Synergistic impacts of ocean acidification and temperature rise on the physiology of marine invertebrates in a latitudinal cline

Auswirkungen synergistischer Effekte von Ozeanversauerung und Temperaturerhöhung auf die Physiologie mariner Invertebraten im latitudinalen Gradienten

Dissertation zur Erlangung des akademischen Grades – Dr. rer. nat. –

dem Fachbereich 2 Biologie / Chemie
der Universität Bremen
vorgelegt von

Zora Mila Colomba Zittier
Diplom-Biologin

Bremen 2017
Gutachter

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

2. Gutachter: Prof. Dr. Bela H. Buck, Universität Bremen
   Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung
   Aquakultur
   Am Handelshafen 12, 27570 Bremerhaven
We wish to pursue the truth no matter where it leads. But to find truth, we need imagination and scepticism both. We will not be afraid to speculate, but we will be careful to distinguish speculation from fact.

The cosmos is full beyond measure of elegant truth; of exquisite interrelationships; of the awesome machinery of nature.

– Carl Sagan –
Contents

Summary III

Zusammenfassung VI

1 Introduction 1

1.1 Thermal tolerance in marine ectothermic animals 1
1.2 CO\textsubscript{2} impacts on marine ectothermic animals 6
1.3 Combined effects of temperature and CO\textsubscript{2} stress 10
1.4 Calcifying model species: \textit{Hyas araneus} and \textit{Mytilus edulis} 11
1.4.1 \textit{H. araneus} 12
1.4.2 \textit{M. edulis} 13
1.5 Aims of the thesis 16

2 Materials & Methods 19

2.1 \textit{Hyas araneus} 20

2.1.1 Animal collection and maintenance 20
2.1.2 Incubation and experimental set up 20
2.1.3 Preparation of animals and experimental protocol 22
2.1.4 Righting response 23
2.1.5 Haemolymph parameters 23
2.1.6 Statistical analysis 24

2.2 \textit{Mytilus edulis} 24

2.2.1 Animal collection and maintenance 24
2.2.2 Incubation and experimental set up 25
2.2.3 Preparation of animals and experimental protocol 28
2.2.4 Oxygen consumption and heart rate 29
2.2.5 Body fluids and tissue samples 29
2.2.6 Statistical analysis 30

3 Publications 33

Publication 1 34

The synergistic effects of increasing temperature and CO\textsubscript{2} levels on activity capacity and acid–base balance in the spider crab, \textit{Hyas araneus}

Publication 2 49

Impact of ocean acidification on thermal tolerance and acid–base regulation of \textit{Mytilus edulis} (L.) from the North Sea

Publication 3 60

Impact of ocean acidification on thermal tolerance and acid-base regulation of \textit{Mytilus edulis} from the White Sea

4 Discussion 75

4.1 Effect of CO\textsubscript{2} and temperature rise on the performance capacity of \textit{Hyas araneus} 76

4.2 Thermal tolerance and the impact of CO\textsubscript{2} on \textit{Mytilus edulis} populations 82

4.2.1 Thermal tolerance and the impact of CO\textsubscript{2} on temperate and subarctic \textit{M. edulis} 83

4.2.2 Common features of the climate-related response 93

4.2.3 Latitudinal differences in the climate-related response 101
5 Conclusion 105
References 107
List of Abbreviations 125
Acknowledgement 127
Erklärung 129
Summary

Ocean warming and acidification, linked to atmospheric carbon dioxide (CO₂) enrichment, occur simultaneously and are two of the main factors driving current ecosystem changes. Yet, their combined impacts on marine organisms are insufficiently understood. It is assumed that organisms adapted to small ranges of environmental parameters such as temperature are more vulnerable to climate change. Calcifying organisms may be particularly susceptible by altered seawater carbonate chemistry that may affect the formation and protection of their shells under such challenging circumstances. Hence, one aim of this thesis is to provide insights on the combined effects of temperature rise and ocean acidification on thermal tolerance, acid-base regulation capacity, and animal performance of the spider crab *Hyas araneus* from the Arctic. Furthermore, the combined effects of ocean warming and acidification may differ between populations along a climate gradient. Identifying the organism responses to multiple drivers on the population level is crucial for realistic predictions on climate-induced effects on marine ecosystems. Thus, potential differences in thermal tolerance and CO₂ sensitivity between populations along a latitudinal cline were examined in the blue mussel *Mytilus edulis*. In a comparative approach the effect of climate change on the physiological performance from the cellular up to whole animal level was investigated with regard to potential differences between (sub) polar and temperate populations.

In order to investigate the effects of temperature on CO₂ sensitivity and associated acid-base status and activity capacity in *H. araneus*, crabs were acclimated at various CO₂ conditions (390 - 3,000 µatm) at two temperatures (1°C spring vs. 4°C summer). Activity capacity was measured as righting response under the different treatment conditions and at a subsequent acute heat exposure (12°C at the respective CO₂ level). Prior to (resting) and after the consecutive stress (two righting trials, heat exposure) haemolymph acid-base status and lactate content were determined. The haemolymph results showed that *H. araneus* was able to substantially accumulate bicarbonate \( [\text{HCO}_3^-] \) to defend its internal milieu under increasing CO₂ levels but failed to maintain haemolymph pH. Warm acclimation (4°C vs. 1°C) exacerbated the impact of CO₂ resulting in a significantly stronger extracellular acidosis and potential restrictions in \( [\text{HCO}_3^-] \) accumulation. This indicates that Arctic *H. araneus* has a low capacity for acid-base regulation especially when hypercapnia is combined with warming.

The righting response of Artic *H. araneus* was not affected by the acclimation temperatures (1°C and 4°C), the rising CO₂ levels and associated acid-base disturbances in the haemolymph, or by the acute heat exposure (12°C) at normocapnia. The combined stress of heat and hypercapnia,
however, caused a functional limitation in all crabs. Warm-acclimated crabs slowed significantly when both stressors were combined but remained comparatively active while cold-acclimated specimen slowed drastically. This difference was likely caused by the stronger increase in temperature for cold- than for warm-acclimated crabs (+11° vs. +8°C).

The consecutive stress of righting and heat exposure had little impact on haemolymph status except for lactate content that increased in all crabs. In cold-acclimated crabs, lactate content reflected the CO₂-dependent activity effort under heat stress (high at control CO₂ and low at high CO₂). In the more active warm-acclimated crabs, lactate content increased with rising CO₂ levels indicating higher energetic costs for activity or exacerbated capacity constraints when hypercapnia is combined with warming. However, even after stress, lactate content remained very low in all crabs indicating a generally low capacity for anaerobic energy production, a phenomenon described for polar cold-adapted animals. The low lactate levels suggest that in all crabs any activity performed was mainly fuelled aerobically. Thus, the drastic slowing of cold-acclimated crabs under combined hypercapnia and heat exposure may not have been caused by constrains in aerobic performance only but also by other implications such as neuronal failure.

Overall, all crabs exposed to temperature extremes suffered from reduced scope of performance, which was intensified by rising CO₂ levels, in line with the concept of oxygen- and capacity-limited thermal tolerance (OCLTT). Arctic *H. araneus* show a high thermal sensitivity that is exacerbated by hypercapnia indicating an adaptation to the stable and cold environment, with the result of a high sensitivity to environmental changes.

On the basis of the OCLTT concept, thermal tolerance, energy metabolism, and acid-base balance at different CO₂ conditions (390 - 1,120 µatm) were investigated in *M. edulis* populations from temperate North Sea and subarctic White Sea using a stepwise warming protocol (3°C/night, starting at 10°C). The results suggest general features in the climate-related physiological response of *M. edulis* populations across a latitudinal gradient. In general, warming (at control CO₂) caused disturbances in haemolymph status (fall in pH and PO₂, rise in PCO₂) that may be associated with setting the pejus temperature (Tₚ, onset of thermal limitation in aerobic scope), in blue mussels. Beginning thermal limitation in the aerobic performance is affirmed by subsequent restrictions in respiratory and cardiac functions. Further warming resulted in a transition to partial anaerobiosis, which defines the critical temperature (Tₖ). The onset of anaerobic metabolism was associated with a breakdown in the respiratory and cardiac functions as well as in the maintenance of the intracellular pH. The revealed physiological characteristics of thermal thresholds of *M. edulis* are in line with the OCLTT concept.
At the acclimation temperature, CO₂ exposure resulted in a largely uncompensated extracellular acidosis while energy turnover and heart rate remained unaffected. The described physiological response patterns to warming under normocapnia were similar under hypercapnia except for the course in respiration rate (MO₂). CO₂ exposure stimulated the respiration rate in the warmth and, thus, shifted the beginning limitation in MO₂ from Tₚ under normocapnia to T₉ under hypercapnia. Whether this elevation of the metabolic rate may be beneficial (adaptation potential) or detrimental (energy depletion) in the long-term needs to be investigated.

Beside the described general features, differences in the environmental stress response were found between *M. edulis* populations along a latitudinal cline. Compared to North Sea mussels, the White Sea population showed a reduced heat tolerance as both upper Tₚ and T₉ were shifted downwards indicating an evolutionary adaptation to the colder habitat. While CO₂ had no effect on thermal limits of temperate mussels, hypercapnia caused a lowering of upper Tₚ and T₉ in subarctic mussels indicating an enhanced vulnerability to environmental change.

The present thesis provides evidence that populations of eurythermal species living at high latitudes exhibit features of cold adaptation with the result of a high sensitivity to environmental change. Moderate hypercapnia can reduce heat tolerance and associated organism performance in ectotherms as shown in Arctic *H. araneus* and subarctic *M. edulis*. However, the impairment was not indicated in temperate *M. edulis*, at least in the short-term. Hence, populations from high latitudes may be more vulnerable and threatened by projected ocean warming and acidification when compared to their temperate conspecifics. The results stress the necessity to account for combined impacts of multiple environmental drivers when assessing a species vulnerability to climate change. Furthermore, latitudinal differences in the climate-related response of species and populations must be considered when projecting future ecosystem changes.
ZUSAMMENFASSUNG

Zusammenfassung


Um den Einfluss der Temperatur auf die CO₂-Sensitivität und den assoziierten Säure-Base-Status der Hämolymphe sowie die Aktivitätsniveau von *H. araneus* zu erfassen, wurden die Krebse unter verschiedenen CO₂-Konzentrationen (390 - 3,000 µatm) bei zwei Temperaturen (1°C Frühling vs. 4°C Sommer) akklimatiert. Ihr Aktivitätsniveau wurde mittels erzwungener Aktivität unter den Inkubationsbedingungen sowie unter akuter Erwärmung (12°C bei entsprechender CO₂-Konzentration) untersucht. Bevor (Ruhephase) und nachdem die Tiere umfangreichem Stress (zwei Tests zum Aktivitätsniveau, Hitzestress) ausgesetzt wurden, wurde der Säure-Base-Status und der Laktatgehalt der Hämolymphe bestimmt.

Die Ergebnisse zeigten, dass *H. araneus* in der Lage war Bikarbonat [HCO₃⁻] zu akkumulieren, um ihren Extrazellularraum unter erhöhten CO₂-Konzentration zu puffern, allerdings gelang es den Tieren nicht, ihren Hämolymphe-pH aufrecht zu erhalten. Die Wärmeakklimation (4°C) verstärkte die Auswirkungen des erhöhten CO₂, was in einer signifikant stärkeren extra-
ZUSAMMENFASSUNG

zellulären Azidose und einer potentiellen Einschränkung der [HCO₃⁻]-Akkumulation resultierte. Dies deutet daraufhin, dass die arktischen *H. araneus* eine niedrige Kapazität der Säure-Base-Regulation aufweisen, besonders wenn Hyperkapnie und Erwärmung gleichzeitig auftreten.

Das Aktivitätsniveau der arktischen *H. araneus* war nicht durch die Akklimations-temperaturen (1° und 4°C), die steigenden CO₂-Konzentrationen und die assoziierte Säure-Base-Störung in der Hämolymphe oder durch die akute Erwärmung (12°C) unter Normokapnie beeinflusst. Der kombinierte Stress von akuter Hitze und Hyperkapnie verursachte jedoch funktionale Einschränkungen in allen Versuchstieren. Warmakklimierte Tiere verlangsamen sich signifikant, wenn beide Stressfaktoren kombiniert wurden, blieben aber verhältnismäßig aktiv verglichen zu den kaltakklimierten Krebsen, welche sich drastisch verlangsamen. Dieser Unterschied wurde wahrscheinlich durch den stärkeren Temperaturanstieg (+11° vs. +8°C) für kaltakklimierte Tiere verursacht.


Alle Versuchstiere, die Temperaturextremen ausgesetzt waren, zeigten eine reduzierte Leistungskapazität, was durch die steigenden CO₂-Konzentrationen verstärkt wurde und mit dem Konzept der Sauerstoff-und Kapazitäts-limitierten Temperaturtoleranz (OCLTT) übereinstimmt. Arktische *H. araneus* zeigten eine hohe Wärmesensitivität, welche durch Hyperkapnie verstärkt wurde und auf eine Adaptation an die stabile und kalte Umwelt der Tiere hinweist, was mit einer hohen Sensitivität gegenüber Umweltveränderung einhergeht.

Auf Basis des OCLTT-Konzeptes wurden Temperaturtoleranz, Energiestoffwechsel und Säure-Base-Staus der Hämolymphe bei verschiedenen CO₂-Konzentrationen (390 - 1,120 μatm)
in Populationen von *M. edulis* von der Nordsee und dem subarktischen Weißen Meer während schrittweiser Erwärmung (3°C/Nacht, beginnend bei 10°C) untersucht.


Unter der Akklimationstemperatur führte die CO$_2$-Exposition zu einer unkompensierten extrazellulären Azidose während der Energieumsatz und die Herzrate unbeeinflusst blieben. Die beschriebene physiologische Reaktion auf Erwärmung unter Normokapnie war dieselben wie unter Hyperkapnie, bis auf den Verlauf der Sauerstoffverbrauchsrate (MO$_2$). CO$_2$-Exposition stimulierte die Respirationsrate unter Erwärmung und verschob die einsetzende Limitierung in MO$_2$ von $T_P$ unter Normokapnie zu $T_C$ unter Hyperkapnie. Ob die Erhöhung der metabolischen Rate langfristig von Vor- oder Nachteil ist (Adaptionspotential oder Energieverbrauch), muss in weiteren Studien untersucht werden.

Neben diesen generellen Merkmalen wurden auch Unterschiede in der Reaktion auf veränderte Klimafaktoren in *M. edulis* Populationen entlang des latitudinalen Gradienten gefunden. Im Vergleich zu Miesmuscheln der Nordsee zeigte die Population aus dem Weißen Meer eine reduzierte Wärmetoleranz. Die obere $T_P$ und $T_C$ waren herabgesetzt, was auf eine evolutionäre Adaptation an das kältere Habitat hinweist. Während die CO$_2$-Exposition keinen Effekt auf Temperaturgrenzen der temperierten Muscheln hatte, führte diese in den subarktischen Muscheln zu einer Erniedrigung der oberen $T_P$ und $T_C$, was auf eine erhöhte Sensitivität für Klimaveränderungen hindeutet.

Die vorliegende Arbeit liefert Hinweise darauf, dass in hohen Breiten lebende Populationen von eurythermen Arten Merkmale der Kälteadaptation aufzeigen, was in einer hohen Sensitivität gegenüber verändernder Umweltparameter resultiert. Moderate Hyperkapnie kann die Temperaturtoleranz und damit verbundene Leistungsfähigkeiten in ektothermen Organismen reduzieren, wie gezeigt am Beispiel der arktischen *H. araneus* und der subarktischen *M. edulis*. Diese
1 Introduction

Coastal regions provide a variety of important ecosystem for all living organisms – biota and humans. They comprise a diverse set of habitat types and biotic communities and are among the most productive environments with a high biological, economic and social value. In addition to the strong impacts by local human activities (e.g. fishery, shipping, tourism and resulting pollutions) marine coastal ecosystems are increasingly threatened by anthropogenic climate change. Temperature is the main environmental factor driving current ecosystem changes by affecting all levels of biological organisation and setting the limits of live (e.g. Kassahn et al. 2009, Pörtner 2010). In addition, the ongoing rise in CO₂ concentrations causes ocean acidification, and already threatens marine organisms (Wootton et al. 2008, Barton et al. 2012, Waldbusser et al. 2013). Ocean acidification is expected to change marine ecosystems substantially in the twenty-first century (Hall-Spencer et al. 2008, Melzner et al. 2009, Wittmann and Pörtner 2013). Survival of organisms will depend on their plasticity in the physiological response but the ability to cope with environmental stress differs between species and even between populations making predictions at ecosystem levels more complicated. Beyond that, current changes in abiotic factors occur concomitantly, yet studies on the interactions of multiple factors on marine organisms are still rare and hardly consider differences between population responses.

The present thesis will provide insights on CO₂ impacts on thermal tolerance, activity and acid-base balance of marine invertebrates, the spider crab *Hyas araneus* from the Arctic and the blue mussel *Mytilus edulis*, in a latitudinal cline along the east Atlantic coast between the North Sea and the White Sea. Identifying how animals respond to multiple drivers on the population level will contribute to our ability to predict climate-induced effects on ecosystem structure and functioning.

1.1 Thermal tolerance in marine ectothermic animals

Already in 1911, Shelford introduced the “Law of Toleration” stating that the abundance and distribution of an ectothermic animal is controlled by its range of tolerance to environmental factors (Shelford 1911, 1913, 1931). This foundational concept later known as “Shelford’s Law of Tolerance” describes that exceeding the minimum or maximum limit of tolerance will result in reduced abundance and finally local (and global) extinction. The environmental temperature is
an ecological key factor shaping the geographic distribution of animals (Varley, 1967, Brett, 1971). During the last century surface temperature of the world’s ocean already rose by approx. 0.3°C due to anthropogenic greenhouse gas emission (Hegerl and Bindoff 2005, Large and Yeager 2012). Current ocean warming has been identified as the main driver of recent poleward shifts in species distribution and ecosystem changes i.e. structure (species composition) and functioning (biomass production and nutrient cycling) (e.g. Parmesan and Yohe 2003, Wethey and Woodin 2008). Thereby temperature affects organisms both, directly by influencing the physiological sensitivity and indirectly by changing their competitiveness and the species-specific phenology – the timing of seasonal activities like spawning and migration. This leads to alterations in the biogeography of organisms and mismatch phenomena in species interactions with associated changes in the community composition (regime shift) and the response of an ecosystem (Edwards and Richardson 2004, Visser and Both 2005, Parmesan 2006, Pörtner and Farrell 2008, Doney et al. 2012). Furthermore, temperature influences organisms at all life-stages and at all levels of biological organisation as it affects the rates of physico-chemical and physiological processes (expressed as the temperature coefficient $Q_{10}$ – the change of rate for a 10°C temperature rise, Krogh 1914). This is especially crucial for ectothermic organisms, in which the temperature of the body changes with the environment directly altering metabolic rates.

In the past 100 years, researchers have discovered some unifying principles of the temperature-dependence in physiological and biochemical mechanisms of ectothermic animals, which reach up to climate-induced changes at the ecosystem level. Build on Shelford’s Law of Tolerance and subsequent modifications (e.g. Southward 1958, Fry 1971, Jones 1971, Schwerdtfeger 1977, Elliot 1981, Frederich and Pörtner 2000, Pörtner 2001, 2002) the concept of oxygen- and capacity-limited thermal tolerance (OCLTT) was developed (see Pörtner 2010, Pörtner et al. 2017 for reviews). The OCLTT concept defines low and high thermal tolerance thresholds in relation to oxygen supply capacity and oxygen demand of ectothermic species, at the same time proposing a systemic to molecular hierarchy of thermal tolerance. The concept states that the performance of an organism is determined by its temperature-dependent aerobic scope – the range between minimum and maximum oxygen consumption, which is directly related to mitochondrial respiration rate and represents the capacity for activity. Accordingly, over a range of optimal temperatures, the available aerobic scope covers all live sustaining mechanisms including feeding, growth, motility and reproduction with a maximum close to the upper pejus temperature (pejus = turning worse, Schwerdtfeger 1977), the limit of the optimum range in the warmth (Fig. 1). On both sides of the thermal window at low and high pejus
**INTRODUCTION**

Fig. 1 Conceptual model of oxygen- and capacity-limited thermal tolerance and the resulting capacity of aerobic performance of the animal (modified from Pörtner 2010). The concept states that the performance of an organism is determined by its temperature-dependent aerobic scope, which is the range between minimum and maximum oxygen consumption. In the optimum range, the available aerobic scope covers all live sustaining mechanisms including growth and reproduction with a maximum close to the upper pejus temperature (Tp). On both sides of the thermal window, at low and high Tp, a first thermal restriction in aerobic scope occurs. At the subsequent critical temperatures (Tc) oxygen supply to tissue is insufficient to maintain life sustaining energy turnover and partial transition to anaerobic metabolism occurs. The denaturation temperatures (Td) indicate the loss of molecular and cellular function. Elevated CO₂ levels may cause a narrowing of thermal windows that may result in constraints of performance capacities (indicated by green arrows). For further details see text.

Temperature (Tp) a first thermal restriction in aerobic scope occurs as the cost of oxygen supply rises and oxygen demand of mitochondrial maintenance further constrains aerobic scope. The mismatch emerges due to a limitation in the circulatory and / or ventilatory performance capacity (Zielinski and Pörtner 1996, Frederich and Pörtner 2000, Peck et al. 2002, Lannig et al. 2004, Melzner et al. 2006a), the processes providing oxygen (uptake and transport) to tissues. Hence, these processes play a major role in determining thermal limits of organisms. The range between low and high Tp defines the ecological thermal tolerance range as it sets the earliest limits to the geographical distribution and boundaries of species and populations as affirmed by observations.
in the field (Pörtner and Knust 2007, Farrell et al. 2008, Jones et al. 2009, Drinkwater et al. 2010, Neuheimer et al. 2011). Beyond pejus limits animals start to exploit their passive tolerance range and successful survival becomes restricted due to the constraint on aerobic performance linked to an arising internal oxygen deficit (hypoxemia). As a result whole animal performance is continuously reduced as seen, for example, in growth rates, activity levels and mortality (Zielinski and Pörtner 1996, Frederich and Pörtner 2000, Mark et al. 2002, Lannig et al. 2004, Melzner 2005, Walther et al. 2009). At the subsequent critical temperatures ($T_C$) oxygen supply to tissue is insufficient to maintain life sustaining energy turnover and some mitochondria switch to anaerobic metabolism. These critical thresholds characterize the limits to the physiological tolerance range of the individual as survival is only possible in the short-term and prolonged periods of exposure will inevitably lead to death. Finally, the denaturation temperature ($T_D$) indicates the loss of molecular and cellular function. $T_D$ can be shifted by protection mechanisms, such as the synthesis of heat shock proteins, antioxidants or antifreeze proteins, if they are possessed by the organism (e.g. Hofmann et al. 2000, Tomanek 2008). Evidence supporting the OCLTT concept was found in various phyla of aquatic (predominantly marine) ectotherms like fishes (van Dijk et al. 1999, Mark et al. 2002, Lannig et al. 2004), crustaceans (Frederich and Pörtner 2000, Metzger et al. 2007, Walther et al. 2009) cephalopods (Melzner et al. 2006), bivalves (Peck et al. 2002, Lannig et al. 2008, 2010) and annelid and sipunculid worms (Zielinski and Pörtner 1996, Sommer et al. 1997, Schröer et al. 2009) but also in air breathing ectotherms like reptiles and birds (see review by Pörtner 2002a and citations therein).

Fig. 2 Schematic picture of the thermal windows of species and populations along a latitudinal cline. Thermal windows are narrow in stenothermal and wide in eurythermal species, reflecting adaptation to different climatic regions. (Modified after Pörtner and Farrell 2008).
INTRODUCTION

Fig. 3 Projected changes in global surface temperatures based on different Representative Concentration Pathways (RCP) (after IPCC 2014). Change in average surface temperature based on multi-model mean projections for 2081–2100 relative to 1986–2005 under the RCP2.6 (left) and RCP8.5 (right) scenarios. The number of models used to calculate the multi-model mean is indicated in the upper right corner of each panel. Stippling (i.e., dots) shows regions where the projected change is large compared to natural internal variability and where at least 90% of models agree on the sign of change. Hatching (i.e., diagonal lines) shows regions where the projected change is less than one standard deviation of the natural internal variability.

