCl enrichment. *Scientific Reports* 7: 41488.

Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH (2014) Evolution in an acidifying ocean. *Trends in Ecology & Evolution* 29: 117–125.

Sunday JM, Fabricius KE, Kroeker KJ, Anderson KM, Brown NE, Barry JP et al. (2017) Ocean acidification can mediate biodiversity shifts by changing biogenic habitat. *Nature Climate Change* 7: 81–85.

Takahashi T, Sutherland SC, Sweeney C, Poisson A, Metzl N, Tilbrook B et al. (2002) Global sea–air CO_2 flux based on climatological surface ocean pCO_2, and seasonal biological and temperature effects. *Deep Sea Research Part II: Topical Studies in Oceanography* 49: 1601–1622.

Takahashi T, Sutherland SC, Wanninkhof R, Sweeney C, Feely RA, Chipman DW et al. (2009) Climatological mean and decadal change in surface ocean pCO_2, and net sea–air CO_2 flux over the global oceans. *Deep Sea Research Part II: Topical Studies in Oceanography* 56: 554–577.

Talmage SC, Gobler CJ (2010) Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. *Proceedings of the National Academy of Sciences* 107: 17246–17251.

Tamayo D, Ibarrola I, Urrutxurtu I, Navarro E (2014) Physiological basis of extreme growth rate differences in the spat of oyster (*Crassostrea gigas*). *Marine Biology* 161: 1627–1637.

Tambutté E, Venn AA, Holcomb M, Segonds N, Techer N, Zoccola D, Allemand D, Tambutté S (2015) Morphological plasticity of the coral skeleton under CO_2-driven seawater acidification. *Nature Communications* 6: 7368.

Taylor CT, Moncada S (2010) Nitric Oxide, Cytochrome C Oxidase, and the Cellular Response to Hypoxia. *Arteriosclerosis, Thrombosis, and Vascular Biology* 30: 643–647.

Taylor JD (1969) The shell structure and mineralogy of the Bivalvia. Introduction. Nuculacea-Trigonacea. *Bulletin of the British Museum Natural History (Zool.)* 3: 1–125.

Tedengren M, Kautsky N (1986) Comparative study of the physiology and its probable effect on size in Blue Mussels (*Mytilus Edulis* L.) from the North Sea and the Northern Baltic Proper. *Ophelia* 25: 147–155.

Telesca L, Peck LS, Sanders T, Thyrring J, Sejr MK, Harper EM (2019) Biomineralization plasticity and environmental heterogeneity predict geographical resilience patterns of foundation species to future change. *Global Change Biology* 00: 1-15.

Thompson RJ, Bayne BL (1974) Some relationships between growth, metabolism and food in the mussel *Mytilus edulis*. *Marine Biology* 27: 317–326.

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. *Marine Biology* 157: 2667–2676.

Thomsen J, Casties I, Pansch C, Körtzinger A, Melzner F (2013) Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global Change Biology* 19: 1017–1027.

Thomsen J, Gutowska M, Saphörster J, Heinemann A, Trübenbach K, Fietzke J et al. (2010) Calcifying invertebrates succeed in a naturally CO_2 enriched coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* 7: 3879–3891.

Thomsen J, Haynert K, Wegner KM, Melzner F (2015) Impact of seawater carbonate chemistry on the calcification of marine bivalves. *Biogeosciences* 12: 4209–4220.

Thomsen J, Himmerkus N, Holland N, Sartoris FJ, Bleich M, Tresguerres M (2016) Ammonia excretion in mytilid mussels is facilitated by ciliary beating. *The Journal of Experimental Biology* 219: 2300–2310.

Thor P, Dupont S (2015) Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. *Global Change Biology* 21: 2261–2271.

Timmins-Schiffman E, Coffey WD, Hua W, Nunn BL, Dickinson GH, Roberts SB (2014) Shotgun proteomics reveals physiological response to ocean acidification in *Crassostrea gigas*. *BMC genomics* 15: 951.

Tomanek L, Zuzow MJ, Ivanina AV, Beniash E, Sokolova IM (2011) Proteomic response to elevated PCO_2 level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *Journal of Experimental Biology* 214: 1836–1844.

Towle EK, Enochs IC, Langdon C (2015) Threatened Caribbean Coral Is Able to Mitigate the Adverse Effects of Ocean Acidification on Calcification by Increasing Feeding Rate. *PLoS ONE* 10: e0123394.

Tunnicliffe V, Davies KTA, Butterfield DA, Embley RW, Rose JM, Chadwick WW (2009) Survival of mussels in extremely acidic waters on a submarine volcano. *Nature Geoscience* 2: 344–348.

Valladares F, Matesanz S, Guilhaumon F, Araújo MB, Balaguer L, Benito Garzón M et al. (2014) The effects of phenotypic plasticity and local adaptation on forecasts of species range shifts under climate change. *Ecology Letters* 17: 1351–1364.

van der Zee EM, van der Heide T, Donadi S, Eklöf JS, Eriksson BK, Olff H, van der Veer HW, Piersma T (2012) Spatially Extended Habitat Modification by Intertidal Reef-Building Bivalves has Implications for Consumer-Resource Interactions. *Ecosystems* 15: 664–673.

van Vuuren DP, Elzen den MGJ, Lucas PL, Eickhout B, Strengers BJ, van Ruijven B, Wonink S, van Houdt R (2007) Stabilizing greenhouse gas concentrations at low levels: an assessment of reduction strategies and costs. *Climatic Change* 81: 119–159.

Vihtakari M, Havenhand J, Renaud PE, Hendriks IE (2016) Variable Individual- and Population- Level Responses to Ocean Acidification. *Frontiers in Marine Science* 3: 331.

Waldbusser GG, Salisbury JE (2014) Ocean Acidification in the Coastal Zone from an Organism's Perspective: Multiple System Parameters, Frequency Domains, and Habitats. *Annual Review of Marine Science* 6: 221–247.

Waldbusser GG, Hales B, Langdon CJ, Haley BA, Schrader P, Brunner EL et al. (2015) Ocean Acidification Has Multiple Modes of Action on Bivalve Larvae. *PLoS ONE* 10: e0128376.

Wang X, Wang M, Jia Z, Wang H, Jiang S, Chen H, Wang L, Song L (2016) Ocean acidification stimulates alkali signal pathway: A bicarbonate sensing soluble adenylyl cyclase from oyster *Crassostrea gigas* mediates physiological changes induced by CO_2 exposure. *Aquatic Toxicology* 181: 124–135.

Wang X, Wang M, Xu J, Jia Z, Liu Z, Wang L, Song L (2017) Soluble adenylyl cyclase mediates mitochondrial pathway of apoptosis and ATP metabolism in oyster *Crassostrea gigas* exposed to elevated CO_2. *Fish and Shellfish Immunology* 66: 140–147.

Wasmund N, Uhlig S (2003) Phytoplankton trends in the Baltic Sea. *ICES Journal of Marine Science* 60: 177–186.

Watson SA, Lefevre S, McCormick MI, Domenici P, Nilsson GE, Munday PL (2014) Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. *Proceedings of the Royal Society B: Biological Sciences*. 281: 20132377.

Watson SA, Southgate PC, Tyler PA, Peck LS (2009) Early larval development of the sydney rock oyster *Saccostrea glomerata* under near-future predictions of CO_2-driven ocean acidification. *Journal of Shellfish Research* 28: 431–437.

Widdicombe S, Spicer JI (2008) Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *Journal of Experimental Marine Biology and Ecology* 366: 187–197.

Wieser W, Krumschnabel G (2001) Hierarchies of ATP-consuming processes: direct compared with indirect measurements, and comparative aspects. *The Biochemical Journal* 355: 389–395.

Wittmann AC, Pörtner HO (2013) Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change* 3: 995–1001.

Wood HL, Sundell K, Almroth BC, Sköld HN, Eriksson SP (2016) Population-dependent effects of ocean acidification. *Proceedings of the Royal Society B: Biological Sciences* 283: 20160163.

Wright JM, Parker LM, O'Connor WA, Scanes E, Ross PM (2018) Ocean acidification affects both the predator and prey to alter interactions between the oyster *Crassostrea gigas* (Thunberg, 1793) and the whelk *Tenguella marginalba* (Blainville, 1832). *Marine Biology* 165: 46.

Zeebe RE, Wolf-Gladrow DA (2001) *CO_2 in seawater: equilibrium, kinetics, isotopes*. Elsevier Oceanographic Series, Vol. 65, p. 346.

Appendix

Publication A1

Evolution of Marine Organisms under Climate Change at Different Levels of Biological Organisation

Ben P. Harvey, Balsam Al-Janabi, Stefanie Broszeit, Rebekah Cioffi, Amit Kumar, Maria Aranguren-Gassis, Allison Bailey, Leon Green, Carina M. Gsottbauer, Emilie F. Hall, Maria Lechler, Francesco P. Mancuso, Camila O. Pereira, Elena Ricevuto, Julie B. Schram, **Laura S. Stapp**, Simon Stenberg and Lindzai T. Santa Rosa

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Review

Evolution of Marine Organisms under Climate Change at Different Levels of Biological Organisation

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Abstract: Research to date has suggested that both individual marine species and ecological processes are expected to exhibit diverse responses to the environmental effects of climate change. Evolutionary responses can occur on rapid (ecological) timescales, and yet studies typically do not consider the role that adaptive evolution will play in modulating biological responses to climate change. Investigations into such responses have typically been focused at particular biological levels (e.g., cellular, population, community), often lacking interactions among levels. Since all levels of biological organisation are sensitive to global climate change, there is a need to elucidate how different processes and hierarchical interactions will influence species fitness. Therefore, predicting the responses of communities and populations to global change will require multidisciplinary efforts across multiple levels of hierarchy, from the genetic and cellular to communities and ecosystems. Eventually, this may allow us to establish the role that acclimatisation and adaptation will play in determining marine community structures in future scenarios.

Keywords: ocean acidification; climate change; acclimation; evolutionary potential; adaptation; biological organisation; biologically-relevant scales

1. Introduction

Evolutionary processes play a fundamental role in the organisational structure of biological systems and the diversity of life [1]. It is possible for evolution to occur on a rapid ecological timescale, that may allow organisms to avoid extinction following environmental change [2]. One environment which is arguably changing faster than others is the marine environment [3], where increasing levels of atmospheric CO_2 are causing the seawater temperature and carbonate chemistry of surface waters to

change at geologically unprecedented rates [4]. Future warming and altered ocean chemistry (broadly termed climate change throughout the present review) are recognised as pervasive and detrimental anthropogenic influences on marine life [5–9]. Climate change is expected to impose strong selection pressure on fitness-related traits, impacting on populations and ecosystems [10–14], and yet most future projections of community dynamics and population persistence in marine organisms do not consider the role of evolution and adaptive capacity [15–17].

The potential for genetic adaptation in response to climate change has been acknowledged [17,18], and adaptive evolution may represent a critical mechanism which could alleviate some of the negative consequences expected with future climate change [19]. However, the relatively limited number of studies means that evidence is still somewhat scarce [20]. A number of recent reviews outline the role of adaptive evolution in the face of climate change, including the need for determining species' capacity for evolutionary adaptation and physiological acclimatisation, the distinctions between evolutionary and phenotypically plastic responses, and summaries of the different experimental approaches (e.g., molecular tools, quantitative genetics, standing genetic variation, and experimental evolution). They also outline possible directions for future research (for reviews, see [16,17,19–23], and references therein). A glossary for some of the terms commonly used in this review is given in Box 1.

Box 1. Glossary for terms used in this article.

Acclimation: Reversible process of an organism to adjust to experimental conditions. When the process is induced by natural environmental changes, it is called **acclimatisation**.

Bottleneck effect: Reduction in population size due to environmental events, leading to a strong reduction of the variation in the gene pool.

Effective population size: Size of a hypothetical ideal population with random mating that corresponds to population genetic processes within the focal wild population.

Epigenetics: Heritable changes in gene regulation processes that are not caused by changes in the DNA sequence.

Evolution: Genetic changes in a population over generations. It is said to be microevolution when these changes occur over relatively short timescales, rather than on geological scales (macroevolution).

Evolutionary rescue: Genetic adaptation of populations that allows them to recover from demographic effects and avoid extinction.

Experimental evolution: Controlled experiment that exposes populations to new environmental conditions for multiple generations to observe for genetic adaptation.

Fitness: The potential for individuals of a given genotype to survive and pass their genes to future generations by influencing either their own reproductive success or that of related individuals.

Genetic adaptation: A process of transgenerational selection of genes to maximise or maintain the relative fitness of a population in a given environment.

Phenotypic buffering: Type of phenotypic plasticity, in which no difference in the response of a trait to a given environment might be observed because plasticity in a physiological process allows an organism to maintain fitness.

Phenotypic plasticity: Phenotypic adjustment to the environment without any genetic change.

Quantitative genetics: Method to partition the observed phenotypic variance among relatives (of known genetic relatedness) into their environmental and genetic components.

Selection: Non-random reproduction or survival of individuals of a particular phenotype.

Studies investigating biological responses to climate change will often be carried out with a particular focus, whether that be physiology, evolutionary biology or community ecology. Such focus naturally means that other interacting facets of eco-evolution are often neglected [24]. Clearly, there are many important inter-disciplinary studies that do bridge this gap (e.g., [25–27]), however, there are still often disparities in the extent (if at all) that adaptive evolution is considered by different disciplines when determining a species' response under a changing environment. Inter-disciplinary work that links eco-evolution through biological hierarchies is not a new concept having been raised by numerous influential comparative physiologists in the 1950s, such as C. Ladd Prosser [28]. We believe this idea bears reiterating, and consider modern science to possess the necessary advancements in technology and communication required to begin incorporating this concept into future research.

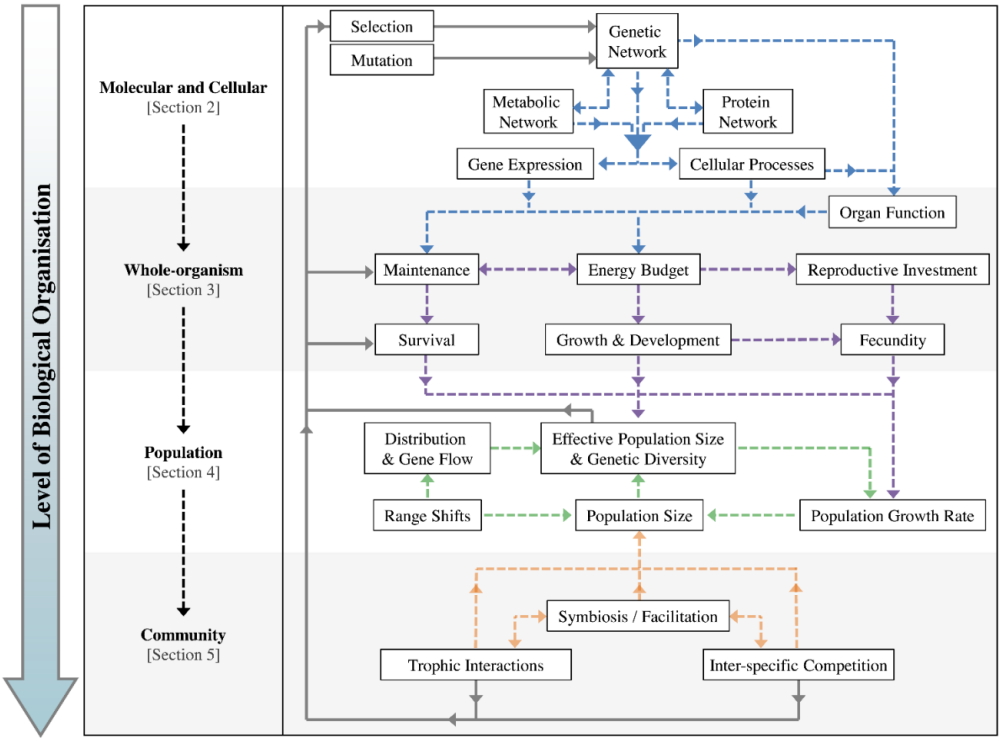
There are a number of factors that mediate evolutionary processes, but their effects are highly dependent on the level of biological organisation that is considered (e.g., intra-individual, whole-organism, population, community and ecosystem, see Figure 1). The underlying mechanisms of how these levels of hierarchy will interact to influence fitness in the face of climate change are poorly understood, but are important in determining whether individual populations and communities will persist at levels comparable to the present day [16].

This review will focus on factors that can modulate adaptive evolution at different levels of biological organisation, by considering the response of marine organisms at these different levels in terms of the consequences for fitness traits (*i.e.*, lifetime reproductive success). We discuss: (1) what molecular and cellular mechanisms exist that can influence fitness and drive adaptive evolution; (2) how changes in life history and behavioural characteristics of organisms can influence lifetime reproductive success; (3) how demographic processes (gene frequencies, population size and turnover) and genetic architecture (heritability, imprinting, genetic correlations and diversity) of the population will influence adaptive evolution; and (4) how changes in species interactions and community composition influence the magnitude and direction of adaptive evolution of populations.

2. Role of Molecular and Cellular Processes in Evolutionary Responses

Molecular and cellular level studies can provide several approaches for improving our understanding of the potential for adaptation in response to climate change. These can include characterising an organism's capacity to acclimatise to changing environmental conditions, as well as establishing a more mechanistic understanding of the response of organisms to abiotic factors at different levels of intra-individual biological organisation, such as the nature of sub-lethal cellular stress [29]. Eventually this might enable us to investigate whether genetic adaptation can occur at a sufficient rate to maintain the physiological functioning required for survival and reproduction, and gain important insights into energy allocation and physiological responses due to climate change, as well as other biotic and abiotic stressors [22]. However, the distribution of a species is shaped by both a species' physiological limits and biotic interactions with co-existing species, and therefore, cellular and molecular studies alone may only provide part of the picture.

Figure 1. Conceptual diagram of the factors modulating evolution at different levels of biological organisation (molecular, cellular, whole-organism, population and community), that will determine the response of marine organisms to future climate change. The arrow on the left represents the increased biological complexity (going from top to bottom). Single-headed arrows indicate the direction of the effect with the level of biological organisation indicated by different colours. Effects originating from the molecular and cellular (dashed blue), whole-organism (dashed purple), population (dashed green) and community (dashed orange). Double-headed arrows indicate that there is feedback between two factors, as well as the effect, and the solid grey arrows indicate a feedback loop. Note that the depiction of factors is conceptual and not comprehensive.



2.1. Biochemical Reactions and Gene Expression

Within the organism, protein activity is often thought to underlie variations in fitness (for discussion, see [30]). Fitness at the biochemical level could be simply considered as the ability of proteins to function (within their respective intra- and extra-cellular setting) in order to integrate the diverse functions of cells and organelles [31]. Proteins are responsible for crucial functions in all biological processes [31], and evolutionary changes can occur through changes in the proteins themselves (e.g., post-translational modifications), the encoding gene(s) of those proteins, or the transcription of those encoding genes [31].

Fitness-related traits can be influenced through genetic variation in these proteins, such as the collinearity of gene mutations, whereby the point mutations in the DNA sequence will correspondingly change the sequence of amino acids in a protein [32]. These biochemical consequences can influence

protein function and in turn, tolerances to environmental conditions [33]. For example, a minor mutation (only two amino-acids out of 334) in a dehydrogenase enzyme in the temperate mussel *Mytilus galloprovincialis* (Lamarck, 1819) resulted in higher thermal tolerance towards warm conditions [34]. Alternatively, enzymes possessing alternative alleles, such as for lactate dehydrogenase-B in cold- and warm-adapted populations of the killifish (*Fundulus heteroclitus* L., 1776) [35], may be able to confer adaptation potential for thermal tolerance through variable allele frequencies.

In order to produce adaptive phenotypes, changes may be required in multiple combinations of alleles [36]. Allelic changes are embedded within genetic networks and hence, will not occur independently to other changes, since any allelic changes at a particular locus will influence only one aspect of a genetic network [37]. These genetic networks essentially consist of the genes which encode the transcription factors as the input for each coding gene, and the *cis*-regulatory modules that control the appropriate phases of expression of these genes [38]. Gene regulatory networks control the expression of genes in any given developmental process [39], including fitness-related traits, and therefore, any changes in the networks could play an important role in adaptive evolution and climate change responses [37].

Environmental effects may cause changes either in specific genes within the network, influencing their gene expression, or affect the gene regulatory network as a whole [40]. Genetic networks will primarily be influenced by current environmental conditions and maternal effects (the latter described in Section 2.3), and these changes will, in turn, alter the protein and metabolic networks that influence gene regulation (*via* a feedback loop reaction) [37]. Changes in genetic networks may influence plastic responses and facilitate adaptive evolution by providing a rapid response to the changing environmental conditions. However, if the genetic regulatory network is influenced by other factors that do not follow the changing environmental conditions, such as photoperiod [41] or even biotic interactions [42] (discussed further in Section 5), then adaptive evolution might require a restructuring of the genetic network in order to conform to the novel environmental conditions [37].

Currently it remains unclear whether the few examples that demonstrate observable adaptive evolution of traits in response to climate change (e.g., body size [43], migration timing [44], thermal responses [45]) are dictated by various independent genes (within their respective genetic networks), or by fewer key regulatory genes within their genetic or metabolic networks. This is important to consider since any changes in the 'upstream' network genes could have extensive and numerous effects on traits [37], and yet the network itself may also provide some redundancy and buffering against perturbations, whereby changes to regulatory genes do not influence the genes they regulate [46]. Eventually, it may be possible to identify common genetic (e.g., collinearity in the gene order between genomes [47]) and physiological mechanisms underlying species responses [17]. However, studies demonstrating a clear link between the genetic variation and phenotypic variation for the majority of traits are scarce (but for example, see [48]). Therefore, any studies of genetic variation should focus on traits with more straightforward or measurable relationships to fitness [17,21].

Establishing the evolutionary significance of cellular-level plasticity (*i.e.*, the changes in the expression levels of stress-related genes, e.g., [49]) requires demonstration of a heritable component of expression variation, or allelic variation in the coding genes themselves [17,50]. Accurately estimating selection responses requires the genetic component of this variation (in regulatory responses) to be related to the fitness of the organism [51,52] in order to ascertain the fitness-related consequences for

the individual and the population. This highlights the need to investigate transcriptome profile responses in terms of survival, fecundity, or other ecologically important traits that determine lifetime reproductive success (but see [30] for a discussion on the limitations in the link between the transcriptome and the phenotype), and importantly, ascertain whether sufficient genetic variation exists in that trait [53].

2.2. Cellular Processes and Organ Function

Cellular and organ functioning during stressful conditions will primarily be dictated by changes at the genomic and biochemical level (Section 2.1). The principal factor determining the underlying cellular stress response (a universally conserved mechanism to protect macromolecules within cells from damage [54]) depends on the extent of stress-induced disturbances (reviewed by [55]). During moderate stress, resources may be shifted from anabolic (e.g., protein biosynthesis) towards vital processes for cellular homeostasis (e.g., ion regulation; [55]) to maintain cellular integrity and ensure short-term survival. However, on longer time scales such shifts may not be feasible and might lead to a reduction in organism performance (e.g., reduced growth rates or fecundity) since the organismal energy budget can be considered as the sum of all cellular energy budgets [55].

Such trade-offs in physiological functions could have important fitness consequences, but may not be apparent when only observing the whole organism level. For instance, a study on the effects of ocean acidification on the reef-building coral, *Acropora millepora* (Ehrenberg, 1834), reported major changes in gene expression and cell physiology long before phenotypic effects were observed, in this case, a decrease in calcification rates [54]. Thus, cellular functioning might play a central role in linking environmental conditions to an organism's fitness [56], and the plasticity and adaptive evolution of cellular processes may be an important influence on species resilience towards changing environmental conditions.

Adaptive evolution in cellular function may be possible through gene duplication [57–59], whereby paralogous genes (*i.e.*, gene copies) that perform a particular function either increase their expression (increased gene dosage) or diverge their functions through mutation [60]. This divergence can be achieved by one of the copies acquiring a new function, or through a partial loss-of-function mutation of both copies that complement each other [61], while retaining the full set of functions (termed sub-functionalisation [60]). This sub-functionalisation is a relatively common mechanism for functionally related proteins [61], such as components of cell signalling pathways, and may facilitate evolution of advantageous traits: e.g., a changed pH optima of proteins [62], a beneficial trait for maintaining acid-base homeostasis in response to ocean acidification.

2.3. Epigenetics and Trans-Generational Plasticity

The environment experienced by an organism can shape the phenotype of their offspring, and is termed trans-generational plasticity (e.g., [63]). Trans-generational plasticity can be due to maternal or paternal effects, genomic imprinting, gene expression or other epigenetic processes. These epigenetic effects (whether a gene is being expressed or not) can be transmitted through the germ line [64], which can allow for transmission through meiosis to the succeeding generation, constituting a heritable, epigenetic change [65]. For example, five weeks exposure to elevated pCO_2 during the reproductive

conditioning of Sydney rock oysters (*Saccostrea glomerata*, Gould 1850) reduced the development time and increased the body size of their larvae through trans-generational plasticity [66].

Mechanisms exist that should allow these epigenetic changes to result in localised changes in the DNA sequence, such as changes in the activity of chromatin-modifying enzymes [65]. Providing they exert the same functional effect, any epigenetic effects can potentially become a genetic change, and exert a selectable phenotypic response [65]. During climate change, the environmental conditions that induce these epigenetic effects (like temperature) will persist (albeit progressively increasing) and therefore with each successive generation, the epigenetic response could actually result in continued DNA change in selected regions of the genome [65].

The gene regulatory network, responsible for many fitness-related traits (Section 2.1), is initiated by maternal transcripts and proteins, which cascade into subsequent gene regulatory interactions [67]. Early genes that function during development (such as for larval morphology) can be influenced by the fitness traits of the maternal parent (e.g., by changes in egg size or provisioning [67]), and therefore it may be possible that parental exposure to climate change can cause DNA (or heritable, epigenetic) changes that promote adaptive evolution in key regulatory genes, or the genetic network as a whole.

3. Role of Whole-Organism Physiological and Behavioural Responses

Marine organisms possess a range of reproductive and developmental strategies that have important implications for their fitness [68]. Different reproductive modes, life histories, and demographic processes can influence these strategies [69–71]. In this section we focus on how climate change, specifically ocean acidification and warming, can influence the physiology and behaviour of the individuals, affecting their survival and fitness. It is important to consider the factors that influence selection at this level of biological organisation in order to link individual phenotypes, which are in turn driven by transcriptional and cellular processes, to population-level effects.

3.1. Maintenance and Energetic Trade-Offs

The capacity to maintain metabolic processes under environmental stress may support (or promote) the retention of particular life history traits (such as reproductive output) that may ultimately determine a species' biogeography [72,73]. A recent study using an *in situ* transplant experiment with polychaetes, found that species capable of maintaining their metabolic rates (under stress) were able to migrate into or even colonise areas characterised by chronically elevated levels of pCO_2 [26]. This high-CO_2 tolerance was achieved in the polychaetes *via* acclimatisation for *Amphiglena mediterranea* (Leydig, 1851) and by adaptation for *Platynereis dumerilii* (Audouin & Milne-Edwards, 1834) [26]. However, such resilience often comes at a cost [74]. The individuals of *P. dumerilii* were smaller in body size compared to nearby populations in lower pCO_2 conditions, attributed to increases in maintenance costs due to a higher mean metabolic rate under chronic exposure to elevated pCO_2. Since the size (in several polychaete species) can determine the maximum numbers of eggs that a female produces, this resilience could result in reduced reproductive output [26]. Although the study did not empirically test this, any reallocation of energy away from reproduction would clearly have important implications for lifetime reproductive success.

Fitness-related traits can be genetically correlated to each other and, depending on strength and direction of selection, influence the potential for adaptive evolution (for more detail, see [17]). Briefly, a positive correlation could include a co-tolerance to multiple stressors (e.g., developing sea urchin larvae obtaining tolerance to low pH and therefore also temperature [75]), or a selection for a particular trait providing tolerance for another trait (e.g., growth and disease resilience in Sydney rock oyster (*S. glomerata*) providing tolerance to high pCO_2, [66]). If the intra-individual physiological mechanisms (Section 2) and an organism's response during climate change are nonlinearly related, then there is a need to understand what physiological trade-offs are occurring that are influencing their fitness related traits. Fitness trade-offs will certainly influence potentially selected traits, if other energetically maintained traits are selected over survival or reproductive output.

3.2. Life-History Stages

Research into physiological responses to climate change has demonstrated that fitness traits, such as reproduction and development, are likely to be disproportionally affected [76–78]. Since natural selection acts upon lifetime reproductive success, climate change can reduce fitness through impacts on early life-history stages, such as an increase in developmental duration or number of defects [79–81]. However, many marine species have complex life histories, and despite early life history stages being considered to be particularly vulnerable to climate change [82], there is increasing evidence that selection pressures act on each life stage differently (e.g., [83]). Phenotypic carry-over effects can also occur between life history stages (as well as trans-generationally, Section 2.3) that could exacerbate or alleviate the impacts on fitness-related traits. For example, exposure to stressful conditions during the larval stage can reduce the juvenile fitness if those conditions continue (e.g., [84]). This may be particularly important given that different stages of ontogeny may utilise different habitats (e.g., [85]) or exhibit different behaviour. Hence, impacts considered on individual life-stages may not accurately estimate the fitness response of a given species [86].

3.3. Behavioural Responses

Organismal behaviour is mediated by multiple external and internal sensory inputs that may be changed directly and indirectly by climate change [87]. The plastic behavioural responses observed in organisms are largely a direct physiological response to a changing environment, since the nervous system is under biochemical and physiological control [88]. Hence, changes in the underlying physiological condition (see Section 2) could influence behavioural performance by constraining an ecologically-relevant behaviour, such as swimming activity [89]. A study in coral reef fish found that small temperature increases (<3 °C) contributed to changes in animal personality (activity, boldness, aggression), thought to be linked to individual responses in energy metabolism [90].

Changing environments can also modulate behaviour by interfering with sensory inputs and neural functioning. For example, elevated levels of pCO_2 are hypothesised to remodel the sensory pathway of the GABA-A system of marine organisms, including the larval clownfish (*Amphiprion percula* Lacepède, 1802), damselfish (*Neopomacentrus azysron* Bleeker, 1877), and gastropod *Gibberulus gibbosus* (Röding, 1798) [91,92], causing sensory and behavioural impairment, including learning ability [93]. This phenomenon is thought to be associated with ion regulatory mechanisms during high

CO_2 exposure (accumulation of intracellular HCO_3^- and Cl^-), which interfere with neurotransmitter functions (for more details, see [92]). Impaired learning regarding the identity of predators during high pCO_2, or diminished detection of the olfactory cues for settlement (for instance) influence fitness by negatively affecting the survivorship of the individual [93,94]. Sensory pathways occur in differing complexities with receptors and messenger systems of different adaptive potential [95]. Hence, knowing the mechanistic pathway of a behavioural response is important for determining the evolutionary potential of an organism or indeed a trait. Linking these pathways with their genes is important for finding out if organisms can adapt, in order to cope behaviourally with environmental stressors [96]. Behavioural traits may be more evolutionary labile than other traits [97], and may contribute to or hinder adaptation [19,98].

4. Role of Population-Level Responses

Focusing on population-level organisation is crucial for connecting the fitness responses of lower levels (individual/population) to changes in higher levels (species/community). The analysis of microevolution in populations requires an understanding of how environmental changes influence evolutionary processes such as gene flow, mutation, genetic drift and natural selection [99]. Historically, the concept and investigation of population level adaptation in the marine environment was largely dismissed; it was assumed that marine connectivity would maintain high levels of gene flow between populations via adult and larval dispersal [100], and so impede local adaptation. However, new evidence compiled by Sanford and Kelly [101] shows that microevolution is not restricted to organisms with low dispersal abilities. Through a literature survey Sanford and Kelly [101] found that 66% of marine invertebrates with planktonic life stages for dispersal, *i.e.*, meroplankton, present highly adaptive differentiation at the population level (e.g. *Haliotis rufescens*, Table 1). Depending on the taxa investigated, the planktonic dispersal stages of the identified (66%) invertebrates experienced brief (up to a few days as with some corals, sea anemones or ascidians) to prolonged (several weeks to longer, some crustaceans and gastropods [100]) planktonic larval durations.

4.1. Demographic Processes

Populations can respond to environmental pressures more rapidly through range shifts and phenotypic plasticity rather than through evolutionary adaptation [102]. Evolutionary responses are likely to vary depending on the cost of adaptation, timescale, life-history and dispersal ability in addition to other factors [19]. Different evolutionary responses have been previously investigated and require a variety of techniques (for a survey of selected reference studies see Table 1). Understanding genetic variation, as well as specific population dynamics, is crucial to explore the potential for evolutionary rescue [103]. For example, populations in isolated environments, such as the Baltic Sea, may also undergo isolation and develop genetic endemism as a result of local extinctions or adaptation by evolutionary rescue [104]. Therefore, population size and genetic variation in the context of the intensity and duration of environmental selection pressures must be considered [105] to identify what part of the population (*i.e.*, the effective population size [106]) contributes to the next generation.

Table 1. Published studies investigating population level evolutionary responses to climate change (including ocean acidification) in marine species.

Taxonomic Affiliation	Response Variable(s)	Driver	Method(s)	Evolutionary Response	Ref.
Spermatophyta: *Zostera marina*	Growth rate Survival	T	F	Genotypic complementarity	[107]
Coccolithophyceae: *Emiliania huxleyi*	Growth rate Production rate: (PIC)	OA	LS	Selection of genotypes Direct positive adaptation	[108]
Gephyrocapsa oceanica	Growth rate Carbon fixation	OA	LS	Selection of genotypes (Adaptation)	[109]
Diatomophyceae: *Thalassiosira pseudonana*	Phytosynthetic efficiency	OA	LS	No adaptation	[110]
Anthozoa: *Acropora millepora*	Thermal and physiological tolerance	T	F	Natural selection	[111]
Pocillopora damicornis	Coral bleaching (thermal tolerance)	T ES	CG	Local adaptation or acclimation	[112]
Bivalvia: *Mytilus trossulus*	Growth rate Survival	T	TE	Possible thermal adaptation	[113]
Gastropoda: *Haliotis rufescens*	Genetic polymorphism	T	SNP	Local adaptation Genetic differentiation	[114]
Polychaeta: *Platynereis dumerilii*	Body size	OA	TE	Genetic adaptation	[26]
Amphiglena mediterranea	Body size	OA	TE	Physiological plasticity	[26]
Amphipoda: *Orchestia gammarellus*	Growth Thermal tolerance	T	LS	Selection	[115]
Cirripedia: *Semibalanus balanoides*	Genetic polymorphism	T D	TE	Balancing selection Local adaptation	[116]
Copepoda: *Tigriopus californicus*	Survival (LT_{50}) Thermal plasticity	T	LS	Low adaptation potential	[117]
Decapoda: *Uca pugnax*	Developmental rate	T	CG	Selection on variation Local adaptation	[118]
Echinoidea: *Heliocidaris erythrogramma armigera*	Hatching success	T	QG	Genotype-by- environment interaction	[119]
Strongylocentrotus purpuratus	Gene expression: thermal resistance	T	CG	Selection of thermally sensitive genes	[120]
Strongylocentrotus purpuratus	Larval body size	OA	CG	Heritability correlates with high-pCO_2	[121]
Centrostephanus rodgersii	Cleavage and gastrulation stage	T OA	QG	Heritable genetic variation for sires	[75]
Centrostephanus rodgersii	Embryonic development	T OA	CG	Varying expansion of population	[122]
Teleostei: *Gadus morhua*	Body shape	T	CG	Counter-gradient variation	[123]
Fundulus heteroclitus	Thermal tolerance	T	LS	Selection Regulation of heat shock proteins	[124]

Notes: Selective driver: abbreviated as T - temperature; OA - ocean acidification; ES - environmental stability; D - desiccation. Method: F - field experiment; LS - laboratory selection experiment; CG - common garden experiment; TE - transplant experiment; SNP - outlier SNP analysis; QG - quantitative genetics.

Populations may have an increased chance of persistence if they react to changing climatic conditions with higher phenotypic plasticity. Should this plasticity occur in a fitness-related trait, then this may present a heritable variation for selection to act upon (e.g., [23,125]). This mechanism would thereby allow for a faster non-mutational selection [126]. Populations that are maladapted to climate change will likely experience an initial decline and thus, a reduced effective population size [127]. Phenotypic buffering, a type of phenotypic plasticity, represents an important mechanism for maintaining population performance under stressful conditions until adaptive evolution can "catch up" and sufficiently improve population fitness [23,128]. For example, genetically diverse populations of the seagrass *Zostera marina* (L., 1758) showed quicker recovery following sub-lethal temperature stress when compared to less diverse populations [107]. This buffering effect was expressed due to the complementarity of different genotypes (e.g., facilitation) that maintained ecosystem functioning, and may promote adaptive evolution [107].

4.2. Environmental Variability

The potential for adaptation under naturally low or fluctuating pH can be studied in regions of upwelling along the continental coast of (Western) North America [129]. A transcriptomic analysis of sea urchin larvae (*Strongylocentrotus purpuratus* Stimpson, 1857) collected from a naturally variable low pH upwelling site revealed that larvae under present day conditions initiated a robust transcriptional response, but only a muted response to near future conditions [130]. These exposures to transient extreme conditions may be sufficient to provide populations with a selection for tolerance (e.g., [131]). However, the question then becomes whether selection for one stressor will provide increased tolerance to another. Quantitative genetics is a technique that may help answer this type of question because it allows partitioning of the observed phenotypic variance of a population among relatives (with known genetic relatedness) into their environmental and genetic components [132], in a synchronic approach (*sensu* [23]). Numerous studies have demonstrated evolutionary adaptive capacity using quantitative genetics (as reviewed in [71]).

In the absence of mutations, adaptive evolution relies on the genetic variation in physiological tolerances [133], this is because in turn, the variation of physiological tolerances influences the likelihood of extinction [121,134]. These tolerance traits in natural populations are termed standing genetic variation, and arguably the most important influence maintaining this adaptive variation is spatially varying selection [135]. For most species, the temperature gradient across their distribution (e.g., 30 °C difference between the pole and equator [121]) will greatly exceed the expected future temperature change (3.7–4.8 °C, [136]). In contrast, pH gradients are often relatively homogenous when compared to predicted change (0.3–0.5 pH units by 2100 [136]; but see [129,137,138]). Therefore, populations may possess greater adaptive variation for temperature tolerance, but have less adaptive variation for pH tolerance [117].

It is crucial to distinguish between microevolutionary (genetic) and phenotypic (plastic) responses at the population level. Many past studies have lacked this focus, but identifying the drivers responsible for changes in fitness traits should be given more attention in future studies (e.g. [19,139]). Non-genetic evidence can also be lacking, missing potential patterns, such as in situations of counter gradient variation whereby genetic and environmental influence can oppose each other [140]. This was the case

for the genetic divergence of body shape between two populations of juvenile Atlantic cod (*Gadus morhua* L., 1758), in which phenotypic differences were mitigated by environmental influences [123]. Even the positive, negative or neutral correlation between two fitness traits may accelerate, slow down, or not impact adaptive evolution [17]. As such, local environmental variability must be considered when determining population responses.

4.3. Modes of Population-Level Response

Examination of time series data reveals evolutionary responses to climate change, such as direct allochronic studies which include a mixture of populations that are on their way to adaptation or extinction (reviewed in depth by [102]). These studies can show that the selection of genotypes is an immediate mechanism of population-level adaptation. Multi-generational analysis of selection of the coccolithophore (*Emiliania huxleyi* (Lohmann) Hay & Mohler, 1967) has provided evidence for evolutionary adaptation responses detected by selection of genotypes and direct positive adaptation to increased pCO_2 by mutation [108]. However, it is important to emphasise that the rate of adaptation for single-celled organisms, due to their fast generation times, will likely differ along with the mechanisms utilised when compared to multi-cellular organisms. Future studies should be optimised by an interdisciplinary approach, including abiotic changes driven by climate change, biological networks, and the relationship between the phenotypic and genetic analysis, for a better understanding of future climate change impacts on the evolution of populations.

5. Community Composition and Interactions

While studies of evolution on single species and populations are already underway (either *in situ* or in the laboratory), the potential of communities and ecosystems to evolve as a unit in response to changing environments has not yet received as much attention. This is partially due to the complex nature of communities. Another important reason is that for several decades, ecological and evolutionary time scales were thought to diverge widely and this has led to very different thought models of evolution and ecology [141]. In particular, it was thought that evolution takes place in time frames that cannot influence ecology, while the effect of ecology on evolution has been studied in some prominent examples. For instance, in the Atlantic cod (*Gadus morhua* L., 1758) fishing pressure led to earlier age at maturation [142]. However, the dynamic effect of evolution on ecology is an emerging field of study since it was recognised that evolution of ecologically relevant traits can influence contemporary communities [143,144].

If community composition is altered, the coevolution between interacting species will be driven and/or modified by their interactions within the community [145,146]. This diffuse coevolution means that the selection of a specific trait in one species may depend on the presence of another species [147], making species identity and uniqueness a plastic response in community-level responses [148]. Therefore, the effects of future climate change on communities will likely be complex [149], and influence the outcomes of competition, facilitation (e.g. [150]) and trophic interactions (e.g., predator-prey [151,152], and plant-herbivore [153,154]).

5.1. Changes to Community Dynamics

The fast population turnover of single-celled phytoplankton represents a great opportunity to study experimental evolution and to quantify evolutionary and plastic responses of populations to future climate change [155]. Phytoplankton communities represent a pivotal role in marine ecosystem functioning [155], forming the base of the marine food web and crucial for global biogeochemical cycles [156]. Under current conditions of dissolved inorganic carbon, many phytoplankton species are not fully saturated for growth and photosynthesis, and therefore, will benefit from the addition of CO_2 (e.g., [157–159]). However, any selection for fast growth, despite providing competitive ability through size (but see [160]), may come at the cost of reduced resilience to pCO_2 [161]. This was shown by a study ([161]) that used genetically distinct isolates of phytoplankton species (sixteen strains of the diatom *Skeletonema marinoi*, Sarno & Zingone 2005 and eight strains of dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen, 1985) and found that slow-growing cultures generally responded positively to elevated pCO_2, while fast-growing cultures either showed neutral or negative responses. Hence, the effects of climate change need to be considered holistically in terms of both ecological performance as well as physiological tolerance.

The enormous diversity of phytoplankton and the variety of environmental stressors makes it unthinkable to experimentally test all the possible trait responses in every phytoplankton group. The difficulty lies in establishing whether this evolutionary potential can be realised, and whether results from laboratory experiments can be related to natural populations (see [155]). Therefore, understanding the mechanistic effects of future climate change on key functional groups (e.g., [162]) will require a deeper understanding, across biological hierarchies, of the direct effects on their physiology (molecular and cellular), basic biology (whole-organism), as well as estimates of gene flow, population size, and recombination rates (population) [155].

In order to extrapolate from the organism and individual species' responses to the community level, we also need to understand the response of the ecological interactions within the community. For example, any increased biomass associated with higher atmospheric CO_2 may be indirectly mediated by the presence of grazers (indirect trophic interactions, e.g., [163]), or regulated by heterotrophs of the same community (e.g., [164]). Similarly, phytoplankton responses associated with climate change can lead to bottom-up control (e.g., [165]), or, due to sufficient food availability to marine organisms may provide physiological homeostasis (e.g., *Mytilus edulis* L.; 1758 [166]). As such, if the effects of climate change differ between similar co-existing species (e.g., [167]), it may indirectly influence selection by causing ecological release; reducing the need for competitive traits.

In addition to the direct effects, future climate change may have indirect effects on other communities. Where CO_2 is a resource for organisms, it can play an important role leading to changes in community competition (e.g., [168]). For example, opportunistic turf- and mat-forming algae have been demonstrated to inhibit other taxa (e.g., [169]) and outcompete kelp recruitment (e.g., [170]), inducing phase shifts. Species in diverse communities tend to have lower effective population sizes compared to when they are in isolation due to the competitive interactions [171]. This typically increases the role of genetic drift compared to selection, and might reduce the rates of adaptive evolution [172]. Climate change might reduce those inter-specific interactions (e.g., bottom-up control releasing resource limitation [165]) and thereby enhance the potential for adaptation, through reductions in genetic drift.

Alternatively, climate change may increase competition (e.g., [173]) and amplify changes in mean population size, increasing extinction risks, as well as decreasing adaptation rates (Figure 1). This may be further exacerbated through co-extinctions, due to increased habitat and biodiversity loss, whereupon one species is dependent on another that is already extinct [174].

The presence of co-occurring species might enable adaptation by initiating coevolutionary interactions (e.g., [175]), however, it has also been suggested that increasing biodiversity may begin to inhibit that subsequent adaptation (e.g. [171]). This is due to an increased number of species in an assemblage, increasing the chance that a current species will possess traits that would predispose the species towards favourable selection under future environmental conditions, and could restrict the opportunity of other co-occurring species to adapt. Species-specific adaptation mechanisms could ultimately feedback to influence ecosystem functioning [143]. For example, three bacterial species that were raised together had higher productivity compared to the same species that adapted isolation [176]. This was due to the inter-specific competition that caused them to select for specialisations in their resource use (niche partitioning [177]), leading to a complementary adaptation [176]. Hence understanding whether the evolutionary potential can be realised will require investigations that utilise realistically diverse assemblages (e.g., [169,173]). It does however, also raise the challenge of understanding whether future ecosystems will become sustained ecosystems (with fewer species that are selected for their favourable traits), or more evolved ecosystems as a whole. This is crucial given the extensive research regarding biodiversity and ecosystem multi-functionality in present day communities (for more details, see [178]).

5.2. Habitat Fragmentation and Biological Invasions

Anthropogenic climate change is expected to reorganise patterns of species diversity [179,180]. One possible approach for investigating the selection response using naturally assembled communities is through the use of natural analogues for future climate change, such as CO_2 vents [26], or coastal upwelling sites [114,181]. These areas provide long-term chronic exposure to novel environmental conditions, and allow experimental work to capture an organism's response in fitness-related traits [26,182], such as reproductive success. Moreover, organism responses will include carry-over effects (between life-history stages and trans-generationally), as well as being influenced by other ecological interactions, such as competition and trophic interactions. Yet, (a caveat) for those species that are not direct-developing, these sites may be confounded by larvae received from outside of the site, with different environmental conditions, likely reducing selection pressure.

For long-lived sessile foundation species, such as reef-building corals, evidence suggests that acclimatisation and adaptation will be essential for population persistence in the face of climate change [25], given that any range shifts are likely to be slow [183]. A recent transplant experiment utilising the table top coral (*Acropora hyacinthus* Dana, 1846) found that acclimatisation and adaptive responses (mirrored in the patterns of gene expression) allowed this faster-growing coral to inhabit areas of the reef that far exceeded their expected temperature tolerances [25]. This tolerance to elevated temperature might be associated with either the coral host (e.g., [48,184]), or their associated *Symbiodinium* (e.g., [185]). In contrast, experimental work investigating the coral reefs at the shallow volcanic CO_2 seeps (in Papua New Guinea) found an overall reduction in diversity and recruitment in

the coral communities pre-acclimated to high pCO_2, thought to be associated with shifts in competitive interactions [173]. This highlights that the adaptive evolution of coral reef communities is possible and driven by abiotic factors (Court Jester hypothesis, [186]), however, community-level interactions (such as the increased competition in high pCO_2) may equal or exceed these fitness-related responses (*i.e.*, survival), and lead to adaptive evolution being driven by biotic factors (Red Queen hypothesis, [186]) Clearly, the relative roles of biotic and abiotic factors will be stressor-specific reaffirming the need to investigate the adaptive evolution responses with multiple stressors in realistic communities.

Biological invasions are important drivers of change in marine communities, particularly coastal communities (e.g., [187,188]). Increases in temperature may facilitate species' range shifts, thereby aiding invasion [188]. One particular example of this is the 'tropicalisation' of the Mediterranean Sea, where, invasions and establishments have been made possible due to increasing annual mean temperatures all year around [189]. The integration of novel species may influence evolutionary processes by altering existing interactions (e.g., [190]) or population growth rates (see [191]). Alternatively, both the native and non-native species may be able to achieve coevolution if their co-existence can maximise their habitat use [192]. Although native species might be able to overcome the invasion of some non-natives, some may become less adapted to the new conditions and be out competed by invasive organisms, which exhibit greater adaptability or the ability to demonstrate strong fitness effects [188].

6. Future Directions

It is inevitable that increasing ocean acidification will be accompanied by changes in other abiotic factors, and therefore interactions with other stressors (*i.e.*, temperature, nutrients, hypoxia or salinity) are extremely likely [193]. For both single and multiple stressors, there is a crucial need to incorporate the potential for adaptation to future climate change, to reliably determine the sensitivity and mechanisms for adaptation of marine organisms.

Adaptation capacity will be driven through a number of mechanisms with different taxonomic and functional groups utilising a variety of processes. Species with large population sizes and fast population turnover rates, such as phytoplankton, are likely to demonstrate the potential to achieve the adaptation rates required for future climate change (e.g., [108]), making them a model species for laboratory experimental evolution. However, these experiments will likely be carried out in the absence of more complex trophic and ecological interactions. In order to clarify the effects of anthropogenic climate change on community- and ecosystem-levels, future research should be directed towards identifying key species, and establishing their interactions with coexisting species, particularly if those ecological interactions vary with season or ontogeny [194,195]. The choice of species could be associated with the needs for either ecosystem's services or functioning, such as the disproportionate role that coccolithophores play in the carbon-cycle, or societal needs, such as for food security, or possibly in an ecological context, being habitat forming or a keystone species.

Given the differential sensitivities and responses of different life-stages, future research needs to identify which life-stages are most affected by climate change and the key interactions (among species and to different climatic scenarios) within ecosystems [196]. The negative results from short-term studies on early life-stages often make it difficult to extrapolate to longer-term impacts [197–199], especially

when multiple stressors interact, since the sensitivity of early life-stages may not be representative when responses are considered across all ontogeny and life-stages. The exposure of previous generations to environmental conditions will influence the response of subsequent generations (*i.e.*, carry-over effects). As such, the use of chronic long term multigenerational experiments should contribute to our understanding of both developmental and trans-generational plasticity [108,155,198]. An additional important consideration is the current local-scale variability of environmental conditions. If the adverse conditions that we expect by the end of the century are already being experienced by marine organisms, and are within the range of the current environmental variability (e.g., CO_2-enriched upwelling, Kiel Fjord, western Baltic Sea [138]), then these transient extreme conditions may result in a pre-selection for tolerance (e.g., [131]). This pre-selection might be achieved through the divergent selection of specific genes in candidate loci (e.g., [114]), and contribute to the maintenance of positive life-history traits.

Phenotypic plasticity may provide the potential for species to achieve sufficient tolerance in the short-term, such that they may actually be able to achieve adaptation to environmental change. To attain a mechanistic understanding of this process will require an interdisciplinary approach, including investigations at different levels of biological hierarchy. This is because the capacity of a species' phenotypic plasticity might be set at the cellular level, for example through changes in oxygen demand via mitochondrial activity. However, it is important to consider that these responses might be first observed through changes in abundance (or distribution), using more phenomenological approaches at the population level. Alternatively, the persistence of a species could be attributed to its dispersal ability and the availability of suitable habitat and hence potential spatial scale of gene flow. As such, research needs to be carried out at biologically-relevant scales. Therefore, a crucial first step in understanding responses at the population level will require linking the intra-individual physiology (e.g., transcriptional and cellular responses) to the fitness-related traits of the whole-organism, in order to more reliably estimate the effects of climate change on contemporary population demographics into the future.

7. Conclusions

Biotic factors such as competition and trophic interactions shape marine communities at local spatial scales and over relatively short timescales. Other extrinsic factors, such as oceanic and atmospheric environmental conditions will influence patterns of biodiversity over longer timescales, and at regional or global scales [186]. Since climate change is occurring rapidly, it is likely that biotic interactions may play a more important role, compared to abiotic factors, when it comes to evolution (*i.e.*, the Red Queen hypothesis [200]). As such, establishing the association between local environmental conditions and the genomic-physiological features of key species, that are known to be influential in communities (including their interactions with co-existing species), should elucidate how community processes will be affected, and whether evolutionary potential can be realised. However, investigating broader spatial scales will require determining the link between the genomic-physiological responses of contemporary populations and population dynamics. This could establish a deeper understanding between the physiological stress responses of marine organisms to both biotic and abiotic factors, and critical (yet often unknown) demographic processes such as effective population size.

Both adaptation and acclimatisation may enable organisms to persist in future oceans, and understanding how factors at different levels of biological hierarchy will influence these important

evolutionary responses to climate change is crucial. Future research needs to investigate biological responses both spatially and temporally, by utilising spatially representative replication across different scientific disciplines and research institutes, in an effort to integrate responses and adaptive mechanisms at regional or global scales. This will help to achieve direct comparisons and a more integrative picture of the responses at the community and ecosystem levels. Only then can we establish whether the future organisational structure of marine ecosystems will resemble the communities of today, and what role acclimatisation and adaptation will play in the persistence of marine organisms.

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Author Contributions

Original concept, drafting and editing manuscript: Ben Harvey. Group leaders: molecular and cellular responses—Amit Kumar; whole-organism—Rebekah Cioffi; population-level responses—Balsam Al-Janabi; community composition and interactions—Stefanie Broszeit. Figure 1—Ben Harvey. All other authors contributed with concept development, writing and commented on the manuscript at all stages.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Schmitz, O.J. Global climate change and the evolutionary ecology of ecosystem functioning. *Annu. N. Y. Acad. Sci.* **2013**, *1297*, 61–72.
2. Bell, G.; Gonzalez, A. Evolutionary rescue can prevent extinction following environmental change. *Ecol. Lett.* **2009**, *12*, 942–948.
3. Burrows, M.T.; Schoeman, D.S.; Buckley, L.B.; Moore, P.; Poloczanska, E.S.; Brander, K.M.; Brown, C.; Bruno, J.F.; Duarte, C.M.; Halpern, B.S.; *et al*. The pace of shifting climate in marine and terrestrial ecosystems. *Science* **2011**, *334*, 652–655.
4. Doney, S.C.; Schimel, D.S. Carbon and climate system coupling on timescales from the precambrian to the anthropocene. *Annu. Rev. Environ. Resour.* **2007**, *32*, 31–66.
5. Halpern, B.S.; Walbridge, S.; Selkoe, K.A.; Kappel, C.V.; Micheli, F.; D'Agrosa, C.; Bruno, J.F.; Casey, K.S.; Ebert, C.; Fox, H.E.; *et al*. A global map of human impact on marine ecosystems. *Science* **2008**, *319*, 948–952.
6. Dupont, S.; Dorey, N.; Thorndyke, M. What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? *Estuar. Coast. Shelf Sci.* **2010**, *89*, 182–185.
7. Kroeker, K.; Kordas, R.; Crim, R.; Singh, G. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecol. Lett.* **2010**, *13*, 1419–1434.
8. Harvey, B.P.; Gwynn-Jones, D.; Moore, P.J. Meta-analysis reveals complex marine biological responses to the interactive effects of ocean acidification and warming. *Ecol. Evol.* **2013**, *3*, 1016–1030.
9. Kroeker, K.J.; Kordas, R.L.; Crim, R.; Hendriks, I.E.; Ramajo, L.; Singh, G.S.; Duarte, C.M.; Gattuso, J.-P. Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Glob. Change Biol.* **2013**, *19*, 1884–1896.
10. Pörtner, H.-O. Physiological basis of temperature-dependent biogeography: Trade-offs in muscle design and performance in polar ectotherms. *J. Exp. Biol.* **2002**, *205*, 2217–2230.
11. Somero, G.N. Linking biogeography to physiology: Evolutionary and acclimatory adjustments of thermal limits. *Front. Zool.* **2005**, *2*, 1.
12. Widdicombe, S.; Spicer, J. Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? *J. Exp. Mar. Biol. Ecol.* **2008**, *366*, 187–197.
13. Somero, G.N. The physiology of climate change: How potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **2010**, *213*, 912–920.

14. Van der Putten, W.H.; Macel, M.; Visser, M.E. Predicting species distribution and abundance responses to climate change: Why it is essential to include biotic interactions across trophic levels. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2010**, *365*, 2025–2034.
15. Stockwell, C.A.; Hendry, A.P.; Kinnison, M.T. Contemporary evolution meets conservation biology. *Trends Ecol. Evol.* **2003**, *18*, 94–101.
16. Munday, P.L.; Warner, R.R.; Monro, K.; Pandolfi, J.M.; Marshall, D.J. Predicting evolutionary responses to climate change in the sea. *Ecol. Lett.* **2013**, *16*, 1488–1500.
17. Sunday, J.M.; Calosi, P.; Dupont, S.; Munday, P.L.; Stillman, J.H.; Reusch, T.B.H. Evolution in an acidifying ocean. *Trends Ecol. Evol.* **2014**, *29*, 117–125.
18. Dupont, S.; Portner, H. Marine science: Get ready for ocean acidification. *Nature* **2013**, *498*, 429.
19. Gienapp, P.; Teplitsky, C.; Alho, J.S.; Mills, J.A.; Merilä, J. Climate change and evolution: Disentangling environmental and genetic responses. *Mol. Ecol.* **2008**, *17*, 167–178.
20. Merilä, J.; Hendry, A.P. Climate change, adaptation, and phenotypic plasticity: The problem and the evidence. *Evol. Appl.* **2014**, *7*, 1–14.
21. Chevin, L.-M.; Lande, R.; Mace, G.M. Adaptation, plasticity, and extinction in a changing environment: Towards a predictive theory. *PLoS Biol.* **2010**, *8*, doi:10.1371/journal.pbio.1000357.
22. Hoffmann, A.A.; Sgrò, C.M. Climate change and evolutionary adaptation. *Nature* **2011**, *470*, 479–485.
23. Reusch, T.B.H. Climate change in the oceans: Evolutionary *versus* phenotypically plastic responses of marine animals and plants. *Evol. Appl.* **2013**, *7*, 104–122.
24. Blondel, J. From biogeography to life history theory: A multithematic approach illustrated by the biogeography of vertebrates. *J. Biogeogr.* **1987**, *14*, 405–422.
25. Palumbi, S.R.; Barshis, D.J.; Traylor-Knowles, N.; Bay, R.A. Mechanisms of reef coral resistance to future climate change. *Science* **2014**, *344*, 895–898.
26. Calosi, P.; Rastrick, S.P.S.; Lombardi, C.; de Guzman, H.J.; Davidson, L.; Jahnke, M.; Giangrande, A.; Hardege, J.D.; Schulze, A.; Spicer, J.I.; *et al*. Adaptation and acclimatization to ocean acidification in marine ectotherms: An *in situ* transplant experiment with polychaetes at a shallow CO_2 vent system. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**, *368*, doi: 10.1098/rstb.2012.0444.
27. Queirós, A.M.; Fernandes, J.A.; Faulwetter, S.; Nunes, J.; Rastrick, S.P.S.; Mieszkowska, N.; Artioli, Y.; Yool, A.; Calosi, P.; Arvanitidis, C.; *et al*. Scaling up experimental ocean acidification and warming research: From individuals to the ecosystem. *Glob. Change Biol.* **2014**, doi:10.1111/gcb.12675.
28. Prosser, C.L. Physiological variation in animals. *Biol. Rev.* **1955**, *30*, 229–261.
29. Somero, G.N. The physiology of global change: Linking patterns to mechanisms. *Annu. Rev. Mar. Sci.* **2012**, *4*, 39–61.
30. Feder, M.E.; Walser, J.C. The biological limitations of transcriptomics in elucidating stress and stress responses. *J. Evol. Biol.* **2005**, *18*, 901–910.
31. Berg, J.M.; Tymoczko, J.L.; Stryer, L. *Biochemistry*; WH Freeman: New York, NY, USA, 2002; p. 1026.
32. Griffiths, A.J.F.; Miller, J.H.; Suzuki, D.T.; Lewontin, R.C.; Gelbart, W.M. Gene-protein relations. In *An Introduction to Genetic Analysis*, 7th ed.; Griffiths, A.J.F., Ed.; WH Freeman: New York, NY, USA, 2000.

33. Kassahn, K.S.; Crozier, R.H.; Pörtner, H.O.; Caley, M.J. Animal performance and stress: Responses and tolerance limits at different levels of biological organisation. *Biol. Rev. Camb. Philos. Soc.* **2009**, *84*, 277–292.
34. Fields, P.A.; Rudomin, E.L.; Somero, G.N. Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): Sequence-function linkages and correlations with biogeographic distribution. *J. Exp. Biol.* **2006**, *209*, 656–667.
35. Powers, D.A.; Schulte, P.M. Evolutionary adaptations of gene structure and expression in natural populations in relation to a changing environment: A multidisciplinary approach to address the million-year saga of a small fish. *J. Exp. Zool.* **1998**, *282*, 71–94.
36. Norry, F.M.; Larsen, P.F.; Liu, Y.; Loeschcke, V. Combined expression patterns of QTL-linked candidate genes best predict thermotolerance in *Drosophila melanogaster*. *J. Insect Physiol.* **2009**, *55*, 1050–1057.
37. Franks, S.J.; Hoffmann, A.A. Genetics of climate change adaptation. *Annu. Rev. Genet.* **2012**, *46*, 185–208.
38. Davidson, E.H. *The Regulatory Genome: Gene Regulatory Networks in Development and Evolution*; Academic Press: San Diego, CA, USA, 2006.
39. Davidson, E.; Levin, M. Gene regulatory networks. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 4935.
40. Williams, S.E.; Shoo, L.P.; Isaac, J.L.; Hoffmann, A.A.; Langham, G. Towards an integrated framework for assessing the vulnerability of species to climate change. *PLoS Biol.* 2008, *6*, e325.
41. Bradshaw, W.E.; Holzapfel, C.M. Light, time, and the physiology of biotic response to rapid climate change in animals. *Annu. Rev. Physiol.* **2010**, *72*, 147–166.
42. Atkinson, N.J.; Urwin, P.E. The interaction of plant biotic and abiotic stresses: From genes to the field. *J. Exp. Bot.* **2012**, *63*, 3523–3543.
43. Gardner, J.L.; Peters, A.; Kearney, M.R.; Joseph, L.; Heinsohn, R. Declining body size: A third universal response to warming? *Trends Ecol. Evol.* **2011**, *26*, 285–291.
44. Kovach, R.P.; Gharrett, A.J.; Tallmon, D.A. Genetic change for earlier migration timing in a pink salmon population. *Proc. R. Soc. Biol. Sci. Ser. B* **2012**, *279*, 3870–3878.
45. Karell, P.; Ahola, K.; Karstinen, T.; Valkama, J.; Brommer, J.E. Climate change drives microevolution in a wild bird. *Nat. Commun.* **2011**, *2*, 208.
46. Runcie, D.E.; Garfield, D.A.; Babbitt, C.C.; Wygoda, J.A.; Mukherjee, S.; Wray, G.A. Genetics of gene expression responses to temperature stress in a sea urchin gene network. *Mol. Ecol.* **2012**, *21*, 4547–4562.
47. Keller, B.; Feuillet, C. Colinearity and gene density in grass genomes. *Trends Plant Sci.* **2000**, *5*, 246–251.
48. Barshis, D.J.; Ladner, J.T.; Oliver, T.A.; Seneca, F.O.; Traylor-Knowles, N.; Palumbi, S.R. Genomic basis for coral resilience to climate change. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 1387–1392.
49. Todgham, A.E.; Hofmann, G.E. Transcriptomic response of sea urchin larvae *Strongylocentrotus purpuratus* to CO_2-driven seawater acidification. *J. Exp. Biol.* **2009**, *212*, 2579–2594.
50. Wray, G.A. The evolutionary significance of *cis*-regulatory mutations. *Nat. Rev. Genet.* **2007**, *8*, 206–216.

51. Barrett, R.D.; Hoekstra, H.E. Molecular spandrels: Tests of adaptation at the genetic level. *Nat. Rev. Genet.* **2011**, *12*, 767–780.
52. Davidson, W.S. Adaptation genomics: next generation sequencing reveals a shared haplotype for rapid early development in geographically and genetically distant populations of rainbow trout. *Mol. Ecol.* **2012**, *21*, 219–222.
53. Edwards, S.V. Next-generation QTL mapping: Crowdsourcing SNPs, without pedigrees. *Mol. Ecol.* **2013**, *22*, 3885–3887.
54. Kaniewska, P.; Campbell, P.R.; Kline, D.I.; Rodriguez-Lanetty, M.; Miller, D.J.; Dove, S.; Hoegh-Guldberg, O. Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE* **2012**, *7*, e34659.
55. Sokolova, I.M.; Frederich, M.; Bagwe, R.; Lannig, G.; Sukhotin, A.A. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Mar. Environ. Res.* **2012**, *79*, 1–15.
56. Seebacher, F.; Franklin, C.E. Determining environmental causes of biological effects: The need for a mechanistic physiological dimension in conservation biology. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2012**, *367*, 1607–1614.
57. Buckley, B.A.; Gracey, A.Y.; Somero, G.N. The cellular response to heat stress in the goby Gillichthys mirabilis: A cDNA microarray and protein-level analysis. *J. Exp. Biol.* **2006**, *209*, 2660–2677.
58. Wapinski, I.; Pfeffer, A.; Friedman, N.; Regev, A., Natural history and evolutionary principles of gene duplication in fungi. *Nature* **2007**, *449*, 54–61.
59. Raffaele, S.; Farrer, R.A.; Cano, L.M.; Studholme, D.J.; MacLean, D.; Thines, M.; Jiang, R.H.Y.; Zody, M.C.; Kunjeti, S.G.; Donofrio, N.M.; *et al*. Genome evolution following host jumps in the Irish potato famine pathogen lineage. *Science* **2010**, *330*, 1540-1543.
60. Hartl, D.L. *Essential Genetics: A Genomics Perspective*; Jones and Bartlett Publishers: London, UK, 2014.
61. Pires-daSilva, A.; Sommer, R.J. The evolution of signalling pathways in animal development. *Nat. Rev. Genet.* **2003**, *4*, 39–49.
62. Garcia-Moreno, B. Adaptations of proteins to cellular and subcellular pH. *J. Biol.* **2009**, *8*, 98.
63. Salinas, S.; Munch, S.B. Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. *Ecol. Lett.* **2012**, *15*, 159–163.
64. Jaenisch, R.; Bird, A. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat. Genet.* **2003**, *33*, 245–254.
65. Turner, B.M. Epigenetic responses to environmental change and their evolutionary implications. *Philos. Trans. R. Soc. B Biol. Sci.* **2009**, *364*, 3403–3418.
66. Parker, L.M.; Ross, P.M.; O'Connor, W.A.; Borysko, L.; Raftos, D.A.; Pörtner, H.-O. Adult exposure influences offspring response to ocean acidification in oysters. *Glob. Change Biol.* **2012**, *18*, 82–92.
67. Garfield, D.A.; Runcie, D.E.; Babbitt, C.C.; Haygood, R.; Nielsen, W.J.; Wray, G.A. The Impact of gene expression variation on the robustness and evolvability of a developmental Gene Regulatory Network. *PLoS Biol.* **2013**, *11*, doi:10.1371/journal.pbio.1001696.
68. Suchanek, T.H. The role of disturbance in the evolution of life history strategies in the intertidal mussels *Mytilus edulis* and *Mytilus californianus*. *Oecologia* **1981**, *50*, 143–152.