The thermal tolerance window is specific for species and their life stages but the width and position on the temperature scale also depends on the climate regime of the respective habitat. Organisms adjust to changing environmental conditions by acclimatization and evolutionary adaptation resulting in shifts of thermal thresholds with seasons and along a latitudinal cline causing population differences (Fig. 2) (Sommer et al. 1997, van Dijk et al. 1999, Lannig et al. 2003, Sokolova and Pörtner 2003, Wittmann et al. 2008, Schröer et al. 2009, Kelly et al. 2012; for reviews see Pörtner et al. 2001, Pörtner 2002a). Hence, tolerance and survival of populations will depend on their physiological plasticity to cope with the challenges of ocean warming. In general, the thermal window is shifted downwards with colder climate conditions, i.e. towards the winter season or with increasing latitude and water depth (Sokolova et al. 2000, Pörtner 2002a, 2002b, Pörtner et al. 2006, Wittmann et al. 2008, Schröer et al. 2009), and is narrowed in polar animals compared to their boreal counterparts (e.g. Sommer et al. 1997, van Dijk et al. 1999, Peck and Conway 2000, Sokolova and Pörtner 2003, Peck et al. 2014). The latter occurs because animals from highest polar latitudes are adapted to a cold and stable environment exhibiting lower functional capacities in many physiological processes, which result in a high sensitivity to environmental stress. Global climate models forecast successive warming during the 21st century for the world’s oceans with considerable greater rates at high latitudes (for an overview see IPCC 2007, 2013). The calculated rates differ but the geographic pattern is
independent of the used scenario (Fig. 3). Together with the fact that polar populations and species have a limited possibility to migrate to more favourable and colder habitats, they are likely at greatest risk of ocean warming. Yet, the consideration of population differences in meta-analyses or ecosystem models on species responses to future climate change are still rare. Identifying the underlying mechanisms of thermal tolerance as well as the thermal window and the physiological plasticity of ecologically important species with respect to population differences are important to understand potential future changes in ecosystems.

1.2 CO₂ impacts on marine ectothermic animals

Carbon dioxide (CO₂) is one of the primary greenhouse gasses in the atmosphere. Anthropogenic CO₂ emission, mainly due to the burning of fossil fuels, caused a rise in the atmospheric partial pressure (PCO₂) from its pre-industrial level of about 280 µatm to about 400 µatm today (Dlugokencky and Tans 2017). Beside its warming effect, CO₂ penetrates the surface ocean and alters the seawater carbonate chemistry. The dissolved free carbon dioxide (CO₂(aq)) reacts with the seawater and forms carbonic acid (H₂CO₃) that dissociates to bicarbonate [HCO₃⁻] and further to carbonate (CO₃²⁻) thereby releasing protons (H⁺), which decrease seawater pH (Eq. 1). The ratio of the carbonate species is determined by the respective equilibrium constants (K₀, K₁ and K₂ in Eq. 1), which depend on temperature, salinity and pressure (Zeebe and Wolf-Gladrow 2001).

\[
\begin{align*}
\text{CO}_2_{(aq)} + \text{H}_2\text{O} & \overset{K_0}{\leftrightarrow} \text{H}_2\text{CO}_3 \overset{K_1}{\leftrightarrow} \text{H}^+ + \text{HCO}_3^- \overset{K_2}{\leftrightarrow} 2 \text{H}^+ + \text{CO}_3^{2-} \\
\text{Eq. 1}
\end{align*}
\]

The ongoing CO₂ uptake from the atmosphere causes a reduction in seawater pH, carbonate level and carbonate saturation level in a process termed ocean acidification. Since the industrial revolution, [H⁺] ions increased already by about 30% representing a decrease in the global surface pH by 0.1 units (Zeebe and Wolf-Gladrow 2001, Caldeira and Wickett 2003, 2005, Orr et al. 2005) (Fig. 4). Scenarios of future CO₂ accumulation projected a decrease in surface ocean pH by another 0.3 - 0.4 units, equalling ~ 700 - 1,100 µatm CO₂, during the 21st century (Meehl et al. 2007, IPCC 2013) and by further 0.4 - 1.0 pH units, equalling ~ 2,000 - 8,000 µatm CO₂, till the year 2300 (Caldeira and Wickett 2005). Inferred from the geological record the current acidification process already represents the fastest one in the past 300 million years (Caldeira and Wickett 2003, Raven et al. 2005, Honisch et al. 2012). That period includes several mass extinctions most notably the Permian-Triassic extinction (~ 252 to 201 million years ago), also
INTRODUCTION

known as the great dying, during which 90 - 95% of marine species were eliminated with an involvement of increased CO₂ concentration (hypercapnia) (Knoll et al. 1996, 2007, Montenegro et al. 2011). Various mainly negative effects of ocean acidification on the physiology of marine organisms have been identified. Impacts on physiological mechanisms like disturbances in acid-base regulation, gene expression and protein synthesis intervene the energy metabolism of the organism (for reviews see Melzner et al. 2009, Whiteley 2011, Wittmann and Pörtner 2013). These led to changes, for instance, in the rates of respiration, growth, calcification and reproduction, resulting in an alteration of whole animal performance and associated behaviour.

Fig. 4 Atmospheric enrichment of CO₂ and resulting ocean pH and PCO₂ (after Feely et al. 2009). Time series of atmospheric CO₂ at Mauna Loa (ppmv) and surface ocean pH and PCO₂ (µatm) at Ocean Station Aloha in the subtropical North Pacific. Note that the increase in oceanic CO₂ over the period of observations is consistent with the atmospheric increase within the statistical limits of the measurements.

When CO₂ enters the animal body by diffusion internal PCO₂ rises causing an increase in [H⁺] and associated decrease in pH (acidosis) in body fluids due to the CO₂ hydration reaction. As cellular processes strongly depend on pH maintenance acid-base balance is crucial for whole animal performance and, thus, is suggested to play a pivotal role in CO₂ resilience of organisms (for review see Pörtner 2008, Melzner et al. 2009). pH shifts in extra- and intracellular compartments are minimized by the so-called non-bicarbonate buffering system of body fluids (Heisler and Piiper 1971, Pörtner and Sartoris 1999, Seibel and Walsh 2003, Melzner et al. 2009). This passive buffering system comprises non-bicarbonate buffers (proteins, amino acids
or organic/inorganic phosphate groups), which bind excess protons resulting in a slight increase of $[\text{HCO}_3^-]$ under elevated $\text{PCO}_2$ according to the chemical equilibrium (Somero 1981, Henry and Wheatly 1992, Seibel and Walsh 2003). However, buffering is insufficient to restore fluid pH during prolonged elevated $\text{PCO}_2$ (hours to days) as it only masks the effect of acidosis (Melzner et al. 2009). Maintaining extra- and intracellular pH over longer terms is only possible by eliminating $[\text{H}^+]$ ions via active ion exchange across cell and epithelia membranes. These mechanisms, termed proton equivalent ion exchange, actively eliminate $[\text{H}^+]$ and accumulate $[\text{HCO}_3^-]$ in exchange of acid-base relevant ions (Truchot 1975, Cameron 1978, Claiborne and Heisler 1986, Seibel and Walsh 2003, Pörtner et al. 2004, Fabry et al. 2008). Active transport is energetically costly and can, thus, reduce aerobic scope for other processes such as growth, reproduction and calcification. Conversely, it is hypothesized that animals with relatively high metabolic rates or activity levels (fish, crustaceans and cephalopod molluscs) also show a higher capacity for pH regulation and, thus, CO$_2$ resilience than more inactive or sessile ones (molluscs, echinoderms and corals) (for reviews see Melzner et al. 2009, Kroeker et al. 2013). A high CO$_2$ sensitivity for corals has long been observed in the field, but was only recently reported for molluscs in natural habitats were shifts towards high-CO$_2$ upwelling conditions caused massive die-offs of larvae (Barton et al. 2012).

Calcifying organisms are considered to be more vulnerable to CO$_2$ stress (Melzner et al. 2009, Wittmann and Pörtner 2013) due to the additional challenges involved in the formation and protection of their calcareous shells and skeletons under the altered seawater carbonate chemistry (Fabry et al. 2008, Doney et al. 2009, Kroeker et al. 2010). Marine calcifiers studied under expected future CO$_2$ levels revealed highly variable (including contrary) CO$_2$ responses of divers processes (e.g. pH regulation, somatic growth, calcification and fertilization) even among related species of the same phylum (e.g. Ries et al. 2009, for reviews see Melzner et al. 2009, Whiteley 2011, Wittmann and Pörtner 2013). For instance, a comparative study of a shallow living crab and its deep-sea counterpart revealed a reduced capacity for acid-base regulation in the latter likely due to lower functional capacities resulting from adaptation to a stable and cold environment (Pane and Barry 2007). Thus, species and populations from cold polar latitudes might also be more sensitive to ocean acidification when compared to boreal ones. In addition, the rate of ocean acidification is considerably greater at high latitudes (Fig. 4), due to the higher solubility of CO$_2$ at lowered temperatures and salinities (freshwater impact from glaciers and sea ice), similar to the geographic pattern described for ocean warming (see section 1.1). This suggests (again) that species and populations from polar latitudes will be under greater threat in the near future than boreal ones.
Fig. 5 Times series of ocean surface pH and projected changes based on different RCPs (after IPCC 2013). (a) Time series (model averages and minimum to maximum ranges) and (b) maps of multi-model surface ocean pH for the scenarios RCP2.6, RCP4.5, RCP6.0 and RCP8.5 in 2081–2100. The maps in (b) show change in global ocean surface pH in 2081–2100 relative to 1986–2005. The number of CMIP5 models (Coordinated Modelling Intercomparison Project Phase 5) to calculate the multi-model mean is indicated in the upper right corner of each panel.
Furthermore, intertidal species like blue mussels might show a higher resilience to elevated CO₂ levels than subtidal or open water species as they are naturally exposed and therefore adapted to highly fluctuating CO₂ concentrations during tidal cycles. One main strategy in response to environmental stress is the so-called metabolic depression, the reduction of aerobic metabolism below the resting rate under control conditions (e.g. review by Guppy et al. 1994). Metabolic depression can be induced by extracellular acidosis (Guppy et al. 1994, Reipschläger and Pörtner 1996, Pörtner, Reipschläger, et al. 1998, Michaelidis et al. 2005). However, this strategy is beneficial only during temporary hypercapnia and can be harmful or even lethal in the long term. In *Mytilus galloprovincialis*, for example, aerobic metabolism was permanently depressed under long-term severe hypercapnia (~5,000 µatm) resulting in an extensive reduction of growth capacity (Michaelidis et al. 2005). Above all, the physiological response of a species also depends on the CO₂ concentration, making general predictions of animal performance and associated changes at the ecosystem level even more difficult. Thus, the underlying mechanisms determining CO₂ resilience needs to be identified with respect to different modes of life and latitudinal differences.

1.3 Combined effects of temperature and CO₂ stress

Marine species and ecosystems have to cope with various climate-related drivers and their potential interactions, which can exacerbate the threat of a single factor. Anthropogenic CO₂ emission poses two of the major threats to global biodiversity and ecosystem functioning. CO₂ is a primary greenhouse gas and its enrichment in the atmosphere causes global warming that already leads to a poleward shift of species distribution and associated ecosystem changes (described in section 1.1). At the same time, CO₂ released into the atmosphere equilibrates with the sea surface causing ocean acidification, which has various impacts on the physiology of marine ectotherms (described in section 1.2). Both factors temperature and CO₂ are identified as the main ocean drivers of present climate change. For realistic predictions of the consequences for ecosystem structures and functioning their combined effects must be considered. Yet, the combined impacts on marine organisms are insufficiently understood.

Previous studies of climate-induced impacts on the physiological performance of marine ectotherms mainly focused on individual factors rather than on combinations and have not considered the capacity of species to adapt to global climate change (hereafter this term refers to ocean warming and acidification). Both CO₂ and temperature individually affect the energy
expenditure of marine ectotherms with consequences for aerobic scope and performance. Therefore, the OCLTT concept (see section 1.1) was extended in such a way that acute CO₂ stress enhances the thermal sensitivity of the organism (Pörtner 2010, 2012). In line with this assumption, the few studies available on combined drivers mainly revealed exacerbated impacts on animal aerobic scope and performance when warming was combined with hypercapnia (Burke 1979, Metzger et al. 2007, Rosa and Seibel 2008, Walther et al. 2009, Lannig et al. 2010, Sheppard Brennand et al. 2010, Schalkhausser et al. 2013). For instance, simultaneous exposure adversely affected activity level, growth performance, larval development and tolerance to extreme temperatures in marine ectotherms. In decapod crustaceans hypercapnia induced a narrowing of the thermal window by a downward shift of upper thermal limits, which coincided with reduced haemolymph oxygen content and cardiac performance (Metzger et al. 2007, Walther et al. 2009).

Constraints on aerobic scope and performance induced by global climate change are likely exacerbated in species and populations, which exhibit low functional capacities such as polar or deep-sea organisms (Pörtner 2001, Seibel and Walsh 2001, 2003, Pörtner et al. 2009). Considering that the rate of both ocean warming and acidification is considerably greater at high latitudes polar species and ecosystems are likely under severe threat in the near future. To gain a comprehensive insight on climate-induced impacts at the population and ecosystem level trade-offs in adaptation processes and in organismic energy budgets of multiple species and populations from distinct geographic zones need to be evaluated in more detail under combined key drivers. With regard to the additional challenge for calcifying organisms, the present work will focus on CO₂ impacts on thermal tolerance and animal performance from (sub) polar vs. boreal specimens of marine calcifiers to deepen our understanding of which species and populations are most likely future winners and losers.

1.4 Calcifying model species: *Hyas araneus* and *Mytilus edulis*

Decapod crustaceans and mussels have a high ecological and economical value. They account for several thousand tonnes of the global shellfish production per year. In ecosystems, they are important constituents influencing trophodynamics and species assemblages. Negative impacts of climate change on these groups could have enormous ecologic and economic consequences. The focus of the present study is on two ecologically relevant, calcifying species from different taxonomic groups, the spider crab *Hyas araneus* (Linnaeus, 1758) and the blue
mussel *Mytilus edulis* (Linnaeus, 1758). Both species are widely distributed along a latitudinal gradient, they live in diverse environmental conditions and exhibit various physiological mechanisms to cope with the ambient challenges. Thus, they are ideal model species to identify potential physiological variation in climatic tolerance at the population level.

1.4.1 *H. araneus*

Crustacea is one of the most dominant and widespread taxon in the oceans (Martin and Davis 2001). The spider crab *Hyas araneus* belongs to the order Decapoda (Fig. 6), the most species-rich order of crustacean with nearly 15,000 extant species that are ecologically and economically important (Wickins and Lee 2002, De Grave et al. 2009). This taxon includes crabs, shrimp, lobsters and crayfish, that are of great economic value as seafood.

![Fig. 6 Spider crab *Hyas araneus* from around Svalbard, Arctic (photo credit: Z. Zittier).](image)

They are primary and secondary consumers and serve as prey for a variety of animals thereby playing a crucial role in controlling the energy flow in diverse ecosystems (e.g. Welsh 1975, Coull and Bell 1983, Field 1983). Due to their ecological importance and wide distribution, decapod crustacean are suitable model species for a broad field in biological research including studies on ocean acidification and in combination with other factors (e.g. Pane and Barry 2007, Small et al. 2010, Walther et al. 2010, Dissanayake and Ishimatsu 2011). In general, decapod crustacean are seen as good acid-base regulators and, thus, as relatively tolerant to increasing ambient CO₂ concentrations (for reviews see Melzner et al. 2009, Whiteley 2011, Wittmann and Pörtner 2013). However, regulation capacity can be highly variable and remains incomplete, especially in species adapted to cold and stable environments (Pane and Barry 2007, Whiteley 2011).
**H. araneus** is widely distributed in the North-East Atlantic and the Arctic Ocean occurring from northern France (English Channel) to Svalbard, Greenland, and Russia (Christiansen 1982, Camus et al. 2002, Avant 2003, Tavares and De Melo 2004, Weslawski et al. 2010, Kaschner et al. 2016). It lives on hard and sandy substrata in the sublittoral zone and up to a depth of 1200 m, but are most common in the upper 50 m (Weslawski et al. 1988, Camus et al. 2002). This benthic crab is a relatively inactive species with a low metabolic rate that may, thus, be vulnerable to future climate change, especially at the edge of its distribution range. The present study focuses on the northern population from Svalbard where **H. araneus** is one of the most dominant decapod (Weslawski et al. 1988, 2010). It is relevant for the ecosystem acting as a scavenger as well as an active predator and serves as a food source for a variety of organisms such as gulls, ducks, seals, and sharks (Dyer 1985, Weslawski et al. 1988, Nickell and Moore 1992, Leclerc et al. 2012).

Around Svalbard, the species experiences temperatures ranging from an average of -1.8° in winter to 6°C in summer (Camus et al. 2002, Svendsen et al. 2002, Mark 2015). For comparison, at its southern range limit in the North Sea ambient temperatures vary seasonally between 3° and 21°C (Wiltshire and Manly 2004). The Svalbard population of this slow-moving, low power species may exhibit an enhanced cold tolerance that in turn lowers their heat tolerance and may, thus, be especially vulnerable to climate change.

1.4.2 *M. edulis*

*Mytilus* (Mollusca, Bivalvia, Mytiloida) is one of the most cosmopolitan genera in the marine realm dominating many coastal ecosystems in temperate waters of the northern and southern hemisphere (Seed 1992, Gosling 2003). It is found in the intertidal and subtidal zone from fully saline to estuarine conditions living sedentary on rocky shores through semi-consolidated sediments attached by byssus threads to stones, other mussels, or any suitable substratum (Gosling 1992, Seed 1992).

*Mytilus spp.* has a tremendous ecological importance, it is a benthic keystone species and an important ecological engineer forming dense mussel beds that increases species richness and influences nutrient and mineral cycling. Mussel beds are highly productive and complex, they provide an important substratum for numerous epibionts and offer food, and shelter for a diverse community of organisms. Thus, mussel beds contribute greatly to coastal ecosystem structure and functioning (e.g. Suchanek 1985, Seed and Suchanek 1992, Saier 2002, Gosling 2003, Fly et al. 2015). As suspension feeders mussels have a diverse diet consisting of phyto- bacterio-,
INTRODUCTION

zooplankton, detritus and dissolved organic matter. Filtered particles that are not ingested are bound in mucus and expelled as pseudofaeces (Dame and Dankers 1988, Mann 1988, Langdon and Newell 1990, Davenport et al. 2000). Mussels extensively enhance removal and sedimentation of inorganic and organic particles of the water column. Their biodeposits promote microbial remineralisation contributing to recycling of nutrients required by primary producers, thus enhancing benthic-pelagic coupling (e.g. Nixon 1981, Kautsky and Evans 1987, Dame and Dankers 1988, Asmus and Asmus 1993). Mussels itself are an important food source for various animals such as snails, sea urchins, sea stars, crabs, fish and birds. Also, due to its sedentary and suspension feeding lifestyle, *Mytilus* spp. is widely used as an indicator organism for monitoring environmental quality (Bayne et al. 1985, Salazar and Salazar 1996, Dondero et al. 2006).

![Common mussel bed](https://example.com/mussel-bed.jpg)

**Fig. 7** Common mussel bed (photo credit: D. Burton / naturepl.com).

Besides their enormous ecological importance, mussels have a great relevance for the global shellfish economy. Mussels have been harvested for centuries and mussel culture dates back to over 2,000 years (MacKenzie et al. 1997, Smaal 2002 Edwards 1997, Smaal 2002). During the last century the landing of mussels grew drastically and aquaculture became increasingly important (Gosling 2003, FAO 2017). Today, the annual global harvest is around 1.8 million tonnes with a value of approx. 3.3 billion US dollars of which the European production accounts for nearly 50% (Smaal 2002, Kerton and Yan 2017).
Due to their sedentary mode of life in the sub- and intertidal as well as their widespread distribution, mussels are naturally exposed to large fluctuations in abiotic factors such as temperature, concentrations of $O_2$ and $CO_2$, salinity and air exposure. Hence, exhibit a high tolerance to a wide range of environmental conditions (see e.g. reviews by Gosling 1992, 2003). However, recent meta-analyses on the sensitivity to ocean acidification of marine ectotherms consider bivalves as particularly vulnerable as they build heavy calcareous shells and exhibit a low metabolic rate, are relatively inactive, and are weak acid-base regulators (Melzner et al. 2009, Hendriks et al. 2010, Wittmann and Pörtner 2013). Future environmental conditions may be more detrimental for populations from the latitudinal range edges where they live close to their tolerance limits and may show a limited physiological plasticity.

Of the Mytilus genus, *M. edulis* is the most prominent and widespread representative found throughout the Atlantic and the East Pacific (Gosling 2003, Berge et al. 2006). At the European coast three lineages of *Mytilus* species exist: *M. edulis*, *M. galloprovincialis*, and *M. trossulus* (McDonald and Koehn 1988, Seed 1992, Bierne et al. 2003, Väinölä and Strelkov 2011, Śmietanka et al. 2013). The distribution of the major taxon *M. edulis* ranges from the White Sea to the Biscay coast of France (Berge et al. 2006, Śmietanka et al. 2009, Väinölä and Strelkov 2011, Hilbish et al. 2012). At the southern range edge, from the British Isles to France, it occurs sympatric with *M. galloprovincialis*, which extents towards warmer waters and inhabits the Mediterranean Sea (Bierne et al. 2003, Hilbish et al. 2012). An overlap of the range of *M. edulis* also exists with *M. trossulus*, which colonises the brackish waters along the Norwegian and Russian coast and occupies the Baltic Sea (Väinölä and Strelkov 2011). In regions where these closely related *Mytilus* species coexist hybridization and gene flow occurs (Seed 1992, Bierne et al. 2003, Śmietanka et al. 2009, 2013, Hilbish et al. 2012). However, pure populations of *M.*
edulis are considered to inhabit the investigation areas of the North Sea and the White Sea of the present thesis (Śmietanka et al. 2009, Väinölä and Strelkov 2011). In the North Sea, mean sea surface temperature is around 4°C during winter and 18°C in summer (Wiltshire and Manly 2004, Wiltshire et al. 2008, Federal Maritime and Hydrographic Agency (BSH) 2016). Average sea surface temperature in the White Sea is –1°C in winter and 15°C in summer (Sukhotin and Berger 2013, Usov et al. 2013). Thus, the subarctic White Sea population might show an enhanced cold tolerance and associated enhanced sensitivity to environmental change.

1.5 Aims of the thesis

The survival and distribution of species at the population level in a changing environment will depend on their ability to cope with this challenge by metabolic and other adjustments. Little is known about the future physiological performance of marine invertebrates, especially of (sub)polar species under global climate change (ocean warming and acidification), and hardly any study examined the thermal window and associated performance capacity on the basis of the OCLTT concept (see section 1.1) in bivalves so far. The focus of the present thesis lies on the physiological response to climate change by marine calcifiers with different modes of life, i.e. active crustaceans considered to have a high metabolic rate and acid-base regulation capacity and bivalve molluscs with low metabolic rates and capacities for pH regulation. The comparative approach contributes to our understanding of the physiological impacts of global climate change on marine organisms from the cellular up to whole animal level with regard to potential differences between polar and temperate species.

Working with the Arctic population of the spider crab Hyas araneus and a sub polar as well as a temperate population of the blue mussel Mytilus edulis as model organisms, the following questions were addressed:

a) What is the effect of ocean warming and acidification on acid-base regulation capacity and associated performance in Arctic H. araneus?

To investigate the effect of temperature on CO₂ sensitivity and associated acid-base balance and activity capacity in Artic H. araneus haemolymph oxygen content, acid-base parameters and the anaerobic metabolite lactate as well as the righting response were determined under different treatment conditions. The effects of various CO₂ concentrations at two temperatures (spring vs.
summer) on the righting response were measured at acclimation temperatures and at a subsequent acute heat exposure. At resting conditions as well as after the combined stress of righting and heat exposure acid-base status as well as oxygen and lactate content were determined in haemolymph. It was hypothesized that increasing oceanic CO₂ levels impair the capacities for acid-base regulation and impair activity performance, especially at the limits of thermal tolerance (Publication 1).

b) What are the differences in the species response to climate change of polar versus boreal decapod crustaceans?

Crustaceans are considered to be good acid-base regulators, yet pH compensation occurs to various degrees. Further, stenothermal polar species exhibit lower functional capacities of many physiological processes than their eurythermal counterparts with potential impairment of acid-base regulation ability. Polar populations of eurythermal species like *H. araneus* might also show features of cold adaptation resulting in a higher sensitivity to climate change. To confirm this assumption the results from the present study (Publication 1) were compared to findings in *H. araneus* populations from boreal areas and discussed with existing literature on crustaceans.

c) What are the thermal limits and associated performance capacities in *M. edulis* from different latitudes? Do they reflect the thresholds defined by the OCLTT concept?

These questions were addressed by determining and comparing the thermal limits and acid-base regulation capacity in *M. edulis* populations from the North Sea (temperate; Publication 2) and the White Sea (sub polar; Publication 3) by the use of the OCLTT concept. Aerobic and anaerobic metabolism, cardiac performance and acid-base status of extra- and intracellular as well as extrapallial fluids were determined during acute warming. It is hypothesized that the sub polar population is more thermally vulnerable due to potential adaptation to a colder and more stable environment resulting in lower functional capacities.

d) How does CO₂ exposure modulate the response to warming in *M. edulis*? Do differences occur in populations along a latitudinal cline?