69. Dawson, M.N. Phylogeography in coastal marine animals: A solution from California? *J. Biogeogr.* **2001**, *28*, 723–736.
70. Sherman, C.D.H.; Hunt, A.; Ayre, D.J. Is life history a barrier to dispersal? Contrasting patterns of genetic differentiation along an oceanographically complex coast. *Biol. J. Linn. Soc.* **2008**, *95*, 106–116.
71. Sunday, J.M.; Crim, R.N.; Harley, C.D.G.; Hart, M.W. Quantifying rates of evolutionary adaptation in response to ocean acidification. *PLoS ONE* **2011**, *6*, e22881.
72. Brown, J.H.; Gillooly, J.F.; Allen, A.P.; Savage, V.M.; West, G.B. Toward a metabolic theory of ecology. *Ecology* **2004**, *85*, 1771–1789.
73. Kooijman, S.A.L.M. *Dynamic Energy Budget Theory for Metabolic Organisation*; Cambridge University Press: Cambridge, UK, 2010; p. 514.
74. Holcomb M.; McCorkle D.C.; Cohen A.L. Long-term effects of nutrient and CO_2 enrichment on the temperate coral *astrangia poculata* (Ellis and Solander, 1786). *J. Exp. Mar. Biol. Ecol.* **2010**, *386*, 27–33.
75. Foo, S.A.; Dworjanyn, S.A.; Poore, A.G.B.; Byrne, M. Adaptive capacity of the habitat modifying sea urchin *Centrostephanus rodgersii* to ocean warming and ocean acidification: Performance of early embryos. *PLoS ONE* **2012**, *7*, e42497.
76. Langenbuch, M.; Pörtner, H.O. High sensitivity to chronically elevated CO_2 levels in a eurybathic marine sipunculid. *Aquat. Toxicol.* **2004**, *70*, 55–61.
77. Kurihara, H. Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. *Mar. Ecol. Prog. Ser.* **2008**, *373*, 275–284.
78. Pörtner, H.-O. Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view. *Mar. Ecol. Prog. Ser.* **2008**, *373*, 203–217.
79. O'Connor, M.I.; Bruno, J.F.; Gaines, S.D.; Halpern, B.S.; Lester, S.E.; Kinlan, B.P.; Weiss, J.M. Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 1266–1271.
80. Arnold, K.; Findlay, H.; Spicer, J.; Daniels, C.; Boothroyd, D. Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). *Biogeosciences* **2009**, *6*, 1747–1754.
81. McDonald, M.R.; McClintock, J.B.; Amsler, C.D.; Rittschof, D.; Angus, R.A.; Orihuela, B.; Lutostanski, K. Effects of ocean acidification over the life history of the barnacle *Amphibalanus amphitrite*. *Mar. Ecol. Prog. Ser.* **2009**, *385*, 179–187.
82. Byrne, M. Impact of ocean warming and ocean acidification on marine invertebrate life history stages: Vulnerabilities and potential for persistence in a changing ocean. *Oceanogr. Mar. Biol.* **2011**, *49*, 1–42.
83. Miller, N.A.; Paganini, A.W.; Stillman, J.H. Differential thermal tolerance and energetic trajectories during ontogeny in porcelain crabs, genus *Petrolisthes*. *J. Therm. Biol.* **2013**, *38*, 79–85.
84. Emlet, R.B.; Sadro, S.S. Linking stages of life history: How larval quality translates into juvenile performance for an intertidal barnacle (*Balanus glandula*). *Integr. Comp. Biol.* **2006**, *46*, 334–346.
85. Werner, E.E.; Gilliam, J.F. The ontogenetic niche and species interactions in size-structured populations. *Annu. Rev. Ecol. Syst.* **1984**, *15*, 393–425.

86. Dupont, S.; Dorey, N.; Stumpp, M.; Melzner, F.; Thorndyke, M.C. Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. *Mar. Biol.* **2012**, *160*, 1835–1843.
87. Ferrari, M.C.O.; Manassa, R.P.; Dixson, D.L.; Munday, P.L.; McCormick, M.I.; Meekan, M.G.; Sih, A.; Chivers, D.P. Effects of ocean acidification on learning in coral reef fishes. *PLoS ONE* **2012**, *7*, e31478.
88. Candolin, U.; Wong, B.B.M. *Behavioural Responses to a Changing World: Mechanisms and Consequences*; Oxford University Press: Oxford, UK, 2012; pp. 208–210.
89. Dissanayake, A.; Ishimatsu, A. Synergistic effects of elevated CO_2 and temperature on the metabolic scope and activity in a shallow-water coastal decapod (*Metapenaeus joyneri*; Crustacea: Penaeidae). *ICES J. Mar. Sci.* **2011**, *68*, 1147–1154.
90. Biro, P.A.; Beckmann, C.; Stamps, J.A. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. *Proc. R. Soc. Biol. Sci. B* **2010**, *277*, 71–77.
91. Watson, S.-A.; Lefevre, S.; McCormick, M.I.; Domenici, P.; Nilsson, G.E.; Munday, P.L. Marine mollusc predator-escape behaviour altered by near-future carbon dioxide levels. *Proc. R. Soc. Biol. Sci. B* **2014**, *281*, doi: 10.1098/rspb.2013.2377.
92. Nilsson, G.E.; Dixson, D.L.; Domenici, P.; McCormick, M.I.; Sørensen, C.; Watson, S.-A.; Munday, P.L. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nat. Clim. Change* **2012**, *2*, 201–204.
93. Chivers, D.P.; McCormick, M.I.; Nilsson, G.E.; Munday, P.L.; Watson, S.-A.; Meekan, M.G.; Mitchell, M.D.; Corkill, K.C.; Ferrari, M.C.O. Impaired learning of predators and lower prey survival under elevated CO_2: A consequence of neurotransmitter interference. *Glob. Change Biol.* **2014**, *20*, 515–522.
94. Munday, P.L.; Dixson, D.L.; Donelson, J.M.; Jones, G.P.; Pratchett, M.S.; Devitsina, G.V.; Døving, K.B. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1848.
95. Goh, C.S.; Bogan, A.A.; Joachimiak, M.; Walther, D.; Cohen, F.E. Co-evolution of proteins with their interaction partners. *J. Mol. Biol.* **2000**, *299*, 283–293.
96. Greenwood, A.K.; Wark, A.R.; Yoshida, K.; Peichel, C.L. Genetic and neural modularity underlie the evolution of schooling behavior in threespine sticklebacks. *Curr. Biol.* **2013**, *23*, 1884–1888.
97. Blomberg, S.P.; Garland, T.; Ives, A.R. Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution* **2003**, *57*, 717–745.
98. Bradshaw, A.D.; McNeilly, T. Evolutionary response to global climatic change. *Ann. Bot. Lond.* **1991**, *67*, 5–14.
99. Bohonak, A.J. Dispersal, gene flow, and population structure. *Q. Rev. Biol.* **1999**, *74*, 21–45.
100. Hellberg, M.E.; Burton, R.S.; Neigel, J.E.; Palumbi, S.R. Genetic assessment of connectivity among marine populations. *Bull. Mar. Sci.* **2002**, *70*, 273–290.
101. Sanford, E.; Kelly, M.W. Local adaptation in marine invertebrates. *Ann. Rev. Mar. Sci.* **2011**, *3*, 509–535.
102. Merilä, J. Evolution in response to climate change: In pursuit of the missing evidence. *BioEssays* **2012**, *34*, 811–818.

103. Gonzalez, A.; Ronce, O.; Ferriere, R.; Hochberg, M.E. Evolutionary rescue: An emerging focus at the intersection between ecology and evolution. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**, *368*, doi:10.1098/rstb.2012.0404.
104. Johannesson, K.; Smolarz, K.; Grahn, M.; André, C. The future of Baltic Sea populations: Local extinction or evolutionary rescue? *Ambio* **2011**, *40*, 179–190.
105. Bell, G. Evolutionary rescue and the limits of adaptation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**, *368*, doi:10.1098/rstb.2012.0080.
106. Cuveliers, E.L.; Volckaert, F.A.M.; Rijnsdorp, A.D.; Larmuseau, M.H.D.; Maes, G.E. Temporal genetic stability and high effective population size despite fisheries-induced life-history trait evolution in the North Sea sole. *Mol. Ecol.* **2011**, *20*, 3555–3568.
107. Reusch, T.B.H.; Ehlers, A.; Hämmerli, A.; Worm, B. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 2826–2831.
108. Lohbeck, K.T.; Riebesell, U.; Reusch, T.B.H. Adaptive evolution of a key phytoplankton species to ocean acidification. *Nat. Geosci.* **2012**, *5*, 346–351.
109. Jin, P.; Gao, K.; Beardall, J. Evolutionary responses of a coccolithophorid *Gephyrocapsa oceanica* to ocean acidification. *Evolution* **2013**, *67*, 1869–1878.
110. Crawfurd, K.J.; Raven, J.A.; Wheeler, G.L.; Baxter, E.J.; Joint, I. The response of *Thalassiosira pseudonana* to long-term exposure to increased CO_2 and decreased pH. *PLoS ONE* **2011**, *6*, e26695.
111. Smith-Keune, C.; Oppen, M. Genetic structure of a reef-building coral from thermally distinct environments on the Great Barrier Reef. *Coral Reefs* **2006**, *25*, 493–502.
112. D'Croz, L.; Maté, J.L. Experimental responses to elevated water temperature in genotypes of the reef coral *Pocillopora damicornis* from upwelling and non-upwelling environments in Panama. *Coral Reefs* **2004**, *23*, 473–483.
113. Yanick, J.F.; Heath, J.W.; Heath, D.D. Survival and growth of local and transplanted blue mussels (*Mytilus trossulus*, Lamark). *Aquac. Res.* **2003**, *34*, 869–875.
114. De Wit, P.; Palumbi, S.R. Transcriptome-wide polymorphisms of red abalone (*Haliotis rufescens*) reveal patterns of gene flow and local adaptation. *Mol. Ecol.* **2013**, *22*, 2884–2897.
115. Gaston, K.J.; Spicer, J.I. Do upper thermal tolerances differ in geographically separated populations of the beachflea *Orchestia gammarellus* (Crustacea: Amphipoda)? *J. Exp. Mar. Biol. Ecol.* **1998**, *229*, 265–276.
116. Schmidt, C.; Heinz, P.; Kucera, M.; Uthicke, S. Temperature-induced stress leads to bleaching in larger benthic foraminifera hosting endosymbiotic diatoms. *Limnol. Oceanogr.* **2011**, *56*, 1587–1602.
117. Kelly, M.W.; Sanford, E.; Grosberg, R.K. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. *Proc. R. Soc. Biol. Sci. B* **2012**, *279*, 349–356.
118. Sanford, E.; Holzman, S.B.; Haney, R.A.; Rand, D.M.; Bertness, M.D. Larval tolerance, gene flow, and the northern geographic range limit of fiddler crabs. *Ecology* **2006**, *87*, 2882–2894.
119. Lymbery, R.A.; Evans, J.P. Genetic variation underlies temperature tolerance of embryos in the sea urchin *Heliocidaris erythrogramma armigera*. *J. Evol. Biol.* **2013**, *26*, 2271–2282.
120. Osovitz, C.J.; Hofmann, G.E. Thermal history-dependent expression of the *hsp70* gene in purple sea urchins: Biogeographic patterns and the effect of temperature acclimation. *J. Exp. Mar. Biol. Ecol.* **2005**, *327*, 134–143.

121. Kelly, M.W.; Padilla-Gamiño, J.L.; Hofmann, G.E. Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus purpuratus*. *Glob. Change Biol.* **2013**, *19*, 2536–2546.
122. Pecorino, D.; Barker, M.F.; Dworjanyn, S.A.; Byrne, M.; Lamare, M.D. Impacts of near future sea surface pH and temperature conditions on fertilisation and embryonic development in *Centrostephanus rodgersii* from northern New Zealand and northern New South Wales, Australia. *Mar. Biol.* **2014**, *161*, 101–110.
123. Marcil, J.; Swain, D.P.; Hutchings, J.A. Countergradient variation in body shape between two populations of Atlantic cod (*Gadus morhua*). *Proc. R. Soc. Biol. Sci. B* **2006**, *273*, 217–223.
124. Fangue, N.A.; Hofmeister, M.; Schulte, P.M. Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *J. Exp. Biol.* **2006**, *209*, 2859–2872.
125. Schaum, E.; Rost, B.; Millar, A.J.; Collins, S. Variation in plastic responses of a globally distributed picoplankton species to ocean acidification. *Nature Clim. Change* **2012**, *3*, 298–302.
126. Pistevos, J.C.A.; Calosi, P.; Widdicombe, S.; Bishop, J.D.D. Will variation among genetic individuals influence species responses to global climate change? *Oikos* **2011**, *120*, 675–689.
127. Willi, Y.; Van Buskirk, J.; Hoffmann, A.A. Limits to the adaptive potential of small populations. *Annu. Rev. Ecol. Evol. Syst.* **2006**, *37*, 433–458.
128. Lande, R. Adaptation to an extraordinary environment by evolution of phenotypic plasticity and genetic assimilation. *J. Evol. Biol.* **2009**, *22*, 1435–1446.
129. Feely, R.A.; Sabine, C.L.; Hernandez-Ayon, J.M.; Ianson, D.; Hales, B. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. *Science* **2008**, *320*, 1490–1492.
130. Evans, T.G.; Chan, F.; Menge, B.A.; Hofmann, G.E. Transcriptomic responses to ocean acidification in larval sea urchins from a naturally variable pH environment. *Mol. Ecol.* **2013**, *22*, 1609–1625.
131. Saderne, V.; Wahl, M. Differential responses of calcifying and non-calcifying epibionts of a brown macroalga to present-day and future upwelling pCO_2. *PLoS ONE* **2013**, *8*, e70455.
132. Leal, S.M. Genetics and Analysis of Quantitative Traits. *Am. J. Hum. Genet*. **2001**, *68*, 548–549.
133. Hughes, A.R.; Inouye, B.D.; Johnson, M.T.J.; Underwood, N.; Vellend, M. Ecological consequences of genetic diversity. *Ecol. Lett.* **2008**, *11*, 609–623.
134. Pespeni, M.H.; Sanford, E.; Gaylord, B.; Hill, T.M.; Hosfelt, J.D.; Jaris, H.K.; LaVigne, M.; Lenz, E.A.; Russell, A.D.; Young, M.K.; *et al*. Evolutionary change during experimental ocean acidification. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 6937–6942.
135. Hedrick, P.W. Genetic Polymorphism in Heterogeneous Environments: The Age of Genomics. *Annu. Rev. Ecol., Evol., Syst.* **2006**, *37*, 67–93.
136. Intergovernmental Panel on Climate Change. *Climate Change 2013—The Physical Science Basis*; Cambridge University Press: Cambridge, UK, 2014.
137. Thomsen, J.; Casties, I.; Pansch, C.; Körtzinger, A.; Melzner, F. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: Laboratory and field experiments. *Glob. Change Biol.* **2012**, *19*, 1017–1027.

138. Thomsen, J.; Gutowska, M.A.; Saphörster, J.; Heinemann, A.; Trübenbach, K.; Fietzke, J.; Hiebenthal, C.; Eisenhauer, A.; Körtzinger, A.; Wahl, M.; *et al*. Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* **2010**, *7*, 3879–3891.
139. Dam, H.G. Evolutionary adaptation of marine zooplankton to global change. *Annu. Rev. Mar. Sci.* **2013**, *5*, 349–370.
140. Conover, D.O.; Present, T.M.C. Countergradient variation in growth rate: Compensation for length of the growing season among Atlantic silversides from different latitudes. *Oecologia* **1990**, *83*, 316–324.
141. Hairston, N.G.; Ellner, S.P.; Geber, M.A.; Yoshida, T.; Fox, J.A. Rapid evolution and the convergence of ecological and evolutionary time. *Ecol. Lett.* **2005**, *8*, 1114–1127.
142. Olsen, E.M.; Heino, M.; Lilly, G.R.; Morgan, M.J.; Brattey, J.; Ernande, B.; Dieckmann, U. Maturation trends indicative of rapid evolution preceded the collapse of northern cod. *Nature* **2004**, *428*, 932–935.
143. Schoener, T.W. The newest synthesis: understanding the interplay of evolutionary and ecological dynamics. *Science* **2011**, *331*, 426–429.
144. Palkovacs, E.P.; Marshall, M.C.; Lamphere, B.A.; Lynch, B.R.; Weese, D.J.; Fraser, D.F.; Reznick, D.N.; Pringle, C.M.; Kinnison, M.T. Experimental evaluation of evolution and coevolution as agents of ecosystem change in Trinidadian streams. *Philos. Trans. R. Soc. B* **2009**, *364*, 1617–1628.
145. Janzen, D.H. When is it coevolution. *Evolution* **1980**, *34*, 611–612.
146. Turcotte, M.M.; Corrin, M.S.; Johnson, M.T. Adaptive evolution in ecological communities. *PLoS Biol.* **2012**, *10*, doi:10.1371/journal.pbio.1001332.
147. Strauss, S.Y.; Sahli, H.; Conner, J.K. Toward a more trait-centered approach to diffuse (co)evolution. *New Phytol.* **2005**, *165*, 81–89.
148. Lai, S.-M.; Liu, W.-C.; Jordán, F. On the centrality and uniqueness of species from the network perspective. *Biol. Lett.* **2012**, *8*, 570–573.
149. Hale, R.; Calosi, P.; McNeill, L.; Mieszkowska, N.; Widdicombe, S. Predicted levels of future ocean acidification and temperature rise could alter community structure and biodiversity in marine benthic communities. *Oikos* **2011**, *120*, 661–674.
150. Diaz-Pulido, G.; Gouezo, M.; Tilbrook, B.; Dove, S.; Anthony, K. High CO_2 enhances the competitive strength of seaweeds over corals. *Ecol. Lett.* **2011**, *14*, 156–162.
151. Ferrari, M.C.O.; McCormick, M.I.; Munday, P.L.; Meekan, M.G.; Dixson, D.L.; Lonnstedt, Ö.; Chivers, D.P. Putting prey and predator into the CO_2 equation—Qualitative and quantitative effects of ocean acidification on predator–prey interactions. *Ecol. Lett.* **2011**, *14*, 1143–1148.
152. Sanford, E.; Gaylord, B.; Hettinger, A.; Lenz, E.A.; Meyer, K.; Hill, T.M. Ocean acidification increases the vulnerability of native oysters to predation by invasive snails. *Proc. R. Soc. Biol. Sci. B* **2014**, *281*, doi: 10.1098/rspb.2013.2681.
153. O'Connor, M.I. Warming strengthens an herbivore-plant interaction. *Ecology* **2009**, *90*, 388–398.
154. Poore, A.G.B.; Graba-Landry, A.; Favret, M.; Sheppard Brennand, H.; Byrne, M.; Dworjanyn, S.A. Direct and indirect effects of ocean acidification and warming on a marine plant–herbivore interaction. *Oecologia* **2013**, *173*, 1113–1124.

155. Collins, S.; Rost, B.; Rynearson, T.A. Evolutionary potential of marine phytoplankton under ocean acidification. *Evol. Appl.* **2014**, *7*, 140–155.
156. Falkowski, P.G.; Fenchel, T.; Delong, E.F. The microbial engines that drive Earth's biogeochemical cycles. *Science* **2008**, *320*, 1034–1039.
157. Iglesias-Rodriguez, M.; Halloran, P.; Rickaby, R.; Hall, I.; Colmenero-Hidalgo, E.; Gittins, J.; Green, D.; Tyrrell, T.; Gibbs, S.; von Dassow, P.; *et al*. Phytoplankton calcification in a high-CO_2 world. *Science* **2008**, *320*, 336–340.
158. Harley, C.D.G.; Anderson, K.M.; Demes, K.W.; Jorve, J.P.; Kordas, R.L.; Coyle, T.A.; Graham, M.H. Effects of climate change on global seaweed communities. *J. Phycol.* **2012**, *48*, 1064–1078.
159. Hurd, C.L.; Hepburn, C.D.; Currie, K.I.; Raven, J.A.; Hunter, K.A. Testing the effects of ocean acidification on algal metabolism: Considerations for experimental designs. *J. Phycol.* **2009**, *45*, 1236–1251.
160. Tatters, A.O.; Schnetzer, A.; Fu, F.; Lie, A.Y.A.; Caron, D.A.; Hutchins, D.A. Short- *versus* long-term responses to changing CO_2 in a coastal dinoflagellate bloom: Implications for interspecific competitive interactions and community structure. *Evolution* **2013**, *67*, 1879–1891.
161. Kremp, A.; Godhe, A.; Egardt, J.; Dupont, S.; Suikkanen, S.; Casabianca, S.; Penna, A. Intraspecific variability in the response of bloom-forming marine microalgae to changed climate conditions. *Ecol. Evol.* **2012**, *2*, 1195–1207.
162. Boyd, P.W.; Rynearson, T.A.; Armstrong, E.A.; Fu, F.; Hayashi, K.; Hu, Z.; Hutchins, D.A.; Kudela, R.M.; Litchman, E.; Mulholland, M.R.; *et al*. Marine phytoplankton temperature *versus* growth responses from polar to tropical waters—Outcome of a scientific community-wide study. *PLoS ONE* **2013**, *8*, e63091.
163. Alsterberg, C.; Eklöf, J.S.; Gamfeldt, L.; Havenhand, J.N.; Sundbäck, K. Consumers mediate the effects of experimental ocean acidification and warming on primary producers. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8603–8608.
164. Witt, V.; Wild, C.; Anthony, K.R.N.; Diaz-Pulido, G.; Uthicke, S. Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef. *Environ. Microbiol.* **2011**, *13*, 2976–2989.
165. Beaugrand, G.; Brander, K.M.; Alistair Lindley, J.; Souissi, S.; Reid, P.C. Plankton effect on cod recruitment in the North Sea. *Nature* **2003**, *426*, 661–664.
166. Melzner, F.; Stange, P.; Trübenbach, K.; Thomsen, J.; Casties, I.; Panknin, U.; Gorb, S.N.; Gutowska, M.A. Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. *PLoS ONE* **2011**, *6*, e24223.
167. Calosi, P.; Rastrick, S.P.S.; Graziano, M.; Thomas, S.C.; Baggini, C.; Carter, H.A.; Hall-Spencer, J.M.; Milazzo, M.; Spicer, J.I. Distribution of sea urchins living near shallow water CO_2 vents is dependent upon species acid-base and ion-regulatory abilities. *Mar. Pollut. Bull.* **2013**, *73*, 470–484.
168. Connell, S.D.; Kroeker, K.J.; Fabricius, K.E.; Kline, D.I.; Russell, B.D. The other ocean acidification problem: CO_2 as a resource among competitors for ecosystem dominance. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2013**, *368*, doi:10.1098/rstb.2012.0442.

169. Kroeker, K.J.; Micheli, F.; Gambi, M.C. Ocean acidification causes ecosystem shifts via altered competitive interactions. *Nat. Clim. Change* **2012**, *3*, 156–159.
170. Connell, S.; Russell, B. The direct effects of increasing CO_2 and temperature on non-calcifying organisms: increasing the potential for phase shifts in kelp forests. *Proc. R. Soc. Biol. Sci. B* **2010**, *277*, 1409–1415.
171. de Mazancourt, C.; Johnson, E.; Barraclough, T.G. Biodiversity inhibits species' evolutionary responses to changing environments. *Ecol. Lett.* **2008**, *11*, 380–388.
172. Johansson, J. Evolutionary responses to climate changes: how does competition affect adaptation? *Evolution* **2008**, *62*, 421–435.
173. Fabricius, K.E.; Langdon, C.; Uthicke, S.; Humphrey, C.; Noonan, S.; De'ath, G.; Okazaki, R.; Muehllehner, N.; Glas, M.S.; Lough, J.M. Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations. *Nat. Clim. Change* **2011**, *1*, 165–169.
174. Dunn, R.R.; Harris, N.C.; Colwell, R.K.; Koh, L.P.; Sodhi, N.S. The sixth mass coextinction: Are most endangered species parasites and mutualists? *Proc. R. Soc. Biol. Sci. B* **2009**, *276*, 3037–3045.
175. Stenseth, N.C.; Smith, J.M. Coevolution in ecosystems: Red queen evolution or stasis? *Evolution* **1984**, *38*, 870–880.
176. Lawrence, D.; Fiegna, F.; Behrends, V.; Bundy, J.G.; Phillimore, A.B.; Bell, T.; Barraclough, T.G. Species interactions alter evolutionary responses to a novel environment. *PLoS Biol.* **2012**, *10*, doi:10.1371/journal.pbio.1001330.
177. Chesson, P. A need for niches? *Trends Ecol. Evol.* **1991**, *6*, 26–28.
178. Byrnes, J.E.; Gamfeldt, L.; Isbell, F.; Lefcheck, J.S.; Griffin, J.N.; Hector, A.; Cardinale, B.J.; Hooper, D.U.; Dee, L.E.; Emmett Duffy, J. Investigating the relationship between biodiversity and ecosystem multifunctionality: Challenges and solutions. *Meth. Ecol. Evol.* **2014**, *5*, 111–124.
179. Pereira, H.M.; Leadley, P.W.; Proença, V.; Alkemade, R.; Scharlemann, J.P.W.; Fernandez-Manjarrés, J.F.; Araújo, M.B.; Balvanera, P.; Biggs, R.; Cheung, W.W.L.; *et al*. Scenarios for global biodiversity in the 21st century. *Science* **2010**, *330*, 1496–1501.
180. Burrows, M.T.; Schoeman, D.S.; Richardson, A.J.; Molinos, J.G.; Hoffmann, A.; Buckley, L.B.; Moore, P.J.; Brown, C.J.; Bruno, J.F.; Duarte, C.M.; *et al*. Geographical limits to species-range shifts are suggested by climate velocity. *Nature* **2014**, *507*, 492–495.
181. Pespeni, M.H.; Chan, F.; Menge, B.A.; Palumbi, S.R. Signs of adaptation to local pH conditions across an environmental mosaic in the California current ecosystem. *Integr. Comp. Biol.* **2013**, *53*, 857–870.
182. Ricevuto, E.; Kroeker, K.J.; Ferrigno, F.; Micheli, F.; Gambi, M.C. Spatio-temporal variability of polychaete colonization at CO_2 vents indicates high tolerance to ocean acidification. *Mar. Biol.* **2014**, *161*, 2909-2919.
183. Hughes, T.P.; Baird, A.H.; Bellwood, D.R.; Card, M.; Connolly, S.R.; Folke, C.; Grosberg, R.; Hoegh-Guldberg, O.; Jackson, J.B.C.; Kleypas, J.; *et al*. Climate change, human impacts, and the resilience of coral reefs. *Science* **2003**, *301*, 929–933.
184. Bellantuono, A.J.; Hoegh-Guldberg, O.; Rodriguez-Lanetty, M. Resistance to thermal stress in corals without changes in symbiont composition. *Proc. R. Soc. Biol. Sci. B* **2012**, *279*, 1100–1107.
185. Barshis, D.; Ladner, J.; Oliver, T.; Palumbi, S.R. Lineage specific transcriptional profiles of *Symbiodinium* spp. unaltered by heat stress in a coral host. *Mol. Biol. Evol.* **2014**, *31*, 1343–1352.

186. Benton, M.J. The Red Queen and the Court Jester: Species diversity and the role of biotic and abiotic factors through time. *Science* **2009**, *323*, 728–732.
187. Drake, J.M.; Lodge, D.M. Hull fouling is a risk factor for intercontinental species exchange in aquatic ecosystems. *Aq. Inv.* **2007**, *2*, 121–131.
188. Walther, G.-R.; Roques, A.; Hulme, P.E.; Sykes, M.T.; Pysek, P.; Kühn, I.; Zobel, M.; Bacher, S.; Botta-Dukát, Z.; Bugmann, H.; *et al*. Alien species in a warmer world: Risks and opportunities. *Trends Ecol. Evol.* **2009**, *24*, 686–693.
189. Bianchi, C.N. Biodiversity issues for the forthcoming tropical Mediterranean Sea. *Hydrobiologia* **2007**, *580*, 7–21.
190. Irwin, A.J.; Finkel, Z.V.; Schofield, O.M.E.; Falkowski, P.G. Scaling-up from nutrient physiology to the size-structure of phytoplankton communities. *J. Plankton Res.* **2006**, *28*, 459–471.
191. Levine, J.M.; Vilà, M.; Antonio, C.M.D.; Dukes, J.S.; Grigulis, K.; Lavorel, S. Mechanisms underlying the impacts of exotic plant invasions. *Proc. R. Soc. Biol. Sci. B* **2003**, *270*, 775–781.
192. Schluter, D. Experimental evidence that competition promotes divergence in adaptive radiation. *Science* **1994**, *266*, 798–801.
193. Halpern, B.S.; Selkoe, K.A.; Micheli, F.; Kappel, C.V. Evaluating and ranking the vulnerability of global marine ecosystems to anthropogenic threats. *Conserv. Biol.* **2007**, *21*, 1301–1315.
194. Barton, A.D.; Pershing, A.J.; Litchman, E.; Record, N.R.; Edwards, K.F.; Finkel, Z.V.; Kiørboe, T.; Ward, B.A. The biogeography of marine plankton traits. *Ecol. Lett.* **2013**, *16*, 522–534.
195. Reusch, T.B.H.; Boyd, P.W. Experimental evolution meets marine phytoplankton. *Evolution* **2013**, *67*, 1849–1859.
196. Russell, B.D.; Wernberg, T.; Mieszkowska, N.; Widdicombe, S.; Hall-Spencer, J.; Connell, S. Predicting ecosystem shifts requires new approaches that integrate the effects of climate change across entire systems. *Biol. Lett.* **2012**, *8*, 164–166.
197. Hendriks, I.E.; Duarte, C.M. Ocean acidification: Separating evidence from judgment—A reply to Dupont *et al. Estuar. Coast. Shelf Sci.* **2010**, *89*, 186–190.
198. Gaylord, B.; Hill, T.M.; Sanford, E.; Lenz, E.A.; Jacobs, L.A.; Sato, K.N.; Russell, A.D.; Hettinger, A. Functional impacts of ocean acidification in an ecologically critical foundation species. *J. Exp. Biol.* **2011**, *214*, 2586–2594.
199. Vihtakari, M.; Hendriks, I.; Holding, J.; Renaud, P.; Duarte, C.; Havenhand, J. Effects of ocean acidification and warming on sperm activity and early life stages of the Mediterranean mussel (*Mytilus galloprovincialis*). *Water* **2013**, *5*, 1890–1915.
200. Van Valen, L. A new evolutionary law. *Evol. Theor.* **1973**, *1*, 1–30.

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Differential impacts of elevated CO_2 and acidosis on the energy budget of gill and liver cells from Atlantic cod, *Gadus morhua*

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Differential impacts of elevated CO_2 and acidosis on the energy budget of gill and liver cells from Atlantic cod, *Gadus morhua*

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ABSTRACT

Ocean acidification impacts fish and other marine species through increased seawater PCO_2 levels (hypercapnia). Knowledge of the physiological mechanisms mediating effects in various tissues of fish is incomplete. Here we tested the effects of extracellular hypercapnia and acidosis on energy metabolism of gill and liver cells of Atlantic cod. Exposure media mimicked blood conditions *in vivo*, either during normo- or hypercapnia and at control or acidic extracellular pH (pH_e). We determined metabolic rate and energy expenditure for protein biosynthesis, Na^+/K^+-ATPase and H^+-ATPase and considered nutrition status by measurements of metabolic rate and protein biosynthesis in media with and without free amino acids (FAA). Addition of FAA stimulated hepatic but not branchial oxygen consumption. Normo- and hypercapnic acidosis as well as hypercapnia at control pH_e depressed metabolic stimulation of hepatocytes. In gill cells, acidosis depressed respiration independent of PCO_2 and FAA levels. For both cell types, depressed respiration was not correlated with the same reduction in energy allocated to protein biosynthesis or Na^+/K^+-ATPase. Hepatic energy expenditure for protein synthesis and Na^+/K^+-ATPase was even elevated at acidic compared to control pH_e suggesting increased costs for ion regulation and cellular reorganization. Hypercapnia at control pH_e strongly reduced oxygen demand of branchial Na^+/K^+-ATPase with a similar trend for H^+-ATPase. We conclude that extracellular acidosis triggers metabolic depression in gill and metabolically stimulated liver cells. Additionally, hypercapnia itself seems to limit capacities for metabolic usage of amino acids in liver cells while it decreases the use and costs of ion regulatory ATPases in gill cells.

1. Introduction

Maintaining acid–base homeostasis is crucial for cellular and thus organism functioning. A wide range of environmental changes such as hypoxia or hypercapnia can cause disturbances of acid–base equilibria. Since the beginning of industrialization the atmospheric carbon dioxide concentration has increased by around 30% and has caused a mean pH reduction of ocean surface waters by around 0.1 units, in a process termed ocean acidification. Forecasts project an average pH decrease by around 0.3–0.4 units by the end of the century and up to 0.8 units by the year 2300, corresponding to an atmospheric PCO_2 increase from 76 Pa (750 μatm) to 101 Pa (1000 μatm) and then 193 Pa (1900 μatm), respectively (Caldeira and Wickett, 2003; Orr et al., 2005; Feely et al., 2009). Thus, environmental hypercapnia and acidification become important drivers in marine ecosystems.

In general, acute hypercapnic exposure of marine ectotherms leads to an increase of PCO_2 in body fluids and tissues associated with a concomitant, potentially transient drop of extra- (pH_e) and intracellular pH (pH_i) depending on PCO_2 and buffer capacity of the respective compartment and tissue (Toews et al., 1983; Heisler, 1993; Larsen et al., 1997; Pörtner et al., 1998; Michaelidis et al., 2005).

The energy metabolism of marine vertebrates and invertebrates seems to be sensitive to acidosis. Depending on the species, severity and duration of the acid–base imbalance, the energy demand of tissues and of whole organism may either increase due to enhanced activity of compensatory mechanisms (Wood et al., 2008; Lannig et al., 2010; Thomsen and Melzner, 2010; Parker et al., 2011; Stumpp et al., 2012) or be depressed as a possible strategy of passive tolerance sustaining survival if disturbances exceed compensatory capacities (Walsh et al., 1988; Reipschläger and Pörtner, 1996; Pörtner et al., 1998; Langenbuch and Pörtner, 2003; Michaelidis et al., 2005; Baker and Brauner, 2012). For example, in the peanut worm *Sipunculus nudus* an extracellular acidosis triggers metabolic depression through a shift to less costly ion-transporters (in terms of ATP stoichiometry) to compensate for the associated intracellular acidosis (Reipschläger and Pörtner, 1996; Pörtner et al., 1998, 2000). The drop of pH_i itself may then have a modulating or depressing effect on intracellular processes as shown for amino acid catabolism and protein biosynthesis, respectively (Langenbuch and Pörtner, 2002;

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Langenbuch et al., 2006). An acute extracellular acidosis also initiated a drop of pH_i in isolated hepatocytes of two Antarctic fish species and a concomitant decrease of hepatic protein biosynthesis (Langenbuch and Pörtner, 2003).

Marine teleosts exhibit high capacities for acid–base-regulation in vivo (Brauner and Baker, 2009). In cod exposed to ~1 kPa CO_2, extra- and intracellular acidosis were fully compensated for within 12 to 24 h, associated with increased extra- and intracellular bicarbonate concentrations (Larsen et al., 1997). The accumulation of bicarbonate is mediated via branchial ion-transport and involves the modulation of acid (H^+) and base (HCO_3^-) transporters at apical and basolateral cellular membranes. Although we still lack a comprehensive understanding of branchial ion regulation, available literature shows that basolateral HCO_3^-/Cl^--ion-exchangers (Claiborne et al., 2002; Evans, 2005; Perry and Gilmour, 2006; Heuer and Grosell, 2014) as well as Na^+/H^+ exchangers (NHE) may be involved in removing excess protons as shown in gills, liver and red blood cells (Walsh and Mommsen, 1992; Baker et al., 2009). Acid removal via NHEs depends on the Na^+ gradient actively built up by Na^+/K^+-ATPase, a major energy (ATP) consumer in most cells. In contrast to freshwater fish, a role for ATP dependent H^+-pumps (V-type-ATPase) during hypercapnia is not clear yet for marine fish. Recent studies rather indicate reduced H^+-ATPase activities during hypercapnic exposure (~0.19 kPa CO_2) in toadfish (Esbaugh et al., 2012).

As a consequence of acid–base disturbances the cellular energy demand of some mechanisms may increase and cause higher whole organism maintenance costs as well as a shift in the use of resources from fitness-related traits (e.g. reproduction) towards homeostasis (Pörtner, 2012; Sokolova et al., 2012). Such reallocation is likely to vary between tissues depending on their function and sensitivity and diverse tissue responses integrate into whole organism effects.

Therefore, our study was designed to improve our mechanistic understanding of the single and combined effects of acid–base variables, namely extracellular pH and PCO_2, on cellular metabolism and the functioning of different tissues (gill, liver) in Atlantic cod, *Gadus morhua*. We chose the liver, as a central metabolic organ, and gills as the major site for ion regulation, and measured cellular oxygen consumption as a proxy for metabolic rate. Normocapnic and hypercapnic conditions were investigated at control pH_e (7.9) and at acidic pH_e (7.0). By repeating the measurement in media supplemented with free amino acids (FAA) we aimed to see whether cellular responses differ compared to "standard" conditions (only glucose, no FAA). To resolve for shifts in cellular energy allocation we determined the respiratory fractions of ATP consuming processes such as protein biosynthesis and Na^+/K^+-ATPase, and in case of the gill, also H^+-ATPase.

2. Material and methods

2.1. Animals

In spring 2012 Atlantic cod (*G. morhua* Linnaeus, $N = 24$, total weight = 87–650 g, total length = 23–45 cm) were caught by trawling in the German bight around Helgoland (54° 09′ 30 N; 7° 52′ 70 E). Fish were directly transferred to the Alfred Wegener Institute in Bremerhaven and kept in aquaria with well-aerated, re-circulated natural seawater at 10 °C, 32 psu and a constant light/dark cycle (12 h:12 h). They were fed twice a week *ad libitum* with frozen *Mytilus edulis* and *Crangon crangon* and were allowed to recover for at least 10 days prior to experimentation.

2.2. Isolation of primary cells

Hepatocytes and gill cells were isolated following modified protocols from Mommsen et al. (1994) and Pärt et al. (1993), respectively. All buffers were placed on ice, sterile filtered and adjusted to *in vivo* blood conditions regarding osmolality (325 mOsm/kg) and pH (7.9 at 10 °C) (Larsen et al., 1997).

Cod were anaesthetized with MS222 (ethyl 3-aminobenzoate methanesulfonate, 0.08 g l^{-1} seawater) and killed with a cut through the spine. Gill arches and liver were excised quickly and transfered to petri dishes containing the respective buffers (liver, buffer 1: 167 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, 10 mM HEPES; gill, buffer 2: 152 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4). For liver, buffer 1 (supplemented with 5 mM EDTA) was injected into the hepatic vascular system until all blood was removed. Afterwards, digestion was initiated by injecting a collagenase-rich buffer (buffer 1 + 1500 U/ml collagenase type 1A + 1% *w/v* bovine serum albumin (BSA)) followed by buffer containing a lower enzyme concentration (buffer 1 + 1125 U/ml collagenase type 1A + 1% *w/v* BSA). In an analogous procedure, gill arches were freed from blood by injecting buffer 2 via the *bulbus arteriosus*. When the perfusate was clear, filaments were cut from the gill arches, finely chopped by use of a pair of scissors and transferred into fresh buffer 2 supplemented with a Trypsin/EDTA solution (0.05% Trypsin and 0.02% EDTA (*v/v*)) to start the digestion of gill filaments. Both tissues were shaken on ice for 60–80 min and gently massaged every 10–15 min using a cut plastic Pasteur pipette until total disintegration was achieved. Digestion of gill tissue was stopped by adding 10% (*v/v*) foetal calf serum (FCS).

Subsequently, each cell suspension was filtered through a two-stage nylon mesh filtering system (250 μm and 100 μm) and cell pellets were collected, washed with respective buffers (liver cells: buffer 1 + 1% BSA (*w/v*) + 1.5 mM $CaCl_2$; gill cells: buffer 2) and concentrated by repeated low-speed centrifugation (100 *g*, 4 °C, 3 min). Both cell suspensions were stored in recovery buffer (Leibovitz 15 with L-glutamine + 5 mM glucose + 200 U/ml penicillin + 200 U/ml streptomycin + 5% (*v/v*) FCS) and were shaken on ice for at least 1 h prior to experimentation to allow cells to recover from handling stress.

A Fuchs–Rosenthal counting chamber and a standard trypan-blue exclusion assay were used to assess cell density and viability. Mean rates for trypan blue exclusion were 97 ± 0.2% for liver and 91 ± 1.3% for gill cells

2.3. Measurements of cellular oxygen consumption and energy allocation

The medium for cell assays was designed to mimic *in vivo* conditions and consisted of buffer 1 complemented by variable concentrations of $NaHCO_3$ and either with free amino acids and glucose (+FAA) at levels found in the hepatic portal plasma of cod (Lyndon et al., 1993; FAA concentrations in μmol l^{-1}: Arg, 90; His, 20; Ile, 49; Leu, 81; Lys, 113; Met, 157; Phe, 48; Thr, 48; Trp, 8; Val, 112; Cys, 31; Tyr, 36; Glu, 286; Gln, 2750; Asp, 61; Asn, 48; Gly, 610; Pro, 70; Ala, 316; Tau, 710; Hyp, 10; Serin, 2750) or without (−FAA) to imitate conditions of a "standard" (glucose, −FAA) metabolic rate (Wieser and Krumschnabel, 2001). Experiments were performed under normocapnic (0.4 kPa PCO_2) and hypercapnic (0.64 kPa PCO_2) conditions at pH_e values of either 7.9 (control, representing normocapnic control and pH_e compensated hypercapnic condition, respectively) or 7.0 (acidic). Normocapnic control values for extracellular PCO_2, pH_e and osmolality (325 mOsm) were set to values found *in vivo* according to Larsen et al. (1997). Assuming a linear relationship (McKenzie et al., 2003; Esbaugh et al., 2012), hypercapnic blood PCO_2 was extrapolated from control values to mimic arterial blood PCO_2 under ocean acidification with a seawater PCO_2 of 0.3 kPa. Oxygen tension of the medium was lowered (12 kPa) to match arterial blood PO_2 of *G. morhua* at 10 °C (Karlsson et al., 2010). Table 1 summarizes the physiochemical conditions for the different experimental assays. Media set to 10 °C in a temperature-controlled water-bath were brought to the desired PO_2 and PCO_2 values by equilibration with gas mixtures provided by a 4-channel multi-gas-controller (647 B, MKS instruments GmbH, Germany). $NaHCO_3$ was added to adjust the different pH values. Required [$NaHCO_3$] concentrations were calculated from the Henderson–Hasselbalch equation by use of pK'' (6.28) and solubility coefficient αCO_2 (0.06 mMol L^{-1} mm Hg^{-1}) determined according to Heisler (1986). Medium pH (NBS scale, National

Table 1
Physiochemical conditions of media under normocapnic or hypercapnic conditions at different extracellular pH (pH_e) (7.9, control or 7.0, acidic).

Parameter	Normocapnia		Hypercapnia	
	pH_e 7.9	pH_e 7.0	pH_e 7.9	pH_e 7.0
pH_e (pH_{nbs})	7.9	7.0	7.9	7.0
PCO_2 (kPa)	0.3	0.3	0.64	0.64
HCO_3^- (mM)	7.7	1.0	12.3	1.6
PO_2 (kPa)	12	12	12	12
Temp. (°C)	10	10	10	10

Bureau of Standards) was checked using a pH meter (WTW, 197-S, Germany).

Measurements of oxygen consumption (5×10^6 cells per assay) were conducted in airtight respiration chambers (Rank Brothers, UK) that were mounted on magnetic stirrers to ensure homogeneity during measurements. Respiration rates were monitored by oxygen microoptodes (needle-type, PreSens GmbH, Germany) that were calibrated at the assay temperature (10 ± 0.5 °C) which was maintained by use of a thermostat (Lauda or Haake, Germany).

In order to determine the energy allocation to cellular energy consumers we injected specific inhibitors using a microlitre glass syringe (Hamilton, Bonaduz, Switzerland) after uninhibited cell respiration had been measured for at least 20–30 min. Inhibitors were applied in the lowest effective concentrations following previous studies (Pörtner et al., 2000; Mark et al., 2005) since overdose can lead to unspecific inhibition as shown for cycloheximide (Wieser and Krumschnabel, 2001). Cycloheximide was used at a final concentration of 100 μM to inhibit protein biosynthesis. Na^+/K^+-ATPase was inhibited via ouabain (6.67 mM) and H^+-ATPase by adding bafilomycin A_1 (0.1 μM). All inhibitors were dissolved in dimethyl sulfoxide. The added volume did not exceed 2% of the total assay volume in order to exclude a possible impact on cellular respiration rates (Mark et al., 2005). Fractional costs of protein biosynthesis were determined in buffer with or without the addition of FAA. Fractional costs of ion regulatory mechanisms were only measured in buffer with FAA. All chemicals were obtained from Sigma Aldrich, Germany.

Cell respiration rates (MO_2 in nmol O_2 min^{-1} 10^6 $cells^{-1}$) as well as oxygen demand of the different energy consuming processes were calculated as described by Lannig et al. (2010).

2.4. Statistical analyses

Data were analysed using Sigma Plot 12.0 (Systat Software Inc.). Outliers were determined according to Nalimov's test (Noack, 1980) and the data were checked for normality (Shapiro–Wilk) and equality of variances. If appropriate, a full two-way ANOVA was performed to unravel the effects of extracellular pH and PCO_2 on cellular respiration rates and on fractional costs of metabolic processes followed by a Holm–Sidak post-hoc test with pairwise comparisons of treatments. In case assumptions for ANOVA were violated or a disordinal interaction between the two factors was detected by ANOVA, treatments were compared pairwise with two tailed *t* tests or Mann–Whitney *U* tests. To account for multiple testing, Bonferroni's correction was applied. In all other cases $P < 0.05$ was accepted to indicate significant differences. To test for the effect of medium composition, the same treatments with or without the addition of FAA were tested in pairwise comparisons using a two-tailed *t* test or a Mann–Whitney *U* test. All data are presented as means ± S.E.M.

3. Results

3.1. Effects of extracellular pH, PCO_2 and FAA on metabolism of isolated gill cells

In branchial cells, neither the supplementation of FAA nor the experimental PCO_2 (Fig. 1A, Table 2) affected cellular respiration rates.

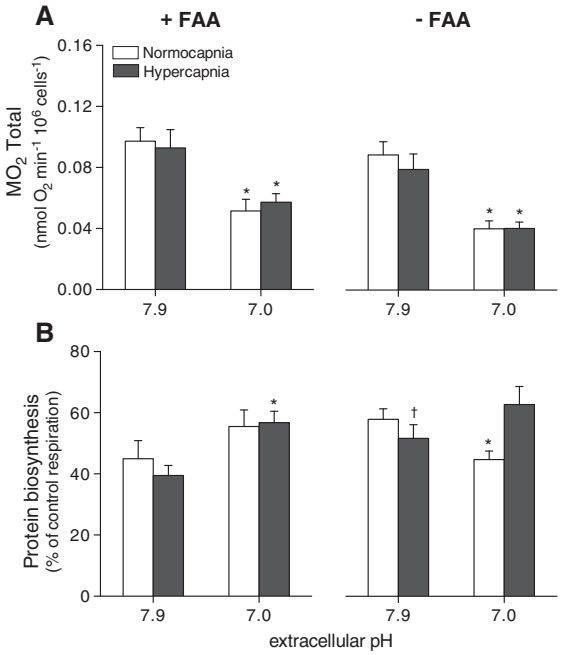

Fig. 1. Oxygen consumption and energy expenditure for protein biosynthesis in gill cells of Atlantic cod. Oxygen consumption (A) and fractional costs of protein biosynthesis (B) in primary gill cells were measured in medium with (+FAA) or without (−FAA) free amino acids (FAA) under normocapnic or hypercapnic conditions at different levels of extracellular pH (pH_e) (7.9, control or 7.0, acidic). * indicates oxygen consumption significantly different from control pH_e at the same PCO_2 and medium composition. # indicates oxygen consumption significantly different from the normocapnic group at the same pH_e and medium composition. † indicates a significant difference between assays in +FAA and −FAA measured at the same PCO_2 and pH_e. Values are means ± S.E.M., N = 11–15 (+FAA) or N = 4–6 (−FAA) per column for oxygen consumption and N = 7–9 (+FAA) or N = 4–6 (−FAA) for fractional costs of protein biosynthesis.

Extracellular acidosis, however, significantly depressed gill cell respiration, independent of substrate supply (Table 2). Under normocapnia, extracellular acidosis significantly depressed gill cell respiration by around 66% in −FAA medium ($P < 0.001$) and by around 50% in + FAA medium ($P < 0.001$). Similarly, hypercapnia combined with extracellular acidosis decreased branchial oxygen consumption by around 55% ($P = 0.018$) and 44% ($P = 0.013$), respectively.

In +FAA medium, pH_e but not hypercapnia significantly affected the fractional oxygen demand of protein biosynthesis in gill cells (Table 2, Fig. 1B). While the fractional energy expense for protein synthesis was similar in normo- and hypercapnic cells at control pH_e (~40%), it increased slightly in normocapnic (by ~8%) and significantly in hypercapnic gill cells (by 17%, $P = 0.016$) when pH_e was low (Fig. 1B, see also Fig. S1 for net oxygen demand).

In −FAA medium, two-way ANOVA revealed a disordinal interaction between pH_e and PCO_2 (Table 2, Fig. 1B). Subsequent testing showed, contrary to findings in +FAA medium, a decrease in fractional (and net, see Fig. S1) energy expense for protein biosynthesis in gill cells during normocapnic acidosis (~57% at pH_e 7.9 vs. ~45% at pH_e 7.0, $P = 0.025$) while it tended to increase under hypercapnic acidosis (~52% at pH_e 7.9 vs. ~63% at pH_e 7.0). Direct comparison of fractional oxygen demand of protein biosynthesis in +FAA compared to −FAA medium showed no differences, except for a higher fraction in −FAA medium at hypercapnic control pH_e (~40%, +FAA vs. ~52%, −FAA; $P = 0.049$).

Hypercapnia significantly affected energy expenditure for Na^+/K^+-ATPase in gill cells of cod (Table 2). At control pH_e, the fractional

Table 2
Two-way ANOVA results performed to investigate effects of PCO_2 and pH_e on oxygen consumption and fractional costs of protein biosynthesis, Na^+/K^+-ATPase and H^+-ATPase in gill cells or hepatocytes from *Gadus morhua* in medium supplemented with (+FAA) or without (−FAA) free amino acids (FAA). Significant results are written in bold.

Tissue	Medium	Parameter	PCO_2 effect			pH_e effect			Interaction		
			F	*df*	*P*	*F*	*df*	*P*	*F*	*df*	*P*
Gill	+FAA	MO_2	*0.005*	*1*	*0.944*	*18.679*	*1*	***<0.001***	*0.284*	*1*	*0.596*
	+FAA	Protein biosynth.	*0.194*	*1*	*0.664*	*8.647*	*1*	***0.007***	*0.512*	*1*	*0.481*
	+FAA	H^+-ATPase	*0.091*	*1*	*0.768*	*0.114*	*1*	*0.741*	*6.685*	*1*	***0.022***
	−FAA	MO_2	*0.357*	*1*	*0.560*	*33.154*	*1*	***<0.001***	*0.409*	*1*	*0.533*
	−FAA	Protein biosynth.	*1.572*	*1*	*0.229*	*0.0469*	*1*	*0.831*	*6.657*	*1*	***0.021***
Liver	+FAA	MO_2	*5.115*	*1*	***0.029***	*21.086*	*1*	***<0.001***	*3.287*	*1*	*0.077*
	+FAA	Na^+/K^+-ATPase	*0.309*	*1*	*0.583*	*10.542*	*1*	***0.003***	*1.118*	*1*	*0.301*
	−FAA	MO_2	*0.191*	*1*	*0.668*	*0.008*	*1*	*0.930*	*0.700*	*1*	*0.415*

oxygen consumption allocated to Na^+/K^+-ATPase dropped significantly from around 53% under normocapnia to 29% under hypercapnia (Fig. 2A, $P < 0.001$, see also Fig. S3 for net oxygen demand). Acidosis per se did not lead to significant changes in energy demand of branchial Na^+/K^+-ATPase even though mean fractional costs under normocapnia were slightly lower at acidic compared to control pH_e (Fig. 2A).

Results for H^+-ATPase revealed a significant disordinal interaction of pH_e and PCO_2 and thus prohibited interpretation of the main factors (Table 2). However, unpaired *t* tests detected a decreasing fraction of oxygen demand for H^+-ATPase at decreasing pH_e, from 26% to 10% during normocapnia ($P = 0.034$) and a reverse, non-significant trend ($P = 0.189$) for the fraction to increase during hypercapnia at low pH_e (Fig. 2B, see also Fig. S3 for net oxygen demand).

3.2. Effects of extracellular pH, PCO_2 and FAA on metabolism of isolated liver cells

Significant effects of FAA, pH_e and PCO_2 on hepatic oxygen consumption rates were observed (Fig. 3A, Table 2, see also Fig. S2 for net oxygen demand). Effects of pH_e and PCO_2 were, however, only visible when cells were metabolically stimulated by FAA supply. At normocapnic control pH_e, oxygen demand of hepatocytes was significantly elevated by 43% in +FAA compared to −FAA medium ($P < 0.001$). In hypercapnic hepatocytes measured in +FAA medium and at control pH_e, we observed a depressed respiration by ~18% compared to normocapnic cells ($P = 0.006$), but respiration rates were still significantly elevated (~33%) compared to cells in −FAA medium ($P < 0.001$). During acidosis the FAA-induced increase of hepatic oxygen demand was reduced further resulting in depressed respiration by around 30% in normocapnic cells at acidic compared to control pH_e ($P < 0.001$), but it was still significantly elevated by 23% compared to respective values obtained in −FAA medium ($P < 0.023$). Hypercapnia exacerbated the effect of low pH_e, as FAA addition had no stimulating effect on hepatic respiration at low pH_e and high PCO_2 (Fig. 3A). Compared to hypercapnic cells at control pH_e, oxygen demand of hypercapnic FAA fed cells at low pH_e was depressed further (~17%), but this effect was non-significant.

For liver cells, measured at control pH_e, only normocapnic cells supplemented with FAA showed a cycloheximide-sensitive fraction (protein biosynthesis) of their cellular oxygen demand (by ~6%, Fig. 3B). For hypercapnic cells at control pH_e and with glucose only

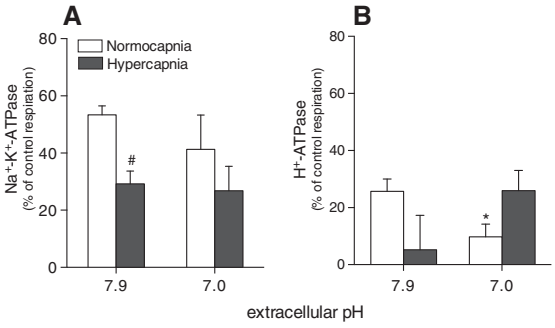

Fig. 2. Energy expenditure for Na^+/K^+-ATPase and H^+-ATPase in gill cells of Atlantic cod. Fractional costs of Na^+/K^+-ATPase (A) and H^+-ATPase (B) in isolated gill cells were measured under normocapnic or hypercapnic conditions at different levels of extracellular pH (pH_e) (7.9, control or 7.0, acidic). Data are presented as percentages of control respiration at the respective extracellular pH. * indicates fractional cost significantly different from control pH_e at the same PCO_2 and medium composition. # indicates fractional cost significantly different from the normocapnic group at the same pH_e and medium composition. Values are means ± S.E.M., $N = 4$–9 per column.

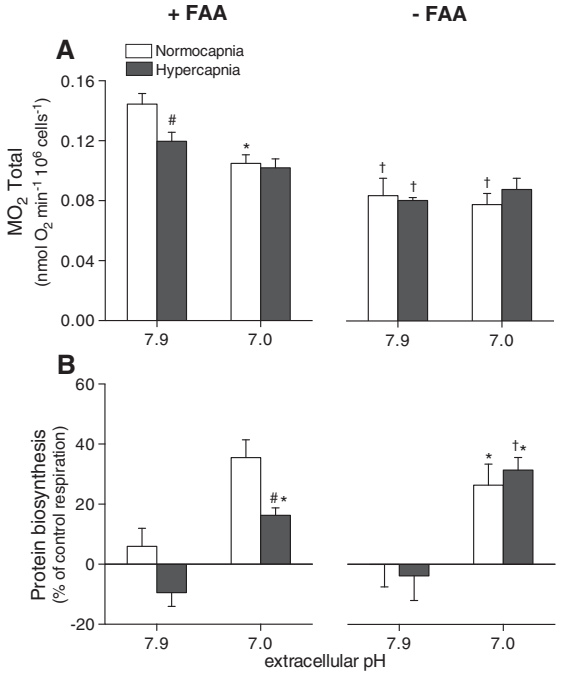

Fig. 3. Oxygen consumption and energy expenditure for protein biosynthesis in liver cells of Atlantic cod. Oxygen consumption (A) and fractional costs of protein biosynthesis (B) of primary liver cells were measured in medium with (+FAA) or without (−FAA) free amino acids (FAA) under normocapnic or hypercapnic conditions at different levels of extracellular pH (pH_e) (7.9, control or 7.0, acidic). * indicates oxygen consumption significantly different from control pH_e at the same PCO_2 and medium composition. # indicates oxygen consumption significantly different from the normocapnic group at the same pH_e and medium composition. † indicates a significant difference between assays in +FAA and −FAA measure at the same PCO_2 and pH_e. Values are means ± S.E.M., $N = 10$–15 (+FAA) or $N = 5$ (−FAA) per column for oxygen consumption and $N = 6$–10 (+FAA) or $N = 4$–6 (−FAA) for fractional costs protein biosynthesis.

(−FAA) we even observed a marginally increased oxygen demand of hepatocytes after applying cycloheximide. In contrast, extracellular acidosis strongly stimulated hepatic protein biosynthesis in −FAA medium (Table 2) independent of the experimental PCO_2 (normocapnia, $P = 0.023$; hypercapnia, $P = 0.001$), leading to 30% elevated fractional costs for protein biosynthesis with declining pH_e. Similarly, in hypercapnic and normocapnic +FAA medium the fractional costs of protein biosynthesis increased under acidosis ($P < 0.001$ and $P = 0.03$, respectively, Fig. 3B). However, the response to cycloheximide was significantly lower in hepatocytes exposed to hypercapnic than to normocapnic acidosis with FAA present ($P = 0.022$). Thus, under hypercapnic acidosis hepatocytes in +FAA medium showed a significantly decreased fractional oxygen demand of protein biosynthesis compared to cells in −FAA medium ($P = 0.009$).

In hepatocytes, the fractional oxygen demand of Na^+/K^+-ATPase responded significantly to pH_e, but not to PCO_2 (Fig. 4, Table 2, see also Fig. S3 for net oxygen demand). At control pH_e, the fractional costs of Na^+/K^+-ATPase were minor (normocapnia = 0.3%; hypercapnia = 2.6%), while they increased under extracellular acidosis, however, changes were significant only in the normocapnic treatments (normocapnia = 20%, $P = 0.004$; hypercapnia = 13%, $P = 0.148$).

4. Discussion

4.1. Effects of extracellular pH, PCO_2 and FAA on metabolism of isolated gill cells

Our data clearly demonstrate that extracellular acidosis (pH_e 7.0) whether normo- or hypercapnic, causes a depression of metabolic rate by around 40% in isolated gill cells of Atlantic cod. This is in line with previous findings that extracellular acidosis causes a down-regulation of metabolism in marine vertebrates and invertebrates at the cell and tissue level (hepatocytes of *Pachycara brachycephalum* and *Lepiodonotothen kempi*, Langenbuch and Pörtner, 2003; body wall of *S. nudus*, Reipschläger and Pörtner, 1996; gill tissue of *Crassostrea virginica*, Willson and Burnett, 2000). Acute studies on the marine invertebrate *S. nudus* revealed that extracellular pH is the key driver for metabolic depression in this species, whereas changes of intracellular pH and PCO_2 alone did not affect metabolic rate (Reipschläger and Pörtner, 1996; Pörtner et al., 1998, 2000). Similarly, an acutely increased

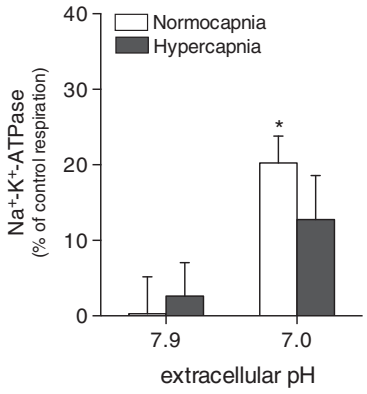

Fig. 4. Energy expenditure for Na^+/K^+-ATPase in liver cells of Atlantic cod. Fractional costs of Na^+/K^+-ATPase of isolated liver cells were measured under normocapnic or hypercapnic conditions at different levels of extracellular pH (pH_e) (7.9, control or 7.0, acidic). Data are presented as percentages of control respiration at the respective pH_e. * indicates fractional cost significantly different from control pH_e at the same PCO_2 and medium composition. # indicates fractional cost significantly different from the normocapnic group within same pH_e. Values are means ± S.E.M., $N = 6–8$ per column.

PCO_2 (~1 kPa) at control pH_e had no effect on oxygen consumption rates of isolated perfused gills from two Antarctic notothenioids (Deigweiher et al., 2009). This matches our findings in gill cells as metabolic rates were not affected by hypercapnia at control pH_e, but were depressed by extracellular acidosis regardless of the experimental PCO_2. Moreover, recent work from our laboratory showed that energy turnover in isolated gills of *G. morhua* is maintained after long-term incubation to elevated seawater PCO_2 at compensated extracellular pH (Kreiss et al., 2014). Taken together, these data indicate that exposure to low pH_e but not to acute or long-term elevated PCO_2 initiates depressed oxygen consumption in gills of Atlantic cod.

Adding FAA did not change branchial oxygen demand suggesting that glucose serves as preferred respiratory substrate over FAA in gill cells of cod (Fig. 1A). An earlier study on primary gill cell cultures of tilapia (seawater-adapted) and toadfish also showed that gill cells from both species oxidized glucose at substantially higher rates than alanine (Perry and Walsh, 1989). ^{14}C-labelled glucose injected into Atlantic cod was in fact mostly utilized in gills (Hemre and Kahrs, 1997).

Our results for branchial protein biosynthesis support earlier studies stating that gill tissue shows high rates of protein biosynthesis in cod (Lyndon and Houlihan, 1998; Carter and Houlihan, 2001). High *in vivo* costs reflect a high rate of protein turnover but also flexibility for functional remodelling in the gills.

Branchial metabolic depression during extracellular acidosis was not associated with a drastic downregulation of protein biosynthesis since the observed changes in net oxygen demand and in associated fractional costs for protein biosynthesis do not fully explain the strong reduction in total oxygen consumption (Fig. 1, S1). At acidic pH_e only normocapnic gill cells in −FAA medium showed a significant reduction (~54%) in fractional energy expenditure for protein biosynthesis whereas it remained unchanged in normocapnic cells in +FAA medium. This might indicate that the availability of extracellular FAA causes maintenance of protein synthesis rates under acidic conditions. Under hypercapnic acidosis, net and fractional oxygen demand of protein biosynthesis were either maintained (−FAA) or even slightly increased (+FAA), possibly reflecting a higher need for cellular reorganization in branchial cells during hypercapnic compared to normocapnic acidosis.

Hypercapnia per se had no effect on gill cell oxygen demand of protein biosynthesis. Energy expenditure at control pH_e was similar in normo- and hypercapnic cells. In isolated gill arches of Atlantic cod respiratory costs of protein biosynthesis and the associated costs of RNA synthesis were also maintained under long-term increased sea water PCO_2 (~0.25 kPa CO_2, Kreiss et al., 2014). In contrast, acute exposure of isolated gill arches of two Antarctic fish species to pH_e compensated hypercapnia led to elevated branchial costs for protein and RNA synthesis (Deigweiher et al., 2009). However, the PCO_2 level applied was higher in the Antarctic study (1 kPa vs. present 0.64 kPa CO_2, reflecting blood PCO_2 during 0.3 kPa CO_2 in seawater), and oxygen demand of Na^+/K^+-ATPase was increased along with the increased respiratory costs of protein and RNA synthesis, contrasting the strongly reduced aerobic costs of Na^+/K^+-ATPase during pH_e compensated hypercapnia in our study.

Generally, costs of Na^+/K^+-ATPase are expected to be high in osmo- and ion-regulating gills due to the central role of this enzyme in sustaining the Na^+-gradient and thus the driving force for many other Na^+-dependent membrane transporters including Na^+/H^+-exchanger. Normocapnic gill cells measured at control pH_e, allocated around 50% of their cellular oxygen demand to Na^+/K^+-ATPase and this fraction dropped to ~30% during hypercapnia (Fig. 2A). This hypercapnia-induced reduction in respiratory costs of Na^+/K^+-ATPase was unexpected, as it seems to contrast the need to remove excess protons, but may indicate an acute compensatory response with reduced involvement of costly Na^+-dependent ion exchange during pH_e-compensated hypercapnia. The present data do in fact support recent findings on the changing role of the Na^+/K^+-pump in teleosts under acute

hypercapnic conditions. In a study on gulf toadfish (*Opsanus beta*) the *in vitro* maximal enzymatic activity of the Na^+/K^+-ATPase decreased after 24 h of compensated hypercapnic acidosis (~0.19 kPa CO_2) but returned to control levels after 72 h of exposure (Esbaugh et al., 2012). In contrast, in Atlantic cod, hypercapnia at compensated pH_e seems to decrease the costs of Na^+/K^+-ATPase not only acutely (this study) but also in the long run (~0.25 kPa CO_2, Kreiss et al., 2014). Interestingly, decreased respiratory costs of the Na^+/K^+-ATPase *in vivo* were associated with unchanged maximal enzyme capacities *in vitro* (Kreiss et al., 2014). Thus, it seems that available branchial capacities of Na^+/K^+-ATPase are not fully exploited in hypercapnia exposed cod.

Similar to the results for Na^+/K^+-ATPase, there was a trend (insignificant) for branchial costs of H^+-ATPase to decrease under compensated hypercapnic acidosis. Overall, costly ATP-demanding ion transport seems to be reduced in hypercapnic gill cells, however, total oxygen demand of gill cells was unchanged. Since oxygen demand of protein biosynthesis was also unchanged from that in normocapnic cells it remains to be investigated, whether other cellular process/processes increased resulting in unchanged overall oxygen demand under pH_e-compensated hypercapnia.