The aim of this approach was to elucidate the effect of CO₂ on oxygen- and capacity limited thermal tolerance windows and the underlying mechanisms in *M. edulis* from different latitudes.
The change of energy expenditure and acid-base regulation capacity in response to acute warming (see above) was determined under realistic CO$_2$ scenarios. As shown for other marine taxa, a downward shift of upper thermal limits with increasing CO$_2$ level is proposed. Further, a main strategy of intertidal organisms to transient environmental changes (e.g. during low tide cycles) is metabolic depression, which is assumed to set in earlier with increasing CO$_2$ level (Publication 3).
2 Materials & Methods

The following experiments were conducted to increase our knowledge of climate-induced impacts on the physiology and associated animal performance of calcifying organisms with regard to acid-base regulation ability and latitudinal differences. The interaction of warming and ocean acidification was studied in the Arctic population of the great spider crab *Hyas araneus* (see Publication 1). Decapod crustaceans are considered to be good acid-base regulators but temperature species may show functional constraints at high latitudes. The role of latitude, ambient temperature variability as well as ocean acidification was analysed in different populations of blue mussels *Mytilus edulis* (see Publication 2 and 3) with a low metabolic rate and pH regulation capacity. An overview of populations used, experimental conditions applied and parameters determined in the present thesis is provided in Table 1. For detailed descriptions please refer to the respective publication.

| Table 1 Overview of species, populations and experiments used in the present thesis. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Species Population Incubation conditions | Acute temperature exposure | Measured parameters |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| *Hyas araneus* Svalbard 1 390 750 1,120 3,000 | 12 (15 min) | Resting at incubation conditions: haemolymph PO$_2$, CCO$_2$, pH, and lactate next day: righting response |
| 4 | | Acute heat exposure: righting response and subsequently haemolymph PO$_2$, CCO$_2$, pH, and lactate |
| *Mytilus edulis* North Sea 10 390 750 1,120 1,120 | 10 – 31 (3°C/night) | haemolymph PO$_2$, PCO$_2$, CCO$_2$ and pH; MO$_2$, heart rate, mantle succinate |
| | | | | |
| White Sea 390 750 1,120 1,120 | 10 – 28 (3°C/night) | haemolymph PO$_2$, PCO$_2$, CCO$_2$ and pH; MO$_2$, intracellular (mantle) pH, PCO$_2$, and succinate |
MATERIALS & METHODS

2.1 Hyas araneus

2.1.1 Animal collection and maintenance

Adult spider crabs, Hyas araneus (carapace size = 3.2 - 5.9 cm), were collected by scientific divers in the Kongsfjorden, Svalbard (78°55´N, 11°56´E) in April - May 2009 during the European Project on Ocean Acidification (EPOCA) Arctic Campaign. Crabs were transferred to the Kings Bay Marine Laboratory in Ny-Ålesund and kept in flow-through aquaria with direct seawater supply from the fjord (0°C, 34.5 psu). They were fed daily with a diet of frozen fish. Feeding was terminated at the start of experimentation to exclude the metabolic effect of food (Widdows 1973a, for a review see Whiteley et al. 2001). Crabs were randomly placed into one of eight experimental tanks and incubated for 12 days under various temperatures and CO₂ concentrations (see Table 1). Only hard-shelled intermoult crabs were used in the experiment.

2.1.2 Incubation and experimental set up

Within the EPOCA Arctic Campaign 2009, seawater from the fjord (from 80 m depth) was pumped in two large reservoir tanks and temperature adjusted to 0.5° (spring) and 4°C (summer; Svendsen et al. 2002), respectively, before being forwarded to several header tanks providing the different CO₂ treatment conditions. A full factorial design was applied for the present study. According to projected CO₂ scenarios (today: 380, towards the year of 2100: 750 and 1,120 and beyond: 3,000 µatm), seawater CO₂ of header tanks was adjusted by bubbling with CO₂ regulated via a pH stat system (Aqua Digital pH-201) (see Fig. 9). The water supply to experimental animal tanks was designed as a flow through system to provide stable CO₂ conditions. Temperature (T), salinity (S), pH, dissolved inorganic carbon (DIC) and total alkalinity (TA) were measured in the header tanks at least every other day. Measurements were carried out using a salinometer (WTW LF197 combination temperature and salinity probe) and a pH meter (Metrohm, 826 pH mobile), which was calibrated on the total scale (pH_total) using Tris/HCl and 2-aminopyridine/HCl buffer solutions. TA was analysed by potentiometric titration (Metrohm Titrand 80) and calculated using a Gran function. DIC was determined using an AIRICA analyser (Marianda, Kiel) calibrated with samples of standard seawater (batch 90). The carbonate chemistry (Table 2) was calculated with the R package seacarb (Lavigne et al. 2008) using TA and either pH or DIC. For a more detailed description of the EPOCA Arctic Campaign set up, see Findlay et al. (2010), for monitoring and calculating the seawater carbonate
MATERIALS & METHODS

chemistry, see Comeau et al. (2009) and for a detailed list of obtained data, see PANGAEA datasets (EPOCA 2009).

An additional 12°C-experimental tank was used to test the response of crabs to acute heat exposure. Temperature was controlled by a thermostat (LAUDA RK 20KS, Lauda-Königshofen) and CO₂ concentrations were set by an MKS mass flow controller (MKS Instruments Deutschland GmbH, München). Seawater pH was measured using a Mettler-Toledo pH meter calibrated to the NBS (National Bureau of Standards) scale (pH_{NBS}) and DIC was determined in 100 µl samples by a CO₂ Analyser (Corning 965, Olympic Analytical Service, Malvern, Worcestershire) calibrated with a serial dilution of a 1 gL⁻¹ CO₂ standard solution (Reagecon Diagnostics Limited, Shannon). Seawater $PCO₂$ was calculated based on measured temperature,
salinity, pH<sub>NBS</sub>, and DIC using the software CO2SYS (Pierrot et al. 2006 using the equilibrium constants of Mehrbach et al. (1973) for the CO<sub>2</sub> / bicarbonate / carbonate system, as refitted by Dickson and Millero (1987) (Table 2).

Table 2 Carbonate chemistry of seawater during the incubation of Arctic spider crab <i>Hyas aranea</i> (<i>1</i>° and <i>4</i>°C) and heat stress experiments (<i>12</i>°C) at different CO<sub>2</sub> concentrations. Data are means ± SE except for the <i>12</i>°C-tank in which values were only measured once because of the short period of its use.

<table>
<thead>
<tr>
<th>PCO&lt;sub&gt;2&lt;/sub&gt; µatm (set)</th>
<th>T °C (set)</th>
<th>pH</th>
<th>PCO&lt;sub&gt;2&lt;/sub&gt; µatm</th>
<th>T °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>380</td>
<td>1</td>
<td>8.08 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>351 ± 10</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>750</td>
<td>1</td>
<td>7.74 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>825 ± 49</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>1,120</td>
<td>1</td>
<td>7.57 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,249 ± 65</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>3,000</td>
<td>1</td>
<td>7.22 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,859 ± 160</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>380</td>
<td>4</td>
<td>8.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>387 ± 18</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>750</td>
<td>4</td>
<td>7.76 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>834 ± 77</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>1,120</td>
<td>4</td>
<td>7.59 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,215 ± 68</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>3,000</td>
<td>4</td>
<td>7.20 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3,094 ± 206</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>380</td>
<td>12</td>
<td>8.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>417</td>
<td>12.2</td>
</tr>
<tr>
<td>750</td>
<td>12</td>
<td>7.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>620</td>
<td>12.5</td>
</tr>
<tr>
<td>1,120</td>
<td>12</td>
<td>7.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>893</td>
<td>12.0</td>
</tr>
<tr>
<td>3,000</td>
<td>12</td>
<td>7.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2539</td>
<td>12.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> = total scale, <sup>b</sup> = NBS (National Bureau of Standards) scale.

2.1.3 Preparation of animals and experimental protocol

At the end of the incubation period (12 days), prebranchial (venous) haemolymph was taken to determine the differences in oxygen and acid-base status of the body fluids of crabs under the various conditions (four CO<sub>2</sub> levels at two temperatures). Samples were drawn anaerobically from the third or fourth walking leg via a surgical needle inserted through the articular membrane of the carpus and either measured immediately (for acid-base parameters and oxygen content) or prepared for further analyses of lactate concentrations as the main anaerobic end product in crustaceans (Albert and Ellington 1985, Gäde and Meinardus-Hager 1986, Spicer et al. 1990). The values were used as a baseline of oxygen and acid-base status for each treatment and
compared to values obtained after the functional challenge of two righting trials and acute heat stress. Crabs were allowed to recover from the first sampling stress for one day, before being subjected to consecutive stressors of (i) righting at the respective incubation treatment (all CO₂ levels at 1° and 4°C) followed by (ii) a 15 min acute heat exposure to 12°C under the respective CO₂ levels and (iii) a second righting trail in the heat before haemolymph was re-sampled.

2.1.4 Righting response

Activity capacity was determined via the righting response, the time an individual requires to right itself after being turned onto its dorsal surface (Cowles and Bogert 1944). One righting trial lasted 5 min with crabs being turned onto their carapace once every 30 s. In case the righting response lasted longer than a 30-s period, the next turn was started at the beginning of the following interval once they have righted themselves. The duration of righting periods of each individual were summed. In case an animal stopped righting during a trial, the residual time span was added to the duration of righting periods. With this procedure, an animal that stopped righting will have a higher value (= slower response) than an animal that kept righting throughout the righting trial even when compared to very slow ones. Obtained datasets of individual righting periods were also used to test if fatigue (continuous slowing) occurred during one trial due to the energy demanding behaviour. Fatigue, however, was not confirmed.

2.1.5 Haemolymph parameters

Haemolymph PO₂, pH and total CO₂ concentration (CCO₂) were analysed immediately after sampling. PO₂ was determined using optodes (TX-3, PreSens GmbH, Regensburg) calibrated in oxygen-free (0%, nitrogen-bubbled) and air-saturated (100%) seawater at the respective temperature. Values given as % air saturation were converted to PO₂ after equation Eq. 2

\[
PO_2 (kPa) = (Patm - PH2O) \times 0.2095 \times (% \text{ air saturation}) / 100 \quad \text{Eq. 2}
\]

where \(Patm\) is the atmospheric pressure (kPa), \(PH2O\) is the temperature-specific water vapour pressure (kPa), calculated after Dejours (1975), and 0.2095 is the proportion of oxygen content in the air. Haemolymph pH measurements were carried out using pH micro glass electrodes (Mettler Toledo InLab micro) calibrated with Radiometer Analytical precision buffers (pH 6.865 and pH 7.413) at the specific experimental temperatures. Haemolymph CCO₂ concentration was determined in 100 µL subsamples using a Corning CO₂ Analyser (see section 2.1.2). Based on
measured parameters haemolymph $PCO_2$ was calculated using the modified version of the Henderson–Hasselbalch equation (Eq. 3).

$$PCO_2 \text{ (kPa)} = \frac{C_{CO_2}}{(10^{pH-pK} \times \alpha_{CO_2} + \alpha_{CO_2})} \quad \text{Eq. 3}$$

where $pK$ is the apparent dissociation constant, and $\alpha_{CO_2}$ is the solubility of CO$_2$ in haemolymph, both calculated after Heisler (1986). Haemolymph bicarbonate was then calculated as follows:

$$[HCO_3^-] \text{ (mM)} = C_{CO_2} - \alpha_{CO_2} \times PCO_2 \quad \text{Eq. 4}$$

For determination of the anaerobic end product lactate, freshly collected haemolymph samples were supplemented 1:1 with ice-cold 15% Trichloracetic Acid (TCA) and stored for 10 min on ice before centrifugation (2 min, 0°C, 10,000 ×g). The supernatant was stored at –80°C until lactate concentration was determined enzymatically using photometric standard tests (Bergmeyer 1985) back at the AWI.

### 2.1.6 Statistical analysis

Results were first checked for outliers using Nalimov’s test (Noack 1980) before data processing was performed. InStat 3.0b and Prism 4.0c (GraphPad Software, Inc.) were used to employ the following tests: Kolmogorov–Smirnov test for normality, one-way analysis of variance (ANOVA) in combination with a Student-Newman-Keuls post hoc test for normally distributed data and nonparametric Kruskal-Wallis and subsequent Dunn’s Multiple Comparisons tests were used when normality was not fulfilled. In R clustering of data due to temperature and CO$_2$ levels was analysed with a principal component analysis and the effect of fatigue within a righting trail of each individual was tested via a Pearson-correlation one-sided test. See Publication 1 for more details. All differences were considered significant if $P < 0.05$. Values are presented as mean values ± standard error (SE).

### 2.2 Mytilus edulis

#### 2.2.1 Animal collection and maintenance

Experimental animals originated from different *Mytilus* populations collected in the wild. Adult mussels (50 to 90 mm shell length) were collected from the subtidal zone of the respective
geographic region. Specimens of two *Mytilus edulis* populations, from the temperate North Sea around Helgoland (54°N), German Bight (June 2009) and the subarctic White Sea near the Kartesh Marine Station (66°N), Russia (July 2011) were transported to the aquarium facilities of the Alfred-Wegener-Institute Helmholtz Centre for Polar and Marine Research (AWI, Bremerhaven, Germany) (Publication 2 and 3, respectively).

Mussels were separated, cleaned from epibionts and maintained in flow-through aquaria with aerated and filtered natural seawater at 10°C and 32 psu for at least one month. Afterwards, mussels were randomly placed in one of four tanks per group and incubated for 2 weeks under different CO2 concentrations (see Table 1). During the acclimation as well as the incubation period, mussels were fed daily ad libitum with freshly hatched *Artemia* larvae and a commercial living algal blend containing *Nannochloropsis*, *Phelodactylum tricornutum* and Chlorella (DT’s Live Marine Phytoplankton, Coralsands, Germany). Feeding was terminated three days before experimentation to eliminate the impact of specific dynamic actions (SDA) on metabolic rates (Widdows 1973a, Gaffney and Diehl 1986). All animal tanks (acclimation, incubation and experimental tanks) were cleaned daily from faeces and pseudo-faeces.

2.2.2 Incubation and experimental set up

Recirculating incubation systems (see Fig. 10) were set up in a temperature control room (10°C) using several reservoirs (450 L) and header tanks (210 L) to provide different CO2 treatment conditions (today: 390 and towards the year 2100: 750 and 1,120 µatm). Water in both tanks was continuously bubbled with the respective air-CO2 mixture via a HTK gas system (HTK Hamburg GmbH, Germany) and circulated between them. In each system, water was supplied from the header tank to the animal tanks (each 15 L, flow through rate ~120 mL min⁻¹), then collected in a basin (210 L, continuously bubbled with the respective air-CO2 mixture) and re-circulated to the reservoir. To keep concentrations of ammonium and nitrite below 0.2 mg L⁻¹, water was exchanged twice a week by disconnecting and subsequently refilling and equilibrating the reservoir for 24 h, while the water from the basin was re-circulated into the header tank.

For experimentation, two animal tanks (80 L, starting with max. 39 animals) and a reservoir (for water exchange) were continuously bubbled with the respective air-CO2 mixture (see Table 1) produced by a MKS system and water temperatures were feedback controlled by a thermostat (LAUDA RP 845, Lauda-Königshofen). Two-thirds of the water were exchanged daily before each temperature rise.
Water chemistry in all animal tanks (incubation, experimental and reservoirs tanks for water changes) was calculated daily via the respective temperature, salinity, pH, and TA or DIC. Measurements were carried out using a salinometer (WTW LF197 combination temperature and salinity probe) and a pH meter (Mettler-Toledo) calibrated at the respective temperature to NBS scale. TA was analysed by potentiometric titration (METROHM Prozessanalytik GmbH & Co, Germany) in experiments with North Sea mussels. In accordance with the latest best practices, in experiments with White Sea mussels DIC instead of TA was analysed by using Seal Analysis SFA QuAAtro; pump Technicon trAAcs 800 TM and measured seawater pH was converted into total scale via measurements of Dickson standards. Seawater $P_{CO_2}$ (incubation: Table 3, experimentation: Table 4) was calculated with CO2SYS as described above (see section 2.1.2).

Fig. 10 Experimental set up for the incubation of $M.\ edulis$. A) Reservoir, B) header tank, C) animal tanks, and D) basein (for more details see text; photo credit: Z. Zittier).
Table 3 Carbonate chemistry of seawater during the incubation of blue mussels, *M. edulis* populations at different CO$_2$ concentrations.

<table>
<thead>
<tr>
<th>Population</th>
<th>PCO$_2$ µatm (set)</th>
<th>T °C (set)</th>
<th>pH</th>
<th>PCO$_2$ µatm</th>
<th>T °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Sea</td>
<td>390</td>
<td>10</td>
<td>8.14 ± 0.03$^a$</td>
<td>443 ± 30</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>10</td>
<td>7.91 ± 0.02$^a$</td>
<td>758 ± 74</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1,120</td>
<td>10</td>
<td>7.81 ± 0.02$^a$</td>
<td>1,037 ± 93</td>
<td>9.7 ± 0.1</td>
</tr>
<tr>
<td>White Sea</td>
<td>390</td>
<td>10</td>
<td>8.04 ± 0.06$^b$</td>
<td>413 ± 53</td>
<td>10.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1,120</td>
<td>10</td>
<td>7.65 ± 0.04$^b$</td>
<td>1,106 ± 111</td>
<td>10.3 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ = NBS (National Bureau of Standards) scale, $^b$ = total scale.

Table 4 Carbonate chemistry of seawater during the experimentation with blue mussels, *M. edulis* populations (acute warming protocol, 3°C/night) at different CO$_2$ concentrations.

<table>
<thead>
<tr>
<th>Population</th>
<th>390 µatm</th>
<th>750 µatm</th>
<th>1,120 µatm</th>
</tr>
</thead>
<tbody>
<tr>
<td>T °C (set)</td>
<td>T °C</td>
<td>pH</td>
<td>PCO$_2$ µatm</td>
</tr>
<tr>
<td>North Sea</td>
<td>10</td>
<td>10.1</td>
<td>8.35$^a$</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>12.8</td>
<td>8.20$^a$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16.3</td>
<td>8.19$^a$</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>18.9</td>
<td>8.20$^a$</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>22.0</td>
<td>8.15$^a$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.9</td>
<td>8.05$^a$</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>27.8</td>
<td>8.11$^a$</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>30.9</td>
<td>8.09$^a$</td>
</tr>
<tr>
<td>White Sea</td>
<td>10</td>
<td>10.1</td>
<td>8.35$^b$</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>12.8</td>
<td>8.20$^b$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>16.3</td>
<td>8.19$^b$</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>18.9</td>
<td>8.20$^b$</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>22.0</td>
<td>8.15$^b$</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>24.9</td>
<td>8.05$^b$</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>27.8</td>
<td>8.11$^b$</td>
</tr>
</tbody>
</table>

$^a$ = NBS (National Bureau of Standards) scale, $^b$ = total scale.
2.2.3 Preparation of animals and experimental protocol

After the incubation period (2 weeks) mussels were transferred to the experimental set up. Eight mussels per treatment were used for parallel measurements of respiration and heart rate. For this approach each experimental tank was equipped four respiration chambers. Mussels were equipped with an integrated plethysmograph infrared sensor (Vishay Semiconductors, CNY70) for non-invasive heart rate recordings. Shell thickness was reduced by grinding, and the photosensor was fixed above the pericardial cavity with cyanacrylate glue and dental wax before mussels were placed in the respective respiration chamber. Recordings of respiration and heart rate were started immediately to observe when mussels had recovered from handling stress (stable recordings). Oxygen consumption (MO₂) and heart rate were determined under resting conditions throughout the applied warming protocol (see below). Several heart recordings proved unreliable due to strong interferences with the grounding of the cooling room electricity and could not be used for further analyses. Other mussels of the respective treatment used for the sampling of tissue and body fluids were randomly distributed across the two experimental tanks and left undisturbed over night. Note that in contrast to MO₂ and heart rate, were same specimens were recorded throughout the experiment, results of other parameters were obtained from different individuals during the warming protocol.

Measurements were started at the acclimation temperature of 10°C, water temperature was then increased overnight by 3°C up to a maximum of 31°C depending of the studied population (for an overview see Table 1, for more details see respective publication). MO₂ and heart rate were determined and body fluids as well as tissue samples were collected during daytime after each temperature step.

Body fluids were sampled anaerobically by gently prying the shell open and, firstly, carefully penetrating the posterior adductor muscle with a surgical needle to draw haemolymph before extrapallial fluid was sampled with a long (8 cm) needle gently inserted between the shell and the pallial attachment of the mantle. Both fluids were immediately analysed for acid-base parameters and gas concentrations (see below). Within 10 min after removal from the aquaria mussels were dissected and mantle tissue was excised, freeze-clamped immediately and stored in liquid nitrogen until further analysis.
2.2.4 Oxygen consumption and heart rate

Oxygen consumption rate was measured using flow-through respirometry with oxygen saturation of the chamber kept at levels above 80% to prevent possible oxygen deprivation effects. After experimentation, mussels were dissected and shell-free dry weight (DW) was determined. MO2 was determined using fiber optic oxygen microoptodes with integrated temperature compensation (TX-3, PreSens GmbH, Regensburg) calibrated in oxygen free (0%, using nitrogen-bubbled seawater or saturated ascorbic acid solution) and air-saturated (100%) seawater at the incubation temperature of 10°C. During the warming protocol the 100%-oxygen values were checked daily to compensate for temperature and hardware drifts. Once drifts by more than 2% were detected optodes were recalibrated at the respective temperature and recorded data were corrected. Parallel-performed blank measurements revealed that background microbial respiration was negligible in this approach likely due to the daily cleaning of respiration chambers. Values were converted to \( P_{O_2} \) (kPa) after equation Eq. 2 (see section 2.1.4) and MO2 was calculated as follows:

\[
MO_2 \text{ (} \mu \text{mol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}) = \frac{(\Delta P_{O_2} \times \beta_{O_2} \times V_f)}{DW} \quad \text{Eq. 5}
\]

where \( \Delta P_{O_2} \) is the difference in oxygen partial pressure between inflowing and outflowing water (kPa), \( \beta_{O_2} \) is the temperature-specific oxygen capacity of water (\( \mu \text{mol O}_2 \text{ L}^{-1} \text{ kPa}^{-1} \)), \( V_f \) is the flow rate (L h\(^{-1}\)) and DW is the shell-free dry weight (g) of the mussel.

Heart rate signals were amplified and digitally recorded using a PowerLab system with Chart v4.1.1 Software (AD Instruments, Spechbach, Germany). Averaged resting heart rate was determined at each temperature by counting the peaks expressed as beats per minute (bpm) over 30 - 90 s intervals within a 3 h period.

2.2.5 Body fluids and tissue samples

Body fluids (haemolymph and extrapallial fluid) were analysed immediately for acid-base parameters and gas concentrations. Extracellular \( P_{O_2} \), \( P_{CO_2} \) (Torr) and pH (NBS scale) were measured using a blood gas analyser from Eschweiler (MT 33, Germany) calibrated at the specific experimental temperature. \( C_{CO_2} \) of body fluids was analysed by gas chromatography (6890N GC System, Agilent Technologies, USA) and bicarbonate concentration was then calculated as described above (Eq. 4, see section 2.1.4).

Mantle tissue samples stored in liquid nitrogen were used to determine anaerobic metabolite concentrations and, in case of White Sea mussels, to measure intracellular pH (pHi) and tissue
concentrations of CO$_2$. Frozen tissues were ground under liquid nitrogen using mortar and pestle. For anaerobic metabolite determinations, tissue powder (~300 mg) was homogenized (0°C, 360 Watt) with ice-cold 0.6 M perchloric acid (PCA) added to a vol/wt ratio of 1 to 5. Precipitated protein was removed by centrifugation (0°C, 2 min at 16,000 g). The extract was neutralized to a pH of ~7.5 by titration with ice-cold 5 M potassium hydroxide (KOH) and centrifuged again to remove precipitated potassium perchlorate. The supernatant was stored at –80°C until prepared for $^1$H-NMR spectroscopy. Samples were dried in a SpeedVac before being resolved in 500 µL D$_2$O containing 1% trimethylsilyl propionate (TSP) as internal reference and transferred to NMR tubes. Fully relaxed high-resolution $^1$H-NMR spectra were recorded using a $^1$H-broadband inverse probe or a HRMAS probe (Bruker Biospin GmbH, Germany). Spectra were post-processed automatically using TopSpin 2.5 (Bruker Biospin GmbH, Germany). After Fourier transformation, baseline and phase of spectra were adjusted. Succinate concentrations were determined by analysing the area under the succinate peak by using the integration routine in TopSpin in the North Sea experiment and by using the program Chenomx 8.1 (Chenomx Inc., Edmonton, Canada) in the White Sea experiment. Measurements of pHi were performed according to the homogenate method developed by Pörtner (1990), using 160 mM potassium fluoride (KF) and 0.1 mM nitrilotriacetic acid (NTA) for the analysis of 200-250 mg tissue. Tissue homogenate pH was determined at the specific experimental temperature using a pH optode (Needle-Type-Housing-pH-Microsensor, PreSens GmbH, Regensburg) calibrated to NBS scale and CCO$_2$ was determined by gas chromatography (Publication 3, Table 3). Intracellular $P$CO$_2$ and [HCO$_3$]$^-$ concentration (Publication 3, Table 4) were then calculated as described above.