In contrast to hypercapnia, extracellular acidosis per se did not affect the branchial fractional respiratory costs of Na^+/K^+-ATPase when comparing costs of normo- and hypercapnic cells between pH_e 7.9 and 7.0 (Fig. 2A). As total oxygen demand of gill cells was depressed during extracellular acidosis this indicates a decreased net oxygen demand of this transporter. Although net oxygen demand was lowered, we determined no statistical difference of transporter costs in normo- and hypercapnic cells between pH_e 7.9 and 7.0. This might, on the one hand, be attributable to a low sample size. On the other hand, the constant fractional costs indicate that metabolic depression in gill cells induced by extracellular acidosis likely reflects the parallel down-regulation of several cellular processes, including acid–base regulation and protein biosynthesis, rather than a strong decrease of any single process.

Taken together, acute adjustments during normocapnic acidosis and compensated or uncompensated hypercapnic acidosis seem to include a shift in the branchial energy allocation reflected in a down-regulation of energy consuming ATPases. High branchial protein biosynthesis rates are maintained and may support rearrangements between transporter densities. As we did not determine intracellular pH due to the limited number of available animals, the question remains open whether extracellular acidosis and hypercapnia mediated the aforementioned effects directly or indirectly via effects of a potential intracellular acidosis. Earlier studies with isolated teleost cells (hepatocytes) emphasize that during acute extracellular acidosis pH_i decreases with decreasing pH_e (Walsh et al., 1988; Langenbuch and Pörtner, 2003). In cod, a hypercapnia-induced drop of pH_e (Δ pH_e ~0.2) led to a significant but transient (around 24 h) decrease in pH_i (Δ pH_i ~0.1) in several tissues (Larsen et al., 1997).

4.2. Effects of extracellular pH, PCO_2 and FAA on metabolism of isolated liver cells

In contrast to findings in gill cells, oxygen demand of normocapnic hepatocytes at control pH_e 7.9 rose by almost 75% upon addition of FAA. Liver is the main organ for amino acid catabolism and anabolism in fish, thus the FAA-induced rise in oxygen consumption is most likely related to the specific use of FAA in liver (Ballantyne, 2001). Goldfish and trout hepatocytes also responded to + FAA medium by significantly increasing respiration rates but, in contrast to our study, along with strongly increased protein biosynthesis (Wieser and Krumschnabel, 2001). In cod hepatocytes, fractional oxygen demand of protein biosynthesis was 6% in + FAA medium, and thus, surprisingly low at normocapnic control pH_e. Ineffectiveness of cycloheximide inhibition can be excluded due to the observed increase in fractional costs of protein biosynthesis (~40%) in response to extracellular acidosis (Fig. 3B). Our findings contrast the observations in hepatocytes from goldfish, trout and other teleost species where protein biosynthesis comprised between 20 and 90% of total oxygen demand (Pannevis and Houlihan, 1992; Smith and Houlihan, 1995; Langenbuch and Pörtner, 2003; Mark et al., 2005). Protein biosynthesis and its plasticity likely vary in a species-dependent manner (Carter and Houlihan, 2001).

As in branchial cells, extracellular acidosis depressed aerobic metabolism of normocapnic hepatocytes, however, only when FAA were added to the medium. Our results are in line with data obtained in hepatocytes of trout and two Antarctic fish species both of which showed depressed metabolic rates in response to an acute lowering of pH_e (Walsh et al., 1988; Langenbuch and Pörtner, 2003). In both studies, pH_i decreased in parallel with a decreasing pH_e. This drop of pH_i likely mediated a strong decrease in protein biosynthesis in hepatocytes of Antarctic fish (Langenbuch and Pörtner, 2003). We observed metabolic depression at low pH_e only in + FAA medium. Compared to cells at control pH_e, acidified liver cells failed to respond to FAA exposure by metabolic stimulation. As outlined above, metabolic stimulation by FAA was not attributable to increased protein biosynthesis. Irrespective of the presence of FAA, both net and fractional oxygen demands of protein biosynthesis were increased during normo- and hypercapnic extracellular acidosis. Thus, the depression of oxygen demand induced by acidosis in FAA stimulated hepatocytes of cod did not involve a reduction in protein biosynthesis (Fig. 3, S2).

The increased energy allocation towards protein biosynthesis during acidic conditions requires explanation. The increase in protein biosynthesis might reflect the stage of cellular reestablishment and reorganization during acute exposure to low pH_e. Acidosis causes the up-regulation of heat shock proteins (HSPs), which in turn may stimulate protein biosynthesis (reviewed by Iwama et al., 1999). The main function of HSPs lies in the conservation of protein integrity, which can be impaired by a drop of the intracellular pH. Interestingly, some Antarctic fish and several cold-adapted species lack an up-regulation of HSPs under stressful conditions (Buckley, 2004; Tomanek, 2010). Thus, up-regulation of HSPs might explain why acidosis stimulated protein biosynthesis in cod hepatocytes compared to the depression found in hepatocytes from Antarctic fish (Langenbuch and Pörtner, 2003). Interestingly, increased hepatic protein biosynthesis and Na^+/K^+-ATPase activity went hand in hand under acidified conditions. Na^+/H^+-exchange plays a central role in hepatic pH_i compensation (Furimsky et al., 1999; Brauner et al., 2004; Ahmed et al., 2006; Huynh et al., 2011), causing higher respiratory costs of Na^+/K^+-ATPase during acute extracellular acidosis.

Fractional costs of Na^+/K^+-ATPase in normo- and hypercapnic cod hepatocytes were relatively low at control pH_e (0.3–2.5%), similar to findings for trout hepatocytes (2.8%, Pannevis and Houlihan, 1992). Higher fractions of respiratory costs of hepatic Na^+/K^+-ATPase were found in Notothenoidei (40–45%, Mark et al., 2005) and *Carassius auratus* (30–40%, Krumschnabel et al., 2001). These differences may be species-specific, but we cannot exclude that closely mimicked *in vivo* conditions of osmolality and FAA levels might have led to minimal costs of hepatic osmoregulation, driven by Na^+/K^+-ATPase.

In light of low respiratory costs of Na^+/K^+-ATPase and protein biosynthesis at control pH_e the question arises which cellular process stimulated metabolic activity during FAA supply and was inhibited during extracellular acidosis. In cod, the liver is the primary organ storing lipids in intracellular droplets (Jobling, 1988). Due to a lipid-rich prey, *de novo* synthesis of lipids is thought to play a minor role in cold-water carnivorous marine teleosts. However, it is well known that cod, due to a low capacity for long-term storage of amino acids, deaminates excess amino acids which then serve as a carbon source for lipid synthesis (Jobling, 1988; Tocher, 2003). We hypothesize that *de novo* synthesis of lipids may occur in hepatocytes supplied with excess FAA at control pH_e, causing high metabolic activity despite low costs of Na^+/K^+-ATPase and protein biosynthesis. In fact, an earlier study showed a high capacity for lipogenesis in isolated hepatocytes of rainbow trout

(Hazel and Sellner, 1979). As most symporters for neutral amino acids are known to be pH-sensitive, low pH_e might hamper transmembrane FAA transport (Shotwell and Oxender, 1983; Ballantyne, 2001). As a consequence, the uptake of FAA — and then lipogenesis — would be inhibited during acidosis. Such phenomena have been observed in rats *in vivo* (Boon et al., 1994). Clearly, future studies need to confirm these hypotheses for fish.

In contrast to observations in gill cells, hypercapnia per se depressed hepatic metabolism, independent of extracellular pH. In metabolically stimulated hepatocytes (+FAA medium) at control pH_e, oxygen consumption of hypercapnic cells was significantly depressed compared to normocapnic cells (Fig. 3A). Furthermore and in contrast to normocapnic hepatocytes, hypercapnic cells showed no increase in metabolism during acidosis at pH_e 7.0 when supplemented with FAA. All of this resembles findings by Walsh et al. (1988) who demonstrated that hypercapnia had a direct negative impact on metabolism of trout hepatocytes, which was independent of extracellular pH and [HCO_3^-]. Interestingly, in a notothenioid (*Notothenia rossii*), hepatic mitochondrial capacities were decreased after long-term hypercapnic exposure at compensated pH_e (0.2 kPa CO_2, Strobel et al., 2012). Such reduction of mitochondrial aerobic scope may become effective when metabolic rate is strongly stimulated such as by FAA addition. Furthermore, at low extracellular pH hypercapnia also depressed the oxygen demand of hepatic protein biosynthesis in + FAA medium indicating that high CO_2 directly affects hepatic capacities of protein biosynthesis in cod.

5. Conclusions

The present study clearly shows tissue-specific metabolic consequences of acute hypercapnia as well as extracellular acidosis in gill and liver cells of Atlantic cod. Although acute responses to acidosis and hypercapnia may differ compared to responses after long-term exposure, our results highlight the importance to investigate different tissues as well as levels of biological organization in order to fully understand the mechanisms determining species sensitivity and tolerance towards environmental changes such as ocean acidification.

pH_e-induced metabolic depression has been identified in various fish and invertebrate species and may thus represent a common mechanism. However, this study and others indicate that this mechanism is used differently by different organs and organisms. While metabolic depression elicited by an extracellular acidosis may be transient due to rapid pH_e compensation *in vivo* (Larsen et al. 1997) we also demonstrate that hypercapnia *per se* limits metabolic capacities of liver cells. In addition to that, hypercapnia decreases the rate of costly ion regulatory ATPases (Na^+/K^+-ATPase, H^+-ATPase) in branchial cells indicating a hypercapnia-induced shift in the branchial energy budget and ion regulation that requires further investigation. Future studies have to elaborate whether the observed changes persist after long-term acclimation as well as how they translate into consequences at organism level.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.05.009.

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References

Ahmed, K.H., Pelster, B., Krumschnabel, G., 2006. Signalling pathways involved in hypertonicity-and-acidification induced activation of Na^+/H^+ exchange in trout hepatocytes. J. Exp. Biol. 209, 3101–3113.
Baker, D.W., Brauner, C.J., 2012. Metabolic changes associated with acid–base regulation during hypercarbia in the CO_2-tolerant chondrostean, white sturgeon (*Acipenser transmontanus*). Comp. Biochem. Physiol. A 161, 61–68.
Baker, D.W., Matey, V., Huynh, K.T., Wilson, J.M., Morgan, J.D., Brauner, C.J., 2009. Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*. Am. J. Physiol. Regul. Integr. Comp. Physiol. 296, R1868–R1880.
Ballantyne, J.S., 2001. Amino acid metabolism. In: Wright, P.A., Anderson, P.M. (Eds.), Fish Physiology: Nitrogen Excretion. Academic Press, pp. 77–108.
Boon, L., Blommaart, P.J., Meijer, A.J., Lamers, W.H., Schoolwerth, A.C., 1994. Acute acidosis inhibits liver amino acid transport: no primary role for the urea cycle in acid–base balance. Am. J. Physiol. 267, F1015–F1020.
Brauner, C.J., Baker, D.W., 2009. Patterns of acid–base regulation during exposure to hypercarbia in fishes. In: Glass, M.L., Wood, S.C. (Eds.), Cardio-Respiratory Control in Vertebrates. Springer, pp. 43–63.
Brauner, C.J., Wang, T., Wang, Y., Richards, J.G., Gonzalez, R.J., Bernier, N.J., Xi, W., Patrick, M., Val, A.L., 2004. Limited extracellular but complete intracellular acid–base regulation during short-term environmental hypercapnia in the armoured catfish, *Liposarcus pardalis*. J. Exp. Biol. 207, 3381–3390.
Buckley, B.A., 2004. Regulation of heat shock genes in isolated hepatocytes from an Antarctic fish, *Trematomus bernacchii*. J. Exp. Biol. 207, 3649–3656.
Caldeira, K., Wickett, M.E., 2003. Anthropogenic carbon and ocean pH. Nature 425, 365-365.
Carter, C.G., Houlihan, D.F., 2001. Protein synthesis. In: Wright, P.A., Anderson, P.M. (Eds.), Fish Physiology: Nitrogen Excretion. Academic Press, pp. 31–76.
Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid–base regulation in fishes: cellular and molecular mechanisms. J. Exp. Zool. 293, 302–319.
Deigweiher, K., Hirse, T., Bock, C., Lucassen, M., Pörtner, H.O., 2009. Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids. J. Comp. Physiol. B. 180, 347–359.
Esbaugh, A.J., Heuer, R., Grosell, M., 2012. Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*. J. Comp. Physiol. B. 182, 921–934.
Evans, D.H., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.
Feely, R.A., Orr, J., Fabry, V.J., Kleypas, J.A., Sabine, C.L., Langdon, C., 2009. Present and future changes in seawater chemistry due to ocean acidification. In: Mcpherson, B.J., Sundquist, E.T. (Eds.), Carbon Sequestration and Its Role in the Global Carbon Cycle. American Geophysical Union, Washington, D. C., pp. 175–188.
Furimsky, M., Moon, T.W., Perry, S.F., 1999. Intracellular pH regulation in hepatocytes isolated from three teleost species. J. Exp. Zool. 284, 361–367.
Hazel, J.R., Sellner, P.A., 1979. Fatty acid and sterol synthesis by hepatocytes of thermally acclimated rainbow trout (*Salmo gairdneri*). J. Exp. Zool. 209, 105–114.
Heisler, N., 1986. Buffering and transmembrane ion transfer processes. In: Heisler, N. (Ed.), Acid–base Regulation in Animals. Elsevier, Amsterdam, pp. 3–47.
Heisler, N., 1993. Acid–base regulation. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press Inc., Boca Raton, pp. 343–377.
Hemre, G.I., Kahrs, F., 1997. ^{14}C-glucose injection in Atlantic cod, *Gadus morhua*, metabolic responses and excretion via the gill membrane. Aquacult. Nutr. 3, 3–8.
Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 307, R1061–R1084.
Huynh, K.T., Baker, D.W., Harris, R., Church, J., Brauner, C.J., 2011. Effect of hypercapnia on intracellular pH regulation in a rainbow trout hepatoma cell line, RTH 149. J. Comp. Physiol. B. 181, 883–892.
Iwama, G.K., Vijayan, M.M., Forsyth, R.B., Ackerman, P.A., 1999. Heat shock proteins and physiological stress in fish. Am. Zool. 39, 901–909.
Jobling, M., 1988. A review of the physiological and nutritional energetics of Cod, *Gadus morhua* L., with particular reference to growth under farmed conditions. Aquaculture 70, 1–19.
Karlsson, A., Heier, L.S., Rosseland, B.O., Salbu, B., Kiessling, A., 2010. Changes in arterial PO_2, physiological blood parameters and intracellular antioxidants in free-swimming Atlantic cod (*Gadus morhua*) exposed to varying levels of hyperoxia. Fish Physiol. Biochem. 37, 249–258.
Kreiss, C.M., Michael, K., Bock, C., Lucassen, M., Pörtner, H.O., 2014. Impact of long-term moderate hypercapnia and elevated temperature on the energy budget of isolated gills of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. A 182C, 102–112.
Krumschnabel, G., Manzl, C., Schwarzbaum, P.J., 2001. Regulation of intracellular pH in anoxia-tolerant and anoxia-intolerant teleost hepatocytes. J. Exp. Biol. 204, 3943–3951.
Langenbuch, M., Pörtner, H.O., 2002. Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid–base variables. J. Exp. Biol. 205, 1153–1160.
Langenbuch, M., Pörtner, H.O., 2003. Energy budget of hepatocytes from Antarctic fish (*Pachycara brachycephalum* and *Lepidonotothen kempi*) as a function of ambient CO_2: pH-dependent limitations of cellular protein biosynthesis? J. Exp. Biol. 206, 3895–3903.
Langenbuch, M., Bock, C., Leibfritz, D., Pörtner, H.O., 2006. Effects of environmental hypercapnia on animal physiology: a ^{13}C NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*. Comp. Biochem. Physiol. A 144, 479–484.
Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M., Bock, C., 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. Mar. Drugs 8, 2318–2339.

Larsen, B.K., Pörtner, H.O., Jensen, F.B., 1997. Extra- and intracellular acid–base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. Mar. Biol. 128, 337–346.
Lyndon, A.R., Houlihan, D.F., 1998. Gill protein turnover: costs of adaptation. Comp. Biochem. Physiol. A 119, 27–34.
Lyndon, A.R., Davidson, I., Houlihan, D.F., 1993. Changes in tissue and plasma free amino acid-concentrations after feeding in Atlantic cod. Fish Physiol. Biochem. 10, 365–375.
Mark, F.C., Hirse, T., Pörtner, H.O., 2005. Thermal sensitivity of cellular energy budgets in some Antarctic fish hepatocytes. Polar Biol. 28, 805–814.
McKenzie, D.J., Piccolella, M., Dalla Valle, A.Z., Taylor, E.W., Bolis, C.L., Steffensen, J.F., 2003. Tolerance of chronic hypercapnia by the European eel *Anguilla anguilla*. J. Exp. Biol. 206, 1717–1726.
Michaelidis, B., Ouzounis, C., Paleras, A., Pörtner, H.O., 2005. Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar. Ecol. Prog. Ser. 293, 109–118.
Mommsen, T.P., Moon, T.W., Walsh, P.J., 1994. Hepatocytes: isolation, maintenance and utilization. In: Hochachka, Mommsen (Ed.), Biochemistry and Molecular Biology of Flshes. Elsevier Science B. V.
Noack, S., 1980. Statistische Auswertung von Mess- und Versuchsdaten mit Taschenrechner und Tischcomputer. Walter de Gruyter, Berlin, New York.
Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.-F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437, 681–686.
Pannevis, M.C., Houlihan, D.F., 1992. The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B. 162, 393–400.
Parker, L.M., Ross, P.M., O'Connor, W.A., Borysko, L., Raftos, D.A., Pörtner, H.-O., 2011. Adult exposure influences offspring response to ocean acidification in oysters. Glob. Change Biol. 18, 82–92.
Pärt, P., Norrgren, L., Bergström, E., Sjöberg, P., 1993. Primary cultures of epithelial cells from rainbow trout gills. J. Exp. Biol. 175, 219–232.
Perry, S.F., Gilmour, K.M., 2006. Acid–base balance and CO_2 excretion in fish: unanswered questions and emerging models*. Respir. Physiol. Neurobiol. 154, 199–215.
Perry, S.F., Walsh, P.J., 1989. Metabolism of isolated fish gill cells: contribution of epithelial chloride cells. J. Exp. Biol. 144, 507–520.
Pörtner, H.O., 2012. Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. Mar. Ecol. Prog. Ser. 470, 273–290.
Pörtner, H.O., Reipschläger, A., Heisler, N., 1998. Acid–base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. J. Exp. Biol. 201, 43–55.
Pörtner, H.O., Bock, C., Reipschläger, A., 2000. Modulation of the cost of pH_i regulation during metabolic depression: a ^{31}P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. J. Exp. Biol. 203, 2417–2428.
Reipschläger, A., Pörtner, H.O., 1996. Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. J. Exp. Biol. 199, 1801–1807.
Shotwell, M.A., Oxender, D.L., 1983. The regulation of neutral amino acid transport by amino-acid availability in animal cells. Trends Biochem. Sci. 8, 314–316.
Smith, R.W., Houlihan, D.F., 1995. Protein synthesis and oxygen consumption in fish cells. J. Comp. Physiol. B. 165, 93–101.
Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15.
Strobel, A., Bennecke, S., Leo, E., Mintenbeck, K., Pörtner, H.O., Mark, F.C., 2012. Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2. Front. Zool. 9 (1), 28.
Stumpp, M., Trübenbach, K., Brennecke, D., Hu, M.Y., Melzner, F., 2012. Resource allocation and extracellular acid–base status in the sea urchin *Strongylocentrotus droebachiensis* in response to CO_2 induced seawater acidification. Aquat. Toxicol. 110–111, 194–207.
Thomsen, J., Melzner, F., 2010. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Mar. Biol. 157, 2667–2676.
Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11, 107–184.
Toews, D.P., Holeton, G.F., Heisler, N., 1983. Regulation of the acid–base status during environmental hypercapnia in the marine teleost fish *Conger conger*. J. Exp. Biol. 107, 9–20.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213, 971–979.
Walsh, P.J., Mommsen, T.P., 1992. Intracellular pH regulation and metabolic interactions in hepatic tissues. J. Exp. Zool. 263, 176–185.
Walsh, P.J., Mommsen, T.P., Moon, T.W., Perry, S.F., 1988. Effects of acid–base variables on in vitro hepatic metabolism in rainbow trout. J. Exp. Biol. 135, 231–241.
Wieser, W., Krumschnabel, G., 2001. Hierarchies of ATP-consuming processes: direct compared with indirect measurements, and comparative aspects. Biochem. J. 355, 389–395.
Willson, L.L., Burnett, L.E., 2000. Whole animal and gill tissue oxygen uptake in the Eastern oyster, *Crassostrea virginica*: effects of hypoxia, hypercapnia, air exposure, and infection with the protozoan parasite *Perkinsus marinus*. J. Exp. Mar. Biol. Ecol. 246, 223–240.
Wood, H.L., Spicer, J.I., Widdicombe, S., 2008. Ocean acidification may increase calcification rates, but at a cost. Proc. R. Soc. Lond. B Biol. Sci. 275, 1767–1773.

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RESEARCH ARTICLE

Intertidal oysters reach their physiological limit in a future high-CO_2 world

Elliot Scanes[1,2,*], Laura M. Parker[2], Wayne A. O'Connor[3], Laura S. Stapp[4] and Pauline M. Ross[2]

ABSTRACT

Sessile marine molluscs living in the intertidal zone experience periods of internal acidosis when exposed to air (emersion) during low tide. Relative to other marine organisms, molluscs have been identified as vulnerable to future ocean acidification; however, paradoxically it has also been shown that molluscs exposed to high CO_2 environments are more resilient compared with those molluscs naive to CO_2 exposure. Two competing hypotheses were tested using a novel experimental design incorporating tidal simulations to predict the future intertidal limit of oysters in a high-CO_2 world; either high-shore oysters will be more tolerant of elevated P_{CO_2} because of their regular acidosis, or elevated P_{CO_2} will cause high-shore oysters to reach their limit. Sydney rock oysters, *Saccostrea glomerata*, were collected from the high-intertidal and subtidal areas of the shore and exposed in an orthogonal design to either an intertidal or a subtidal treatment at ambient or elevated P_{CO_2}, and physiological variables were measured. The combined treatment of tidal emersion and elevated P_{CO_2} interacted synergistically to reduce the haemolymph pH (pH_e) of oysters, and increase the P_{CO_2} in the haemolymph (P_{e,CO_2}) and standard metabolic rate. Oysters in the intertidal treatment also had lower condition and growth. Oysters showed a high degree of plasticity, and little evidence was found that intertidal oysters were more resilient than subtidal oysters. It is concluded that in a high-CO_2 world the upper vertical limit of oyster distribution on the shore may be reduced. These results suggest that previous studies on intertidal organisms that lacked tidal simulations may have underestimated the effects of elevated P_{CO_2}.

KEY WORDS: Emersion, Ocean acidification, Hypercapnia, Multiple stressors, Mollusc

INTRODUCTION

Sessile marine molluscs living in the intertidal zone are periodically exposed to air (emersion) as the tide recedes. When emersed, marine bivalves close their shell, which restricts gas exchange and can result in internal hypercapnia and extracellular acidosis (Burnett, 1988; Truchot, 1990). When hypercapnic, normal physiological processes of bivalves are impeded, and metabolism is slowed until normocapnia returns (Burnett, 1988; Greenway and Storey, 1999; David et al., 2005). Intertidal emersion also reduces the time available to sessile organisms for feeding, waste excretion and other vital processes (Truchot, 1990).

Oysters are ubiquitous sessile molluscs found on many temperate shorelines, extending from the subtidal shore to the upper limits of the intertidal shore, where they provide ecosystem services ranging from complex habitat provision (Underwood and Barrett, 1990; Gutiérrez et al., 2003; Cole et al., 2007) to water quality improvement and benthic–pelagic coupling (Newell et al., 2005). The time oysters spend emersed is dependent on where they are located on the shore. Oysters in the subtidal zone are rarely, or never, emersed. Oysters in the intertidal zone, however, experience regular periods of emersion. Those oysters living at the upper level of the intertidal zone experience the longest periods of emersion and potentially greater respiratory and metabolic acidosis associated with hypercapnia. This emersion results in greater regular changes to the acid–base balance of intertidal oysters compared with subtidal oysters (Dugal, 1939; Burnett, 1988; Truchot, 1990).

Oysters and other molluscs have been identified as highly vulnerable to increasing CO_2 concentrations of the Earth's oceans in a process known as ocean acidification (Fabry et al., 2008; Gazeau et al., 2013). Ocean acidification occurs when CO_2 emissions are absorbed by the oceans, forming a weak acid and reducing oceanic pH (Caldeira and Wickett, 2003, 2005). Compared with pre-industrial levels, the mean pH of surface ocean waters has declined by more than 0.1 units (Caldeira and Wickett, 2005; Raven et al., 2005) and the Intergovernmental Panel on Climate Change (Houghton, 2001; Solomon, 2007; Collins et al., 2013) has predicted that the pH of ocean surface waters will fall a further 0.3–0.5 units (pH 7.8–7.6) by 2100 and 0.7–0.77 units (pH 7.4–7.43) by 2300, assuming median emission scenarios (Caldeira and Wickett, 2003, 2005; Solomon, 2007; Collins et al., 2013). Ocean acidification has been shown to alter the fundamental physiological functions of molluscs, especially acid–base regulation (Pörtner et al., 2004; Parker et al., 2013). This can then further impact a wide range of morphological and physiological characteristics of molluscs, including shell formation (Fabry, 2008; Fabry et al., 2008; Doney et al., 2009), and restrict the energy available for growth, reproduction, immune responses and homeostasis (Pörtner et al., 2004; Fabry et al., 2008; Doney et al., 2009; Parker et al., 2013). These negative effects extend to the larval stages of marine molluscs, which are known to be especially vulnerable to ocean acidification (Parker et al., 2010; Ross et al., 2011; Scanes et al., 2014). Larval growth, settlement and metamorphosis of molluscs has been shown to be negatively impacted by ocean acidification (Ross et al., 2011; Gazeau et al., 2013), and these effects are exacerbated in the presence of other environmental stressors (Ko et al., 2014; Cole et al., 2016). In all ontogenetic stages, molluscs are poor acid–base regulators compared with other taxa. They have a limited capacity to actively accumulate HCO_3^- in order to buffer their extracellular fluids against changes in pH (Melzner et al., 2009).

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RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

Paradoxically, however, while the effects of ocean acidification are predicted to be most severe for molluscs living at the limits of their physiological stress tolerance (Sokolova, 2013; Gazeau et al., 2013), it has also been shown that molluscs living in environments naturally high in CO_2 are more resilient (Thomsen et al., 2010; Parker et al., 2012, 2015). The resilience of organisms to environmental stress can be mediated through plastic responses, genetic adaption and relocation (e.g. to refugia) (Williams et al., 2008; Dawson et al., 2011; Bellard et al., 2012). Pre-exposure to elevated P_{CO_2} changes the responses of some organisms including bivalves to elevated P_{CO_2} (Thomsen et al., 2010; Parker et al., 2012, 2015). When mussels (*Mytilus edulis*) were collected from a region of the Baltic Sea naturally high in CO_2, they were found to be more tolerant to laboratory exposure to elevated P_{CO_2} than *M. edulis* from control areas (Thomsen et al., 2010). Exposure of adult oysters to elevated CO_2 also has positive carry-over effects on offspring. Parker et al. (2012) exposed adult *Saccostrea glomerata* to elevated P_{CO_2} and found their larvae to be more resilient to elevated P_{CO_2}. When these larvae reached adult maturity, they retained those traits and were still more tolerant of elevated P_{CO_2} (Parker et al., 2015), perhaps because of transgenerational plasticity (Ross et al., 2016). Tolerance to ocean acidification has also been observed in organisms that experience greater levels of internal metabolic CO_2 (Melzner et al., 2009). Ectotherms capable of sustaining prolonged physical activity experience an accumulation of metabolic CO_2 as a result of muscle activity. Their ability to regulate this CO_2 and the associated acidosis is believed to explain the apparent tolerance of active organisms to ocean acidification (Melzner et al., 2009).

For oysters, the degree of stress experienced varies across their distribution. Individuals at the upper intertidal shore heights experience severe hypercapnia during emersion (Dugal, 1939; Burnett, 1988; Truchot, 1990), whereas oysters in the subtidal shore experience less hypercapnia. It is predicted that ocean acidification will make it more difficult for intertidal invertebrates to return to normocapnia during periods of immersion that follow emersion (Rastrick et al., 2014). This suggests that intertidal oysters, particularly those high on the shore, will experience greater stress from ocean acidification and have a lower capacity for resilience. Alternatively, because the physiological effects of emersion are similar to those experienced under ocean acidification, oysters living in a high-CO_2 environment may be more resilient to ocean acidification, as has been found in other studies (Thomsen et al., 2010; Parker et al., 2012, 2015; Ross et al., 2016).

The Sydney rock oyster, *Saccostrea glomerata* (Gould 1850), is found in south eastern Australia and is common from shallow subtidal to intertidal habitats in estuaries and protected embayments (Nell, 2001). To determine whether Sydney rock oysters will reach their intertidal limit in a future high-CO_2 world, two competing theories were tested: either oysters will reach their physiological and intertidal limit when exposed to elevated P_{CO_2} or, alternatively, oysters in the upper intertidal zone will be resilient to elevated P_{CO_2}. It was hypothesised that: (1) an intertidal environment would exacerbate the hypercapnic effects of elevated P_{CO_2} on oysters and (2) oysters from the high-intertidal zone will be more resilient to elevated P_{CO_2} and diurnal emersion compared with those from the subtidal zone.

MATERIALS AND METHODS

Location, collection and acclimation

Experimental *S. glomerata* individuals were collected from two locations and two different shore heights (high-intertidal and subtidal) on the shore within Port Stephens, NSW, Australia (location 1: 32°32′42.25″S, 152°03′42.69″E; location 2; 32°41′54.04″S, 152°03′27.71″E). Oysters from each location were pooled together according to their respective shore heights to better ensure genetic diversity. Each location was composed of sloping rocky shore, dominated by large flat surfaces of granite, giving a consistent gentle slope down to the water. At each location, two zones of shore height (high-intertidal and subtidal) were established by measuring the shore height on the rocky shore above Indian Spring Low Water (ISLW). The subtidal zone was identified as being 0.1–0.4 m above ISLW, and the high-intertidal zone as 1.1–1.5 m above ISLW. One hundred individuals were collected per zone and location by gently levering aggregations off the substratum.

Following collection, oysters were taken to the Port Stephens Fisheries Institute (PSFI, NSW, Australia), where they were cleaned of fouling organisms and separated into individual oysters. All experimental seawater (hereafter, FSW) used at PSFI was collected from Little Beach within Port Stephens (32°42′42.75″S, 152°9′26.48″E) and filtered to 1 µm. All experiments were conducted at a constant temperature of 22°C. The oysters from each zone were then transferred to separate 750 l header tanks filled with FSW. It was calculated from observations and measurements of diurnal tidal fluctuations that the high-intertidal zone received approximately 9 h emersion followed by 3 h immersion, and the subtidal zone received 12 h (constant) immersion on an average diurnal 12 h tidal cycle. The collected oysters remained in four 750 l tanks for 1 week to acclimate under a simulated tidal treatment of either 9 h emersion followed by 3 h immersion (for oysters collected from the high-intertidal shore) or constant submergence (for oysters collected from the subtidal shore).

Experimental treatments

A fully orthogonal design was used to test our hypotheses. Oysters were collected from both high-intertidal and subtidal zones, and were then exposed to all possible combinations of two tidal treatments and two P_{CO_2} scenarios. *Saccostrea glomerata* closes its valves when exposed to the air during emersion; this prevents any gaseous exchange with the air while emersed (Potter and Hill, 1982).

Twelve 750 l header tanks were divided among the two experimental tidal treatments: six intertidal and six subtidal. Within each tidal treatment, three header tanks were held at elevated P_{CO_2} (1000 µatm) and three at ambient P_{CO_2} (400 µatm). Each header tank circulated FSW through two 50 l tanks suspended above it, one of which held 16 oysters collected from the high-intertidal zone, while the other held 16 oysters from the subtidal zone. Therefore, each combination of tidal treatment×P_{CO_2}× intertidal/subtidal collection zone was replicated three times across three different header tanks (n=3). Experimental tidal treatments were the same as described previously for acclimation. The intertidal treatment consisted of 9 h emersion followed by 3 h immersion, and the subtidal treatment consisted of 12 h constant immersion. In all header tanks, FSW was pumped from the header tank into the replicate tanks via a spray bar, where it overflowed back to the header tank. Oysters were kept slightly elevated on a mesh stage in all tanks. To emulate tidal conditions, a small hole was drilled in the bottom of all 50 l tanks. When the allocated immersion time for the intertidal treatment (3 h) had been reached, pumps circulating water to the 50 l tank were switched off via an electronic timer (Hager electro Pty Ltd, Glendenning, NSW, Australia), and the FSW slowly drained out of the tank back into the header to simulate a naturally retreating tide. Following emersion (9 h),

Journal of Experimental Biology

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

pumps automatically refilled the tank. Oysters in the subtidal treatment remained submerged under flowing FSW at all times. Oysters were allowed 10 days to acclimate to their tanks and new tidal treatments prior to the introduction of CO_2. Following the introduction of CO_2, oysters remained in experimental treatments for 3 weeks.