2.2.6 Statistical analysis

Results were first checked for outliers using Nalimov’s test (Noack 1980) before further data processing was performed using InStat 3.0b and R. Data were tested for normally distribution and two-way ANOVA in combination with a Tukey’s post hoc test was performed to analyse effects of temperature and CO$_2$ level and possible interactions thereof. If normality was not fulfilled data were log-transformed prior to analysis. The breakpoint temperature indicating the discontinuity in the temperature dependence of MO$_2$ is usually determined using Arrhenius plots and the least squares method. However, such an analysis was impossible because of a limited number of test temperatures beyond the potential breakpoint, which are necessary to determine the second regression line (Sokal and Rohlf 1995). Alternatively, the phase change was
determined by a sigmoidal function that describes the exponential increase of MO$_2$ with rising temperature, and the subsequent limitation in the warmth, when the maximum curvature indicates a breakpoint temperature. MO$_2$ data were best fitted by a five-parameter logistic function. Note that a sigmoidal function is adequate for describing a limitation in the rise of the MO$_2$ rate (when the course deviates from the exponential relationship towards a plateau) and cannot take account of a decrease in rate after reaching a maximum. Therefore, respiration rates that decreased when compared to the previous temperature step were excluded from the breakpoint analyses. See Publication 2 and 3 for more details. All differences were considered significant if $P<0.05$. Values are presented as mean values ± standard deviation (SD), if not stated otherwise.
3 Publications

List of publications and authors’ contributions

Publication 1

I conceived and designed the experiment together with TH and HOP. The experiment was conducted and analysed by myself in cooperation with TH. The first draft of the manuscript was written by myself and revised together with all co-authors.

Publication 2

I developed the ideas for this experiment together with HOP. The experiment was conducted and analysed by myself with support by CB for NMR measurement. The data were interpreted by myself together with CB and GL. The first draft of the manuscript was written by myself and revised together with CB, GL and HOP.

Publication 3

I developed the ideas for this study together with HOP. The experiment was conducted and analysed by myself with support by CB for NMR measurement and by NSH for pH measurement. The first draft of the manuscript was written by myself and revised together with all co-authors.

[Note: The manuscript of Publication 3 was replaced by the published article.]
Publication 1

The synergistic effects of increasing temperature and CO₂ levels on activity capacity and acid–base balance in the spider crab, *Hyas araneus*

Zora M. C. Zittier, Timo Hirse and Hans-O. Pörtner

2013

Marine Biology

160(8): 2049-2062
The synergistic effects of increasing temperature and CO₂ levels on activity capacity and acid–base balance in the spider crab, *Hyas araneus*

Zora M. C. Zittier · Timo Hirse · Hans-O. Pörtner

Received: 27 January 2012 / Accepted: 7 September 2012 / Published online: 14 October 2012
© Springer-Verlag 2012

Abstract With global climate change, ocean warming and acidification occur concomitantly. In this study, we tested the hypothesis that increasing CO₂ levels affect the acid–base balance and reduce the activity capacity of the Arctic spider crab *Hyas araneus*, especially at the limits of thermal tolerance. Crabs were acclimated to projected oceanic CO₂ levels for 12 days (today: 380, towards the year 2100: 750 and 1,120 and beyond: 3,000 l atm) and at two temperatures (1 and 4 °C). Effects of these treatments on the righting response (RR) were determined (1) at acclimation temperatures followed by (2) righting when exposed to an additional acute (15 min) heat stress at 12 °C. Prior to (resting) and after the consecutive stresses of combined righting activity and heat exposure, acid–base status and lactate contents were measured in the haemolymph. Under resting conditions, CO₂ caused a decrease in haemolymph pH and an increase in oxygen partial pressure. Despite some buffering via an accumulation of bicarbonate, the extracellular acidosis remained uncompensated at 1 °C, a trend exacerbated when animals were acclimated to 4 °C. Prior to (resting) and after the consecutive stresses of combined righting activity and heat exposure, acid–base status and lactate contents were measured in the haemolymph. Under resting conditions, CO₂ caused a decrease in haemolymph pH and an increase in oxygen partial pressure. Despite some buffering via an accumulation of bicarbonate, the extracellular acidosis remained uncompensated at 1 °C, a trend exacerbated when animals were acclimated to 4 °C. The additional combined exposure to activity and heat had only a slight effect on blood gas and acid–base status. Righting activity in all crabs incubated at 1 and 4 °C was unaffected by elevated CO₂ levels or acute heat stress but was significantly reduced when both stressors acted synergistically. This impact was much stronger in the group acclimated at 1 °C where some individuals acclimated to high CO₂ levels stopped responding. Lactate only accumulated in the haemolymph after combined righting and heat stress. In the group acclimated to 1 °C, lactate content was highest under normocapnia and lowest at the highest CO₂ level in line with the finding that RR was largely reduced. In crabs acclimated to 4 °C, the RR was less affected by CO₂ such that activity caused lactate to increase with rising CO₂ levels. In line with the concept of oxygen and capacity limited thermal tolerance, all animals exposed to temperature extremes displayed a reduction in scope for performance, a trend exacerbated by increasing CO₂ levels. Additionally, the differences seen between cold- and warm-acclimated *H. araneus* after heat stress indicate that a small shift to higher acclimation temperatures also alleviates the response to temperature extremes, indicating a shift in the thermal tolerance window which reduces susceptibility to additional CO₂ exposure.

Introduction

Global change causes ocean warming and acidification (reduction in pH), due to the atmospheric accumulation of anthropogenic CO₂ and its penetration into the oceans. Since pre-industrial times, the partial pressure of atmospheric CO₂ (PCO₂) has already increased from 280 μatm to present-day values of around 390 μatm. The enrichment of the oceans with atmospheric CO₂ has already caused a pH reduction in surface waters by more than 0.1 units (Orr et al. 2005; Blackford and Gilbert 2007), with projected future decrements of 0.3–0.5 units (equalling ~ 600–1,000 μ atm CO₂) by 2100 under IPCC emission scenarios, reaching a potential extreme of 1.4 units by 2300 (Zeebe and Wolf-Gladrow 2001; Caldeira and Wickett 2003).
Current ecosystem changes are largely driven by temperature (e.g. Pörtner and Farrell 2008; Pörtner 2010), whereas the impact of increasing CO₂ in biotic communities is still far from clear. Ocean acidification is known to have several negative effects on marine organisms. In animals, disturbances of acid–base regulation, metabolism and reduced protein synthesis have been reported, based on shifts in energy allocation associated with reductions in somatic growth, reproduction and calcification (Pörtner 2004, 2006, 2008). Earlier research applied high CO₂ concentrations (5,000–20,000 pμatm) to elucidate the mechanisms underlying responses to CO₂ exposure in various marine environments and to potential scenarios of CO₂ disposal in the oceans (Caldeira et al. 2006). More recent efforts focused on CO₂ effects in the context of ocean acidification scenarios with the goal of realistic projections of ecosystem changes. Available results reveal highly variable CO₂ responses, even between related species of the same phylum. As a hypothesis, the degree of (extracellular) acidosis and thus the capacity of acid–base regulation have been seen as crucial in defining the resistance of marine species to elevated ambient CO₂ levels (Pörtner 2008). Furthermore, climate change confronts organisms with various combinations of stressors, like increased temperature, CO₂ and hypoxia and their potential interaction. All stressors and combinations thereof affect energy turnover and contribute to ecosystem responses with consequences for food web structures.

Crustaceans have been model species in investigating the effects of ocean acidification and the synergisms with other factors (Metzger et al. 2007; Walther et al. 2009; Small et al. 2010; Dissanayake and Ishimatsu 2011). The few studies available on the capacity of acid–base regulation in crustaceans reveal a general ability to compensate for haemolymph acid–base disturbances. However, compensation occurs to various degrees and may remain incomplete (for review, see Whiteley 2011). Efficient iono-regulators completely compensated extracellular acidosis via proton equivalent ion exchange and associated bicarbonate accumulation (P[CO₂] of 3,000–10,000 pμatm up to 30 days) (Pane and Barry 2007; Spicer et al. 2007; Dissanayake et al. 2010; Small et al. 2010). During the exposure to higher P[CO₂] levels (10,000–60,000 pμatm), compensation started to fail within days to weeks or remained incomplete, accompanied by substantial mortality (Spicer et al. 2007). In a comparative study of a shallow-water (Cancer magister) and a deep-sea crab (Chionoecetes tanneri), Pane and Barry (2007) demonstrated fast effective acid–base regulation within 24 h at 8,000 pμatm P[CO₂] in the shallow-water crab, whereas extracellular acidosis remained almost uncompensated in the deep-sea crab and even completely uncompensated when hypercapnia was combined with hypoxia (~10 % O₂ saturation). The reduced acid–base regulatory capacity in deep-sea crabs is likely due to their adaptation to a stable and cold environment and might also be seen in species from polar environments. Shifts in energy allocation due to CO₂ responses will reduce fitness and might be exacerbated in polar regions where organisms are adapted to stable environmental conditions and display lower functional capacities in many physiological processes, with the result of a high sensitivity to environmental stressors. For example, the enhanced sensitivity to thermal stress was mirrored in a downward shift and a narrowing of the thermal tolerance window in polar animals when compared to boreal species and populations (e.g. Pörtner 2001; Pörtner et al. 2009).

Earlier findings in marine invertebrates and fishes indicated that oxygen supply became limited under heat stress (Zielinski and Pörtner 1996; Sommer et al. 1997; van Dijk et al. 1999). This insight led into the development of a temperature tolerance model in a crustacean species (Frederich and Pörtner 2000), and consecutively the concept of oxygen and capacity limited thermal tolerance (OCLTT) (Pörtner 2001, 2002, 2010). According to the OCLTT concept, the capacity for oxygen supply of an animal is maximal in its optimum temperature range (T[O]) between upper and lower pejus thresholds (T[º]), with performance being maximal close to upper pejus limits. The optimum range characterizes the ecological temperature tolerance range and the earliest limits to the biogeographical range of populations as confirmed in a comparison of field and laboratory findings in fishes (Pörtner and Knust 2007). Surpassing the T[º] either during cooling or warming leads to a progressive internal oxygen deficit (hypoxaemia), resulting from a capacity limitation of oxygen supply through ventilation and circulation. A limitation in aerobic scope arises resulting in a loss of whole organism performance including decreases in activity level, reproduction and growth (physiological tolerance range of the individual). The more extreme critical temperatures (T[C]) are then characterized by the onset of anaerobic metabolism. Beyond pejus limits, tolerance and survival of an animal become progressively more time limited. It has been hypothesized that the impact of CO₂ reduces aerobic scope and the performance of organisms especially at thermal extremes and thus narrows their thermal window (Pörtner and Farrell 2008). This hypothesis is built on analyses of oxygen partial pressures in the haemolymph and changes in heart rate in the edible crab Cancer pagurus (Metzger et al. 2007) and is confirmed by findings in the spider crab Hyas araneus from the North Sea (Walther et al. 2009), in barnacles (Findlay et al. 2010b) and coral reef fishes (Munday et al. 2009).

Disturbances in performance may also affect ecologically relevant behaviours like escape from predators and
thereby affect fitness and survival. Exposure to high CO₂ levels (10,000 μatm, 10 days) led to a significant decrease in swimming ability in the shiba shrimp, *Metapenaeus joyneri*, likely due to a decrease in metabolic scope (Dis-sanayake and Ishimatsu 2011). In hypercapnia-exposed (12,000 μatm) hermit crabs, *Pagurus bernhardus*, movement and antennular flapping rate were reduced accompa-
nied by disturbances in selection behaviour for optimal gastropod shells used for protection (De la Haye et al. 2011). These effects were found under very high CO₂ levels and still have to be demonstrated to occur under realistic oceanic PCO₂ scenarios. Disturbances in behav-ioural performance may include changes in predator-prey interactions or competitiveness and thus alter biotic com-munity dynamics and food web structures (Hale et al. 2011).

Little is known about how polar crustaceans or popu-
lations are affected by the synergetic effects of ocean warming and acidification. Therefore, the present study was undertaken to investigate the combined effects of temperature and CO₂ on acid-base balance, activity capacity and associated metabolic processes and perfor-
rance in the marine spider crab, *H. araneus* (Linnaeus 1758). The cold-eurythermal spider crab is distributed in the North Atlantic from the English Channel up to Svalbard where it is one of the most prominent brachyuran crabs. Comparative findings in *H. araneus* larvae from the North Sea (54°N) and around Svalbard (79°N) revealed a decrease in development rate and survival with rising temperature in the Arctic population suggesting a trend towards features of permanent cold adaptation (Walther et al. 2010). This might involve a reduced ability to acclimatize to environmental stressors. CO₂-induced effects on acid-base balance and activity (righting) were investigated after 12 days of incubation under realistic future CO₂ scenarios at two acclimation temperatures and after acute heat stress to elucidate the ecological perfor-
mance of polar crabs in the context of global climate change.

**Methods**

**Animal collection and maintenance**

Adult spider crabs, *H. araneus* (carapace size = 3.2–5.9 cm), were collected by divers in the Kongsfjorden, Sval-
bard (78°55′N, 11°56′E), from the end of April to the begin-
ing of May 2009. Crabs were transferred into the aquaria of the Kings Bay Marine Laboratory in Ny-Åles-
und, kept in natural seawater (0 °C, 34.5 psu), and fed a diet of frozen fish ad libitum. Feeding was terminated at the start of experimentation. Six crabs were placed into one of the eight experimental tanks (30 L) and incubated for 12 days under various temperature and CO₂ concentrations (see below). Only hard-shelled intermoult crabs were used in the experiment.

**Experimental set up**

Within the European Project on Ocean Acidification (EPOCA) Arctic Campaign 2009, the set up for CO₂ incubation was constructed using several header tanks (200 L) to provide the different treatment conditions according to projected scenarios of oceanic CO₂ levels (today: 380, towards the year 2100: 750 and 1,120 and beyond: 3,000 μatm). Seawater from the fjord (from 80 m depth) was pumped into two large reservoir tanks, and temperature was adjusted to 0.5 °C and forwarded to the respective header tanks. Each header tank was bubbled with CO₂ as regulated via a pH stat system (Aquastar Digital pH201). For a more detailed description see Findlay et al. (2010a). The water supply to experimental tanks was designed as a flow through system (~120 mL min⁻¹). Temperature (T), salinity (S), pH, dissolved inorganic carbon (DIC) and total alkalinity (TA) were measured in the header tanks at least every other day. Measurements were carried out using a salinometer (WTW LF197 com-bination temperature and salinity probe) and a pH meter (Metrohm, 826 pH mobile), which was calibrated on the total scale using Tris/HCl and 2-aminoypyridine/HCl buffer solutions. TA was analysed by potentiometric titration (Metrohm Titrand 80) and calculated using a Gran func-
tion. DIC was determined using an APERCA analyser (Marianda, Kiel) calibrated with samples of standard sea-
water (batch 90). The carbonate chemistry (Table 1) was calculated with the R package seacarb (Lavigne et al. 2008) using TA and either pH or DIC. For detailed description of monitoring and calculating the carbonate system, see Comeau et al. (2010).

In addition to the EPOCA incubation system, we set up a 12 °C-tank equilibrated to the respective CO₂ concen-
trations for acute tests in “heat”-exposed animals. Tem-
perature was controlled by a thermostat (LAUDA RK 20KS, Lauda-Königshofen), and gas concentrations were controlled by an MKS mass flow controller (MKS Instruments Deutschland GmbH, München). The partial pressure of CO₂ in seawater (PCO₂) was calculated based on mea-
sured temperature, salinity, pH (NBS scale, Mettler-Toledo pH meter) and DIC (Coming 965 CO₂ Analyser, Olympic Analytical Service, Malvern, Worcestershire) with the software CO₂SYS (Pierrot et al. 2006) using the equilib-
rium constants of Mehrbach et al. (1973) for the CO₂/ bicarbonate/carbonate system, as refitted by Dickson and Millero (1987) and using that for KSO₄ as provided by Dickson (1990) (Table 1).
Experimental protocol

At the end of the acclimation period (12 days), prebranchial (venous) haemolymph was sampled from the third or fourth walking leg and either measured immediately (for acid–base parameters and blood gas concentrations) or prepared for further analyses of lactate concentrations as the main anaerobic end product in crustaceans (Albert and Bogert 1944). Righting experiments are used to examine activity capacity. Furthermore, recorded righting periods were used occasionally during warm water entry to this open Arctic system. Haemolymph measurements were intended to monitor and compare the blood gas and acid–base status: (1) at rest to provide baseline values under acclimated conditions (all CO₂ levels at 1 and 4 °C) and (2) after consecutive stressors ((a) RR at the respective incubation temperatures (5 min), (b) acute heat stress (15 min) and (c) the second run of RR at 12 °C (5 min) under the respective CO₂ levels). This would provide information on whether changes in blood gas and acid–base status occurred under future oceanic scenarios and were behind the functional challenge.

Determination of activity capacity

Activity capacity was determined via the RR, which is defined as the time an individual requires to right itself (e.g. Schembri 1981; Penn and Brockmann 1995) and forced activity (righting) capacity (e.g. Frederich et al. 2000; Wittmann et al. 2012). RRs were measured over 5 min with crabs being turned onto their carapace once every 30 s. The time required for righting was recorded and summed. If an animal took longer than 30 s to turn waiting was extended by the next 30-s period before the animal was exposed to the next turn. Accordingly, time-limited recovery periods were interspersed. This procedure led to the summation of righting periods. In case an animal stopped righting before the end of experimentation, the residual time span was also added. Thus, an animal that stopped righting will have a higher RR value (=slower response) than an animal that rights throughout the experimentation even compared to very slow ones. Overall, this approach proved useful to monitor activity capacity. Furthermore, recorded righting periods were used

### Table 1

<table>
<thead>
<tr>
<th>CO₂ concentrations</th>
<th>PCO₂ μatm (set)</th>
<th>T °C (set)</th>
<th>pH</th>
<th>PCO₂ μatm</th>
<th>T °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>380</td>
<td>1</td>
<td>8.08 ± 0.01</td>
<td>351 ± 10</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>1</td>
<td>7.74 ± 0.02</td>
<td>825 ± 49</td>
<td>0.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>1,120</td>
<td>1</td>
<td>7.57 ± 0.02</td>
<td>1,249 ± 65</td>
<td>0.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3,000</td>
<td>1</td>
<td>7.22 ± 0.02</td>
<td>2,859 ± 160</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>4</td>
<td>8.05 ± 0.02</td>
<td>387 ± 18</td>
<td>4.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>4</td>
<td>7.76 ± 0.03</td>
<td>834 ± 77</td>
<td>3.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>1,120</td>
<td>4</td>
<td>7.59 ± 0.02</td>
<td>1,215 ± 68</td>
<td>4.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3,000</td>
<td>4</td>
<td>7.20 ± 0.03</td>
<td>3,094 ± 206</td>
<td>4.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>380</td>
<td>12</td>
<td>8.10²</td>
<td>417</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>12</td>
<td>7.99³</td>
<td>620</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>1,120</td>
<td>12</td>
<td>7.79⁴</td>
<td>893</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>3,000</td>
<td>12</td>
<td>7.36⁵</td>
<td>2,559</td>
<td>12.0</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SE except for the 12 °C-tank in which values were only measured once because of the short period of its use.

* Total scale, a NBS (National Bureau of Standards) scale
to test for symptoms of fatigue (continuous slowing) within one righting trial of each individual.

Determination of haemolymph oxygen and acid–base parameters

Haemolymph (minimum 0.5 mL) was analysed immediately for PO₂ and pH using optodes (TX-3, PreSens GmbH, Regensburg) and pH micro glass electrodes (Mettler Toledo InLab micro) calibrated at the specific experimental temperature. PO₂-Optodes were calibrated in oxygen-free (0 %, N₂ bubbled) and air-saturated (100 %) seawater. Values given as % air saturation were converted to P

Calcium ions (Ca²⁺) were measured with a microflow system (Lissajous, Munich) and a measurement cell (Eppendorf AG, Hamburg). The supernatant was stored at 4 °C, Fig. 1a) and warm- (4 °C, Fig. 1b) acclimated spider crabs, the RR under normocapnia showed similar values (37.3 ± 1.35 s, 38.0 ± 1.57 s, respectively). In cold-acclimated crabs, righting response period and the cycle number. The clustering of the data due to temperature and CO₂ levels is supported by a principal component analysis (PCA; not shown). Differences were considered significant if P < 0.05. Values are presented as mean ± SE.

Results

Activity capacity

The mean RR of H. araneus incubated at different temperatures and CO₂ treatments prior to and after an additional 12 °C heat stress (for 15 min) is displayed in Fig. 1. RR was measured in seconds; thus, small values mean a fast and larger values a slow response. In cold- (1 °C, Fig. 1a) and warm- (4 °C, Fig. 1b) acclimated spider crabs, the RR under normocapnia showed similar values (37.3 and 40.0 s, respectively). In cold-acclimated crabs, righting

Table 2 Total carbon dioxide of haemolymph (CO₂) prior to and after the additional stress of combined activity and heat exposure (12 °C) for cold- (1 °C) and warm (4 °C)-acclimated H. araneus at different CO₂ concentrations

<table>
<thead>
<tr>
<th>CO₂ level (µatm)</th>
<th>CO₂ (mM) of cold-acclimated crabs</th>
<th>CO₂ (mM) of warm-acclimated crabs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 °C</td>
<td>12 °C</td>
</tr>
<tr>
<td>390</td>
<td>5.82 ± 0.31</td>
<td>8.09 ± 0.21</td>
</tr>
<tr>
<td>750</td>
<td>11.44 ± 0.58</td>
<td>11.83 ± 1.18</td>
</tr>
<tr>
<td>1,120</td>
<td>12.74 ± 1.29</td>
<td>11.50 ± 1.68</td>
</tr>
<tr>
<td>3,000</td>
<td>15.10 ± 1.35</td>
<td>14.55 ± 2.40</td>
</tr>
</tbody>
</table>
activity is largely independent of CO₂ treatments (67.6 ± 7.7 s under 750 μatm, 68.7 ± 7.4 s under 1,120 μatm and 61.4 ± 8.5 s under 3,000 μatm). In warm-acclimated crabs, hypercapnia also led to no detectable effect on RR. A significant reduction in righting activity only occurred after the acute heat stress of 12 °C, more with a temperature change by 11 °C than by 8 °C, and only under elevated CO₂ levels. Starting from normocapnia (40.8 ± 6.9 s), the RR of cold-acclimated crabs exposed to 12 °C decelerated via 106.5 ± 27.9 s under 750 μatm up to 191.7 ± 30.8 s under 1,120 μatm and 191.5 ± 19.6 s under 3,000 μatm. Compared to the acclimation temperature, the RR under heat stress was significantly slowed in crabs incubated at 1,120 and 3,000 μatm (P < 0.001 and P < 0.001, respectively), and some individuals even stopped righting. In warm (4 °C)-acclimated crabs, heat stress led to a significant slowing in RR under high CO₂ levels (46.3 ± 5.5 vs. 78.8 ± 4.0 s under 1,120 μatm, ANOVA, F(1,77) = 4.75, P < 0.05 and 37.8 ± 2.7 vs. 77.8 ± 9.9 s under 3,000 μatm, P < 0.01). However, exposure to 12 °C did not lead to differences between CO₂ treatments.

No fatigue (continuous slowing) occurred during the righting trails of all crabs (data not shown). The decreased righting activity found under hypercapnia at 12 °C was rather caused by a mixture of faster and slower righting events (and in rare cases by a ‘sudden’ stop in righting).

Lactate formation

No lactate accumulation occurred in the haemolymph at acclimation temperatures of 1 and 4 °C (Fig. 2) at rest, regardless of CO₂ treatments. Activity and additional heat exposure to 12 °C led to minor lactate accumulation in all crabs, apparently in a CO₂-dependent pattern. The trends are opposite between acclimation groups. In cold (1 °C)-acclimated crabs, the levels of lactate were highest under normocapnia (25.0 ± 10.7 μM) and dropped with rising CO₂ concentration to 14.9 ± 2.9 μM at 3,000 μatm. In contrast, in warm (4 °C)-acclimated crabs, levels of lactate were lowest under normocapnia (7.3 ± 1.3 μM) and increased with CO₂ up to 31.4 ± 16.1 μM under 3,000 μatm. Due to high inter-individual variability of these overall low values, significant differences between control and exercised animals were found in crabs incubated at 1 °C and 750 μatm only (Kruskal–Wallis test, H(2) = 21.45, P < 0.05).

Haemolymph PO₂

The oxygen partial pressure of the haemolymph (PO₂) (Fig. 3) at rest was similar in cold- and warm-acclimated spider crabs under normocapnia, with values at 1.62 ± 0.22 and 1.79 ± 0.45 kPa, respectively. Moderate hypercapnia (up to 1,120 μatm) did not affect haemolymph PO₂, but levels were significantly elevated at 3,000 μatm. The haemolymph PO₂ level rose to 3.52 ± 0.8 kPa (ANOVA, F(1,78) = 7.02, P < 0.01) in cold-acclimated crabs and up to 2.75 ± 0.38 kPa (ANOVA, F(1,77) = 4.91, P < 0.05) in warm ones. The integrated stress of righting and heat exposure to 12 °C caused a general lowering of haemolymph PO₂. The decrease was significant in all crabs incubated at 3,000 μatm (1.59 ± 0.19 kPa, ANOVA, F(1,78) = 7.02, P < 0.01 for cold- and 0.67 ± 0.19 kPa, ANOVA, F(1,77) = 4.91, P < 0.001 for warm-acclimated crabs).

Haemolymph acid–base status

Haemolymph pH of cold- and warm-acclimated H. araneus at rest decreased with increasing ambient CO₂ levels. In cold-acclimated crabs, the pH fell from 8.18 ± 0.03 at 3,000 μatm (ANOVA, F(1,72) = 12.01, P < 0.05) (Fig. 4a). The values at 1,120 μatm appear unusually high in this group and are mirrored in lower PCO₂ values. In warm (4 °C)-acclimated crabs, the levels of lactate were highest under normocapnia (25.0 ± 10.7 μM) and dropped with rising CO₂ concentration to 14.9 ± 2.9 μM at 3,000 μatm. In contrast, in warm (4 °C)-acclimated crabs, levels of lactate were lowest under normocapnia (7.3 ± 1.3 μM) and increased with CO₂ up to 31.4 ± 16.1 μM under 3,000 μatm. Due to high inter-individual variability of these overall low values, significant differences between control and exercised animals were found in crabs incubated at 1 °C and 750 μatm only (Kruskal–Wallis test, H(2) = 21.45, P < 0.05).
crabs, the pH fell progressively and significantly from 8.16 ± 0.02 under normocapnia via 8.05 ± 0.00 at 1,120 l atm (ANOVA, $F(7,36) = 23.04$, $P < 0.05$) to 7.85 ± 0.05 at 3,000 l atm ($P < 0.001$) (Fig. 4b). Values of cold- and warm-acclimated crabs are comparable under normocapnia; however, the drop in pH with increasing CO$_2$ was more pronounced in the 4°C/C176C-group causing significantly reduced values compared to the 1°C/C176C-acclimated group at 1,120 l atm and 3,000 l atm (ANOVA, $F(7,34) = 27.74$, $P < 0.001$ and $P < 0.001$, respectively). After righting activity and heat stress, the pH pattern remained similar to resting in the cold-acclimated group. pH decreased with increasing hypercapnia from 8.22 ± 0.00 (normocapnia) to 8.10 ± 0.03 (at 3,000 μatm, ANOVA, $F(7,33) = 12.01$, $P < 0.01$). In the 4°C-group, no significant differences were found between CO$_2$ treatments. Here, the acidosis seen under resting conditions and elevated CO$_2$ levels was actually reduced at 1,120 and 3,000 μatm (8.21 ± 0.02, ANOVA, $F(7,34) = 27.74$, $P < 0.01$ and 8.05 ± 0.03, $P < 0.001$, respectively). The partial pressure of carbon dioxide in the haemolymph (PCO$_2$) of *H. araneus* showed a general increase with increasing CO$_2$ concentration, with and without the integrated stress of activity and heat exposure (Fig. 4). In cold-acclimated *H. araneus* at rest, haemolymph PCO$_2$ increased significantly from 0.09 ± 0.01 kPa (normocapnia) up to 0.34 ± 0.03 kPa at 3,000 μatm (ANOVA, $F(7,35) = 19.47$, $P < 0.001$). In warm-acclimated crabs, PCO$_2$ increased from 0.12 ± 0.01 kPa (normocapnia) up to 0.51 ± 0.07 kPa at 3,000 μatm (ANOVA, $F(7,35) = 17.15$, $P < 0.001$). The stronger increase seen in crabs at 4 °C led to significantly higher PCO$_2$ values at high CO$_2$ levels when compared to crabs at 1 °C (ANOVA, $F(7,35) = 21.78$, $P < 0.001$). After activity and heat stress, haemolymph PCO$_2$ of cold-acclimated crabs remained significantly increased...
with 0.29 ± 0.04 at 1,200 µatm (ANOVA, $F_{(7,35)} = 17.15$, $P < 0.05$) and 0.47 ± 0.05 kPa at 3,000 µatm ($P < 0.001$) versus 0.16 ± 0.01 kPa under normocapnia. Compared to resting conditions, a significant increase in haemolymph $P_{CO2}$ occurred under high CO2 levels ($P < 0.05$ at 1,120 µatm, $P < 0.01$ at 3,000 µatm). In warm-acclimated crabs, the patterns remained similar after additional stress with a significantly increased haemolymph $P_{CO2}$ at 3,000 µatm (ANOVA, $F_{(7,38)} = 19.47$, $P < 0.01$). Here, the integrated stress of righting and heat exposure led to a significant drop in $P_{CO2}$ at 3,000 µatm ($P < 0.01$) compared to resting conditions. Furthermore, the observed drop in $P_{CO2}$ under high CO2 levels led to significantly decreased values compared to cold-acclimated crabs after combined righting and heat stress (ANOVA, $F_{(7,38)} = 13.35$, $P < 0.05$ at 1,200 and 3,000 µatm).

Hydramolymph bicarbonate levels ([HCO3−]) at rest increased with rising CO2 levels in all crabs. In cold-acclimated crabs, [HCO3−] increased from 5.8 ± 0.3 mM at normocapnia via 11.3 ± 0.6 mM at 750 µatm (ANOVA, $F_{(7,34)} = 9.25$, $P < 0.01$) and 12.7 ± 1.3 mM at 1,200 µatm ($P < 0.01$) up to 16.7 ± 1.3 mM at 3,000 µatm ($P < 0.001$). [HCO3−] levels of warm-acclimated crabs at rest were enhanced in all CO2 treatments (750 µatm: 13.8 ± 0.8 mM, ANOVA, $F_{(7,38)} = 7.08$, $P < 0.001$; 1,120 µatm: 9.5 ± 0.3 mM; 3,000 µatm: 13.5 ± 1.5 mM, $P < 0.001$) compared to normocapnia (6.4 ± 0.3 mM).
After the integrated stress of activity and heat exposure, the patterns remained similar, but differences between CO₂ treatments resulted less clear. Within the group acclimated to 1 °C, bicarbonate was still significantly enhanced above normocapnic values (8.0 ± 0.2 mM) reaching 16.3 ± 1.7 mM under 3,000 μatm (ANOVA, \( F_{(3,34)} = 9.25, \ P < 0.001 \)). In warm (4 °C)-acclimated crabs, differences between CO₂ treatments became non-significant after exposure to additional righting and heat stress when bicarbonate content rose slightly under normocapnia but dropped under 3,000 μatm.

Discussion

To our knowledge, this is the first report investigating acid–base status together with energy-demanding behaviour (righting) in the context of global climate change (ocean warming and acidification). Furthermore, very little is known about the effects of ocean acidification on crustaceans at high polar latitudes. In H. araneus, none of the applied environmental factors temperature (1 and 4 °C) or CO₂ (580 (control), 750, 1,120 and 3,000 μatm) affected the RR of Arctic specimens. Moreover, the CO₂-induced increase in the CO₂ partial pressure (PCO₂) of haemolymph or decrease in pH (before righting, discussed below) did also not affect the RR. Only among cold-acclimated crabs under hypercapnia, a small trend towards a decreasing activity capacity (slowing) is indicated under hypercapnia (with temperature unchanged). Additional heat stress (12 °C, 15 min) did also not affect the RR under normocapnia. However, when exposed to moderately hypercapnic conditions above 750 μatm, significant slowing occurred in both cold- (1 °C) and warm (4 °C)-acclimated groups. Cold-acclimated crabs responded more strongly to heat stress combined with hypercapnia than warm-acclimated ones with a much stronger reduction in righting activity, and few individuals even stopped responding. In contrast, warm-acclimated crabs were significantly slower after heat stress but were still relatively active even under the highest CO₂ concentrations applied (78 s for warm-acclimated vs. 192 s for cold-acclimated crabs at 3,000 μatm). These findings indicate that the two groups became functionally limited under hypercapnia, but to different degrees. This implies that thermal constraints come into play, which are exacerbated by the synergistic effects of elevated seawater PCO₂.

Previous findings indicate that activity capacity of crustaceans is reduced in the cold. At the respective temperature, cold-acclimated crabs slowed faster during continuous forced activity than warmer ones (Burke 1979). Furthermore, crabs become increasingly slower with decreasing temperature, and Antarctic crustaceans even showed lower activity levels when compared to temperate species (Rebach 1974; Young et al. 2006). From changes in oxygen consumption and lactate production (anaerobic metabolism), Burke (1979) even calculated a higher energy demand for activity in the cold. The reduction in activity is also accelerated once animals are brought to both the low or high edges of their thermal window (Wittmann et al. 2012). Cold-acclimated spider crabs in our experiments slowed strongly after heat stress but only when combined with hypercapnia. Our results indicate that the chosen heat stress temperature of 12 °C might be close to the upper critical temperature (Tc) of cold-acclimated crabs. This is supported by the haemolymph data, which showed lowered oxygen partial pressures (PO₂) and raised lactate levels after activity and heat stress (discussed below).

Previous experiments had demonstrated a downward shift of the upper Tc with hypercapnia in crabs like H. araneus (Metzger et al. 2007; Walther et al. 2009). In the context of the present data, it becomes clear that such shift mirrors a reduction of performance capacity at thermal extremes. Furthermore, temperature limits are lower in winter- or cold-acclimated animals than in summer- or warm-acclimated ones (Hopkin et al. 2006; Pörtner and Farrell 2008; Somero 2011). The warm-acclimated specimens have likely shifted their upper thermal limits to higher temperatures. Therefore, they are less sensitive to the superimposed hypercapnia levels. Higher PCO₂ levels than applied here would probably lead to a dampanging of the RR across all temperatures. For example, Dissanayake and Ishimatsu (2011) found a reduction in swimming speed in the prawn Metapenaeus japonicus under severe hypercapnia (10,000 μatm), independent of acclimation temperature.

Extended acclimation time has the potential to change, possibly improve, animal response during long-term chronic CO₂ exposure. For example, in the sea urchin, Strongylocentrotus droebachiensis, fecundity was significantly decreased under elevated PCO₂ (1,200 μatm) after 4 month of acclimation, while no difference was found in females acclimated for 16 month (Dupont et al. 2012). In contrast, for adult crustacean, harmful effects on survival were reported under long-term exposure (Kurhara et al. 2008). However, as exposure to extreme temperatures is seasonal and transient, such long-term acclimation may not play a similar role for interpreting the present data.

According to the OCLTT concept (see introduction), thermal limitation occurs progressively from the onset of a fall in aerobic scope at pejus limits to the onset of anaerobic metabolism at critical limits. In both acclimation groups under resting conditions, prebranchial (venous) haemolymph PO₂ values were well in the range of literature data for marine crustaceans independent of applied PCO₂ levels (Taylor and Wheatly 1980: 2.3 kPa; Taylor et al. 1981: 0.7–2.7 kPa; Booth et al. 1982: 1.0 kPa; Defur 1983: 2.3–3.7 kPa).
et al. 1983: 2.5 kPa; Whiteley et al. 1997: 7.7 kPa; Watt et al. 1999: ca 1–2.7 kPa). In both groups, PO2 increased under the highest PCO2 applied, indicating enhanced ventilatory effort stimulated by CO2, a phenomenon well described for crustaceans (Massabau and Burtin 1985).

Haemolymph PO2 was generally lower after righting and heat stress reflecting an increase in oxygen demand as expected due to activity and rising temperature. At 3,000 μatm compared to normocapnia, cold-acclimated crabs showed an increase whereas warm-acclimated crabs showed a decrease in PO2. These differences reflect the different degrees of slowing in righting activity and accordingly, efforts in muscular activity. The RR at 12 °C was minor in 1 °C-crabs at high CO2, such that elevated PO2 levels were maintained.

Both the measured PO2 values and the small degree of lactate accumulation overall indicate that oxygen limitation beyond the anaerobic threshold was small during the short-term exposure to 12 °C. Legeay and Massabau (1999) reported for Carcinus maenas that even an arterial haemolymph PO2 of 0.5 kPa did not lead to the onset of anaerobic metabolism. This limiting PO2 would be higher after exercise due to metabolic stimulation. After activity and heat exposure, venous haemolymph PO2 values of all spider crabs were found between 0.67 and 1.59 kPa and according to the small degree of lactate formation seen in the present experiments, not far beyond the threshold for anaerobic metabolism. This conclusion indicates that the crabs at 12 °C were close to their upper critical temperature. Accordingly, a limitation in functional scope was observed indicating a loss of aerobic scope, especially under elevated PCO2. This limitation became visible as a slowing of the RR. The validity of this conclusion is emphasized even more by the fact that warmer temperatures should have led to faster righting instead of slowing, provided a normal Q10 value of around 2–3 applies.

Lactate accumulation in the haemolymph neither occurred at the acclimation temperatures nor under hypercapnic conditions when crabs were resting. After combined activity and heat stress, the values increased slightly and became highly variable. In general, warm-acclimated crabs showed a rise in lactate levels with increasing hypercapnia levels. In contrast, cold-acclimated crabs developed the highest levels under normocapnia and the lowest for the highest CO2 level. As with haemolymph PO2, these differences can be explained by the different levels of righting activity, indicating that a small degree of lactate formation is related to the level of motor activity, with slower righting causing even less lactate accumulation.

In cold-acclimated crabs, activity effort was highest under normocapnia and lowest under high PCO2. In warm-acclimated crabs where the RR was similar after heat stress under the different CO2 treatments, lactate accumulated progressively with increasing acidification. These results may indicate higher energetic costs of activity under hypercapnia. However, even with combined activity and heat stress, lactate levels of all crabs remained relatively low.

Measured lactate concentrations in the haemolymph of Arctic H. araneus after stress were much lower than found in previous reports on crabs. Haemolymph lactate was found to reach values of 0.5–10.1 mM after exercise (Phillips et al. 1977; Burke 1979; Booth et al. 1982; Wheatly et al. 1986). Such studies were mainly done on shallow-water crustaceans from temperate latitudes. There is only one study of a polar crab (Whiteley et al. 1997) and a comparable deep-sea crab (Pane and Barry 2007), both revealing lactate levels far below those of their temperate or shallow-water counterparts. The low lactate levels together with the relatively high PO2 values found in the present study indicate a general emphasis on aerobic metabolism and that the RR was mainly covered aerobically. Our findings imply that the activity effort occurred within limits set by available aerobic scope which was less in the cold-acclimated than the warm-acclimated specimens at 12 °C. Certainly, the observed strong slowing in cold-acclimated crabs under high CO2 levels might also involve other factors shaping activity capacity such as effects on neurological functions. Behavioural changes under elevated CO2 levels in fact indicate the existence of such neurological disturbances (Bibby et al. 2007; De la Haye et al. 2011; Dixson et al. 2010).

The sequence of mechanisms eliciting such effects remains unexplored. One overarching hypothesis is that CO2 effects are mediated through disturbances in acid–base status, specifically reduced extracellular pH (Pörtner et al. 2004, 2005, Pörtner 2008). CO2-exposed crabs in fact showed elevated haemolymph PCO2 values paralleled by decreased haemolymph pH when compared to controls (Fig. 4), although haemolymph pH in spider crabs fell less (<0.32 units, discussed below) than seawater pH (~0.85 units). This observation indicates that H. araneus is able to defend its internal milieu under hypercapnia, through non-bicarbonate buffering and proton equivalent ion exchange, leading to [HCO3−] accumulation in the haemolymph. However, the acidosis remained only partially compensated throughout the experimental period especially when combined with warming (4 vs. 1 °C). In general, the additional challenges of activity and heat (12 °C) had no further effects on acid–base adjustments except that warm-acclimated crabs incubated at high CO2 levels (1,120 and 3,000 μatm) revealed a significant increase in haemolymph pH, likely due to enhanced release of CO2 during activity and, as a consequence, reduced haemolymph PCO2 values.

The impact of hypercapnia on acid–base status was larger in warm than in cold-acclimated crabs. The patterns
CO2 release during activity and metabolic alkalization by acclimated specimens thus may have involved enhanced (Portner 1987). The reduced acidosis in warm (4°C) by glycolytic acidification as in the present experiments consumption during hydrolysis remains uncompensated for substantial mortality (Spicer et al. 2007). In species with a compensation remains incomplete and is accompanied by extracellular acidosis was compensated for by substantial bicarbonate accumulation (12 mM). In the deep-sea crab, the bicarbonate level only reached 3 mM, and the extracellular acidosis remained almost uncompensated (Pane and Barry 2007). The reduced capacity for acid–base regulation in the deep-sea crab was explained by its cold-stenothermal adaptation. The findings of our study suggest that a similarly low capacity might characterize polar species. Despite a significant accumulation rate of bicarbonate (up to 16 mM), possibly due to the longer time span of experimentation (12 days vs. 24 h), Arctic spider crabs failed to compensate for acid–base disturbances even under moderate hypercapnia (>750 μatm). The results imply a low capacity of acid–base regulation in this Arctic population of H. araneus. Furthermore, they support the findings by Walther et al. (2010) suggesting a trend towards features of permanent cold adaptation in the Arctic population. Moreover, the drop in haemolymph pH was exacerbated when hypercapnia was combined with warming to 4°C. The low pH regulation capacity may enhance sensitivity to elevated CO2 partial pressures, especially at temperature extremes. Finally, a finding to be explained is that the acidosis is stronger but the effect on activity capacity less in the warm-acclimated specimens. This observation can be explained by the beneficial effect of warm acclimation on heat resistance and performance, which cannot be compensated for by the stronger acidosis.

Summary and conclusion

Overall, the synergistic effects of ocean acidification and warming reduce organism performance. All crabs exposed to temperature extremes suffered from reduced scope for performance, which was exacerbated by increasing CO2 concentrations. These findings are consistent with a role of extracellular acidosis in mediating this effect (Portner 2008). These findings are also in line with OCLTT. The impact was more extreme in cold- (1°C) than in warm (4°C)-acclimated crabs brought to the same extreme temperature (12°C). The strong reduction in righting activity observed under high PCO2 levels (>750 μatm) might involve disturbances in neurological functions or neuromuscular transmission yet to be identified. H. araneus is able to compensate for the acidosis but not completely, likely due to a low capacity for acid–base regulation visible especially in warm-acclimated crabs. Exposure to extreme warming bouts in combination with elevated PCO2 levels as predicted by 2100 and 2300 may thus have negative effects on acid–base regulation and behavioural performance of H. araneus. This may still be true for the more moderate changes that would progressively develop with the expected rise in mean temperatures. Reduced activity
capacity has negative implications for organism fitness including the capacity for reproduction, foraging and antipredator behaviour.

Acknowledgments. This work is a contribution to the “European Project on Ocean Acidification” (EPOCA) which received funding from the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no 211884. EPOCA is endorsed by the International Programmes IMBER, LOICZ and SOLAS. This project was also supported by the Helmholtz Graduate School for Polar and Marine Research (POLMAR) and the German program on ocean acidification (BIOACID) funded by the BMBF. The authors like to thank Helen Findlay and Hannah Wood of the Plymouth Marine Laboratory (PML) for setting up the CO2 incubation system and Jean-Pierre Gattuso, Frederic Gazeau, Stevee Combeau and Marie-Dominique Pirzy from the Laboratoire d’Océanographie de Villefranche (LOV) for water chemistry analyses. We also thank Kings Bay and the France-German Arctic Research Base AWIPEV on Svalbard for logistic support as well as the scientific divers of the Alfred-Wegener Institute (AWI) especially supervisor Max Schwanitz. The authors are grateful to Stephan Frickenhaus (AWI) for statistical support and discussions. Zora Zittier wishes to thank Christian Bock (AWI) for his encouragement.

References

Limnaeus C (1758) Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. 10th edn. Tomus 1, Holmaiue (Laurentius Salvii), Stockholm


Impact of ocean acidification on thermal tolerance and acid–base regulation of *Mytilus edulis* (L.) from the North Sea

Zora M.C. Zittier, Christian Bock, Gisela Lannig and Hans O. Pörtner

2015

Journal of Experimental Marine Biology and Ecology

473:16-25
Impact of ocean acidification on thermal tolerance and acid–base regulation of Mytilus edulis (L.) from the North Sea

Zora M.C. Zittier⁎, Christian Bock, Gisela Lannig, Hans O. Pörtner

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

ARTICLE INFO

Article History:
Received 24 March 2015
Received in revised form 31 July 2015
Accepted 1 August 2015
Available online xxxx

Keywords:
Warming
Oxygen consumption
Heart rate
Succinate
Extracellular pH
NMR spectroscopy

ABSTRACT

Anthropogenic climate change confronts marine organisms with rapid trends of concomitant warming and CO2 induced ocean acidification. The survival and distribution of species partly depend on their ability to exploit their physiological plasticity during acclimatization. Therefore, in laboratory studies the effects of simulated future ocean acidification on thermal tolerance, energy metabolism and acid–base regulation capacity of the North Sea population of the blue mussel Mytilus edulis were examined. Following one month of pre-acclimation to 10 °C and control CO2 levels, mussels were exposed for two weeks to control and projected oceanic CO2 levels (390, 750 and 1120 μatm) before being subjected to a stepwise warming protocol between 10 °C and 31 °C (+ 3 °C each night). Oxygen consumption and heart rates, anaerobic metabolite levels and haemolymph acid–base status were determined at each temperature. CO2 exposure left oxygen consumption rate unchanged at acclimation temperature but caused a somewhat stronger increase during acute warming and thus mildly higher Q10-values than seen in controls. Interestingly, the thermally induced limitation of oxygen consumption rate set in earlier in normocapnic than in hypercapnic (1120 μatm CO2) mussels (25.2 °C vs. 28.8 °C), likely due to an onset of metabolic depression in the control group following warming. However, the temperature induced increase in heart rate became limited above 25 °C in both groups indicating an unchanged pejus temperature regardless of CO2 treatment. An upper critical temperature was reached above 28 °C in both treatments indicated by the accumulation of anaerobic metabolites in the mantle tissue, paralleled by a strong increase in haemolymph POCO2 at 31 °C. Ocean acidification caused a decrease in haemolymph pH. The extracellular acidosis remained largely uncompensated despite some bicarbonate accumulation. In all treatments animals developed a progressive warming-induced extracellular acidosis. A stronger pH drop at around 25 °C was followed by stagnating heart rates. However, normocapnic mussels enhanced bicarbonate accumulation at the critical limit, a strategy no longer available to hypercapnic mussels. In conclusion, CO2 has small effects on the response patterns of mussels to warming, leaving thermal thresholds largely unaffected. High resilience of adult North Sea mussels to future ocean acidification indicates that sensitivity to thermal stress is more relevant in shaping the response to future climate change.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Coastal regions host a variety of ecosystems that are increasingly threatened by anthropogenic climate change. Current climate change confronts marine organisms with multiple stressors such as increasing temperature, CO2 and hypoxia (Pörtner et al., 2014). Temperature is the main factor shaping the performance of marine animals as it affects organisms in all life-stages and at all levels of biological organisation. There is evidence that temperature-induced impacts on marine ectothermic animals are based on unifying principles of physiological response, which led to the concept of oxygen- and capacity-limited thermal tolerance (OCLTT; for review, see Pörtner, 2010). This concept links physiological principles of thermal tolerance to climate-driven ecosystem changes. According to the OCLTT concept, the oxygen supply of an organism is maximal in the optimum temperature range (T0) between upper and lower pejus thresholds, with performance being maximal close to upper pejus limits. This range characterizes the ecological thermal tolerance range where availability of aerobic energy is maximal for all physiological functions including growth, development and reproduction and therefore determines the geographical distribution of species and populations. In temperate zone species surpassing either an upper or lower pejus temperature (Tp), leads to a mismatch between oxygen uptake and demand as circulation and/or ventilation reach their capacity limits resulting in internal hypoxemia of the organism and thus
progressively reduced aerobic performance and whole animal fitness, visible in a reduction of growth, reproduction etc. The next thresholds are the critical temperatures (Tc) when oxygen supply to tissues becomes insufficient to maintain energy expenditure resulting in a transition to anaerobic energy metabolism.

While temperature is the main factor driving current ecosystem changes (Poloczanska et al., 2014), impacts of increasing CO2 concentrations (causing ocean acidification, i.e. reductions in seawater pH, carbonate levels and carbonate saturation values) are expected to become increasingly involved, especially in upwelling areas (e.g. Barton et al., 2012). Future ocean acidification has the potential to disturb life-sustaining processes in marine organisms like growth, reproduction, energy metabolism and acid-base regulation. Especially calcifying organisms, like bivalves, are projected to be adversely affected due to the additional challenges involved in forming and protecting their calcareous shells and skeletons under the altered seawater carbonate chemistry (Fabry et al., 2008; Donny et al., 2009; Kroeker et al., 2010). Furthermore, the capacity for acid-base regulation is crucial for the scope of whole animal performance (Pörtner, 2008), but, in contrast to fish and crustaceans, bivalves and echinoderms are regarded to be weak acid-base regulators (for review, see Melzer et al., 2009; Parker et al., 2011). These circumstances gain increasing attention as several calcifiers are important species in coastal ecosystem functioning and moreover for shellfish economies. In case of the North Sea, beds of Mytilus edulis provide substratum for various epibionts and offer shelter and food for a diverse community of organisms. A glimpse into the future is provided by massive die-offs of shellfish larvae due to shifts towards high-CO2 upwelling conditions over the last years in coastal regions of the northeast Pacific Ocean (Barton et al., 2012).