CO_2 monitoring

The two P_{CO_2} levels used in this study (400 µatm, 1000 µatm; based on the multi-model average projection by the IPCC for 2100; Collins et al., 2013) were a mean ambient pH_{NBS} of 8.19±0.02 and a mean pH_{NBS} at elevated CO_2 levels of 7.84±0.0035. The elevated CO_2 level was maintained using a pH negative feedback system (Aqua Medic, Aqacenta Pty Ltd, Kingsgrove, NSW, Australia; accuracy ±0.01 pH units). To determine the pH level corresponding to P_{CO_2} levels, total alkalinity (TA) was quantified at each water change using triplicate Gran-titration (Gran, 1952). Following the titration, the TA and chosen P_{CO_2} levels were entered into a CO_2 system calculation program (CO_2 SYS; Lewis et al., 1998), using the dissociation constants of Mehrbach et al. (1973), and the pH level corresponding with the desired P_{CO_2} level was calculated. Seawater physiochemical variables including pH_{NBS}, TA and salinity were measured at each water change (Table S1) – the desired pH values corresponding with P_{CO_2} levels were then recalculated accordingly. Food grade CO_2 was bubbled directly into the header tanks via a CO_2 reactor to ensure proper mixing and, in turn, reduce pH. A pH probe connected to a controlling computer was placed within each tank (probes were calibrated each water change using NBS buffers). When the desired pH level was reached, the delivery of CO_2 was automatically stopped by a computer signal to a solenoid valve. Each header tank set to elevated P_{CO_2} was controlled by its own independent pH controlling system. The pH values of each tank were monitored daily, and the pH electrode of each controlling system was checked daily against another calibrated pH probe (NBS buffers, WTW 3400*i*).

Animal husbandry

Every second day, each header tank received a complete water change. A second set of header tanks were filled and equilibrated to the temperature and, if appropriate, P_{CO_2} level of corresponding tanks already housing oysters. The 50 l tanks containing oysters were then transferred to a clean header tank (of the same treatment variables), ensuring they were out of the water for only a short amount of time. Tanks were completely drained and then scrubbed clean using Virkon S solution (Antec Corp, North Bend, WA, USA). Oysters were fed each day an algal mixture consisting of 50% *Chaetoceros muelleri* and 50% *Tisochrysis luteas* at a concentration equivalent to 2×10^9 *T. luteas* cells oyster^{-1} day^{-1} (Nell and O'Connor, 1991). Animal ethics approval was not required for this study.

Measurement of haemolymph variables

To determine the effects of experimental treatments on oyster haemolymph acid–base variables following 3 weeks of exposure, three oysters were randomly taken from each replicate tank. Oysters in the intertidal treatment were sampled at the end of both their allocated immersion and emersion times. Oysters were then immediately opened without rupturing the pericardial cavity. Haemolymph samples were drawn from the interstitial fluid filling the pericardial cavity chamber of an opened oyster using a sealed 1 ml needled syringe. A 0.2 ml sample was drawn carefully to avoid aeration of the haemolymph. Half of the sample was then immediately transferred to an Eppendorf tube where pH_e of the sample was measured at 20°C using a micro-pH probe (Metrohm 827 biotrode). The remaining haemolymph was transferred to a gas analyser (CIBA Corning 965) to determine total CO_2 concentration (C_{CO_2}). The micro-pH probe was calibrated prior to use with NBS standards at the acclimation temperature and the gas analyser was calibrated following the manufacturer's guidelines. Three oysters per replicate tank from the intertidal treatment were sampled after their entire allocated 3 h immersion cycle and another three oysters after their full 9 h emersion cycle. Three oysters were sampled per replicate tank in the subtidal treatment. Partial pressure of CO_2 in haemolymph (P_{e,CO_2}) and concentration of HCO_3^- in the haemolymph ($[HCO_3^-]_e$) were calculated from the C_{CO_2} using the modified Henderson–Hasselbalch equation (Eqns 1 and 2) according to Heisler (1984, 1986) as found in Riebesell et al. (2010), where the molarity of dissolved species was 1.033 mol l^{-1} (seawater; Hammer et al., 2011), $[Na^+]$ was 0.55 mol l^{-1} (measured previously) and protein concentration of *S. glomerata* was 0.05 g^{-1} l^{-1} (Peters and Raftos, 2003):

$$P_{e,CO_2} = C_{CO_2} \times (10^{pH_e - pK'''} \times \alpha + \alpha)^{-1}, \quad (1)$$

where P_{e,CO_2} is the calculated P_{CO_2} in haemolymph (mmHg), C_{CO_2} is the measured total CO_2 concentration in haemolymph (mmol l^{-1}), α is the physical solubility of CO_2 and pK''' is the apparent dissociation constant of carbonic acid in body fluids (after Heisler, 1986); and:

$$[HCO_3^-]_e = C_{CO_2} - (\alpha_{CO_2} \times P_{e,CO_2}), \quad (2)$$

where C_{CO_2} is the measured total CO_2 concentration in haemolymph (mmHg), α_{CO_2} is the solubility of CO_2 in haemolymph (calculated after Heisler, 1984, 1986) (0.0346 mmol l^{-1} mmHg^{-1}) and P_{e,CO_2} is the calculated P_{CO_2} in haemolymph (mmHg).

Standard metabolic rate and condition index

Standard metabolic rate (SMR) was determined using a closed respiratory system (Parker et al., 2012) when oysters were immersed. Following 3 weeks under experimental conditions, two individuals were randomly selected from each replicate tank for measurements. Oysters were placed in individual 500 ml airtight chambers filled with FSW set to the corresponding P_{CO_2} of that treatment. Each chamber was fitted with a fibre-optic O_2 probe (PreSens dipping probe DP-PSt3, AS1 Ltd, Regensburg, Germany). The probes were calibrated using a two-point calibration (0% and 100% air-saturated FSW) and all measurements were done at the experimental temperature of 22°C. The time taken to reduce the percentage oxygen saturation of seawater in the chamber from 100% to 80% was recorded. A 'blank' chamber containing only FSW was set up for each treatment to test for bacterial respiration. The change in this chamber over the duration of oyster measurements was negligible and therefore not included in the SMR calculation. Time was only recorded when oysters were actively respiring (time during which oxygen levels were decreasing). Prior to these SMR measurements, food was withheld for 24 h to remove any variability associated with digestive metabolism, and individuals were only measured following their allocated immersion time. Following the measurements, oysters were removed from the chambers, opened and tissue was separated from the shell. Both tissue and shells were dried in an oven at 70°C for 72 h then weighed using an electronic balance (±0.001 g). SMR was calculated for each individual using

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

Eqn 3:

$$\mathrm{SMR} = \frac{[V_r \times \Delta C_{O_2,w}]}{\Delta t \times M_b}, \quad (3)$$

where SMR is oxygen consumption normalised to 1 g of dry tissue mass (mg O_2 g^{-1} dry tissue mass h^{-1}), V_r is the volume of the respiratory chamber minus the volume of the oyster (l), $\Delta C_{O_2,w}$ is the measured change in water oxygen concentration (mg O_2 l^{-1}), Δt is time (h) and M_b is the dry tissue mass (g) (Parker et al., 2012). Condition index was calculated as the ratio of shell mass (as a proxy for shell volume) to somatic mass using Eqn 4:

$$\mathrm{CI} = \left(\frac{M_b}{M_s}\right) \times 100, \quad (4)$$

where CI is condition index, M_b is dry tissue mass (g) and M_s is dry shell mass (g).

Shell growth

At the beginning of the experimental exposure, five oysters were randomly selected from each replicate tank and their shell length (antero-posterior measurement) was taken using digital Vernier callipers (±0.01 mm). This procedure was then repeated following 3 weeks of experimental exposure. For each replicate, the mean shell length of the five oysters at the beginning was subtracted from the mean shell length of the five oysters at the end of the exposure, to give mean shell growth (mm) per replicate tank.

Data analysis

To test for differences among haemolymph variables (pH_e, P_{e,CO_2}, $[HCO_3^-]_e$; n=3), condition index (n=3) and SMR (n=3) during immersion, data were analysed using an orthogonal nested 4-way ANOVA. P_{CO_2} treatment (ambient or elevated) was the first factor, tidal treatment (subtidal or intertidal) the second factor, shore collection height (subtidal or high-intertidal) the third factor and tank the fourth factor. Measurements taken during emersion were analysed using a 3-way orthogonal ANOVA where CO_2 (ambient or elevated) was the first factor, shore collection height (subtidal or high-intertidal) the second factor and tank the third factor. To compare emersion and immersion measurements taken from oysters in an intertidal cycle, a 4-way orthogonal nested ANOVA was used, where cycle (immersion versus emersion) was the first factor, CO_2 (ambient or elevated) was the second, shore collection height (subtidal or high-intertidal) was the third and tank was the fourth factor. Shell growth (n=3) was analysed using a 3-way ANOVA where CO_2 (ambient or elevated) was the first factor (fixed and orthogonal), tidal treatment (subtidal or intertidal) was the second (fixed and orthogonal) and shore collection height (subtidal or high-intertidal) was the third (fixed and orthogonal). In all analyses except shell growth, the first three (or two in the case of emersion measurements) factors were fixed and orthogonal, and the tank (TA) factor was random and nested in the three (or two) other factors. All data met Cochran's test for homogeneity of variance without transformation prior to analysis and were analysed using Gmav 5 software (Underwood et al., 2002). SNK tests were performed *post hoc* to determine the source of variation among means (Underwood, 1996). Mean results for measured variables were graphed using Microsoft Excel 2007, with error bars indicating s.e.m. Experimental factors that were not significant (α>0.1; Underwood, 1996) were pooled for some figures to give a mean of the combined non-significant factors.

RESULTS

Haemolymph variables

pH_e

Overall, the pH_e of oysters experiencing an intertidal cycle was higher when they were submerged in the water (immersed) rather than out of the water (emersed), i.e. following 3 h of immersion, the pH_e of oysters was significantly higher than it was following 9 h of emersion (Fig. 1A). Oysters that were emersed for 9 h had a similar pH_e in ambient and elevated P_{CO_2} treatments, which ranged between 6.8±0.04 and 6.79±0.05, respectively (ANOVA, P>0.5).

When oysters were immersed, pH_e was reduced at elevated (pH_e=7.4±0.04) compared with ambient (pH_e=7.58±0.02) P_{CO_2}, regardless of whether oysters were held in a subtidal or intertidal treatment (Fig. 1A). Further, when oysters were immersed, pH_e was lowest at elevated P_{CO_2} in the intertidal treatment (CO_2×tidal treatment interaction; ANOVA, $F_{1,16}$=5.52, P=0.032; Fig. 1A), i.e. the pH_e of oysters in the intertidal treatment remained lower than that in the subtidal treatment for oysters immersed and exposed to elevated P_{CO_2}. There was no significant difference in the pH_e of oysters collected from the subtidal or high intertidal shore (P>0.1).

P_{e,CO_2}

The mean P_{e,CO_2} of oysters was approximately three times greater when oysters were emersed (P_{e,CO_2}=0.67±0.06 kPa) compared with when they were immersed in water (P_{e,CO_2}=0.18±0.03 kPa; ANOVA, $F_{1,16}$=146.45, P<0.001). Further, when oysters were emersed, the P_{e,CO_2} in the haemolymph was greatest at elevated P_{CO_2} (P_{e,CO_2}=0.75±0.081 kPa) compared with the ambient P_{CO_2} treatment (P_{e,CO_2}=0.59±0.038 kPa; ANOVA, $F_{1,8}$=15.81, P=0.0041) (Fig. 1B). Oysters that were immersed and in an elevated P_{CO_2} treatment also had a significantly greater P_{e,CO_2} than those in the ambient treatment (ANOVA, $F_{1,16}$=12.82, P=0.0025).

Oysters collected from the high-intertidal zone had greater P_{e,CO_2} levels than those collected from the subtidal zone during emersion (cycle×shore height interaction; ANOVA, $F_{1,16}$=6.05, P=0.025).

$[HCO_3^-]_e$

There were no significant effects of any factors (ANOVA, P>0.07) on the $[HCO_3^-]_e$ of oysters except for among tanks ($F_{16,48}$=2.35, P=0.01), when measured during immersion. This suggests variability in $[HCO_3^-]_e$ among tanks. During immersion, there was a trend for $[HCO_3^-]_e$ of oysters to be greater in the subtidal compared with the intertidal treatment. Oysters collected from the high shore had a trend for greater $[HCO_3^-]_e$ under elevated P_{CO_2} (Fig. 1C).

Whole-organism measurements

SMR

The SMR of oysters was significantly increased by elevated compared with ambient P_{CO_2} (ANOVA, $F_{1,16}$=11.03, P=0.0043) and intertidal compared with subtidal treatment (ANOVA, $F_{1,16}$=8.97, P=0.0086). The greatest SMR was observed in the combined elevated P_{CO_2} and intertidal treatment (Fig. 2). The oysters collected from the subtidal shore increased their SMR when transferred to an intertidal treatment, whereas oysters from the high-intertidal shore did not change their metabolic rate (significant shore height×tidal treatment interaction; ANOVA, $F_{1,16}$=5.32, P=0.035; Fig. 2).

Condition index

Oysters in the subtidal treatment had significantly greater condition index (ANOVA, $F_{1,16}$=8.77, P=0.0092) compared with oysters in

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

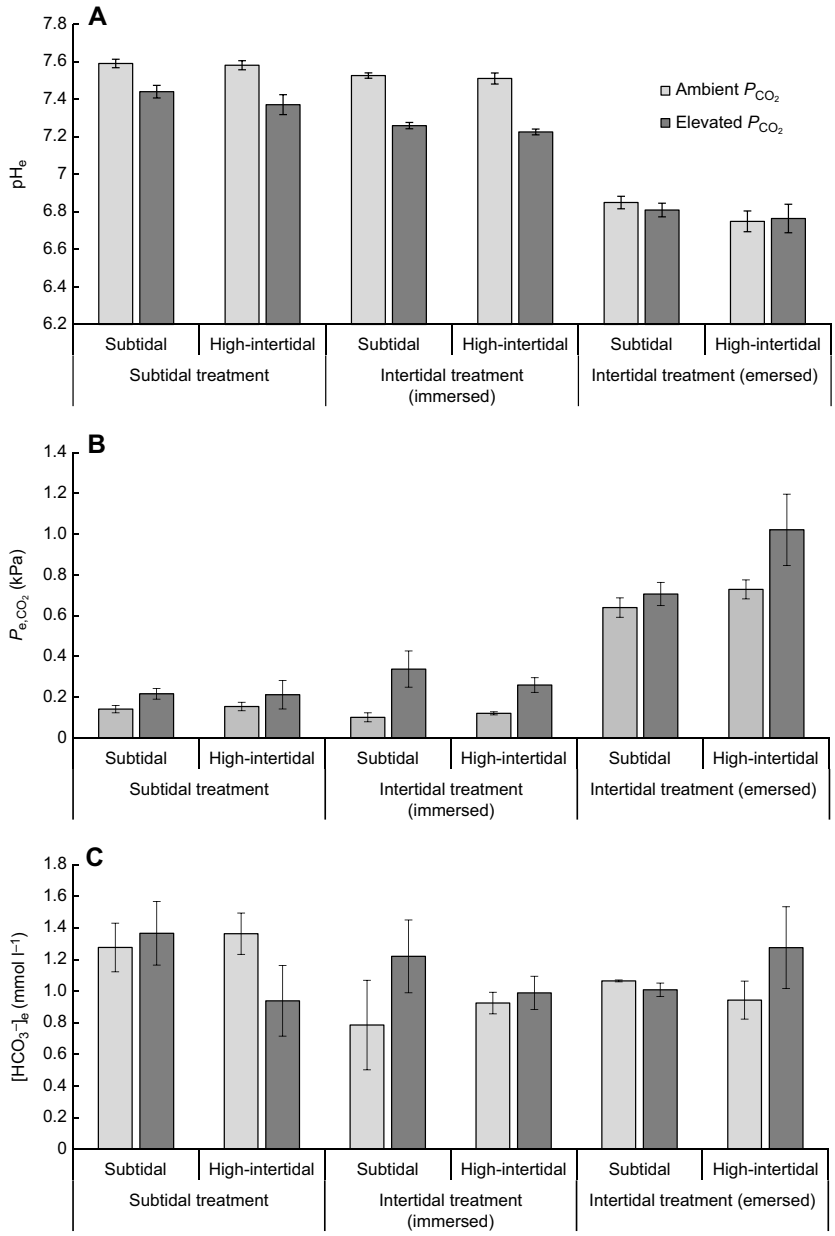

Fig. 1. Haemolymph variables of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. (A) Haemolymph pH (pH_e), (B) P_{CO_2} in the haemolymph (P_{e,CO_2}) and (C) the concentration of HCO_3^- in the haemolymph ($[HCO_3^-]_e$) were measured in the subtidal treatment and in the intertidal treatment during immersion and emersion at (400 µatm) and elevated (1000 µatm) P_{CO_2}. 'Subtidal' and 'High-intertidal' on the *x*-axis indicate the shore height at which oysters were collected; the tidal treatment that oysters were placed into is indicated below (subtidal treatment: constant immersion; intertidal treatment: 3 h immersion, 9 h emersion). Bars represent means±s.e.m. (*n*=9).

Journal of Experimental Biology

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

Fig. 2. Standard metabolic rate (SMR) of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. SMR was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2}. Shore collection heights and tidal treatments as per Fig. 1. Measurements of SMR could not be taken during emersion as SMR can only be measured when immersed. Bars represent means±s.e.m. (n=6). Different letters represent significant differences (P<0.05, *post hoc* SNK) between subtidally collected oysters in different tidal treatments. Pairwise comparisons were conducted on significant factors following a 4-way ANOVA.

the intertidal treatment (Fig. 3). Condition index was not dependent on where oysters were collected on the shore or their P_{CO_2} exposure (Fig. 3).

Shell growth

Oysters in the subtidal treatment had significantly greater shell growth compared with oysters in the intertidal treatment (Riebesell et al., 2010; $F_{1,16}$=9.17, P=0.008; Fig. 4). Mean shell growth was lowest in the oysters in the intertidal treatment at ambient P_{CO_2} (Fig. 4). There was no significant effect of any other factor on shell growth.

DISCUSSION

This study found the acid–base balance of *S. glomerata* to be dependent on both tidal treatment (either intertidal or subtidal) and exposure to elevated P_{CO_2}. When oysters were kept in the intertidal treatment, they experienced a significantly greater reduction in pH_e at elevated P_{CO_2} compared with oysters that were kept in the subtidal treatment. These oysters also displayed a significantly greater increase in SMR and P_{e,CO_2}. These results support our first hypothesis: an intertidal environment exacerbates the hypercapnic effects of elevated seawater P_{CO_2}. The height on the shore where oysters were collected had no effect on pH_e. There was some effect of collection height on SMR and P_{e,CO_2}, although this was not sufficiently strong to provide support for our second hypothesis: oysters collected from the high-intertidal shore are more resilient than those from the subtidal shore to the effects of extracellular hypercapnia associated with emersion and elevated P_{CO_2}. We have shown that the impact of ocean acidification on the acid–base balance of oysters is greater when oysters are in an intertidal environment. For sessile organisms inhabiting a gradient of stress, such as oysters on the intertidal shore, future ocean acidification will

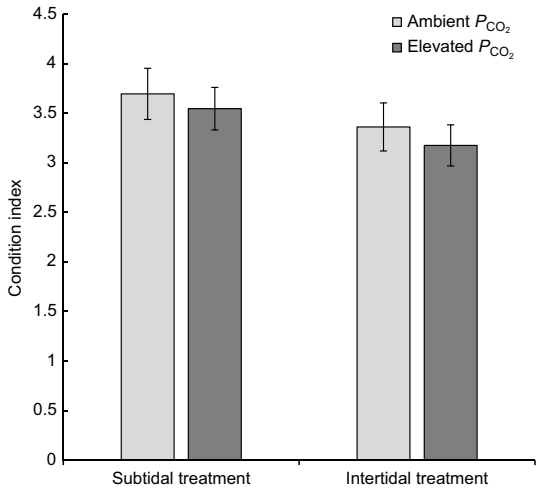

Fig. 3. Condition index of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. Condition index was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2} for subtidal and intertidal treatments (see Fig. 1). Bars represent means±s.e.m. (n=12). The shore height factor was pooled to give a combined mean.

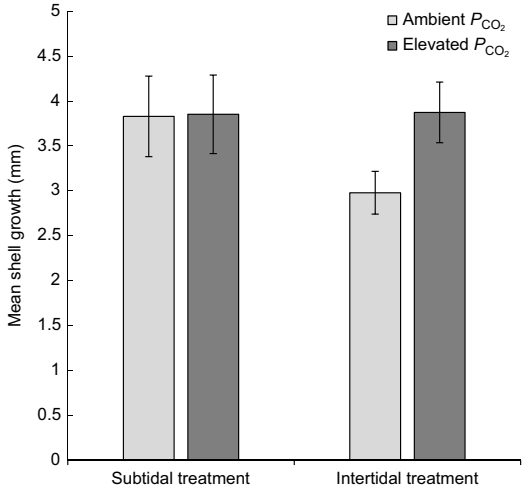

Fig. 4. Mean shell growth of oysters in the subtidal and intertidal treatments after 3 weeks of experimental exposure. Shell growth was measured at ambient (400 µatm) and elevated (1000 µatm) P_{CO_2} for subtidal and intertidal treatments (see Fig. 1). Bars represent means±s.e.m. (n=30). The shore height factor was pooled to give a combined mean.

Journal of Experimental Biology

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

have a differential effect across the distribution. In a future acidified ocean, during emersion at low tide, crabs and other organisms capable of locomotion will be able to escape and seek refugia (Rastrick et al., 2014). Sessile organisms such as oysters, however, fixed to the rocky shore and unable to relocate may experience a contraction in their vertical range.

The impact of elevated CO_2 and intertidal air exposure on *S. glomerata*

Under ambient P_{CO_2}, adult *S. glomerata* experienced significant hypercapnia following emersion. When emersed for 9 h, P_{e,CO_2} rose to a level three times higher than that of oysters that were immersed. Further, oysters exposed to elevated P_{CO_2} experienced an even greater rise of P_{e,CO_2} while emersed compared with those in the ambient treatment. Bivalves such as oysters will close their valves during emersion, limiting respiration. As the organism excretes metabolic CO_2 via aerobic and then anaerobic mechanisms, hypercapnic conditions develop and extracellular acidosis occurs (Burnett, 1988; Truchot, 1990). While P_{e,CO_2} did not differ between the subtidal treatment and intertidal treatment during immersion, it is likely that the continual cost of 'defending' the increase in P_{e,CO_2} experienced in the intertidal treatment during emersion will have negative consequences for other fitness-sustaining processes (i.e. immune response, reproduction, shell and somatic growth; Michaelidis et al., 2005).

The hypercapnia experienced during emersion caused pH_e to fall by 0.6 units under both ambient and elevated P_{CO_2}. This fall was not completely compensated for once the oysters were immersed, with the oysters in the intertidal cycle maintaining a significantly lower pH_e than those in the subtidal cycle at ambient P_{CO_2}. Furthermore, these effects were exacerbated by elevated P_{CO_2}, as the lowest pH_e recorded during immersion was in the combined intertidal and elevated P_{CO_2} treatments. The multiple stressor effect of intertidal treatment and elevated P_{CO_2} was greater than what was predicted by a multiplicative model of the product of the individual effects of either intertidal or elevated P_{CO_2} treatment. Therefore, this interactive effect on pH_e of the two stressors could be considered a synergistic effect or as, Folt et al. (1999) describes, a 'multiplicative synergism'.

Decreasing pH_e in response to periods of emersion-induced hypercapnia is well established (e.g. Truchot and Duhamel-Jouve, 1980; Burnett, 1988). Further, a decrease of pH_e in response to ocean acidification in marine invertebrates is also a well-reported phenomenon (e.g. Lannig et al., 2010; Parker et al., 2013; Schalkhausser et al., 2013). In this study, the pH_e of oysters dropped approximately 0.2 units when they were kept subtidally and under elevated P_{CO_2}. This is comparable to other studies investigating the effects of ocean acidification on bivalve molluscs (Michaelidis et al., 2005; Lannig et al., 2010; Schalkhausser et al., 2013) including *S. glomerata* (Parker et al., 2013, 2015). However, we found that the addition of an intertidal treatment to the elevated P_{CO_2} treatment caused the pH_e of oysters to fall another 0.15 units during immersion. Rastrick et al. (2014) found that the physiological recovery from hypercapnia in the crab *Necora puba* associated with emersion was delayed by elevated P_{CO_2}. Extended periods of decreased pH_e are known to cause a significant reduction in protein synthesis (Kwast and Hand, 1996; Reid et al., 1997), which ultimately leads to decreased somatic growth (Michaelidis et al., 2005). The consistently lower pH_e in oysters in the intertidal and elevated P_{CO_2} treatments means those oysters are likely to have lower somatic growth because of the greater cost of homeostasis (Lannig et al., 2010; Parker et al., 2013).

Bivalves are known to have a limited capacity to compensate for extracellular acid–base disruptions (Schalkhausser et al., 2013). The buffering of extracellular fluids is mostly achieved in bivalves by dissolution of the shell to release HCO_3^- ions (Lindinger et al., 1984), although such shell dissolution is potentially unsustainable in the long term, especially when environmental conditions are unfavourable (Melzner et al., 2011). There were no significant effects of any treatments on the $[HCO_3^-]_e$ of oysters. The $[HCO_3^-]_e$ data were at times quite variable; however, there was a trend for greater $[HCO_3^-]_e$ during emersion and under elevated P_{CO_2}. Lannig et al. (2010) observed only a small increase in $[HCO_3^-]_e$ in the oyster *C. gigas* at a pH of 7.7 (comparable to this study), whereas Michaelidis et al. (2005) observed an increase in $[HCO_3^-]_e$ in the mussel *Mytilus galloprovincialis* when exposed to a pH of 7.3. It has been suggested that a high degree of acidosis is required for bivalves to undergo shell dissolution (Lannig et al., 2010).

An increase in SMR was observed under elevated P_{CO_2} and also when oysters were in an intertidal treatment. The oysters that were in the combined elevated P_{CO_2} and intertidal treatment experienced the greatest increase in P_{e,CO_2} and the lowest pH_e. Increased SMR under elevated P_{CO_2} is believed to occur in response to the increased cost of homeostasis (Fabry et al., 2008; Pörtner, 2008; Portner and Farrell, 2008). Consistently lower pH_e as experienced by oysters in the intertidal and elevated P_{CO_2} treatment may cause greater energy expenditure in homeostatic processes (i.e. defending intracellular pH; Fabry et al., 2008; Pörtner, 2008). Not only do water-breathing intertidal organisms need time immersed in water to excrete waste gases (Truchot, 1990) but also they use this time to feed. The lower condition and decreased growth of oysters in the intertidal treatment potentially reflects reduced opportunities for feeding and the additional metabolic cost which ultimately results in an imbalance of energy supply and demand (Pörtner et al., 2004).

Effect of shore collection height on the response of *S. glomerata* to elevated P_{CO_2}

Oysters were collected from two tidal heights (subtidal and intertidal) to determine whether oysters that had experienced a lifetime of acclimation to frequent emersion and internal acidosis were more resilient to elevated P_{CO_2}. The shore height where oysters were collected did not interact with tidal or elevated P_{CO_2} treatment on the pH_e and P_{e,CO_2} of oysters when measured during immersion. There were, however, some interactions of shore height with tidal treatment on the SMR of immersed oysters and P_{e,CO_2} of emersed oysters. When oysters collected from the subtidal shore were placed into an intertidal treatment, they increased their SMR to a level greater than that of oysters collected from the high-intertidal shore. Oysters collected from the high-intertidal shore did not adjust their SMR when under ambient P_{CO_2} and in either the subtidal or intertidal treatment. During emersion, oysters collected from the high-intertidal shore were shown to have a greater level of P_{e,CO_2} compared with subtidally collected oysters.

Other studies have found alterations in bivalve metabolism in response to tidal heights. Mussels (*M. edulis*) were shown to have differential metabolic indices such as glycogen stores and metabolic enzymes at different tidal heights on the shore (Lesser, 2016). These metabolic indices converged when mussels were transplanted to a common tidal cycle (Lesser, 2016). In another study on *M. edulis*, mussels transplanted from a subtidal to an intertidal treatment were shown to change their SMR within 14 days to suit their new environment (Widdows and Shick, 1985). When mussels acclimated to a subtidal environment were subjected to emersion, they were slower at repaying their accumulated 'oxygen debt'

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

compared with mussels acclimated to an intertidal environment (Widdows and Shick, 1985). Widdows and Shick (1985) also concluded that *M. edulis* that were intertidally acclimated were more efficient at metabolising food while emersed.

The greater SMR found in this study in subtidally collected oysters placed in the intertidal cycle suggests that these oysters are possibly trying to repay their oxygen debt accumulated during emersion, and metabolise food during their time immersed. The lower SMR while immersed and greater P_{e,CO_2} while emersed of oysters of high-intertidal origin may be the result of their lifetime acclimation to this environment. They are potentially more efficient at repaying an oxygen debt, and continue to metabolise ingested food while emersed.

The findings of this and previous studies (Widdows and Shick, 1985; Lesser, 2016) in relation to changing metabolisms of intertidal bivalves when transplanted, suggest that oysters are highly plastic (Collicutt and Hochachka, 1977; Greenway and Storey, 1999; Hamdoun et al., 2003; Ernande et al., 2004; David et al., 2005; Zhang et al., 2012). Plastic responses allow for an immediate response to cope with, and potentially overcome, a stressor (West-Eberhard, 1989). Although plastic responses are essential to coping with stress, they do come at an energetic cost (Koehn and Bayne, 1989; Van Buskirk and Steiner, 2009) and are not always sufficient to prevent death (Visser and Both, 2005). Despite the high plasticity of oysters (Zhang et al., 2012), they still have physiological limits that can be breached (Potter and Hill, 1982) and are especially vulnerable in early life stages (Dove and O'Connor, 2007; Parker et al., 2010). Here, it was found that any acclimation to a high-intertidal environment is likely to be due to a plastic acclimatory response, rather than adaption. In broadcast spawning organisms such as oysters, larval dispersal decreases the capacity to adapt to local conditions and increases the prevalence of phenotypic plasticity (Parsons, 1997; Kinlan and Gaines, 2003).

The common practice for ocean acidification experiments is to maintain organisms under a subtidal regime whilst exposing them to elevated P_{CO_2} (Riebesell and Gattuso, 2015). There is a paucity of studies that have measured the response of marine molluscs to simulated tidal scenarios (Gazeau et al., 2013; Rastrick et al., 2014). One major criticism of ocean acidification research is that the experimental environment is too static and not analogous to the 'real world' (Riebesell and Gattuso, 2015). Previous investigations into intertidal organisms neglecting tidal patterns may have underestimated the effects of ocean acidification on 'real world' populations.

Fate of high-shore oysters

Although oysters can be highly plastic, such plasticity in responses that allow them to withstand stress are energetically costly, reducing their condition and growth, and impacting their future fitness and capacity for resilience. The cost of homeostasis is likely to be greatest for those oysters on the high shore, where feeding time is also limited and emersion is most severe. This may result in energy trade-offs affecting gamete production (Rijnsdorp, 1990; Lester et al., 2004), somatic growth (Michaelidis et al., 2005) and immune responses (Bibby et al., 2008), which has implications for larval settlement and recruitment (Connell, 1985), and adult growth (Pörtner et al., 2004). When tidal emersion is coupled with other stressors such as increased temperature and disease, the resilience of oysters may be further reduced (Potter and Hill, 1982; Dwyer and Burnett, 1996; Willson and Burnett, 2000).

The combination of tidal emersion and elevated P_{CO_2} was found to be synergistic and sublethal rather than lethal. It is likely that under future levels of elevated P_{CO_2}, oysters may reach their physiological limit in the intertidal zone, and may not be able to exist as high on the shore as they currently do. Oysters are essential habitat-forming organisms in temperate intertidal systems, and provide a range of ecosystem services (Gutiérrez et al., 2003; Cole et al., 2007). Across the world, oyster reefs are already in decline. This experiment has shown that those oysters found in the high intertidal zone will be most vulnerable to ocean acidification. We conclude that in a high-CO_2 world, the upper vertical limit of oyster distribution on the shore may be reduced.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.S. was involved in the development of the concept, experimental design and setup, and data analysis. L.M.P. was also involved in the development of the concept, experimental design, running of experiments, measurement of organisms and collection of data. L.S.S. was involved in the collection of data. W.A.O. provided facilities and support, as well as contributing to experimental design. P.M.R. was responsible for supervision of the experiment, development of the concept and experimental design. All authors contributed to the writing of this manuscript.

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Supplementary information

Supplementary information available online at
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References

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. and Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecol. Lett.* **15**, 365-377.

Bibby, R., Widdicombe, S., Parry, H., Spicer, J. and Pipe, R. (2008). Effects of ocean acidification on the immune response of the blue mussel, *Mytilus edulis*. *Aquatic Biol.* **2**, 67-74.

Burnett, L. E. (1988). Physiological responses to air exposure: acid-base balance and the role of branchial water stores. *Am. Zool.* **28**, 125-135.

Caldeira, K. and Wickett, M. E. (2003). Oceanography: anthropogenic carbon and ocean pH. *Nature* **425**, 365-365.

Caldeira, K. and Wickett, M. E. (2005). Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res.* **110**, C09S04.

Cole, V. J., Chapman, M. G. and Underwood, A. J. (2007). Landscapes and life-histories influence colonisation of polychaetes to intertidal biogenic habitats. *J. Exp. Mar. Biol. Ecol.* **348**, 191-199.

Cole, V. J., Parker, L. M., O'Connor, S. J., O'Connor, W. A., Scanes, E., Byrne, M. and Ross, P. M. (2016). Effects of multiple climate change stressors: ocean acidification interacts with warming, hyposalinity, and low food supply on the larvae of the brooding flat oyster *Ostrea angasi*. *Mar. Biol.* **163**, 125.