One main strategy of marine invertebrates to survive short periods of elevated CO2 exposure passively is the so-called metabolic depression with an associated reduction in ventilation (Guppy et al., 1994; Langenbuch and Pörtner, 2002; Michaelidis et al., 2005). However, this strategy may result harmful in the long term. Michaelidis et al. (2005) found permanently depressed aerobic metabolism in Mytilus galloprovincialis over 90 d of severe hypercapnia at ~5000 μatm leading to a 50% reduction in growth rates. Even hypercapnia of ~2000 μatm caused a significant reduction of oxygen consumption, clearance and ingestion rates in clams Ruditapes decussatus (Fernández-Reiriz et al., 2011). Further studies found no effect of CO2 levels projected by 2100 (~2000 μatm) on oxygen consumption rates of bivalves (Lannig et al., 2010; Fernández-Reizit et al., 2012; Liu and He, 2012; Schuhhauser et al., 2012). In M. edulis from the Baltic (Kiel Fjord) no reduction in oxygen consumption was found even at 2500 μatm (Thomsen and Melzner, 2010). However, elevated background CO2 levels in the fjord indicate that this population may be adapted to high CO2 levels as it was then collected in a 2100 μatm scenario. The capacity for acid-base regulation is crucial for the survival of M. edulis individuals exposed to acute warming. CO2 adaptation capacity of M. edulis is provided by massive die-offs of shellfish larvae due to shifts towards high-CO2 upwelling conditions over the last years in coastal regions of the northeast Pacific Ocean (Barton et al., 2012).

2. Material & methods

2.1. Animal collection and maintenance

Wild type adult mussels, M. edulis (50 to 90 mm shell length) were collected from the subtidal zone around Helgoland, German Bight in June 2009 after the main larval peak. Seawater CO2 levels of this area are relatively stable throughout the year and similar to atmospheric levels (average ≤400 μatm) (e.g. Thomas et al., 2007). Mussels were transported in tanks that were constantly flooded with North Sea water to the Alfred-Wegener-Institute for Polar and Marine Research (AWI, Bremerhaven, Germany) within 24 h after collection by the research vessel Utthörn. Mussels were separated, cleaned from epibionts and maintained in aerated and filtered natural seawater from the North Sea at 10 °C and a salinity of 32 in the aquarium facility of the AWI. Following pre-acclimation for at least one month, mussels were randomly placed in one of four tanks per group (15 L, max. 18 animals) and incubated for 2 weeks under different CO2 concentrations (see below). Mussels were fed daily al lith with freshly hatched Artemia larvae as suitable diet, e.g. Davenport et al. (2000) and a commercial living algal blend containing Nannochloropsis, Phaeodactylum tricornutum and Chlorell (DT’s Live Marine Phytoplankton, Coralsands, Germany). To avoid interference with postprandial metabolism (e.g. Bayne and Scullard, 1977; Gaffney and Diehl, 1986) feeding was terminated three days before experimentation. All animal tanks (acclimation, incubation and experimental tanks) were cleaned daily from faeces and pseudo-faeces.

2.2. Incubation and experimental set up

For CO2 incubations, systems were set up in a temperature control room (10 °C) using several reservoirs (450 L) and header tanks (210 L) to provide different treatment conditions according to projected scenarios of oceanic CO2 levels (today: 390 and towards the year 2100: 750 and 1120 μatm). Water was circulated between the reservoir and the header tank; both were continuously bubbled with the respective air-CO2 mixture via a H&K gas system (Hamburg, Germany). From the header tank, water was supplied to the animal tanks (15 L) at a flow rate of ~120 ml/min, thereby progressively cleaned from epibionts and incubated for 2 weeks under different CO2 concentrations (Poloczanska et al., 2014). Each animal tank contained four respiration chambers. Water was exchanged twice a week to reconnect the respiration chambers from the system. Subsequently, the system was refilled and equilibrated for 24 h while the water from the basin was re-circulated into the header tank. The experimental setup comprised two animal tanks (80 L, starting with 36 animals) and a reservoir (for water exchange), all temperatures were feedback-controlled by a thermostat (AUADA RP 845, Lauda-Königshofen) and continuously bubbled with the respective air-CO2 mixture produced by a MGS mass flow controller (MGS Instruments Deutschland GmbH, München). Each animal tank contained four respiration chambers. Water was exchanged before each temperature rise.

Temperature (T), salinity (S), pH, and total alkalinity (TA) were measured daily in all animal tanks (incubation, experimental and reservoirs tanks for water changes) for determining the water chemistry. Measurements were carried out using a salinometer (WTW LF 317; combination temperature and salinity probe) and a pH meter (NBS scale, Mettler-Toledo pH meter). TA was analysed by potentiometric titration (METROM Prozessanalytik GmbH & Co. Germany). The partial pressure of CO2 in seawater (PO2) was calculated based on the measured parameters using the CO2SYS program (Pierrot et al., 2006) after equilibrium constants of Mehrbach et al. (1973) for the CO2-bicarbonate/carbonate system, as refitted by Dickson and Millero (1987) and used for KSO4 as provided by Dickson (1990) (incubation: Table 1, experiment: Table 2).
2.3. Preparation of animals and experimental protocol

After CO₂-exposure mussels were transferred into the experimental setup. Eight mussels per treatment (N = 72) were used for parallel measurements of oxygen consumption and heart rate. Shell thickness was reduced by grinding and a plethysmograph infrared sensor (Vishay Semiconductors, CN70) was placed above the pericardial cavity for non-invasive heart rate measurements. Mussels were interspersed through the lid of the respiration chamber, superglued to the shell and covered with dental wax. Mussels were then placed into respiration chambers within the experimental tanks. In order to monitor recovery from handling stress recordings of oxygen consumption and heart rate were started immediately. After stable readings was obtained (after 3 to 7 h depending on individual; see Fig. 1) data were collected for analyses. Other mussels of the respective treatment (for the sampling of tissues and body fluids) were left undisturbed at least overnight after being transferred to the experimental tanks.

Measurements were started at control temperature (10 °C) and temperature was increased by 3 °C every night. Temperature was increased 2 months later under control (390 μatm; pH NBS 8.14 ± 0.03) data were collected for analyses. Other mussels of the respective treatment (for the sampling of tissues and body fluids) were left undisturbed at least overnight after being transferred to the experimental tanks.

Table 1. Carbonate chemistry of seawater during the incubation of blue mussels, M. edulis, at different CO₂ concentrations. (390, 750, 1120 μatm). Total alkalinity was very stable throughout the experimental period with 2411 ± 7 μatm; see Fig. 1) data were collected for analyses. Other mussels of the respective treatment (for the sampling of tissues and body fluids) were left undisturbed at least overnight after being transferred to the experimental tanks.

animals were treated in the same way making the data of nonomaccinic and hyperomaccinic animals comparable and differences in the response can, thus, be attributed to the CO₂ levels applied. Moreover, all mussels were in good shape also at the end of the 2nd run, as confirmed by condition indices (CI, calculated as: dry meat weight [g] × 1000 / shell length [cm]) of 4.96 ± 1.03 at 390 μatm and 4.67 ± 0.91 at 1120 μatm that lay well in the range of previously findings for M. edulis (cf. Lundbye et al., 1997).

Table 2. Carbonate chemistry of seawater during the incubation of blue mussels, M. edulis, at constant temperature (10 °C) and temperature was increased by 3 °C every night. Temperature was increased 2 months later under control (390 μatm; pH NBS 8.14 ± 0.03) data were collected for analyses. Other mussels of the respective treatment (for the sampling of tissues and body fluids) were left undisturbed at least overnight after being transferred to the experimental tanks.

2.4. Determination of oxygen consumption and heart rate

Oxygen consumption (MO₂) measurements were conducted following Van Dijk et al. (1999) using flow-through respirometry. Briefly, the flow rate (3-46 mL min⁻¹) was set in a way that mussels consumed less than 20% of the O₂ from the water. Throughout the experiment only readings of lowest metabolic rates, stable for at least 40 min (standard metabolic rate, SMR, see Fig. 1) were used for analyses. After the experiment, mussels were dissected to determine shell-free dry weight (DW). MO₂ was measured using oxygen optodes with integrated temperature compensation (TX-3, PreSens GmbH, Regensburg). Optodes were calibrated in oxygen-free (0%, N₂ bubbled) and air-saturated (100%) seawater. The 100%-oxygen values were checked daily to compensate for temperature and hardware drifts. Once air saturation values deviated from calibrations by more than 2%, recorded data were corrected and a new two-point calibration was performed. Values given at 5% air saturation were converted to

\[ \text{PO}_2 (\text{kPa}) = (\text{Patm} - \text{Ph}_{\text{O}_2}) \times 2095 \times (\% \text{air saturation}) / 100 \]

where Patm is the atmospheric pressure (kPa), PhO₂ is the temperature-specific water vapour pressure (kPa), calculated after

\[ \text{PO}_2 (\text{kPa}) = (\text{Patm} - \text{Ph}_{\text{O}_2}) \times 2095 \times (\% \text{air saturation}) / 100 \]

where Patm is the atmospheric pressure (kPa), PhO₂ is the temperature-specific water vapour pressure (kPa), calculated after
Dejours (1975), and 0.2095 is the fraction of oxygen in air. $MO_2$ was calculated as follows:

$$MO_2 \frac{\mu mol O_2 g^{-1} dL^{-1}}{= (\Delta P_{O_2} - (O_2 + V_4)/DW).$$

where $\Delta P_{O_2}$ is the difference in oxygen partial pressure between inflowing and outflowing water ($kPa$), $O_2$ is the temperature-specific oxygen capacity of water ($\mu mol O_2 L^{-1} kPa^{-1}$), $V_4$ is the flow rate ($L h^{-1}$) and $DW$ is the shell-free dry weight (g) of the mussel. Heart rate was determined using a PowerLab system with Chart 4.1.1 Software (AD Instruments, Spechbach, Germany). Averaged heart rate was determined at each temperature by counting the peaks expressed as beats per minute (bpm) over 30–90 s intervals within a 3 h period. Unfortunately, some heart recordings became unreliable at high temperatures possibly due to grounding problems, reducing the available sample size above 22 °C.

2.5. Determination of gas and acid-base status in haemolymph and extrapallial fluid

Haemolymph and extrapallial fluid were analysed immediately after sampling. $P_{O_2}, P_{C_0_2}$ and $pH$ were measured using a blood gas analyser (MT 33, Eschweiler, Germany) calibrated at the specific experimental temperature. Total $CO_2$ concentration ($CO_2 T$, Table 3) of body fluids was determined by gas chromatography (Agilent 6890N GC System, Agilent Technologies, USA). Bisubstrate concentrations ([HCO$_3^-$]) were calculated from measured $CO_2$ (nmol) and $P_{O_2}$ ([Tor]) as follows:

$$[HCO_3^-] = CO_2 \alpha (\alpha CO_2 + P_{O_2}),$$

where $\alpha CO_2$ is the solubility of $CO_2$ in the body fluid (nmol Tor$^{-1}$) calculated after Heisler (1986).

2.6. Tissue extraction and determination of metabolites

Tissue succinate concentrations were determined as follows. Mantle tissue was powdered under liquid nitrogen using a mortar and pestle. Tissue powder (~300 mg) was homogenized (0 °C, 360 W) with ice-cold 0.6 M perchloric acid (PCA) added to a vol/wt ratio of 1 to 5. Precipitated protein was removed by centrifugation (0 °C, 2 min at 16,000 g). The extract was neutralized to a pH of ~7.5 with 5 M potassium hydroxide and cold 0.6 M perchloric acid (PCA) added to a vol/wt ratio of 1 to 5. Precipitated protein was removed by centrifugation. The supernatant was stored at ~8 °C until further analysis. Samples were dried in a SpeedVac for 1H NMR spectroscopy. Prior to measurements dried extracts were resolved in 500 μL D$_2$O containing 1% trimethylsilyl propionate (TSP) as internal reference and transferred to 5 mm NMR tubes, resulting in a final concentration of 0.3 g initial tissue powder per mL. Fully relaxed high-resolution 1H NMR spectra were recorded using an inverse 1H-band probe (1H,3B) on a 400 MHz 9.4 T WBB NMR spectrometer with Avance electronics (Bruker Biospin GmbH, Germany) similar to Lanning et al. (2010). Acquisition parameters were as follows: $TD = 16 k$, $NS = 128$, $DS = 2$, $SW = 4.8 k$, $AQ_1 = 1.7 k$, $DI = 12 k$ with a constant receiver gain of RG 203 to ensure comparability of samples. Spectra were post-processed automatically using TopSpin 2.5 (Bruker Biospin GmbH, Germany). All data were zero filled to 64 k and processed with an exponential multiplication of 0.5 Hz prior to Fourier transformation. After phase and baseline correction, spectra were calibrated to TSP at 0.0 ppm. Succinate concentrations were determined by analysing the area under a singlet peak at 2.4 ppm, corresponding to the chemical shift of succinate at pH 7.5, using the integration routine in TopSpin.

2.7. Statistical analysis

Before data were processed with R outliers were removed by using Nalimov’s test (Noack, 1980). Data were analysed with a Shapiro-Wilk test for normality. Two-way analysis of variance (ANOVA) in combination with Tukey’s post hoc test was performed to analyse effects of temperature and $CO_2$ level and possible interactions thereof. Normality was not fulfilled for haemolymph $P_{O_2}$ data, therefore, data were log-transformed prior to analysis. Arhenius break temperature indicates the discontinuity in the temperature dependence of $MO_2$, at which a significant change in the slope of the plot occurs (Sokal and Rohlf, 1995). However, breakpoint analysis was impossible because a limited number of temperatures resulted above potential breakpoints in the warmth to be able to calculate linear regressions by the least-square method (Sokal and Rohlf, 1995). Hence, the phase change was determined using a sigmoidal function that describes the exponential increase of $MO_2$ with rising temperature, and the subsequent limitation in the warmth, when maximum curvature at a value of zero for the second derivative of the model indicates the breakpoint temperature. Differences were considered significant if $P < 0.05$. Values are presented as means ± SD, N = 5–8 unless stated otherwise.

3. Results

Oxygen consumption rates ($MO_2$) of $M. edulis$ (Fig. 2) were similar under normocapnia (390 atm: $7.1 ± 2.0 \mu mol O_2 h^{-1} g^{-1} DW^{-1}$) and hypercapnia (750 atm: $6.6 ± 1.4; 1120 atm$: $7.1 ± 1.1 \mu mol O_2 h^{-1} g^{-1} DW^{-1}$) at acclimation temperature ($10 °C$). Warming from 10 to 28 °C led to a progressive and significant increase in $MO_2$ under both normocapnia and hypercapnia, which resulted in a somewhat higher oxygen consumption rate under elevated $CO_2$ (390 atm: $25.19 °C$) than under 1120 atm; $24 °C$ (750 atm: $25.19 °C$) and $1120 atm$: $26.91 °C$ (750 atm: $24 °C$). After the last temperature rise from 28 °C to 31 °C only 43% (3 out of 7) of the animals under normocapnia and 63% (5 out of 8) under hypercapnia showed a further increase in oxygen consumption rate, while $MO_2$ values in the other animals decreased to 31 °C. After the last temperature rise from 28 °C to 31 °C only 43% (3 out of 7) of the animals under normocapnia and 65% (5 out of 8) under hypercapnia showed a further increase in oxygen consumption rate, while $MO_2$ values in the other animals decreased to 31 °C. After the last temperature rise from 28 °C to 31 °C only 43% (3 out of 7) of the animals under normocapnia and 63% (5 out of 8) under hypercapnia showed a further increase in oxygen consumption rate, while $MO_2$ values in the other animals decreased to 31 °C.
changes occurred (Fig. 3), however, the additional increase to 31 °C resulted in a significant accumulation of succinate under both normocapnia and hypercapnia (390 μM: 0.12 ± 0.03 a.u. at 10° vs. 0.61 ± 0.33 at 31 °C, P < 0.001; 1120 μM: 0.08 ± 0.03 a.u. at 10° vs. 0.61 ± 0.56 at 31 °C, P < 0.001). Accordingly, two-way ANOVA identified a main effect only for temperature (P < 0.001).

Oxidation and acid-base status determined in extrapallial fluids were similar to the ones in haemolymph (Table 4), therefore only haemolymph data are described here. Haemolymph P:\textsubscript{CO\textsubscript{2}} showed high inter-individual variability. Two-way ANOVA suggested a significant effect of temperature and of the interaction between temperature and CO\textsubscript{2} on haemolymph P:\textsubscript{CO\textsubscript{2}} (P < 0.001 and P = 0.044, respectively). However, post hoc analyses did not reveal significant differences during warming when compared to acclimation temperature (10 °C) or between the CO\textsubscript{2} treatments at a specific temperature likely due to high inter-individual variability (Fig. 4A). In normocapnic mussels haemolymph P:\textsubscript{CO\textsubscript{2}} shows a trend to decrease during warming from 116.4 ± 5.2 Torr at 10 °C to 85.0 ± 29.2 Torr at 31 °C, which resembles the decline under hypercapnia when initial P:\textsubscript{CO\textsubscript{2}} values at 10 °C were somewhat lower than in control P:\textsubscript{CO\textsubscript{2}} incubations (750 μM: from 102.6 ± 12.2 Torr at 10 °C to 80.4 ± 10.5 at 22 °C; 1120 μM: from 100.1 ± 13.1 Torr at 10 °C to 87.2 ± 22.8 at 31 °C). Post hoc tests confirmed a significant decrease only between 13° and 31 °C under normocapnia, again due to the high variability of data.

Haemolymph P:\textsubscript{CO\textsubscript{2}} did not show significant differences between CO\textsubscript{2} treatments throughout the experimental period (Fig. 4B). While no changes occurred during warming from 10 to 28 °C, further warming to 31 °C resulted in a significant increase of haemolymph P:\textsubscript{CO\textsubscript{2}} in both normocapnic and hypercapnic animals, with a somewhat stronger increase in the former ones (390 μM: 0.93 ± 0.23 at 10 °C vs. 10.83 ± 4.57 Torr at 31 °C, P < 0.001; 1120 μM: 1.77 ± 0.52 at 10 °C vs. 7.79 ± 3.01 Torr at 31 °C, P < 0.001).

Haemolymph pH of M. edulis displayed different patterns depending on CO\textsubscript{2} treatment during acute warming between 10 °C and 31 °C (Fig. 4C). pH was significantly affected by temperature, CO\textsubscript{2} treatment and their interaction (P < 0.001, P = 0.003, P = 0.016, respectively). pH values remained highest under normocapnia. They fell significantly during the first temperature rise from 7.65 ± 0.06 at 10 °C to 7.39 ± 0.05 at 13 °C (P = 0.005), and remained relatively stable thereafter until they dropped significantly from 7.38 ± 0.08 at 22 °C to 7.01 ± 0.25 at 25 °C (P < 0.001), with no further change until 31 °C (7.02 ± 0.05). In contrast, haemolymph pH under 750 μM of hypercapnia started with a value of 7.41 ± 0.12 at 10 °C (P = 0.028), significantly lower than under normocapnia, but reached 7.34 ± 0.11 at 22 °C, similar to the value seen under normocapnia. Mussels under hypercapnic conditions of 1120 μM started with a pH of 7.16 ± 0.11 at 10 °C (P = 0.008) and reached 7.30 ± 0.22 at 22 °C followed by a drop to 7.10 ± 0.11 at 25 °C, significantly lower than at acclimation temperature (P < 0.001), with no further changes thereafter. A sudden drop in haemolymph pH between 22 °C and 25 °C thus occurred independent of CO\textsubscript{2} treatment. As data between 10 °C and 22 °C are the results from the 1st run and the ones between 25 °C and 31 °C from the 2nd run (see experimental protocol in Section 2.3) it seemed conceivable that the observed drop resulted from combining the two data sets. However, occasional measurements performed below 25 °C in the 2nd...
run checked for the comparability of the data sets and these values confirmed the existence of the overproportional drop (data not shown).

At 10 °C haemolymph bicarbonate concentration ([HCO₃⁻]) increased significantly from 1.48 ± 0.18 under normocapnia to 2.24 ± 0.07 mM at 1120 μatm (P = 0.047). During acute warming from 10 °C to 31 °C further changes in haemolymph [HCO₃⁻] depended on the CO₂ treatment. In normocapnic mussels between 10 °C and 28 °C, haemolymph [HCO₃⁻] remained relatively constant around 1.48 ± 0.18 mM and increased significantly to a maximum of 3.50 ± 1.00 mM at 31 °C (P = 0.001). In contrast, haemolymph [HCO₃⁻] concentrations under 750 μatm varied slightly from 1.66 ± 0.14 at 10 °C to 1.39 ± 0.23 mM at 19 °C and 1.64 ± 0.27 mM at 22 °C. Haemolymph [HCO₃⁻] at 1120 μatm decreased progressively from 2.24 ± 0.07 at 10 °C to a minimum of 1.49 ± 0.07 mM at 19 °C and started to increase thereafter to 2.37 ± 0.67 mM at 31 °C, significantly higher than found at 19 °C, 22 °C and 25 °C (P = 0.007, P = 0.037, P = 0.026, respectively). Two-way ANOVA identified a significant interaction between temperature and CO₂ treatment (P < 0.001) and a main effect of temperature (P < 0.001). At control temperature, haemolymph [HCO₃⁻] concentration was significantly higher under hypercapnia (1120 μatm, P = 0.047) than normocapnia, despite the lower pH value under hypercapnia. The difference was eliminated during acute warming until temperature rose from 28 °C to 31 °C, which resulted in a significantly higher [HCO₃⁻] level in normocapnic mussels than in hypercapnic ones (P = 0.001).

Fig. 5 (normocapnia, 390 μatm) and Fig. 6 (hypercapnia, 1120 μatm) depict the comparison between haemolymph pH on the one hand and heart rate (A) or oxygen consumption (B), on the other hand. It should be noted that heart rates above 22 °C were only available from N = 1–2 animals per treatment (see figures and Section 2.4), such that statistical analysis was only possible for data from 10 °C to 22 °C. Hypercapnia at 750 (not shown) and 1120 μatm had no significant effect on heart rate of M. edulis between 10 °C and 22 °C, when compared to normocapnia. Regardless of CO₂ treatment, acute warming between 10 °C and 22 °C caused heart rates to rise progressively to significantly higher values at 19 °C than at acclimation temperature (P = 0.001 at 390, P = 0.011 at 750, P = 0.001 at 1120 μatm). Two-way ANOVA suggested a significant effect of temperature (P < 0.001) and CO₂ (P < 0.001) but the post hoc test did not confirm any differences between the three CO₂ treatments. During further warming, the increase in heart rate became limited above or at 25 °C under both normocapnia and hypercapnia (1120 μatm). While heart rate under hypercapnia levelled off above 25 °C, rates under normocapnia levelled off and started to decrease at 31 °C. The limitation to a further increase in heart rate occurred when haemolymph pH had suddenly dropped below 7.3 regardless of CO₂ treatment (Figs. 5A and 6A). In normocapnic mussels the same pattern was found for oxygen consumption: when haemolymph pH had dropped the limitation to a further rise in MO₂ became effective (Fig. 5B). In contrast, hypercapnic mussels showed a further exponential increase in MO₂ until 28 °C regardless of acidosis (Fig. 6B).

4. Discussion

The aim of this study was to investigate the interacting effects of temperature and CO₂ levels according to near future ocean acidification scenarios, and to interpret the data by use of the OCLTT concept.

4.1. Thermal limits under normocapnia

Oxygen consumption rate (MO₂) of M. edulis at acclimation temperature (10 °C) ranged between 4.4 and 9.8 pmol O₂ h⁻¹ g DW⁻¹ under normocapnia (390 μatm). Rates were found well in the range of previously published data for M. edulis (Okumus and Stirling, 1994; Schüter and Johansen, 1994; Sukhotin and Pörtner, 2001). Acute
warming from 10 °C to 31 °C resulted in a progressive rise in MO2 following a Q10-value (10–28 °C) of 2.19. During the last temperature rise from 28 °C to 31 °C, 43% (3 of 7) of the animals were able to further increase their MO2 while the others showed a decrease. This suggests that about half of the animals had exceeded their critical temperature limit, likely due to lower individual performance capacity. These findings of anaerobic metabolite accumulation and haemolymph P\textsubscript{CO2} confirmed that the critical temperature (TC) was reached above 28 °C (discussed below).

Interestingly, a phase change in the MO2 rise during acute warming was found at a calculated breakpoint temperature of 25.2 °C (Fig. 2A, vertical line). This breakpoint indicates the onset of a limitation in oxygen supply, and might involve metabolic depression. According to the OCLTT concept the progressive reduction in the scope for oxygen supply may be caused by a rise in baseline energy demand, paralleled by a capacity limit of cardiac performance reached at the pejus temperature (TP) (Pörtner, 2001). In accordance, a limitation in heart rate was also observed above 25 °C (see below). The present findings allow to put changes in extracellular pH into this context. The correlation between haemolymph pH on the one hand and oxygen consumption or heart rate, on the other hand (see Fig. 5) indicates that a sudden drop in extracellular pH between 22 °C and 25 °C might have influenced the phase change in haemolymph pH on the one hand and oxygen consumption or heart rate, on the other hand (see Fig. 5) indicates that a sudden drop in extracellular pH between 22 °C and 25 °C might have influenced the phase change in haemolymph pH on the one hand and oxygen consumption or heart rate, on the other hand (see Fig. 5)

A heart rate of 13 ± 2 bpm at acclimation temperature (10 °C) is in good agreement with data published previously for M. edulis at similar salinities and temperatures (Braby and Somero, 2006; Widdows, 1973b). Upon acute warming, heart rate increased progressively up to 25 °C and levelled off thereafter (indicating TP, see discussion above) before a decrease was found at 31 °C. The onset of a drop in heart rate indicates the progressive break down of the circulatory system and thus TC (~28 °C) (Frederich and Pörtner, 2000) supporting the conclusions from the MO2 data. The temperature-dependent heart rates of the present study are in line with findings by Widdows (1973b) in M. edulis acclimated at 15 °C. These animals displayed a progressive increase in heart rate up to 25 °C followed by a drastic drop at 30 °C.

The critical temperature is the physiological limit where oxygen supply becomes insufficient resulting in a transition to anaerobic metabolism (for review, see Pörtner, 2010). The onset of anaerobic metabolism is indicated by the accumulation of succinate, a key anaerobic metabolite of bivalve mitochondria (Zurburg and Kluytmans, 1980; Sukhotin and Pörtner, 1999; Hines et al., 2007). In fact, a sharp
In haemolymph PCO₂ occurred at the critical temperature. M. edulis started to defend its internal milieu against further acidification by [HCO₃⁻] accumulation. In mussel leading to a slightly but not significantly higher Q₁₀ value (28 °C) than in control animals (2.49 vs. 2.19, respectively). For comparison, oysters (Crassostrea gigas) showed significantly higher Q₁₀-values after acute warming (5–25 °C, 5 °C 48 h) under hypercapnia (PCO₂ = 1500 µatm) compared to normocapnia (Lannig et al., 2010). In their study animals were exposed to long-term hypercapnia (26–55 days vs. 14 days in the present study) before experimentation, which had no impact on oyster metabolic rates but resulted in a decreased body condition index. This suggests reduced growth efficiency and, hence, indicating shifts in energy allocation, possibly exacerbated by starvation. Similarly, long-term moderate hypercapnia at constant temperatures had no impact on metabolic rates in blue mussels but reduced shell growth rates (M. edulis: Berte et al., 2006, 2009; 1500 µatm; Thomsen and Melzner, 2010, 1000–4000 µatm). M. galloprovincialis: Michaelidis et al., 2005, 5000 µatm). Hence, longer exposure times to hypercapnia would likely exacerbate the effect of warming on energy demand resulting in a significantly increased Q₁₀ than under normocapnia. These findings suggest that the hypercapnic Tc was reached at the same temperature as under normocapnia, confirmed by anaerobic metabolite and haemolymph PCO₂ data (see below). However, the MO₂ course under hypercapnia showed a somewhat higher mean slope and consequently does not reflect metabolic depression in all animals, at least up to 28 °C. The data indicate an increase in variability in the response. The discontinuity in MO₂ under hypercapnia occurred at a calculated breakpoint temperature of 28.84 °C and thus, close to the Tc. In contrast, the discontinuity in normocapnic animals occurred at lower temperature (25.15 °C), was attributed to metabolic depression and interpreted to mark onset of the pejus range. The Tc of hypercapnic mussels seems to be similar as haemolymph pH dropped during the temperature rise from 22 °C to 25 °C followed by a restriction of cardiac performance beyond 25 °C (see Fig. 6) as seen in normocapnic mussels. Overall, the data indicate that during warming, CO₂ exposed animals remained in a somewhat more active state than those under normocapnia.
Extrapolating from the discussion above small increments in CO₂ may prevent early metabolic depression during warming.

The question arises how initial hypercapnia in M. edulis can prevent metabolic depression and thereby support tolerance to external warming. Other studies in fact report a CO₂ dependent stimulation of mechanisms strengthening resilience. For example, hypercapnia reportedly induces a release of adenosine into the haemolymph of crabs and thereby stimulates cardiac performance (e.g. Stegen and Grieshaber, 2001). The fact that heart rate did not increase indicates that this hypothesis does not apply to the blue mussel (see Fig. 6). Findings in mammals show that CO₂ can stimulate neuronal function (Dulla et al., 2005). There are no such findings reported for mussels, leaving the exact mechanism behind putative CO₂ induced metabolic stimulation obscure.

The results of the present study imply that the circulatory system of M. edulis reached its temperature-induced capacity limit above 25 °C in both groups, indicating the onset of the pejus range at this temperature. The Tc of M. edulis was unaffected by moderate hypercapnia and found above 28 °C when succinate concentration rose sharply and regardless of CO₂ treatment. In crabs and crustaceans, it could recently be shown that hypercapnia led to a downward shift of the upper critical temperature (Metzger et al., 2007: Cancer pagurus at 10,000 ppm; Walther et al., 2005: Hex panum at 710 and 3000 ppm). No such shift could be observed in the present study, which might again be explained by the different habitats and pre-adaptation of Mytilus to life in the sub- and intertidal zone with highly fluctuating CO₂ concentrations and temperatures. Similar to permanently submersed cephalopods and fish, crustaceans regulate their internal milieu rather well (for review, see Whiteley, 2011). A higher energetic effort in acid-base regulation may lead to thermal tolerance shifts responding more strongly to hypercapnia in crabs than in the bivalve.

Hypercapnic exposure caused a reduced haemolymph pH in M. edulis. The extracellular acidosis remained uncompensated despite some compensation visible in a significantly increased [HCO₃⁻] level. The same patterns were reported for other bivalves (Michaelidis et al., 2005; Lannig et al., 2010) and lower marine invertebrates (Pörtner et al., 1998).

Although no significant impact of increasing CO₂ level was detected on haemolymph [HCO₃⁻], neither at acclimation temperature nor during warming, values were increased under hypercapnia at control temperatures and match the ones found by Thomsen et al. (2010) under similar conditions. During warming, levels increased suddenly above 28 °C at the critical temperatures in all treatments. Nevertheless, heat stress under normocapnia resulted in higher internal CO₂ accumulation above 28 °C due to acidosis and less gas exchange. A stronger respiratory acidosis in normocapnic animals was prevented by significant [HCO₃⁻] accumulation. This indicates potential use of residual acid-base regulation capacity under normocapnia, which was not available to hypercapnic mussels in the warmth. Future studies have to show whether metabolically depressed animals have the ability to sustain critical conditions longer than mussels e.g. under hypercapnia, when metabolic depression is prevented.

5. Conclusion

Overall, physiological transitions observed during warming of North Sea blue mussels M. edulis mirror the tolerance thresholds as defined by the OCLTT concept. The warming induced fall in haemolymph pH may be involved in setting the pejus temperature (Tₚₑ, onset of falling aerobicscope) at 25 °C by dampening heart rate regardless of CO₂ treatment. The critical temperature found above 28 °C (Tc, onset of anoxic metabolicism) also remained unaffected by moderate hypercapnia indicating that the population studied may be resilient to CO₂ excalitations, possibly related to its pre-adaptation to life in the intertidal zone. While normocapnic mussels showed a somewhat earlier limitation in temperature-dependent oxygen consumption rate (breakpoint temperature 25.2 °C) and actively defended their internal milieu when reaching critical limits most hypercapnic mussels remained in an active state (breakpoint temperature 24.8 °C) and failed to significantly increase their haemolymph [HCO₃⁻] level in the warmth. Thus, CO₂ exposure modulated the response to warming by somewhat reducing the degree of metabolic depression in the heat. It remains to be explored whether this effect is adaptive or reduces the capacity to sustain fitness in a highly variable environment. Especially during longer periods of exposure the latter may be harmful for sustained ecological performance of M. edulis.

Author contributions

Conceived and designed the experiments: ZMCZ and HOP. Performed the experiments: ZMCZ, with the help of CB. Analysed and interpreted the data: ZMCZ, with the help of CB and CL. Contributed reagents/materials/analysis tools: ZMCZ, CB, and HOP. Wrote the paper: ZMCZ, CB, and HOP. Revision of the paper: ZMCZ, GL, and HOP.

Acknowledgements

This work is a contribution to the “European Project on Ocean Acidification” (EPOCA) which received funding from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement no 211384. EPOCA is endorsed by the International Programmes IMBER, LOICZ and SOLAS. This project was also supported by the Helmholtz Graduate School for Polar and Marine Research (POLMAR). The authors would like to thank Stephan Frickenhaus for statistical support. [SES]

References


Publication 3

Impact of ocean acidification on thermal tolerance and acid-base regulation of *Mytilus edulis* from the White Sea

Zora M.C. Zittier, Christian Bock, Alex A. Sukhotin, N. Sören Häfker and Hans O. Pörtner

2018

Polar Biology

41(11): 2261-2273
Impact of ocean acidification on thermal tolerance and acid–base regulation of *Mytilus edulis* from the White Sea

Z. M. C. Zittier · C. Bock · A. A. Sukhotin · N. S. Häfker · H. O. Pörtner

Received: 3 October 2017 / Revised: 13 June 2018 / Accepted: 14 June 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract
Ocean warming and acidification are two important environmental drivers affecting marine organisms. Organisms living at high latitudes might be especially threatened in near future, as current environmental changes are larger and occur faster. Therefore, we investigated the effect of hypercapnia on thermal tolerance and physiological performance of sub-Arctic *Mytilus edulis* from the White Sea. Mussels were exposed (2 weeks) to 390 µatm (control) and 1120 µatm CO₂ (year 2100) before respiration rate (MO₂), anaerobic metabolite (succinate) level, haemolymph acid–base status and intracellular pH (pHi) were determined during acute warming (10–28 °C, 3 °C over night). In normocapnic mussels, warming induced MO₂ to rise exponentially until it levelled off beyond a breakpoint temperature of 20.5 °C. Concurrently, haemolymph PCO₂ rose significantly > 19 °C followed by a decrease in PO₂ indicating the pejus temperature (Tₚ, onset of thermal limitation). Succinate started to accumulate at 28 °C under normocapnia defining the critical temperature (Tₐ). pHi was maintained during warming until it dropped at 28 °C, in line with the concomitant transition to anaerobiosis. At acclimation temperature, CO₂ had only a minor impact. During warming, MO₂ was stimulated by CO₂ resulting in an elevated breakpoint of 25.8 °C. Nevertheless, alterations in haemolymph gases (> 16 °C) and the concomitant changes of pHi and succinate level (25 °C) occurred at lower temperature under hypercapnia versus normocapnia indicating a downward shift of both thermal limits Tₚ and Tₚ by CO₂. Compared to temperate conspecifics, sub-Arctic mussels showed an enhanced thermal sensitivity, exacerbated further by hypercapnia, indicating their potential vulnerability to environmental changes projected for 2100.

Keywords Global warming · Population comparison · Energy metabolism · Anaerobiosis · Extra- and intracellular acid–base status · ¹H-NMR spectroscopy

Introduction
Future ocean warming and acidification may adversely affect many marine organisms. Earlier studies of thermal constraints in marine ectotherms led to the concept of oxygen- and capacity-limited thermal tolerance (OCLTT; for review, see Pörtner 2010). According to the OCLTT concept, oxygen supply to tissue and thus aerobic performance of the organism is optimized in a limited temperature range supporting maximal growth, reproduction and development success. On both sides of the temperature range thermal limitation first occurs at low and high pejus temperatures (Tₚ) by an emerging mismatch in oxygen supply versus demand due to insufficient capacities of ventilation and circulation. Aerobic scope is further reduced at critical temperatures (Tₐ) when anaerobic metabolism sets in. These sublethal temperature limits may be ecologically relevant as the onset of reduced animal performance relates to the geographical distribution of species and populations (Deutsch et al. 2008).

Ocean acidification has the potential to disturb processes like growth, reproduction, calcification, energy metabolism and acid–base regulation in marine organisms. It has been hypothesised that the degree of (extracellular) acidosis and
thus the capacity of acid–base regulation plays a key role in setting the sensitivity of marine ectotherms to elevated CO₂ tensions (Pörtner 2008). Marine invertebrates, which are characterized by poor acid–base regulation capacity (Miles et al. 2007; Mezner et al. 2009; Thomsen et al. 2010; Whiteley 2011), are considered to be adversely affected by ocean acidification. This particularly concerns calcifiers, like bivalves, due to the sensitivity of shell formation and preservation at reduced pH (Fabry et al. 2008; Doney et al. 2009; Kroeker et al. 2010).

Changes in CO₂ concentrations and temperature occur concomitantly and studying the interaction of these factors is crucial in light of ongoing climate and ecosystem changes (IPCC 2014). Elevated CO₂ concentrations (hypercapnia) may narrow the thermal window of ectotherms by a shift of sublethal thermal limits (for review, see Pörtner 2012) with projected consequences for the range of geographical distribution and associated shifts in ecosystem structure and functioning (e.g. Findlay et al. 2010; Hale et al. 2011). The role of CO₂ in shaping thermal tolerance may differ between organisms according to their regional climate and natural habitat. Recently thermal thresholds as defined by the OCLTT concept were identified in intertidal blue mussels Mytilus edulis from the North Sea under control and hypercapnic conditions (Zittier et al. 2015). In this population, sublethal thermal limits (Tₐ and T₊) remained largely unaffected by hypercapnic exposure (1120 µatm). Nevertheless, hypercapnia altered the response to warming by preventing metabolic depression in the warmth.

Vulnerability to environmental stress may differ between populations and in general may rise with increasing latitude due to adaptation to colder temperatures (e.g. Pörtner 2001; Pörtner et al. 2009; Zittier et al. 2013). Different seasonal temperature extremes and variabilities along a latitudinalcline may relate to different thermal windows of populations. The White Sea is a subpolar sea in northern Russia characterized by a colder climate than the temperate North Sea causing population specific thermal adaptations in marine invertebrates such as gastropods and lugworms (Hummel et al. 1997; Sommer et al. 1997; Tschischka et al. 2000; Sokolova and Pörtner 2003; Schröer et al. 2009). Identifying latitudinal differences in the physiological performance of populations is therefore crucial for predicting future changes in community structures and ecosystem functioning in the world’s oceans.

The aim of our study was to determine the thermal window and acid–base regulation capacity of M. edulis from the White Sea and investigate the effects of ocean acidification under realistic CO₂ scenarios, for comparison with the respective findings in mussels from the North Sea (Zittier et al. 2015). It is hypothesised that the sub-Arctic population has a reduced tolerance to warming and elevated CO₂ levels when compared to temperate conspecifics. Mussels were incubated at oceanic control (390 µatm) CO₂ levels and those projected for the end of century (1120 µatm), for two weeks before exposure to acute warming. Oxygen consumption, acid–base status of haemolymph and extrapallial fluid as well as intracellular pH and anaerobic metabolite accumulation in mantle tissue were investigated.

Materials and methods

For comparable results mussel maintenance, incubation, experimentation as well as the experimental procedure followed those outlined in a recent study on M. edulis from the North Sea (Zittier et al. 2015) and are described briefly below.

Animal collection and maintenance

Mytilus edulis (50–85 mm shell length) were collected from a small area in the shallow subtidal zone in the White Sea, Russia (12 °C, 25 PSU; 66°20′14.27″N, 33°38′12.10″E), and transported to the Alfred-Wegener-Institute for Polar and Marine Research (AWI, Bremerhaven, Germany). Mussels were cleaned from epibionts and pre-acclimated (10 °C, 32 PSU) for at least one month. They were then incubated for 2 weeks under control and elevated CO₂ concentrations (see below). As M. edulis can adapt to a wide range of salinities within days (e.g. Gosling 1992, 2003) the difference in salinity between habitat and laboratory conditions should be negligible in the present study and, thus, the natural North Sea water available at the institute was used. Mussels were fed daily ad libitum with freshly hatched Artemia larvae (Davenport et al. 2000) and a commercial living algal blend (DT’s Live Marine Phytoplankton, Coralsands, Germany). Feeding was terminated three days before experimentation to avoid interference with postprandial metabolism (e.g. Bayne and Scullard 1977; Gaffney and Drieh 1986).

Incubation and experimental set up

The incubation system and experimental set up were the same as used in Zittier et al. (2015). Briefly, each incubation system was located in a temperature control room (10 °C) containing a reservoir (450 L) and header tank (210 L) both continuously bubbled (HTK gas system, Hamburg, Germany) with the control (390 µatm) or the projected oceanic CO₂ concentration for the end of century (1120 µatm). From the header tank water was supplied to the animal tanks (15 L, flow-through rate ~120 mL min⁻¹), and then collected in a basin (210 L, continuously bubbled with the respective air-CO₂ mixture) and re-circulated to the reservoir. Water was exchanged twice a week by disconnecting the reservoir from the circuit to subsequently refill and equilibrate it for...
24 h. The experimental set up consists of two animal tanks (each with 33 animals at the start) and a reservoir (for water exchange) continuously bubbled with the respective air-CO₂ mixture (MKS Instruments Deutschland GmbH, München) and water temperature was feedback controlled by a thermostat (LAUDA RP 845, Lauda-Königshofen). Water was exchanged daily before each temperature rise.

Water chemistry in all animal tanks (incubation, experimental and reservoir tanks for water changes) was calculated daily via the respective values of temperature (T), salinity (S), pH and total dissolved inorganic carbon (DIC). Measurements were carried out using a salinometer (WTW LF197 combination temperature and salinity probes) and a pH metre (Mettler-Toledo pH metre) calibrated at the respective temperature to NBS scale that was converted into total scale via measurements of Dickson standards. DIC was analysed with Seal Analysis SFA QuAAtro; pump Technicon trAArs 800 TM. The partial pressure of CO₂ in seawater (PCO₂) was calculated using CO2SYS (equilibrium constants of Mehrbach et al. 1973; reftled by Dickson and Miller 1987; Pierrot et al. 2006). The physicochemical parameters of seawater are presented in Tables 1 (incubation) and 2 (experimentation).

**Preparation of animals and experimental protocol**

After the CO₂-incubation period, mussels (n = 66 per treatment) were transferred to the experimental setup. Eight mussels per treatment were used for respiration measurements. Recordings of respiration were started immediately after the mussel was placed in a respiration chamber within the experimental tank. Data for analysis were collected after recovery from handling stress (stable recordings) and after an acclimation period of at least 5 h to each temperature rise. Other mussels in the experimental tanks (for tissue and blood fluid sampling) were left undisturbed overnight.

Starting from the control temperature of 10 °C, temperature was increased by 3 °C every night until 28 °C was reached. In addition to the recording of respiration, blood and tissue samples were collected at each temperature step from mussels not subjected to analyses of respiration. Haemolymph was withdrawn from the posterior adductor muscle and extrapallial fluid was sampled from the extrapallial space and immediately analysed for acid–base parameters and gas levels. Afterwards, mantle tissue was excised, freeze-clamped immediately and stored in liquid nitrogen until further analysis of intracellular acid–base status and succinate content (see below). For a more detailed description of the procedures see also Zittier et al. (2015).

Mussels were kept unfed throughout the experimental period to avoid postprandial rise in metabolism. Reduced food supply can modulate the metabolic rate and stress resistance of animals (Melzner et al. 2011) but a pre-experiment, that followed the same experimental procedure as in the present study except that temperature was kept constant, did not affect the respiration rate indicating no effect by time or lack of food during the chosen experimental period, at least at constant temperature. However, normocapnic and hypercapnic mussels from the White Sea (present study) as well as the North Sea (Zittier et al. 2015) were all treated in the same way such that the results are comparable and differences can be attributed to the seawater CO₂ level and the ones between the two populations to the adaptation to different climate regimes of their habitats.

**Determination of respiration rate**

Respiration rate (MO₂) was measured as resting oxygen consumption using only readings of the lowest metabolic rates maintained for at least 40 min (see Zittier et al. 2015). Respiration rate was determined by oxygen optodes with integrated temperature compensation (TX-3, PreSens GmbH, Regensburg) using flow-through respirometry following Van

---

### Table 1 Physicochemical parameters of seawater (mean ± SD) during control (390 µatm) and CO₂-incubation (1120 µatm) of blue mussels, Mytilus edulis at 10 °C

<table>
<thead>
<tr>
<th>PCO₂ (µatm)</th>
<th>T (°C)</th>
<th>pH total</th>
<th>PCO₂ (µatm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>390</td>
<td>10.2 ± 0.2</td>
<td>8.04 ± 0.06</td>
<td>413 ± 53</td>
<td>13</td>
</tr>
<tr>
<td>1120</td>
<td>10.3 ± 0.2</td>
<td>7.65 ± 0.04</td>
<td>1106 ± 111</td>
<td>11</td>
</tr>
</tbody>
</table>

### Table 2 Physicochemical parameters of seawater during the acute warming protocol (3 °C over night) with blue mussels, Mytilus edulis under normocapnia (390 µatm) and CO₂-exposure (hypercapnia; 1120 µatm)

<table>
<thead>
<tr>
<th>T (°C) (set)</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
<th>T (°C) (set)</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH total</td>
<td>PCO₂ (µatm)</td>
<td>pH total</td>
<td>PCO₂ (µatm)</td>
<td>pH total</td>
</tr>
<tr>
<td>10</td>
<td>10.1</td>
<td>7.89</td>
<td>602.0</td>
<td>10.2</td>
<td>7.75</td>
</tr>
<tr>
<td>13</td>
<td>13.2</td>
<td>7.87</td>
<td>652.6</td>
<td>13.2</td>
<td>7.68</td>
</tr>
<tr>
<td>16</td>
<td>16.1</td>
<td>7.94</td>
<td>550.3</td>
<td>16.1</td>
<td>7.64</td>
</tr>
<tr>
<td>19</td>
<td>19.2</td>
<td>7.92</td>
<td>600.2</td>
<td>19.2</td>
<td>7.67</td>
</tr>
<tr>
<td>22</td>
<td>22.2</td>
<td>7.99</td>
<td>505.2</td>
<td>22.1</td>
<td>7.66</td>
</tr>
<tr>
<td>25</td>
<td>25.0</td>
<td>8.02</td>
<td>458.2</td>
<td>25.1</td>
<td>7.57</td>
</tr>
<tr>
<td>28</td>
<td>28.0</td>
<td>8.00</td>
<td>490.0</td>
<td>28.0</td>
<td>7.64</td>
</tr>
</tbody>
</table>
Hypercapnia (Normocapnia = Dw, ÷ Hypercapnia × g Dw) was determined. MO_2 was calculated as follows:

\[ \text{MO}_2 (\text{µmol O}_2 \text{ h}^{-1} \text{ g DW}^{-1}) = (\Delta \text{PO}_2 \times \beta \text{O}_2 \times \text{V}_g) + \text{DW}, \]

where \( \Delta \text{PO}_2 \) is the difference in partial pressure between in- and out-flowing water (kPa), \( \beta \text{O}_2 \) is the oxygen capacity of water (µmol O_2 L^{-1} kPa^{-1}), \( \text{V}_g \) is the flow rate (L hr^{-1}) and DW is the shell-free dry weight (g) of the mussel.

**Determination of extra- and intracellular gas and acid–base status**

Haemolymph and extrapallial fluid were analysed immediately after sampling as in Zittier et al. (2015). Extracellular \( \text{P} \text{O}_2, \text{PCO}_2 \) (mmHg) and pH (NBS scale) were measured using a blood gas analyser from Eschweiler (MT 33, Germany) calibrated at the specific experimental temperature. Total CO_2 concentration (C\text{CO}_2, mmol L^{-1}) was determined by gas chromatography (Table 3).

Body fluids was analysed by gas chromatography (6890 N GC System, Agilent Technologies, USA, for reference see below). Bicarbonate concentrations \( [\text{HCO}_3^-] \) were calculated as follows:

\[ [\text{HCO}_3^-] (\text{mM}) = \text{PCO}_2 - (\alpha \text{PCO}_2 \times \text{PCO}_2), \]

where \( \alpha \text{PCO}_2 \) is the solubility of CO_2 in the body fluid (mmol L^{-1} mmHg^{-1}) calculated after Heisler (1986).

Intracellular pH (pHi) and tissue concentrations of CO_2 were measured in mantle tissue samples stored in liquid nitrogen. Samples were analysed using the homogenerate method developed by Pörner et al. (1990), using 160 mmol L^{-1} potassium fluoride (KF) and 0.1 mmol L^{-1} nitritotriacetic acid (NTA) for the analysis of 200–250 mg tissue. pH was determined at the respective experimental temperature using a pH optode (Needle-Type-Housing-pH-Microsensor, PreSens GmbH, Regensburg) and C\text{CO}_2 was determined by gas chromatography (Table 3). Intracellular PCO_2 and [HCO_3^-] concentration (Table 4) were then calculated as described above.