Collicutt, J. M. and Hochachka, P. W. (1977). The anaerobic oyster heart: coupling of glucose and aspartate fermentation. *J. Comp. Physiol.* **115**, 147-157.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J.-L., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W., Johns, T. and Krinner, G. (2013). Long-term climate change: projections, commitments and irreversibility. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (ed. T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P. M. Midgley), pp. 1029-1136. Cambridge, NY, USA: Cambridge University Press.

Connell, J. H. (1985). The consequences of variation in initial settlement vs. post-settlement mortality in rocky intertidal communities. *J. Exp. Mar. Biol. Ecol.* **93**, 11-45.

David, E., Tanguy, A., Pichavant, K. and Moraga, D. (2005). Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *FEBS J.* **272**, 5635-5652.

Dawson, T. P., Jackson, S. T., House, J. I., Prentice, I. C. and Mace, G. M. (2011). Beyond predictions: biodiversity conservation in a changing climate. *Science* **332**, 53-58.

RESEARCH ARTICLE Journal of Experimental Biology (2017) 220, 765-774 doi:10.1242/jeb.151365

Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A. (2009). Ocean acidification: the other CO_2 problem. *Mar. Sci.* **1**, 169-192.
Dove, M. C. and O'Connor, W. A. (2007). Salinity and temperature tolerance of Sydney rock oysters *Saccostrea glomerata* during early ontogeny. *J. Shellfish Res.* **26**, 939-947.
Dugal, L.-P. (1939). The use of calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. *J. Cell. Comp. Physiol.* **13**, 235-251.
Dwyer, J. I., III and Burnett, L. E. (1996). Acid-base status of the oyster *Crassostrea virginica* in response to air exposure and to infections by *Perkinsus marinus*. *Biol. Bull.* **190**, 139-147.
Ernande, B., Boudry, P., Clobert, J. and Haure, J. (2004). Plasticity in resource allocation based life history traits in the Pacific oyster, *Crassostrea gigas*. I. Spatial variation in food abundance. *J. Evol. Biol.* **17**, 342-356.
Fabry, V. J. (2008). Marine calcifiers in a high-CO_2 ocean. *Science* **320**, 1020-1022.
Fabry, V., Langdon, C., Balch, W., Dickson, A., Feely, R., Hales, B., Hutchins, D., Kleypas, J., Sabine, C. and Atkinson, M. J. (2008). Present and future impacts of ocean acidification on marine ecosystems and biogeochemical cycles. Report of the Ocean Carbon and Biogeochemistry Scoping Workshop on Ocean Acidification Research held 9-11 October 2007, La Jolla, CA, 64 pp.
Folt, C. L., Chen, C. Y., Moore, M. V. and Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnol. Oceanogr.* **44**, 864-877.
Gazeau, F., Parker, L. M., Comeau, S., Gattuso, J.-P., O'Connor, W. A., Martin, S., Pörtner, H.-O. and Ross, P. M. (2013). Impacts of ocean acidification on marine shelled molluscs. *Mar. Biol.* **160**, 2207-2245.
Gran, G. (1952). Determination of the equivalence point in potentiometric titrations. Part II. *Analyst* **77**, 661-671.
Greenway, S. C. and Storey, K. B. (1999). The effect of prolonged anoxia on enzyme activities in oysters (*Crassostrea virginica*) at different seasons. *J. Exp. Mar. Biol. Ecol.* **242**, 259-272.
Gutiérrez, J. L., Jones, C. G., Strayer, D. L. and Iribarne, O. O. (2003). Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos* **101**, 79-90.
Hamdoun, A. M., Cheney, D. P. and Cherr, G. N. (2003). Phenotypic plasticity of HSP70 and HSP70 gene expression in the Pacific oyster (*Crassostrea gigas*): implications for thermal limits and induction of thermal tolerance. *Biol. Bull.* **205**, 160-169.
Hammer, K. M., Kristiansen, E. and Zachariassen, K. E. (2011). Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae). *Mar. Environ. Res.* **72**, 135-142.
Heisler, N. (1984). Acid-base regulation in fishes. *Fish Physiol.* **10**, 315-401.
Heisler, N. (1986). Comparative aspects of acid-base regulation. In *Acid-Base Regulation in Animals*, pp. 397-450. Amsterdam: Elsevier.
Houghton, J. T. (2001). *Climate Change 2001: The Scientific Basis*. Cabridge, UK: Cambridge University Press.
Kinlan, B. P. and Gaines, S. D. (2003). Propagule dispersal in marine and terrestrial environments: a community perspective. *Ecology* **84**, 2007-2020.
Ko, G. W. K., Dineshram, R., Campanati, C., Chan, V. B. S., Havenhand, J. and Thiyagarajan, V. (2014). Interactive effects of ocean acidification, elevated temperature, and reduced salinity on early-life stages of the pacific oyster. *Envrion. Sci. Technol.* **48**, 10079-10088.
Koehn, R. K. and Bayne, B. L. (1989). Towards a physiological and genetical understanding of the energetics of the stress response. *Biol. J. Linn. Soc.* **37**, 157-171.
Kwast, K. E. and Hand, S. C. (1996). Oxygen and pH regulation of protein synthesis in mitochondria from *Artemia franciscana* embryos. *Biochem. J.* **313**, 207-213.
Lannig, G., Eilers, S., Pörtner, H. O., Sokolova, I. M. and Bock, C. (2010). Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas*—changes in metabolic pathways and thermal response. *Mar. Drugs* **8**, 2318-2339.
Lesser, M. P. (2016). Climate change stressors cause metabolic depression in the blue mussel, Mytilus edulis, from the Gulf of Maine. *Limnol. Oceanogr.* **61**, 1705-1717.
Lester, N. P., Shuter, B. J. and Abrams, P. A. (2004). Interpreting the von Bertalanffy model of somatic growth in fishes: the cost of reproduction. *Proc. R. Soc. B Biol. Sci.* **271**, 1625-1631.
Lewis, E., Wallace, D. and Allison, L. J. (1998). *Program Developed for CO_2 System Calculations*. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee.
Lindinger, M., Lauren, D. and McDonald, D. (1984). Acid-base balance in the sea mussel, *Mytilus edulis*. III: Effects of environmental hypercapnia on intra-and extracellular acid-base balance. *Mar. Biol. Lett.* **5**, 371-381.
Mehrbach, C., Culberson, C. H., Hawley, J. E. and Pytkowicz, R. M. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* **18**, 897-907.
Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H.-O. (2009). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.
Melzner, F., Stange, P., Trübenbach, K., Thomsen, J., Casties, I., Panknin, U., Gorb, S. N. and Gutowska, M. A. (2011). Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the Blue mussel *Mytilus edulis*. *PLoS ONE* **6**, e24223.
Michaelidis, B., Ouzounis, C., Paleras, A. and Pörtner, H. O. (2005). Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* **293**, 109-118.
Nell, J. A. (2001). The history of oyster farming in Australia. *Mar. Fish. Rev.* **63**, 14-25.
Nell, J. A. and O'Connor, W. A. (1991). The evaluation of fresh algae and stored algal concentrates as a food source for Sydney rock oyster, *Saccostrea commercialis* (Iredale & Roughley), larvae. *Aquaculture* **99**, 277-284.
Newell, R. I., Fisher, T., Holyoke, R. and Cornwell, J. (2005). Influence of eastern oysters on nitrogen and phosphorus regeneration in Chesapeake Bay, USA. In *The Comparative Roles of Suspension-Feeders in Ecosystems* (ed. R. F. Dame and S. Olenin), pp. 93-120. Dordrecht: Springer inc.
Parker, L. M., Ross, P. M. and O'Connor, W. A. (2010). Comparing the effect of elevated pCO_2 and temperature on the fertilization and early development of two species of oysters. *Mar. Biol.* **157**, 2435-2452.
Parker, L. M., Ross, P. M., O'Connor, W. A., Borysko, L., Raftos, D. A. and Pörtner, H.-O. (2012). Adult exposure influences offspring response to ocean acidification in oysters. *Global Change Biol.* **18**, 82-92.
Parker, L. M., Ross, P. M., O'Connor, W. A., Pörtner, H. O., Scanes, E. and Wright, J. M. (2013). Predicting the response of molluscs to ocean acidification. *Biology* **2**, 629-651.
Parker, L. M., O'Connor, W. A., Raftos, D. A., Pörtner, H.-O. and Ross, P. M. (2015). Persistence of positive carryover effects in the oyster, *Saccostrea glomerata*, following transgenerational exposure to ocean acidification. *PLoS ONE* **10**, e0132276.
Parsons, K. E. (1997). Role of dispersal ability in the phenotypic differentiation and plasticity of two marine gastropods. *Oecologia* **110**, 461-471.
Peters, R. and Raftos, D. A. (2003). The role of phenoloxidase suppression in QX disease outbreaks among Sydney rock oysters (*Saccostrea glomerata*). *Aquaculture* **223**, 29-39.
Pörtner, H. O. (2008). Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar. Ecol. Prog. Ser.* **373**, 203-217.
Pörtner, H. O. and Farrell, A. P. (2008). Physiology and climate change. *Science* **322**, 690-692.
Pörtner, H. O., Langenbuch, M. and Reipschläger, A. (2004). Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *J. Oceanogr.* **60**, 705-718.
Potter, M. A. and Hill, B. J. (1982). Heat mortality in the Sydney rock oyster, *Saccostrea (Crassostrea) commercialis* and the effectiveness of some control methods. *Aquaculture* **29**, 101-108.
Rastrick, S. P. S., Calosi, P., Calder-Potts, R., Foggo, A., Nightingale, G., Widdicombe, S. and Spicer, J. I. (2014). Living in warmer, more acidic oceans retards physiological recovery from tidal emersion in the velvet swimming crab, *Necora puber*. *J. Exp. Biol.* **217**, 2499-2508.
Raven, J., Caldeira, K., Elderfield, H., Hoegh-Guldberg, O., Liss, P., Riebesell, U., Shepherd, J., Turley, C. and Watson, A. (2005). *Ocean Acidification Due to Increasing Atmospheric Carbon Dioxide*. London, UK: The Royal Society.
Reid, S. D., Dockray, J. J., Linton, T. K., McDonald, D. G. and Wood, C. M. (1997). Effects of chronic environmental acidification and a summer global warming scenario: protein synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **54**, 2014-2024.
Riebesell, U. and Gattuso, J.-P. (2015). Lessons learned from ocean acidification research. *Nat. Clim. Change* **5**, 12-14.
Riebesell, U., Fabry, V. J., Hansson, L. and Gattuso, J.-P. (2010). *Guide to Best Practices for Ocean Acidification Research and Data Reporting*. Luxembourg: Publications Office of the European Union.
Rijnsdorp, A. D. (1990). The mechanism of energy allocation over reproduction and somatic growth in female North Sea plaice, *Pleuronectes platessa* L. *Neth. J. Sea. Res.* **25**, 279-289.
Ross, P. M., Parker, L., O'Connor, W. A. and Bailey, E. A. (2011). The impact of ocean acidification on reproduction, early development and settlement of marine organisms. *Water* **3**, 1005-1030.
Ross, P. M., Parker, L. and Byrne, M. (2016). Transgenerational responses of molluscs and echinoderms to changing ocean conditions. *ICES J. Mar. Sci.* **73**, 537-549.
Scanes, E., Parker, L. M., O'Connor, W. A. and Ross, P. M. (2014). Mixed effects of elevated pCO_2 on fertilisation, larval and juvenile development and adult responses in the mobile subtidal scallop *Mimachlamys asperrima* (Lamarck, 1819). *PLoS ONE* **9**, e93649.
Schalkhausser, B., Bock, C., Stemmer, K., Brey, T., Pörtner, H.-O. and Lannig, G. (2013). Impact of ocean acidification on escape performance of the king scallop, *Pecten maximus*, from Norway. *Mar. Biol.* **160**, 1995-2006.
Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integr. Comp. Biol.* **53**, 597-608.
Solomon, S. (2007). *Climate Change 2007: The Physical Science Basis: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press.

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Thomsen, J., Gutowska, M. A., Saphörster, J., Heinemann, A., Trübenbach, K., Fietzke, J., Hiebenthal, C., Eisenhauer, A., Körtzinger, A., Wahl, M. et al. (2010). Calcifying invertebrates succeed in a naturally CO_2 enriched coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* **7**, 3879-3891.

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. *Annu. Rev. Physiol.* **52**, 61-74.

Truchot, J.-P. and Duhamel-Jouve, A. (1980). Oxygen and carbon dioxide in the marine intertidal environment: diurnal and tidal changes in rockpools. *Respir. Physiol.* **39**, 241-254.

Underwood, A. J. (1996). *Experiments in Ecology: their Logical Design and Interpretation using Analysis of Variance*. Cambridge, UK: Cambridge University Press.

Underwood, A. J. and Barrett, G. (1990). Experiments on the influence of oysters on the distribution, abundance and sizes of the gastropod *Bembicium auratum* in a mangrove swamp in New South Wales, Australia. *J. Exp.Mar. Biol. Ecol.* **137**, 25-45.

Underwood, A., Chapman, M. and Richards, S. (2002). *GMAV-5 for Windows. An Analysis of Variance Programme*. Sydney: Centre for Research on Ecological Impacts of Coastal Cities, Marine Ecology Laboratories, University of Sydney.

Van Buskirk, J. and Steiner, U. K. (2009). The fitness costs of developmental canalization and plasticity. *J. Evol. Biol.* **22**, 852-860.

Visser, M. E. and Both, C. (2005). Shifts in phenology due to global climate change: the need for a yardstick. *Proc. R. Soc. B Biol. Sci.* **272**, 2561-2569.

West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* **20**, 249-278.

Widdows, J. and Shick, J. M. (1985). Physiological responses of *Mytilus edulis* and *Cardium edule* to aerial exposure. *Mar. Biol.* **85**, 217-232.

Williams, S. E., Shoo, L. P., Isaac, J. L., Hoffmann, A. A. and Langham, G. (2008). Towards an integrated framework for assessing the vulnerability of species to climate change. *PLoS Biol.* **6**, e325.

Willson, L. L. and Burnett, L. E. (2000). Whole animal and gill tissue oxygen uptake in the Eastern oyster, *Crassostrea virginica*: Effects of hypoxia, hypercapnia, air exposure, and infection with the protozoan parasite Perkinsus marinus. *J. Exp. Mar. Biol. Ecol.* **246**, 223-240.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H. et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490**, 49-54.

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Naturally acidified habitat selects for ocean acidification–tolerant mussels

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ECOLOGY

Naturally acidified habitat selects for ocean acidification–tolerant mussels

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Ocean acidification severely affects bivalves, especially their larval stages. Consequently, the fate of this ecologically and economically important group depends on the capacity and rate of evolutionary adaptation to altered ocean carbonate chemistry. We document successful settlement of wild mussel larvae (*Mytilus edulis*) in a periodically CO_2-enriched habitat. The larval fitness of the population originating from the CO_2-enriched habitat was compared to the response of a population from a nonenriched habitat in a common garden experiment. The high CO_2–adapted population showed higher fitness under elevated P_{CO_2} (partial pressure of CO_2) than the non-adapted cohort, demonstrating, for the first time, an evolutionary response of a natural mussel population to ocean acidification. To assess the rate of adaptation, we performed a selection experiment over three generations. CO_2 tolerance differed substantially between the families within the F_1 generation, and survival was drastically decreased in the highest, yet realistic, P_{CO_2} treatment. Selection of CO_2-tolerant F_1 animals resulted in higher calcification performance of F_2 larvae during early shell formation but did not improve overall survival. Our results thus reveal significant short-term selective responses of traits directly affected by ocean acidification and long-term adaptation potential in a key bivalve species. Because immediate response to selection did not directly translate into increased fitness, multigenerational studies need to take into consideration the multivariate nature of selection acting in natural habitats. Combinations of short-term selection with long-term adaptation in populations from CO_2-enriched versus nonenriched natural habitats represent promising approaches for estimating adaptive potential of organisms facing global change.

INTRODUCTION

Ocean acidification, caused by rising atmospheric CO_2 concentrations due to excess fossil fuel burning, severely affects many marine organisms (*1*). Calcifying organisms are especially affected by the change of ocean chemistry because their ability to form calcified structures is reduced. Bivalves are among the most vulnerable taxonomic groups because their $CaCO_3$-containing shells protect the animal from predation. In particular, their larval stages suffer from substantial reductions in growth and survival under elevated P_{CO_2} (partial pressure of CO_2) (*2*–*5*). This is likely a consequence of the high calcification rates during the formation of the first larval shell (*6*, *7*). Although the benthic life stage is able to compensate for the negative impact even of highly elevated P_{CO_2} (~3000 µatm) when food supply is abundant, early larval development is completely fueled by the limited energy provided by the egg and thus represents an important ecological bottleneck (*6*, *7*). Bivalves of the genus *Mytilus* have important ecological roles in boreal, benthic ecosystems and can contribute by up to 90% to epibenthic biomass in coastal habitats (*8*). In addition, their high economic value for aquaculture has stimulated a number of recent studies to estimate their adaptation potential to future ocean conditions (*4*, *9*–*11*). However, the relatively long generation time of bivalve species complicates multigenerational (MG) studies. Consequently, most studies until now have estimated evolutionary potential by quantifying variation of fitness-relevant traits such as growth during early development within and between locally adapted populations (*10*, *12*, *13*) or assessed transgenerational phenotypic plasticity (*10*). These studies did not provide a uniform picture on the potential of bivalves to adapt to ocean acidification. Modeling the rate of adaptation in a single population based on larval shell size variations within the first 60 hours of development under elevated P_{CO_2} (1000 µatm) suggested a low potential for adaptation when extrapolated over 50 generations (*8*). To capture longer time periods, comparison of populations naturally experiencing differing carbonate system conditions offers useful proxies for estimating adaptation [space-for-time substitution (*14*)]. In one study, the growth response of field-collected juvenile mytilid mussels originating from two populations differed under elevated P_{CO_2}, indicating local adaptation (*12*). In another, larval shell development of two *Mytilus* species was similar during exposure to varying carbonate system treatments, although one species originated from a habitat that encounters upwelling events associated with elevated P_{CO_2} (*13*). Although these studies suggested that bivalves can potentially adapt to rising P_{CO_2}, they lack a formal estimation of genetic versus nongenetic sources of variation. For example, transgenerational acclimation to elevated P_{CO_2} can substantially modulate fitness of offspring as observed in fish (*15*). In the oyster *Saccostrea glomerata*, a 5-week exposure of parental animals to elevated P_{CO_2} during gametogenesis enhanced the development and growth rates of F_1 and even F_2 offspring under acidified conditions (*9*, *16*). Selective breeding for aquaculture purposes substantially increased the productivity of *S. glomerata* and resulted in 25% improved growth within two to four generations (*17*). The higher developmental rates of this breeding line were also maintained under elevated P_{CO_2} compared to wild-type oysters (*10*). Therefore, rapid evolutionary responses in bivalves may enable adaptation to ocean acidification, but these MG selection studies using continuously elevated P_{CO_2} as selective agent are lacking so far.

To fill this gap, we performed a 3-year MG experiment to test whether the blue mussel *Mytilus edulis* can successfully adapt to ocean acidification and to estimate which mechanisms contribute to rapid evolutionary responses. The tested population inhabits the seasonally acidified Kiel Fjord, western Baltic Sea, which is characterized by low pH and elevated P_{CO_2} levels during the reproductive period of the species

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(*18*, *19*). This experiment was supported by field monitoring of carbonate chemistry variation in relation to mussel settlement patterns. In addition, we compared the Baltic population in a common garden experiment to the response of mussels from the North Sea, which is characterized by less variable pH conditions and higher seawater alkalinity due to higher salinity (*20*).

To investigate the time scale of adaptation to ocean acidification, we conducted two experiments to compare long-term adaptation between populations and processes of short-term adaptation within a population. We hypothesized that Baltic mussels have already adapted to high-CO_2 seawater and would better tolerate simulated ocean acidification than North Sea mussels. Furthermore, we hypothesized that selection for ocean acidification–tolerant specimens would increase the fitness of their offspring when exposed to acidified conditions.

RESULTS

Field carbonate chemistry monitoring and larval settlement (Baltic Sea)

Monitoring of pH in Sylt and Kiel Fjord revealed higher and more stable pH in the North Sea habitat compared to the habitat of the Baltic population (fig. S1). Monthly mean pH in Sylt remained above 8, with maximum values recorded during spring bloom in April (Fig. 1A). In contrast, mean pH values declined to about 7.7 during the upwelling period in summer and autumn in Kiel Fjord. Our monitoring of mussel settlement on weekly deployed panels and continuously logged seawater P_{CO_2} revealed that bivalve larvae survived and settled in Kiel Fjord, which is characterized by elevated and fluctuating P_{CO_2}. The hourly averaged P_{CO_2} was 1087 ± 537 µatm and ranged between 266 and 2861 µatm over the whole monitoring period from mid-July to mid-September 2012 (Fig. 1B). Despite such high and fluctuating environmental P_{CO_2}, Baltic mussels settled successfully, with a peak of more than 1000 larvae settled per panel in early August (Fig. 1C) at elevated P_{CO_2} levels similar to those predicted for the average surface ocean of 2100. Because P_{CO_2} fluctuated rapidly due to upwelling events in Kiel Fjord, environmental conditions experienced by different larval cohorts differed significantly (fig. S2). Earlier settlers of the July cohort experienced only moderately elevated P_{CO_2} because larvae avoided the first pronounced upwelling peak at the beginning of August (Fig. 1C) (mean P_{CO_2}, 826 µatm; range, 266 to 1502 µatm). In contrast, larvae that settled at the end of August experienced P_{CO_2} levels between 443 and 2861 µatm (mean, 1191 µatm) during a calculated 27-day larval phase (Fig. 1C). Larvae settling in mid-September were again exposed to lower and more stable P_{CO_2} levels (larval phase, 25 days; mean, 859 µatm; range, 427 to 2225 µatm) (Fig. 1C). The number of days August and September cohorts were exposed to daily mean P_{CO_2} values above 1000 µatm differed, with 17 and 5 days corresponding to 63 and 20% of their estimated whole planktonic life phase, respectively.

Population comparison experiment (Baltic Sea versus North Sea)

The formation of the first larval shell [prodissoconch I (PD I)] (fig. S3) (*21*) was strongly delayed in both North Sea and Baltic Sea populations at high P_{CO_2}, which resulted in significantly reduced shell length compared to larvae from the control P_{CO_2} [two-way analysis of variance (ANOVA): population: $F = 1.6$, $P > 0.05$; P_{CO_2}: $F = 112.1$, $P < 0.01$]. However, Baltic mussel larvae were less affected and showed a smaller shell length reduction compared to North Sea larvae at elevated P_{CO_2} [−24% (Baltic Sea) versus −38% (North Sea) shell length compared to respective controls; population × P_{CO_2}: $F = 6.5$, $P < 0.05$] (Fig. 2A).

Growth patterns translated well into observed survival. Here, survival at 390-µatm P_{CO_2} did not differ between the two populations, but Baltic larval survival was higher at elevated P_{CO_2} (two-way ANOVA: population: $F = 0.8$, $P > 0.05$; P_{CO_2}: $F = 10.9$, $P < 0.01$; population × P_{CO_2}: $F = 6.9$, $P < 0.05$) (Fig. 2C and table S1). Subsequent shell growth rates were similar in Baltic larvae exposed to 390- and 2400-µatm P_{CO_2} (Fig. 2B; no data for North Sea larvae at 2400-µatm P_{CO_2} because of high mortality).

Three-year MG experiment (Baltic Sea)

F_0 and F_1 generation (2012).

The MG experiment utilized controlled genetic crosses of mussels collected from the more P_{CO_2}-tolerant Baltic Sea population to select for CO_2-tolerant and CO_2-sensitive families, which were used to elucidate the relative contribution of genetic and nongenetic environmental factors enabling adaptation to ocean acidification (Fig. 3B). PD I size was strongly reduced in F_1 larvae exposed to elevated P_{CO_2} and declined

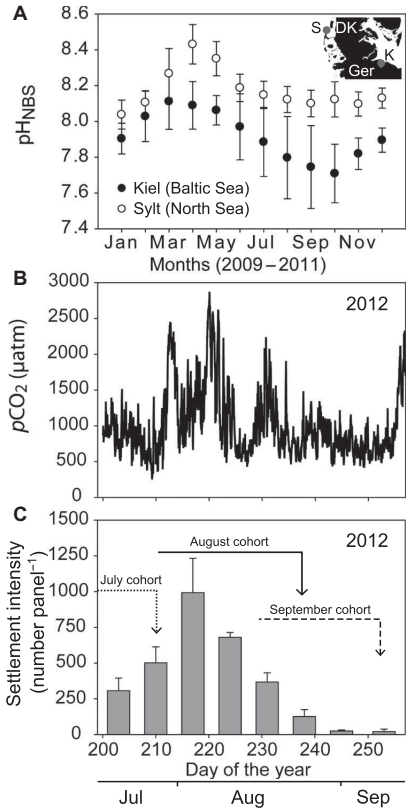

Fig. 1. Habitat carbonate system variability and juvenile settlement. (**A**) Averaged monthly pH values recorded from 2009 to 2011 in the habitats of the two tested populations. The inset depicts the location of the two habitats in North Sea and Baltic Sea (S, Sylt; K, Kiel; DK, Denmark; Ger, Germany). (**B**) Continuously measured seawater P_{CO_2} in summer 2012 in Kiel Fjord. (**C**) Mussel larval settlement intensity on panels between July and September. The arrows correspond to the estimated planktonic phases of larvae settled in July, August, and September. Values are means ± SD.

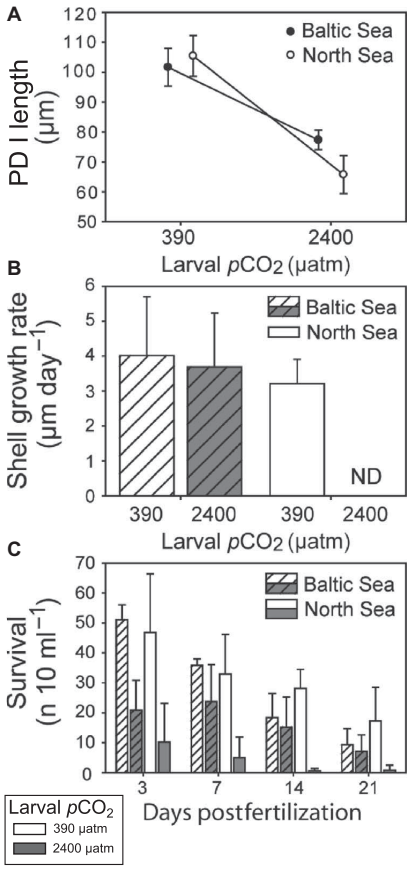

Fig. 2. Larval performance of Baltic Sea and North Sea populations exposed to elevated P_{CO_2}. (**A**) PD I length of both populations declined at high P_{CO_2}, but Baltic larval size was less affected (n = 22 to 73). (**B**) Daily shell growth was similar for both populations and P_{CO_2} treatments [no data (ND) for North Sea larvae at high P_{CO_2} due to low survival on days 14 and 21]. (**C**) Survival rapidly declined in North Sea larvae exposed to elevated P_{CO_2}, whereas Baltic Sea larvae were less affected by elevated P_{CO_2} from day 7 forward. Values are means ± SD; numbers in bracket state the number of measured individuals per P_{CO_2} treatment.

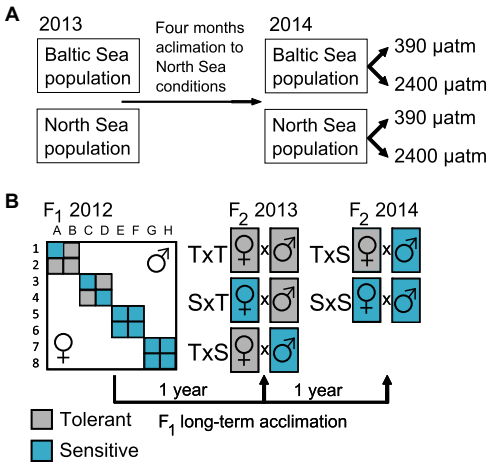

Fig. 3. Flow chart of the experimental approach. (**A**) Collection of mussels from North Sea and Baltic Sea population, acclimation to North Sea conditions from December 2013 to April 2014, and subsequent transfer, spawning, and exposure to two P_{CO_2} treatments. (**B**) Crossing schemes for F_1 and F_2 generation. In 2012, F_0 animals (eight dams and eight sires) were crossed pairwise to generate 16 full-sib F_1 families nested within four half-sib groups. Settled mussels were subsequently acclimated for 1 and 2 years. In 2013, F_2 was generated by performing single-specimen crosses using pure F_1 tolerant lines as well as tolerant and sensitive F_1 families selected in, and long-term acclimated to, 390-, 1120-, and 2400-µatm (tolerant only) P_{CO_2}. In 2014, F_2 was generated with three dams from four families of each line (tolerant from 390- and 2400-µatm P_{CO_2} acclimation and sensitive from 390-µatm P_{CO_2} acclimation only) crossed with one male from the tolerant family E6.

from 112 ± 6 µm at 390 µatm to 94 ± 7 µm and 78 ± 8 µm at 1120 and 2400 µatm, respectively (ANOVA: P_{CO_2}: F = 165.1, P < 0.001) (Fig. 4A). Family-specific PD I shell length varied substantially, and the calculated heritability for this trait was 0.56 [confidence interval (CI), 0.27 to 0.81], 0.47 (CI, 0.24 to 0.81), and 0.53 (CI, 0.23 to 0.83) at 390-, 1120-, and 2400-µatm P_{CO_2}, respectively. Subsequently, larvae from all P_{CO_2} treatments grew at comparable rates and thus reached similar sizes at the end of the planktonic phase (Fig. 4C). Because of the large variance in final larval survival between families, our study showed no significant difference in larval survival between 390- and 1120-µatm P_{CO_2}, but did reveal a drastic reduction at 2400 µatm (Fig. 4E and table S2). Similarly, larvae from all families successfully settled at the two lower P_{CO_2} levels, but only the offspring of five families (classified as "tolerant" families A2, B1, B2, C4, and D3) metamorphosed into juveniles at 2400 µatm (Fig. 3B). Successful metamorphosis and thus tolerance correlated positively with PD I size because shell length was slightly larger in tolerant compared to sensitive families at 2400 µatm (82 ± 5 µm versus 76 ± 8 µm) (two-way ANOVA: sensitive versus tolerant: F = 1.5, P > 0.05; P_{CO_2}: F = 365.7, P < 0.001; sensitive versus tolerant × P_{CO_2}: F = 5.5, P < 0.05) (Fig. 4A).

Following settlement, juveniles of all families were transferred into a flow-through experimental system and raised for 1 year (2012–2013) at the respective P_{CO_2} until the next spawning season (Table 1). During that time, no mortality was observed and F_1 juveniles from all families grew to shell sizes of about 25 mm within 1 year irrespective of P_{CO_2} treatment or family type [390 µatm, 24.7 ± 3.7 mm; 1120 µatm, 26.0 ± 2.7 mm; and 2400 µatm, 24.8 ± 3.0 mm (tolerant families only)] (ANOVA: F = 0.216, P > 0.05).

First F_2 generation (2013).

Crosses of F_1 specimens were carried out to test (i) whether developmental acclimation of F_1 families conferred environment-specific benefits in relation to offspring P_{CO_2} (that is, transgenerational plasticity) and (ii) whether tolerance has a genetic component that could be crossed into the genetic background of sensitive families (Fig. 3B). Maternal investment, measured as egg production of F_1 dams and egg diameter, did not change under elevated P_{CO_2} (fig. S4). Fertilization success was not significantly affected by P_{CO_2} levels, irrespective of whether it was assayed in tolerant or sensitive families. In crosses between tolerant F_1 parents (T×T), PD I size of F_2 larvae was similar at control P_{CO_2}, irrespective of parental rearing history. However, at high P_{CO_2}, PD I sizes were larger for offspring from tolerant F_1 families raised at elevated P_{CO_2} compared to larvae from control P_{CO_2}–treated parents (see parental P_{CO_2} × offspring P_{CO_2} interaction effects in table S2). Shell size of offspring from F_1 dams selected

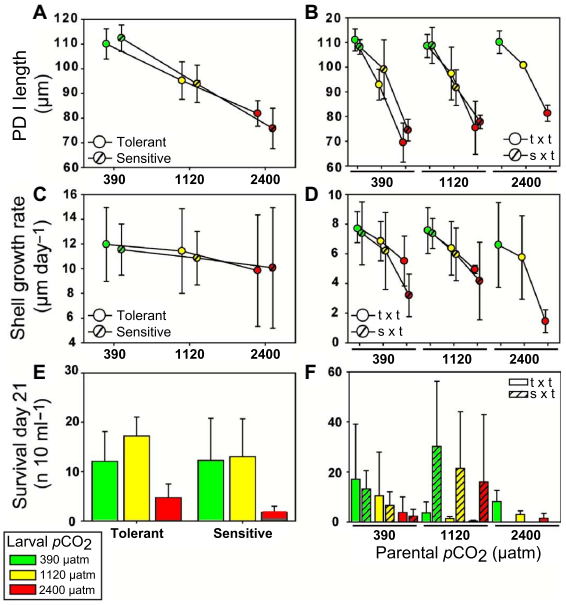

Fig. 4. Larval performance of F_1 and F_2 animals exposed to elevated P_{CO_2}. (**A**) PD I length declined with increasing P_{CO_2} but remained larger in tolerant F_1 families (n = 314 to 620) and (**B**) partly recovered in tolerant (T×T) but not sensitive (S×T) F_2 offspring from high P_{CO_2}–treated parents (n = 902 to 1314). (**C**) Larval daily shell growth at elevated P_{CO_2} was not affected in F_1 offspring (**D**) but decreased in F_2 larvae and further deteriorated with high-CO_2 exposure of parental animals. (**E**) Larval survival decreased at high P_{CO_2} but was higher in tolerant families compared to sensitive F_1 families. (**F**) Parental selection and long-term acclimation at high P_{CO_2} did not improve survival in the F_2 generation. Values are means ± SD; numbers in brackets state the number of measured individuals per P_{CO_2} treatment.

and raised under 2400-µatm P_{CO_2} increased by 11.9 µm or 17% compared to that of offspring of 390-µatm acclimated F_1 dams (Fig. 4B and table S2). In contrast to the F_1 generation, F_2 larval growth rates in general were slower and particularly reduced at high P_{CO_2} and additionally declined when raised from F_1 exposed to elevated P_{CO_2} during long-term acclimation (Fig. 4D and table S2). Furthermore, larger PD I size of F_2 larvae at 2400-µatm P_{CO_2} had no positive effect on larval survival, in contrast to observations in the F_1 generation (Fig. 4F and table S2). Selection of tolerant phenotypes in the F_1 generation thus only had a positive transgenerational effect on PD I size but did not improve the mean population fitness of their F_2 offspring. Rather, offspring from mothers acclimated at control P_{CO_2} conditions (390 µatm) showed higher survival rates, indicating no positive effects of parental acclimation to high P_{CO_2} on offspring survival (table S2). In contrast to the results for crosses between tolerant families (T×T), crosses between tolerant mothers and sensitive fathers (S×T) resulted in similar PD I sizes of F_2 larvae, irrespective of parental P_{CO_2} treatment (Fig. 4B and table S2). Crosses between tolerant mothers and sensitive fathers showed an increased survival when compared to T×T crosses, especially at 1120-µatm P_{CO_2} (Fig. 4F and table S2).

Second F_2 generation (2014).

The observed responses for fecundity, PD I, larval growth rates, and survival were largely confirmed when generating the second F_2 generation from the same F_1 animals in the subsequent year (Fig. 3B and fig. S5). When kept for another year in the experimental system at their respective P_{CO_2} treatment (2013–2014), fecundity and egg sizes were affected neither by parental P_{CO_2} treatment nor by family type (sensitive/tolerant F_1 families; figs. S4, B and D, and S5A). F_2 offspring from high P_{CO_2}–selected parental F_1 animals showed a nonsignificant trend toward larger PD I size (fig. S5B and table S3). Shell growth rates were reduced in all high P_{CO_2}–treated larvae but were again affected neither by parental acclimation P_{CO_2} treatment nor by family type (fig. S5C). Similarly, survival of larvae was negatively affected by elevated P_{CO_2} and not improved by selection of tolerant F_1 parents (T×T) or the prolonged high-P_{CO_2} acclimation of F_1 animals (fig. S5D and table S3).

DISCUSSION

The high sensitivity of bivalve larvae to elevated P_{CO_2} (*3*, *4*) suggests that selective pressures should be strong and populations should rapidly adapt to the prevailing local P_{CO_2} levels. However, evidence for this hypothesis is circumstantial (*9*, *12*, *13*). We used two different approaches to study the adaptation potential of mussels to ocean acidification. First, we performed a population comparison (PC) experiment to test for existing differences in tolerance to ocean acidification. Second, we assessed how rapidly tolerance can be acquired by selection of tolerant phenotypes or transgenerational plasticity in an MG experiment.

Population comparison experiment (Baltic Sea versus North Sea)

In an experimental common garden approach, larval performance of North Sea mussels under low and elevated P_{CO_2} was compared to that of larvae from the Baltic Sea population. On the phenotypic level, adaptation to elevated P_{CO_2} in Baltic mussels was indicated by increased survival under elevated P_{CO_2} and higher capacity to maintain PD I formation rates compared to the more sensitive North Sea mussels. The experiment revealed that naturally and locally deviating ocean carbonate chemistry characteristics influence the responses of blue mussel populations to experimental ocean acidification, most likely reflecting local adaptation to prevailing environmental conditions on longer time scales (*22*–*24*). In general, larval calcification was strongly impaired by elevated P_{CO_2}; this impairment was even more pronounced than reported at comparable P_{CO_2} for fully marine populations (*5*, *25*). This reflects our choice of the highly selective environment in the brackish Baltic Sea, where conditions for calcification are less favorable (*7*). Calcification of bivalve larvae is not directly affected by P_{CO_2}, but is sensitive to lowered pH and availability of inorganic carbon (HCO_3^-; C_T) as a substrate for calcification, which correlates with seawater Ω [calcium carbonate saturation state (*5*, *7*, *26*)]. The low alkalinity and thus low C_T concentrations of the Baltic Sea result in lowered carbon availability and Ω and therefore synergistically enhance the negative effects of elevated P_{CO_2} on larval calcification (*7*). As a result of this intensified selection pressure, Baltic mytilid mussels have successfully adapted to adverse conditions for calcification.

Calcification of PD I coincides with the highest relative calcification rates of all bivalve life stages, which makes this ontogenetic stage most vulnerable to external carbonate system perturbations (*5*–*7*). The correlation of higher calcification rates and survival of tolerant Baltic mussels suggests that PD I calcification is mechanistically linked to survival and therefore directly to fitness. PD I sizes were similar in both populations under control P_{CO_2} when the external carbonate chemistry did not limit calcification; thus, growth and development were potentially limited by

Table 1. Carbonate chemistry during the larval experiments and the long-term acclimation. pH on total scale and C_T were measured (n = 163), and A_T, P_{CO_2}, [CO_3^{2-}], and $\Omega_{Aragonite}$ were calculated using CO2SYS. NBS, National Bureau of Standards.

	Temperature (°C)	Salinity (g kg^{-1})	P_{CO_2} treatment (µatm)	C_T (µmol kg^{-1})	pH (total scale)	Measured pH (NBS scale)	A_T (µmol kg^{-1})	P_{CO_2} (µatm)	[CO_3^{2-}] (µmol kg^{-1})	$\Omega_{Aragonite}$
F_1 larvae 2012	17.7 ± 0.1	15.5 ± 0.3	390	1802 ± 46	7.97 ± 0.02	8.17 ± 0.07	1884 ± 51	508 ± 14	76.6 ± 4.9	1.23 ± 0.08
			1120	1889 ± 78	7.65 ± 0.01	7.75 ± 0.06	1897 ± 76	1128 ± 82	38.6 ± 1.2	0.62 ± 0.02
			2400	1995 ± 50	7.39 ± 0.03	7.46 ± 0.05	1944 ± 51	2114 ± 108	22.6 ± 1.4	0.36 ± 0.02
F_2 larvae 2013	17.1 ± 0.2	16.0 ± 0.4	390	1916 ± 86	8.03 ± 0.20	8.16 ± 0.07	2026 ± 90	476 ± 181	99.8 ± 14.8	1.60 ± 0.24
F_2 larvae 2014			1120	2056 ± 54	7.64 ± 0.15	7.69 ± 0.01	2063 ± 87	1264 ± 167	44.1 ± 33.5	0.71 ± 0.53
			2400	2078 ± 27	7.43 ± 0.15	7.43 ± 0.02	2032 ± 28	2093 ± 379	28.7 ± 15.2	0.46 ± 0.24
	18.8 ± 0.1	16.0 ± 0.2	390	1875 ± 12	8.05 ± 0.03	8.19 ± 0.03	1989 ± 17	440 ± 24	99.6 ± 5.3	1.62 ± 0.09
Long-term acclimation (2012–2014)			2400	2039 ± 25	7.40 ± 0.07	7.55 ± 0.07	1991 ± 13	2160 ± 358	25.0 ± 4.3	0.41 ± 0.07
	11.4 ± 4.3	15.1 ± 2.1	390	2044 ± 125	7.84 ± 0.07	8.02 ± 0.06	2064 ± 122	734 ± 108	46.2 ± 12.1	0.71 ± 0.20
			1120	2108 ± 118	7.57 ± 0.05	7.71 ± 0.07	2068 ± 118	1381 ± 136	25.7 ± 6.7	0.40 ± 0.11
PC			2400	2258 ± 257	7.33 ± 0.06	7.44 ± 0.09	2146 ± 253	2515 ± 382	15.7 ± 4.6	0.24 ± 0.07
	15.5 ± 0.1	28.5 ± 0.2	390	2160 ± 14	8.02 ± 0.02	8.16 ± 0.01	2334 ± 14	462 ± 26	136.0 ± 6.0	2.14 ± 0.09
			2400	2411 ± 30	7.33 ± 0.01	7.46 ± 0.01	2357 ± 29	2588 ± 45	31.4 ± 0.6	0.49 ± 0.01

other physiological processes. Because PD I is formed before development of the larval feeding apparatus, a substantial fraction of the limited energy stored in the bivalve egg is needed for shell formation even under favorable carbonate system conditions (*6*, *27*). Because calcification generates protons, which need to be excreted by means of an active transport process, disproportional up-regulation of shell formation could challenge the larval energy budget. A more efficient energy allocation into PD I formation may thus explain larger and maintained PD I size under elevated P_{CO_2} in the adapted Baltic compared to North Sea mussels.

Three-year MG experiment (Baltic Sea)

Although common garden experiments offer a means to test for the existence of local adaptation, only experiments performed over multiple generations can give insights into the rate and mechanistic basis of the adaptation process. Earlier studies using oysters as model organisms revealed that parental preexposure to elevated P_{CO_2} resulted in faster growth and development of larvae under high P_{CO_2} when compared to larvae generated from parents that were acclimated to control conditions (*9*). Therefore, transgenerational phenotypic plasticity needs to be considered as an important factor that can modulate the response of bivalves to ocean acidification (*28*).

In our MG experiment performed with the Baltic Sea population, we observed a large variance in response to elevated P_{CO_2} among the CO_2-sensitive and CO_2-tolerant families. Our high-resolution environmental P_{CO_2} monitoring in the habitat of the population revealed rapidly fluctuating P_{CO_2}. Thus, different cohorts of larvae can be exposed to either high or low P_{CO_2} during the sensitive planktonic larval phase, indicating that not all individuals from the Kiel Fjord population were selected in a high-P_{CO_2} environment. It is likely that this environmental heterogeneity selects for maintenance of variance of CO_2 tolerance and genetic diversity in this population. The role of temporal heterogeneity of selection pressures for maintaining genetic diversity has historically been underestimated, although the scaling of phenotypic change with time strongly suggests that fluctuating selection pressures are the rule rather than the exception (*29*). Especially when generations overlap and selection pressures vary across life stages, fixation of alleles by selective sweeps becomes unlikely (*30*). The high sensitivity of larvae to elevated P_{CO_2} compared to adult mussels along with several yearly cohorts observed in the fjord fits this condition for maintaining genetic diversity. The low predictability of selective environmental P_{CO_2} levels in the Baltic population makes tracking of these fluctuations by heritable trait changes unlikely and should rather select for bet hedging (*31*) or mechanisms of

plasticity, particularly for transgenerational plasticity with the early life stages that are affected here (*10*, *32*).

In both experiments (the MG and PC experiments), selection for tolerance to high P_{CO_2} correlated with a higher capacity to reach larger PD I sizes in the F_1 generation. Calculated heritabilities for this trait (0.23 to 0.83) were within the range of values previously reported for mytilid larvae [0.09 to 0.9 (*8*, *33*)]. The relatively larger PD I size of tolerant compared to sensitive families was also passed on to the F_2 generation, thus showing a heritable component. This suggests that the ability to form the PD I shell even under adverse environmental conditions can be an important fitness trait. Although transgenerational plasticity could partly compensate the negative effects of elevated P_{CO_2} levels for PD I formation rates within one generation (Fig. 4B), the absence of an effect on F_2 survival implies that PD I size alone cannot be used as a trait for reliable modeling of the evolutionary response of population mean fitness (*9*). Similar results were obtained for oysters when selection of larvae under 856-µatm P_{CO_2} did not improve the survival of their F_2 offspring under the same P_{CO_2} treatment (*16*). Increased performance observed in marine organisms under moderately elevated P_{CO_2} can probably be attributed to transgenerational phenotypic plasticity (TGP). TGP has been suggested to function as a short-term buffering mechanism to alleviate the effects of adverse environments before genetic adaptation can fill the fitness deficit. TGP has been shown to even persist over several generations in a range of species (*10*, *15*, *34*, *35*). TGP can manifest itself in altered animal performance with beneficial effects on growth and fecundity (*34*–*37*) or, in the case of bivalves, via modification of shell formation processes (*38*). More specifically, TGP can modulate, for example, respiratory capacity (aerobic scope) by acting upon mitochondrial properties. TGP thereby enables animals to adjust crucial physiological processes to the changed environment (*35*–*37*, *39*). Maternal effects can play a central role in passing TGP from one generation to the next (*35*, *37*). The rapid recovery of the PD I size of offspring from high P_{CO_2}–treated dams under acidified conditions (Fig. 4A) could result from such maternally driven TGP as well. In contrast, the absence of a positive effect on F_2 survival suggests that F_1 larval fitness is dependent on specific combinations of genotypes and nonheritable components.

Although significant adaptive responses may not necessarily be detectable on the whole-organism level within the three generations investigated in this study, they likely have contributed to the higher fitness of the Kiel Fjord population compared to North Sea mussels over longer time scales. Although high-CO_2 fluctuations in this habitat have increased only within recent decades as a result of eutrophication, adverse conditions for calcification due to lower alkalinity compared to the North Sea have prevailed for thousands of years (*20*, *40*). The high mortality of bivalves during the sensitive larval phase and the very high effective population size of mussels in the Baltic Sea (*41*) should have efficiently selected for beneficial mutations that increased population fitness. In support of this view, changed allele frequencies in response to elevated P_{CO_2} have been observed in sea urchin larvae within only 7 days of exposure (*24*). In our study, selection of tolerant F_1 specimens did not improve F_2 survival, which corresponds to findings obtained with oysters (*16*). However, selective breeding of high-yield oysters for aquaculture purposes resulted in significantly improved ocean acidification tolerance as a side effect within just four generations (*10*, *17*). The absence of a beneficial effect of selection in our study could be due to the small number of individuals used for the genetic crosses, which reduced the standing genetic variation present in the F_1 generation. However, a large standing variation is needed as a prerequisite for selection (*24*). Consequently, future experiments would need to use a larger number of individuals or families to lower the risk of detrimental genetic drift to more closely resemble the genetic variability present in populations, enabling rapid adaptation (*42*). This is particularly important for coastal habitats such as Kiel Fjord, which are characterized by large abiotic variability that could lead to high genetic variation within a single population.

In conclusion, several lines of evidence suggest a potential of *Mytilus* populations to adapt to elevated CO_2. This conclusion is supported by (i) the different sensitivity of Baltic Sea and North Sea populations in response to a natural P_{CO_2} gradient and (ii) a heritable component of calcification performance in early larval development observed in the MG experiment. Mussel larvae from the Baltic were characterized by higher CO_2 tolerance that correlated with higher ability to form the PD I shell under CO_2 stress. In concurrence with these data, our MG experiment revealed that selection for settlement in high-P_{CO_2} environments correlated with retention of PD I formation capabilities in F_1 animals. However, selection of tolerant F_1 phenotypes and long-term acclimation of F_1 specimens in our MG study did not significantly improve F_2 offspring survival. Consequently, prediction of adaptation potential based on short-term experiments and single traits within a population and generation appears to be highly speculative. Future experiments need to be performed over multiple generations to obtain a detailed understanding of the rate of adaptation and the underlying mechanisms to predict whether adaptation will enable marine organisms to overcome the constraints of ocean acidification.

MATERIALS AND METHODS

Kiel Fjord seawater P_{CO_2} was continuously monitored using a HydroC CO_2 sensor [Kongsberg Maritime AS (*43*)] mounted on a floating platform in about 1-m water depths. Abundance of settled bivalve larvae was assessed weekly on 5 cm × 5 cm manually roughed, replicated polyvinyl chloride panels (n = 4) suspended in the fjord in about 50-cm water depth.

For the PC experiment, *M. edulis* from Kiel Fjord (Baltic Sea) were transferred to List/Sylt (North Sea) and suspended in net cages along with North Sea specimens to acclimate to North Sea conditions. Acclimation lasted from December 2013 to April 2014 when all specimens were transferred back to Kiel and used for spawning the next day.

For the MG experiment, adult *M. edulis* were collected in Kiel Fjord in 2012 and kept overnight in a flow-through seawater setup under control conditions. Spawning was induced by a moderate heat shock (5°C) using heaters. Parental (F_0) animals (eight dams, A to H; eight sires, 1 to 8) were crossed pairwise in a reduced North Carolina I cross under control conditions to generate 16 full-sib families within four half-sib groups. Embryos were transferred into three experimental P_{CO_2} levels (390, 1120, or 2400 µatm). All families with successful settlement at 2400-µatm P_{CO_2} were considered as tolerant (5 of 16), and the remaining families (11 of 16) were termed "sensitive." Juveniles were transferred to a flow-through setup under constant P_{CO_2} until the next spawning season. The setup consisted of a header tank, which steadily supplied the experimental aquaria with seawater from Kiel Fjord. A *Rhodomonas* suspension was pumped into the header using a peristaltic pump and provided food to the experimental aquaria. Each aquarium was separately aerated with pressurized air with a P_{CO_2} of either 390, 1120, or 2400 µatm. Animals grew to average sizes of about 25 mm and sexual maturity within 1 year.

In 2013, individual crosses of F_1 specimens were carried out within tolerant families (dams: A2, B1, C4 × sires: D3) and between tolerant and sensitive families (tolerant dams: A2, B1, C4 × sensitive sires: E6; sensitive dams: F5, G7, H8 × tolerant sires: D3). The sex bias in mussels

(*44*) resulted in only 4 of the 16 families with male offspring and 12 exclusively female families, which reduced the number of potential crosses. P_{CO_2} during fertilization and the larval phase corresponded to tolerant sires' acclimation P_{CO_2} (exception tolerant dams × sensitive sires), and larvae were exposed to all P_{CO_2} levels in a fully crossed experimental design. In 2014, the response of 390- or 2400-µatm acclimated tolerant families and 390-µatm acclimated sensitive families was compared. For this purpose, eggs from three individuals from each of four tolerant dam families (A2, B1, B2, and C4) acclimated to 390- or 2400-µatm P_{CO_2} or females from the sensitive dam families (F5, G7, H7, and H8) acclimated to 390-µatm P_{CO_2} were pooled in equal numbers. Eggs were fertilized at 390- or 2400-µatm P_{CO_2}, corresponding to the treatment P_{CO_2} during the larval phase, with the sperm of one sire of family E6 acclimated to 1120 µatm.

Larval experiments were performed in a constant-temperature room at 19°C (population experiment, 16°C) at the Helmholtz Centre for Ocean Research Kiel. For fertilization, excess sperm was added, which does not allow testing for effects of P_{CO_2} on fertilization success. After assessing fertilization success in replicated measurements, embryos were transferred into the experimental units at an initial density of 10 larvae ml^{-1}. Experimental units were filled with 0.2-µm filtered seawater from Kiel Fjord. Weekly, 60% of the water volume was exchanged. In 2012 and 2013, larvae were fed daily with *Isochrysis* and *Rhodomonas* (days 7 to 21) or with *Rhodomonas* only (2014) cultured in F/2 or Provasoli enriched seawater (PES) medium, respectively. Larval survival and shell growth were assessed on days 2, 7, 14, and 21 after fertilization. Samples were immediately fixed, or living specimens were collected individually using a pipette, counted and subsequently fixed in 4% paraformaldehyde, and buffered with 4 mM $NaHCO_3$. Pictures of eggs and larvae were taken under a stereomicroscope with a MicroPublisher 3.3 RTV camera and analyzed for shell length using the Image-Pro Plus 5.0.1 software.

Monitoring of pH_{NBS}, salinity, and temperature was carried out twice or once a week in the larval experiments and the juvenile long-term acclimation, respectively. Weekly, water samples were analyzed for C_T using an AIRICA CT analyzer (Marianda) or for A_T using a Metrohm 862 Compact Titrosampler. Sample pH was determined on NBS or total scale using seawater buffers. Carbonate system speciation was calculated using the CO2SYS program using published $KHSO_4$, K_1, and K_2 dissociation constants (*45*–*47*).

Statistical tests are based on replicate means and were analyzed by ANOVA using R. Genetic variation of size was based on individual measurements and was analyzed by generalized linear mixed models (GLMMs) containing animal and replicate tank as random effects. See the Supplementary Materials for a detailed method description.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/4/e1602411/DC1

Supplementary Materials and Methods

fig. S1. Geographic origin of the two tested populations from Kiel Fjord in the Baltic Sea and the island of Sylt in the North Sea.

fig. S2. Analysis of the P_{CO_2} data from Fig. 1B on P_{CO_2} levels experienced by larvae settling in July, August, and September in Kiel Fjord (54°19.8'N; 10°9.0'E).

fig. S3. Picture of an *M. edulis* larva, with an approximate shell length of 120 µm, at the PD I stage 2 days after fertilization.

fig. S4. Egg diameter and fecundity of F_0 and F_1 dams.

fig. S5. F_1 egg diameter and F_2 larval performance in 2014.

table S1. Statistical analyses of population experiment.

table S2. Main effect contrasts from Bayesian GLMMs.

table S3. Statistical analyses of the transgenerational experiment in 2014.

References (*48*–*54*)

REFERENCES AND NOTES

1. K. J. Kroeker, R. L. Kordas, R. Crim, I. E. Hendriks, L. Ramajo, G. S. Singh, C. M. Duarte, J.-P. Gattuso, Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Glob. Chang. Biol.* **19**, 1884–1896 (2013).
2. H. Kurihara, T. Asai, S. Kato, A. Ishimatsu, Effects of elevated pCO_2 on early development in the mussel *Mytilus galloprovincialis*. *Aquat. Biol.* **4**, 225–223 (2008).
3. S. C. Talmage, C. J. Gobler, Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of larval shellfish. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 17246–17251 (2010).
4. F. Gazeau, L. M. Parker, S. Cormeau, J.-P. Gattuso, W. A. O'Connor, S. Martin, H.-O. Pörtner, P. M. Ross, Impacts of ocean acidification on marine shelled molluscs. *Mar. Biol.* **160**, 2207–2245 (2013).
5. G.G. Waldbusser, B. Hales, C. J. Langdon, B. A. Haley, P. Schrader, E. L. Brunner, M. W. Gray, C. A. Miller, I. Gimenez, Saturation-state sensitivity of marine bivalve larvae to ocean acidification. *Nat. Clim. Change* **5**, 273–280 (2014).
6. G. G. Waldbusser, E. L. Brunner, B. A. Haley, B. Hales, C. J. Langdon, F. G. Prahl, A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. *Geophys. Res. Lett.* **40**, 2171–2176 (2013).
7. J. Thomsen, K. Haynert, K. M. Wegner, F. Melzner, Impact of seawater carbonate chemistry on the calcification of marine bivalves. *Biogeosciences* **12**, 4209–4220 (2015).
8. P. Enderlein, M. Wahl Dominance of blue mussels versus consumer-mediated enhancement of benthic diversity. *J. Sea Res.* **51**, 145–155 (2004).
9. J. M. Sunday, R. N. Crim, C. D. G. Harley, M. W. Hart, Quantifying rates of evolutionary adaptation in response to ocean acidification. *PLOS ONE* **6**, e22881 (2011).
10. L. M. Parker, P. M. Ross, W. A. O'Connor, L. Borysko, D. A. Raftos, H.-O. Pörtner, Adult exposure influences offspring response to ocean acidification in oysters. *Glob. Chang. Biol.* **18**, 82–92 (2012).
11. J. M. Sunday, P. Calosi, S. Dupont, P. L. Munday, J. H. Stillman, T. B. H. Reusch, Evolution in an acidifying ocean. *Trends Ecol. Evol.* **29**, 117–125 (2014).
12. C. Duarte, J. M. Navarro, K. Acuña, R. Torres, P. H. Manríquez, M. A. Lardies, C. A. Vargas, N. A. Lagos, V. Aguilera, Intraspecific variability in the response of the edible mussel *Mytilus chilensis* (Hupe) to ocean acidification. *Estuaries Coasts* **38**, 590–598 (2015).
13. G.G. Waldbusser, B. Hales, C. J. Langdon, B. A. Haley, P. Schrader, E. L. Brunner, M. W. Gray, C. A. Miller, I. Gimenez, G. Hutchinson, Ocean acidification has multiple modes of action in bivalve larvae. *PLOS ONE* **10**, e0128376 (2015).
14. T. B. H. Reusch, Climate change in the oceans: Evolutionary versus phenotypically plastic responses of marine animals and plants. *Evol. Appl.* **7**, 104–122 (2014).
15. G.M. Miller, S.-A. Watson, J. M. Donelson, M. I. McCormick, P. L. Munday, Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. *Nat. Clim. Change* **2**, 858–861 (2012).
16. L. M. Parker, W. A. O'Connor, D. A. Raftos, H.-O. Pörtner, P. M. Ross, Persistence of positive carryover effects in the oyster, *Saccostrea glomerata*, following transgenerational exposure to ocean acidification. *PLOS ONE* **10**, e0132276 (2015).
17. J. A. Nell, B. Perkins, Evaluation of progeny of fourth generation Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) breeding lines. *Aquacult. Res.* **36**, 753–757 (2005).
18. J. Thomsen, M. A. Gutowska, J. Saphörster, A. Heinemann, K. Trübenbach, J. Fietzke, C. Hiebenthal, A. Eisenhauer, A. Körtzinger, M. Wahl, F. Melzner, Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* **7**, 3879–3891 (2010).
19. C. Pansch, I. Schaub, J. Havenhand, M. Wahl, Habitat traits and food availability determine the response of marine invertebrates to ocean acidification. *Glob. Chang. Biol.* **20**, 765–777 (2014).
20. J. Beldowski, A. Löffler, B. Schneider, L. Joensuu, Distribution and biogeochemical control of total CO_2 and total alkalinity in the Baltic Sea. *J. Mar. Syst.* **81**, 252–259 (2010).
21. T. R. Waller, Functional morphology and development of veliger larvae of the European oyster, *Ostrea edulis* Linné. *Smithson. Contrib. Zool.* **328**, 1–70 (1981).
22. P. Calosi, S. P. S. Rastrick, C. Lombardi, H. J. de Guzman, L. Davidson, M. Jahnke, A. Giangrande, J. D. Hardege, A. Schulze, J. I. Spicer, M.-C. Gambi, Adaptation and acclimatization to ocean acidification in marine ectotherms: An in situ transplant experiment with polychaetes at a shallow CO_2 vent system. *Philos. Trans. R. Soc. B* **368**, 20120444 (2013).
23. M. W. Kelly, J. L. Padilla-Gamiño, G. E. Hofmann, Natural variation and the capacity to adapt to ocean acidification in the keystone sea urchin *Strongylocentrotus purpuratus*. *Glob. Chang. Biol.* **19**, 2536–2546 (2013).
24. S. R. Palumbi, D. J. Barshis, N. Trylor-Knowles, R. A. Bay, Mechanisms of reef coral resistance to future climate change. *Science* **344**, 895–898 (2014).
25. R. K. Bechmann, I. C. Taban, S. Westerlund, B. F. Godal, M. Anberg, S. Vingen, A. Ingvarsdottir, T. Baussant, Effects of ocean acidification on early life stages of shrimp

(*Pandalus borealis*) and mussel (*Mytilus edulis*). *J. Toxicol. Environ. Health A* **74**, 424–438 (2011).
26. L. T. Bach, Reconsidering the role of carbonate ion concentration in calcification by marine organisms. *Biogeosciences* **12**, 4939–4951 2015).
27. A. Lucas, C. Rangel, Detection of the first larval feeding in *Crassostrea gigas*, using the epifluorescence microscope. *Aquaculture* **30**, 369–374 (1983).
28. S. A. Foo, M. Byrne, Acclimatization and adaptive capacity of marine species in a changing ocean. *Adv. Mar. Biol.* **74**, 69–116 (2016).
29. P. W. Messer, S. P. Ellner, N. G. Hairston Jr., Can population genetics adapt to rapid evolution? *Trends Genet.* **32**, 408–418 (2016).
30. S. Ellner, N. G. Hairstone Jr., Role of overlapping generations in maintaining genetic variation in a fluctuating environment. *Am. Nat.* **143**, 403–417 (1994).
31. L. N. S. Shama, Bet hedging in a warming ocean: Predictability of maternal environment shapes offspring size variation in marine sticklebacks. *Glob. Chang. Biol.* **21**, 4387–4400 (2015).
32. C. K. Ghalambor, J. K. McKay, S. P. Carroll, D. N. Reznick, Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* **21**, 394–407 (2007).
33. J. E. Toro, L. I. Paredes, Heritability estimates of larval shell length in the Chilean blue mussel *Mytilus chilensis*, under different food densities. *Aquat. Living Resour.* **9**, 347–350 (1996).
34. A. Rodriguez-Romero, M. D. Jarrold, G. Massamba-N'Siala, J. I. Spicer, P. Calosi, Multi-generational responses of a marine polychaete to a rapid change in seawater pCO_2. *Evol. Appl.* **9**, 1082–1095 (2015).
35. L. N. S. Shama, A. Strobel, F. C. Mark, K. M. Wegner, Transgenerational plasticity in marine sticklebacks: Maternal effects mediate impacts of a warming ocean. *Funct. Ecol.* **28**, 1482–1493 (2014).
36. P. Thor, S. Dupont, Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. *Glob. Chang. Biol.* **21**, 2261–2271 (2015).
37. L. N. S. Shama, F. C. Mark, A. Strobel, A. Lokmer, U. John, K. M. Wegner, Transgenerational effects persist down the maternal line in marine sticklebacks: Gene expression matches physiology in a warming ocean. *Evol. Appl.* **9**, 1096–1111 (2016).
38. S. C. Fitzer, M. Cusack, V. R. Phoenix, N. A. Kamenos, Ocean acidification reduces the crystallographic control in juvenile mussel shells. *J. Struct. Biol.* **188**, 39–45 (2014).
39. J. M. Donelson, P. L. Munday, M. I. McCormick, C. R. Pitcher, Rapid transgenerational acclimation of a tropical reef fish to climate change. *Nat. Clim. Change* **2**, 30–32 (2012).
40. J. Thomsen, I. Casties, C. Pansch, A. Körtzinger, F. Melzner, Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: Laboratory and field experiments. *Glob. Chang. Biol.* **19**, 1017–1027 (2013).
41. K. Johannesson, C. André, Life on the margin: Genetic isolation and diversity loss in a peripheral marine ecosystem, the Baltic Sea. *Mol. Ecol.* **15**, 2013–2029 (2006).
42. R. D. H. Barrett, D. Schluter, Adaptation from standing genetic variation. *Trends Ecol. Evol.* **23**, 38–44 (2008).
43. P. Fietzek, B. Fiedler, T. Steinhoff, A. Körtzinger, In situ quality assessment of a novel underwater pCO_2 sensor based on membrane equilibration and NDIR spectrometry. *J. Atmos. Oceanic Tech.* **31**, 181–196 (2014).
44. E. Kenchington, B. MacDonald, L. Cao, D. Tsagkarakis, E. Zouros, Genetics of mother-dependent sex ratio in blue mussels (*Mytilus spp.*) and implications for doubly uniparental inheritance of mitochondrial DNA. *Genetics* **161**, 1579–1588 (2002).
45. A. G. Dickson, F. J. Millero, A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Res.* **34**, 1733–1743 (1987).
46. A. G. Dickson, Standard potential of the reaction: AgCl(s) +1/H_2(g) = Ag(s) + HCl(aq), and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K. *J. Chem. Thermodyn.* **22**, 113–127 (1990).
47. C. Mehrbach, C. H. Culberson, J. E. Hawley, R. M. Pytkowic, Measurement of apparent dissociation-constants of carbonic acid in seawater at atmospheric-pressure. *Limnol. Oceanogr.* **18**, 897–907 (1973).
48. N. Rayssac, F. Pernet, O. Lacasse, R. Tremblay, Temperature effect on survival, growth and triacylglycerol content during the early ontogeny of *Mytilus edulis* and *M. trossulus*. *Mar. Ecol. Prog. Ser.* **417**, 183–191 (2010).
49. M. Sprung, Physiological energetics of mussel larvae (*Mytilus edulis*). I. Shell growth and biomass. *Mar. Ecol. Prog. Ser.* **17**, 283–293 (1984).
50. H. Stuckas, K. Stoof, H. Quesada, R. Tiedemann, Evolutionary implications of discordant clines across the Baltic *Mytilus* hybrid zone (*Mytilus edulis* and *Mytilus trossulus*). *Heredity* **103**, 146–156 (2009).
51. S. S. Mathiesen, J. Thyrring, J. Hemmer-Hansen, J. Berge, A. Sukhotin, P. Leopold, M. Bekaert, M. K. Sejr, E. E. Nielsen, Genetic diversity and connectivity within *Mytilus* spp. in the subarctic and Arctic. *Evol. Appl.* **10**, 39–55 (2017).
52. F. Melzner, J. Thomsen, W. Koeve, A. Oschlies, M. A. Gutowska, H. W. Bange, H. P. Hansen, A. Körtzinger, Future ocean acidification will be amplified by hypoxia in coastal habitats. *Mar. Biol.* **160**, 1875–1888 (2013).
53. A. G. Dickson, C. L. Sabine, J. R. Christian, *Guide to Best Practices for Ocean CO_2 Measurements* (PICES Special Publications, 2007), vol. 3, 191 pp.
54. J. D. Hadfield, MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *J. Stat. Softw.* **33**, 1–22 (2010).

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