**Tissue extraction and determination of metabolites**

Tissue succinate concentrations were determined in perchloric acid (PCA) extracts from mantle tissues using 1H-NMR spectroscopy as described in Zittier et al. (2015). In the North Sea mussels succinate concentration had not been affected by hypercapnia and had only increased at the highest temperature and independent of CO_2. Therefore, in the present study succinate was determined at the beginning of the warming trial and at the two highest temperatures (25° and 28°C). Following the procedure by Zittier et al. (2015) tissue extracts were dried in a SpeedVac for spectroscopy and, prior to measurements, resolved in D_2O containing 1% trimethylsilyl propionate (TSP) as internal reference. Fully relaxed high-resolution 1H-NMR spectra were recorded on a 400 MHz 9.4T WB NMR spectrometer with Avance III electronics (Bruker Biospin GmbH, Germany) as described in Zittier et al. (2015). Spectra were post-processed automatically using TopSpin 2.5 (Bruker Biospin GmbH, Germany). All data were zero filled to 64 k and processed with an exponential multiplication of 0.3 Hz prior to Fourier

---

**Table 3** Total carbon dioxide (C\text{CO}_2, mmol L^{-1}) of haemolymph and mantle tissue in blue mussels, Mytilus edulis under normocapnia (390 µm) and hypercapnia (1120 µm) during acute warming (3°C over night)

<table>
<thead>
<tr>
<th>T (°C) (set)</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haemolymph</td>
<td>Mantle</td>
</tr>
<tr>
<td>10</td>
<td>1.91 ± 0.11</td>
<td>2.15 ± 0.33</td>
</tr>
<tr>
<td>13</td>
<td>1.88 ± 0.15</td>
<td>1.45 ± 0.22</td>
</tr>
<tr>
<td>16</td>
<td>1.23 ± 0.03</td>
<td>1.74 ± 0.14</td>
</tr>
<tr>
<td>19</td>
<td>1.45 ± 0.22</td>
<td>1.46 ± 0.16</td>
</tr>
<tr>
<td>22</td>
<td>1.37 ± 0.17</td>
<td>1.42 ± 0.35</td>
</tr>
<tr>
<td>25</td>
<td>1.23 ± 0.33</td>
<td>2.11 ± 0.14</td>
</tr>
<tr>
<td>28</td>
<td>1.67 ± 0.08</td>
<td>1.96 ± 0.25</td>
</tr>
</tbody>
</table>

Mean ± SD, n = 5–8

**Table 4** Intracellular partial pressure of carbon dioxide (PCO_2) and bicarbonate content ([HCO_3^-]) of mantle tissue in blue mussels, Mytilus edulis under normocapnia (390 µm) and hypercapnia (1120 µm) during acute warming (3°C over night)

<table>
<thead>
<tr>
<th>T (°C) (set)</th>
<th>Normocapnia</th>
<th>Hypercapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCO_2 (µm)</td>
<td>[HCO_3^-] (mmol L^{-1})</td>
</tr>
<tr>
<td>10</td>
<td>6.20 ± 1.26</td>
<td>1.83 ± 0.29</td>
</tr>
<tr>
<td>16</td>
<td>5.76 ± 0.58</td>
<td>1.51 ± 0.07</td>
</tr>
<tr>
<td>22</td>
<td>9.08 ± 0.69</td>
<td>1.79 ± 0.13</td>
</tr>
<tr>
<td>25</td>
<td>17.24 ± 2.87</td>
<td>1.34 ± 0.12</td>
</tr>
<tr>
<td>28</td>
<td>18.30 ± 4.03</td>
<td>1.30 ± 0.27</td>
</tr>
</tbody>
</table>

Mean ± SD, n = 5–8
transformation. Metabolites were identified from calibrated spectra and succinate concentrations were quantified using Chenomx 8.1 (Chenomx Inc., Edmonton, Canada).

**Statistical analysis**

All data were first checked for outliers using Nalimov’s test and, after removal, results were further analysed using R (R Core Team 2014). After testing the data for normality and homogeneity of variance, two-way analysis of variance (ANOVA; R function: aov()) in combination with a Tukey’s post hoc test (Tukey Honestly Significant Difference, R function: TukeyHSD()) was performed to analyse effects of temperature and CO₂ level and possible interactions thereof (see Online Resource 1 for an overview of ANOVA results). The breakpoint temperature of MO₂ indicates the deviation from the exponential relationship with temperature when the slope of the curve changes significantly (phase change), which is usually determined by Arrhenius breakpoint analysis (Sokal and Rohlf 1995). However, this was impossible because the number of temperatures that resulted above potential breakpoints in the warmth is insufficient to calculate linear regressions by the least-square method when stated otherwise. Differences were considered significant if \( p \leq 0.05 \). Values are presented as mean ± standard deviation (SD), \( n = 5–8 \) unless stated otherwise.

**Results**

At control temperature (10°C) respiration rate (MO₂) was similar under normocapnia and hypercapnia (Fig. 1). During warming, MO₂ increased progressively before it levelled off beyond a specific breakpoint temperature (phase change). Under normocapnia the course of MO₂ (Fig. 1a, black circles, solid curve) yields a calculated breakpoint temperature of 20.5°C (Fig. 1a, vertical line). This breakpoint temperature was mirrored in patterns of haemolymph PCO₂ and P0₂ of normocapnic mussels (see below). Interestingly, the MO₂ of normocapnic mussels first showed a consistent increase (\( Q_{10} = 10–22°C = 2.6 \)) before differences among individuals developed above 22°C, which are not reflected in any other trait measured. Above 22°C 37.5% (3 out of 8) of the mussels revealed a progressive decline in MO₂ with further warming (Fig. 1a, grey circles) while the others showed a continued increase (Fig. 1a, white circles) (these “sub-groups” were not used for statistical analyses). In contrast, all hypercapnic mussels showed similar responses during warming. The increasing MO₂ (\( Q_{10} = 25°C = 2.5 \)) started to level off above 25°C in all hypercapnic individuals and \( Q_{10} \) fell below 1.6 resulting in a calculated breakpoint temperature of 25.84°C (Fig. 1b, vertical broken line). Assessing the average response of normocapnic and hypercapnic mussels, a significant main effect of temperature on MO₂ was found (2-way ANOVA, \( F(6,88) = 34.27, p < 0.0001 \)). Oxygenation and acid–base status were similar in haemolymph and extrapallial fluid of all experimental
groups during the entire protocol in line with earlier findings (see discussion). Therefore, only haemolymph data are described in the following. Haemolymph $P_{O_2}$ levels (Fig. 2a) under normocapnia started at $103.9 \pm 19.7$ mmHg, $n = 7$ at 10 °C and mean values remained relatively stable with warming until a decrease occurred above 22 °C towards a $P_{O_2}$ of $81.2 \pm 12.5$ mmHg, $n = 5$ at 28 °C ($\Delta P_{O_2} = -22.7$). In contrast, haemolymph $P_{O_2}$ under hypercapnia started at a somewhat higher level of $117.9 \pm 8.7$ mmHg, $n = 6$ at 10 °C, mean values remaining relatively stable just until 19 °C and decreasing rapidly thereafter, resulting in significantly reduced $P_{O_2}$ at $19 \pm 5$ at 28 °C (Tukey HSD, $p < 0.001$; $\Delta P_{O_2} = -44.7$) compared to 10 °C. Two-way ANOVA identified a significant interaction between temperature and $P_{CO_2}$ treatment (2-way ANOVA, $F(6,82) = 2.24, p = 0.047$) and a main effect of temperature (2-way ANOVA, $F(6,82) = 8.35, p < 0.0001$).

Depending on $P_{CO_2}$ treatment haemolymph $P_{CO_2}$ displayed different patterns during acute warming (Fig. 2b) and was significantly affected by temperature (2-way ANOVA, $F(6,79) = 43.61, p < 0.0001$), $P_{CO_2}$ treatment (2-way ANOVA, $F(1,79) = 17.65, p < 0.0001$) and the interaction thereof (2-way ANOVA, $F(6,79) = 14.14, p < 0.0001$). Starting with similar values at 10 °C (390 µatm: 7.22 ± 0.09, n = 7; 1120 µatm: 7.18 ± 0.07, n = 7). Two-way ANOVA indicated a significant effect by temperature (2-way ANOVA, $F(6,79) = 33.55, p < 0.0001$) and $CO_2$ treatment (2-way ANOVA, $F(1,79) = 66.29, p < 0.0001$). Albeit non-significant, an accidental leak in the MKS system in the beginning of the experiment led to increased seawater $P_{CO_2}$ and $CO_2$ levels during the first two days (10 and 13 °C) of normocapnic experimentation (Table 2) compared to control conditions (Table 1). This resulted in enhanced haemolymph $CO_2$ level (Table 3) and, thus, calculated [HCO$_3^-$] concentration (Fig. 2d; grey symbols). Besides a potential, slight impact on haemolymph $P_{CO_2}$ and pH values at 13 °C (cf. Figure 3), other measured traits remained unaffected by the accidental leak (including pH). Careful evaluation of all present results and the comparative findings from the North Sea study...
(Zittier et al. 2015) suggest that the short-time alterations in seawater chemistry at the beginning of the warming trial under normocapnia did not influence the course of measured traits at higher temperatures. Considering haemolymph CO₂ and [HCO₃⁻] under normocapnia, both levels had been reduced at 16 °C, when seawater PCO₂ was restored again, and remained stable thereafter providing evidence that the elevated values at 10 and 13 °C are an acute response to the accidental elevation in CO₂ and could thus be safely excluded from statistical analysis. At 16 °C haemolymph [HCO₃⁻] levels were similar in both CO₂ treatments and remained stable during warming until a sudden rise occurred at 28 °C (390 µatm: 1.17 ± 0.03 mmol L⁻¹, n = 5 at 16 °C vs. 1.55 ± 0.05 mmol L⁻¹, n = 5 at 28 °C; 1120 µatm: 1.08 ± 0.13 mmol L⁻¹, n = 6 at 10 °C vs. 2.03 ± 0.26 mmol L⁻¹, n = 6 at 28 °C, Tukey HSD, p < 0.0001) (Fig. 2). Two-way ANOVA suggested a significant effect of temperature (2-way ANOVA, F(6,62) = 12.06, p < 0.0001), CO₂ treatment (2-way ANOVA, F(1,62) = 8.13, p = 0.006) and the interaction thereof (2-way ANOVA, F(6,62) = 2.99, p = 0.025). A post hoc test did not reveal any differences between CO₂ treatments until the final temperature of 28 °C was reached, where the rise in [HCO₃⁻] level was stronger under hypercapnia than under normocapnia (Tukey HSD, p = 0.02).

Succinate levels in mantle tissue were unaffected by CO₂ exposure at control temperature (Fig. 3). During warming, no changes in succinate levels were found under normocapnia until a sudden increase was detected at the final temperature of 28 °C. Under hypercapnia succinate levels of mantle tissue were already elevated at 25 °C and levelled off thereafter resulting in a lower final concentration than under normocapnia. Independent of the treatment, all mussels showed high inter-individual variability when succinate increased (390 µatm: 6.43 ± 1.69 arbitrary units (a.u.) at 10 °C vs. 14.50 ± 6.91 a.u. at 28 °C, Tukey HSD, p < 0.05; 1120 µatm: 4.67 ± 1.72 a.u. at 10 °C vs. 8.83 ± 5.73 a.u. at 28 °C). Two-way ANOVA suggested a main effect of temperature (2-way ANOVA, F(4,41) = 4.08, p = 0.007), as well as a significant interaction between temperature and CO₂ treatments (2-way ANOVA, F(2,41) = 3.52, p = 0.04).

Intracellular pH (pHi) remained unaffected by CO₂ exposure at the control temperature (Fig. 4; 390 µatm: 7.03 ± 0.01, n = 5; 1,120 µatm: 6.96 ± 0.14, n = 6) and values remained more or less unchanged during warming until a threshold temperature was reached. pHi remained unchanged under normocapnia until a sudden and significant drop occurred at the final temperature of 28 °C (6.51 ± 0.05, n = 6, Tukey HSD, p < 0.0001). Values under hypercapnia dropped significantly already at 25 °C (6.53 ± 0.07, n = 6, Tukey HSD, p < 0.0001) and decreased further to the minimum value of 6.45 ± 0.04 at 28 °C. Thus, compared to values seen under normocapnia, pH levels of hypercapnic mussels were significantly lower at 25 °C (Tukey HSD, p < 0.0001) before their converged again at the next higher temperature. Accordingly, a significant effect of temperature (2-way ANOVA, F(4,44) = 115.15, p < 0.0001), CO₂ treatment (2-way ANOVA, F(1,44) = 40.81, p < 0.0001) and the interaction thereof (2-way ANOVA, F(3,44) = 19.92, p < 0.0001) were suggested.

Discussion

Thermal limits under normocapnia

Mytilus edulis from the White Sea (sub-Arctic) under normocapnia displayed an oxygen consumption rate (MO₂) of 9.43 µmol O₂ h⁻¹ g DW⁻¹ at the acclimation temperature of 10 °C. These rates are well in the range of those reported...
previously for *M. edulis* (Schlüter and Johansen 1994; Sukhotin and Pörtner 2001; Thomesen and Melzner 2010; Zittier et al. 2015). During acute warming MO₂ increased progressively until a phase change, indicating a beginning thermal limitation, at a calculated breakpoint temperature of 20.5 °C (Fig. 1). Interestingly, beyond 22 °C the respiratory responses to warming began to vary between individuals, which was not reflected in any other measured trait. While 5 out of 8 mussels were able to increase their MO₂ until the final temperature was reached (Q₁₀ (10–28 °C) = 2.4), others showed a decline in MO₂ with further warming and the Q₁₀ value (22–28 °C) fell close to zero. This partially contradicts the findings in sub-Arctic and high-Arctic *M. edulis* populations from Greenland, where MO₂ of all mussels declined above 21 °C (Thyring et al. 2015). However, the differences in metabolic rates in the warmth found in the present study had no influence on both upper thermal threshold *Tₚ* (onset of thermal limitation) and *T_c* (onset of anaerobic metabolism), which were the same in all normocapnic animals (see below), at least in the short-term. Hence, they might reflect differing performance capacities to cope with temperature stress. Recent publications provide increasing evidence that inter-individual variation in basic fitness-related traits, such as activity, metabolic rates, growth performance, physiological and behavioural responses to stressors is biologically meaningful (Sukhotin et al. 2003; Tamayo et al. 2011; Calosi et al. 2013; for review see Careau et al. 2008) and should be taken into account when interpreting experimental results. Further studies are necessary to identify the cause and potential long-term effects of the inter-individual differences in metabolic responses to warming seen in the present study.

According to the OCLTT concept (see introduction), a mismatch between oxygen uptake and demand develops in the warmth beyond the pejus temperature as circulation and/or ventilation reach their capacity limits. In combination with rising oxygen demand, these functional constraints result in internal hypoxemia of the organism, which contributes to progressively reduce aerobic performance. Haemolymph PCO₂ increased significantly in all mussels during warming from 19 to 22 °C followed by the decline in haemolymph PO₂ from 22 °C onward (discussed in detail below) in line with the onset of a fall in aerobic scope and thus *Tₚ*. The average breakpoint temperature of MO₂ was found at 20.5 °C, which is close to the suggested *Tₚ* for all individuals based on the changes in haemolymph status. Thus, the phase change in MO₂ was indicative for the *Tₚ* under normocapnia.

The picture was clearer in North Sea mussels, as the onset of thermal limitation in MO₂ occurred at similar temperatures distinctly below *Tₚ* in all individuals (Zittier et al. 2015). This limitation may have involved metabolic depression induced by the observed drop in haemolymph pH, and a concomitant limitation in heart rate rise. Hence, the phase change in MO₂ in North Sea mussels is indicative for the *Tₚ* as defined by the OCLTT concept. This also supports our conclusion for the White Sea and North Sea populations that the phase change in MO₂, combined with alterations in haemolymph status, can act as indicators for the upper *Tₚ* under normocapnia, even if variable between White Sea individuals. The putative *Tₚ* of sub-Arctic *M. edulis* matches the highest habitat temperature presently experienced by mussels in the White Sea where mean summer surface temperature is 15 °C with extreme temperatures reaching 20 °C (Berger et al. 2001; Dale and Prego 2003; Sukhotin and Berger 2013; Usov et al. 2013).

The critical temperature limit is defined by the onset of anaerobic metabolism when oxygen supply becomes insufficient to cover all energy expenditure aerobically. At *Tₚ*, the main anaerobic metabolite in bivalves, succinate (Zurburg and Klaytmans 1980; Sukhotin and Pörtner 1999; Himes et al. 2007) accumulated significantly in mantle tissue during warming from 25 to 28 °C in all specimens (Fig. 3). Intracellular pH (pHi) of mantle tissue remained almost constant and independent of temperature whereas extracellular pH (pHe) decreased progressively with warming (see below). pH was maintained during warming trial until a strong acidosis set in above 25 °C. Thus, the sudden drop in pHi occurred in parallel to the onset of anaerobic metabolism, thereby co-defining *T_c*. These findings are in line with common results reported for marine ectotherms. For several species differences between temperature-induced pH changes in extra- and intra-cellular compartments were demonstrated (e.g. Walsh et al. 1994; Butler and Day 1993; Sommer et al. 1997). In general pH decreases only slightly with temperature in ectothermic animals and only an extreme decrease is interpreted to indicate critical temperature limits (e.g. Van Dijk et al. 1999; Sartoris et al. 2003; Melzner et al. 2006), which is supported by our data.

In *M. edulis* from the North Sea a reduction in aerobic performance indicating *Tₚ* occurred around 25 °C and anaerobic metabolites defining *T_c* accumulated significantly > 28 °C (Zittier et al. 2015). In the White Sea population (present study) a reduction in aerobic performance occurred already around 20 °C and anaerobic metabolism set in > 25 °C. Both populations were studied under identical environmental temperature regimes. Thus, seasonal impacts should be negligible and differences between populations should mainly be due to their adaptation to different environmental temperature regimes. Thus, both upper thermal limits *Tₚ* and *T_c* were shifted downwards by several degrees in the sub-Arctic population. These findings are in line with evolutionary temperature adaptation to the colder environment in the sub-Arctic population. The thermal window of a species shifts with seasonal acclimatisation or latitudinal adaptation to different temperatures (Sommer et al. 1997; Chapple et al. 1998; Van Dijk et al. 1999; Sommer and Pörtner 2002; Wittmann et al. 2003).
In general, the thermal window is shifted to low temperatures in animals from high latitudes when compared to temperate species and populations (e.g. Pörtner 2001).

Gas and acid-base status of haemolymph and extrapallial fluid were similar in all experimental groups as previously found in the *M. edulis* populations from the North Sea (Zitter et al. 2015) and the Baltic Sea (Thomsen et al. 2010) as well as in *Mytilus galloprovincialis* from the Mediterranean Sea (Gazeau et al. 2014), emphasizing that both fluids are characterized by a similar carbonate system. Therefore, only haemolymph data will be discussed here. As demonstrated for several marine taxa including bivalves, a thermally induced onset of anaerobic metabolism is caused by the reduction in blood PO$_2$ below levels sufficient to sustain oxygen diffusion to mitochondria at elevated demand (Frederich and Pörtner 2000; Peck et al. 2002; Lannig et al. 2004). For example, in the Antarctic bivalve *Latterula elliptica* acute warming led to a sudden and drastic drop in haemolymph PO$_2$ by > 70% from 78.4 (control) to 20.3 mmHg when $T_p$ was reached (Peck et al. 2002). In fact, the decreasing haemolymph PO$_2$ of mussels in the present study indicates that oxygen uptake from haemolymph is not fully compensated anymore by increased respiratory and/or circulatory performance. However, the decrease was less drastic and resulted only in a reduction of around 20% from 103.9 (control) to 81.2 mmHg at $T_p$ (see Fig. 2a), which reflects the finding in North Sea population (Zitter et al. 2015). As discussed by Zitter et al. (2015), the difference to the blood PO$_2$ pattern reported by Peck et al. (2002) might be related to where the analysed haemolymph was drawn from. In contrast to sampling from the pericardium (Peck et al. 2002), haemolymph was sampled from the posterior adductor muscle in both *Mytilus* studies and is assumed to contain a mixture of pre- and post-branchial haemolymph (Booth et al. 1984; Walsh et al. 1984) that might disguise an oxygen limitation on the venous side and at the cellular level.

In parallel, a sharp increase in haemolymph P$_{CO_2}$ occurred above 19 °C. Highly elevated haemolymph P$_{CO_2}$ can be related to insufficient CO$_2$ release at high metabolic rates or may result from depressed ventilatory and circulatory activities due to cellular metabolic depression. Both suggest a reduction in aerobic scope during temperature stress incurring a $T_p$ of > 19 °C. A second sharp increase in haemolymph P$_{CO_2}$ occurred in parallel to the onset of anaerobic metabolism and, thus, at $T_C$. Here, extracellular acidosis may exacerbate a rise in P$_{CO_2}$ through titration of carbonates, e.g. from the shell. This pattern of P$_{CO_2}$ had not previously been reported.

In contrast to pH, pH decreased progressively during the warming protocol and no active regulation, indicated by bicarbonate [HCO$_3^-$] accumulation, was found until the final temperature of 28 °C when extracellular [HCO$_3^-$] increased slightly (Fig. 2d). Some reduction of pHe would be expected from the alphabet principle (~ 0.017 units °C$^{-1}$; Reeves 1972). If pHe is reduced more strongly, as seen in the present study, this can elicit metabolic depression (e.g. Reipschläger and Pörtner 1996; Pörtner and Bock 2000). The phase change in MO$_2$ coincided with the fall in pHe from 7.43 at 19 °C to 7.39 at 22 °C and the subsequent reduced MO$_2$ of some animals at 25 °C coincided with a pHe of 7.33. The strong reduction in MO$_2$ of some animals suggests that a pHe of < 7.4 can induce metabolic depression in sub-Arctic *M. edulis*. In the North Sea study (Zitter et al. 2015), pHe did not decrease progressively but stepwise during warming. A strong drop in pHe from 7.38 at 22 °C to 7.01 at 25 °C occurred in parallel to the limitation in MO$_2$ rise of all individuals. This led to the suggestion that a pHe of at least < 7.3 is necessary to elicit a depression of aerobic metabolism. The present findings support this conjecture; however, the pH threshold inducing metabolic depression may not only differ between species but also between populations and might be influenced by temperature and adaptation to high CO$_2$ environments (cf. Michaelidis et al. 2005; Thomsen and Melzner 2010). For example, blue mussels from the western Baltic Sea showed control metabolic rates in laboratory experiments even under 4000 µatm resulting in a pHe of 7.1 (Thomsen and Melzner 2010). However, this population regularly faces extreme environmental P$_{CO_2}$ values up to 2300 µatm during summer and autumn (Thomsen et al. 2010).

In summary, our results indicate a downward shift in both upper thermal limits pejus and critical at high latitudes (White Sea vs. North Sea: $T_p$ − 20 °C vs. 25 °C, $T_C$ − 28 °C vs. 31 °C). The reduced heat tolerance in White Sea mussels is likely the result of evolutionary adaptation to the sub-Arctic climate. Considering that current environmental change develops more rapidly and strongly at high latitudes future warming may pose a high risk for the sub-Arctic population. According to IPCC scenarios, the projected temperature rise for the year 2100 of this region is + 6 °C (IPCC 2007) suggesting that future summer temperatures may exceed the pejus limit of the White Sea population. This, in turn, might result in reduced abundance of blue mussels in the intertidal or shallow subtidal zones in the White Sea with implications for the structure, function and services of this ecosystem.

**CO$_2$ effects on thermal limits**

Moderate hypercapnic exposure (1120 µatm) of *M. edulis* from the White Sea had no impact on MO$_2$ compared to normocapnia (Fig. 1b), a finding in line with results reported for other *Mytilus* populations (Thomsen et al. 2010; Zitter et al. 2015). During acute warming under hypercapnia, MO$_2$ rose progressively in all individuals until the final temperature of
28 °C. The calculated breakpoint temperature indicating the onset of the limitation in MO2 rise was 25.8 °C. Succinate accumulated in mantle tissue already at 25 °C defining the critical temperature Tc. Hence, the phase change in MO2 under hypercapnia was shifted to the critical limit while the one under normocapnia occurred at the pejus limit (see “Discussion” section; Fig. 1). As the temperature response in MO2 changed with CO2 exposure the breakpoint cannot be used as a general indicator for a specific thermal threshold. This matches the findings of the North Sea study (Zittier et al. 2015) where hypercapnic animals during warming remained in a more active mode and the limitation in MO2 indicated Tc while mussels under normocapnia showed a limitation around Tp likely due to an onset of metabolic depression. Mild hypercapnia can apparently prevent metabolic depression in M. edulis (Zittier et al. 2015), however, the exact mechanism remains obscure. A higher metabolic rate stimulated by hypercapnia (1500 µatm) during acute warming has also been reported for oysters (Lannig et al. 2010). In crustaceans CO2 stimulated cardiac performance through adenosine release (Stegen and Grieshaber 2001) but led to an early breakdown of heart rate when combined with acute warming (Walther et al. 2009). However, heart rate of M. edulis from the North Sea stagnated during warming, independent of seawater CO2 concentration (Zittier et al. 2015). Long-term CO2 exposure of M. edulis (8 weeks, ~480 to 3800 µatm) without a thermal challenge showed that aerobic metabolism is first increasing and then declining with rising CO2 (Thomsen and Melzner 2010). A study on the metabolic profile of M. edulis revealed that long-term CO2 exposure (90 days at ~2400 and ~3800 µatm) reduced succinate content in mantle tissue and thus (anaerobic) energy turnover, whereas succinate was strongly increased under the extreme levels of ~24,000 µatm (Ellis 2013). In these studies it was assumed that moderate hypercapnia increases aerobic metabolism most probably to compensate for an increased cellular energy demand while higher CO2 levels lead to a limitation in aerobic metabolic rate. This is also in line with findings in M. galloprovincialis where exposure to ~5000 µatm at unchanged temperature led to metabolic depression (Michaelidis et al. 2005), which did not occur at lower levels of ~1100 µatm (Gazeau et al. 2014). However, the mechanisms resulting in metabolic stimulation by moderate hypercapnia are not yet clear and require further study.

The accumulation in succinate defining the critical limit was already seen at 25 °C compared to 28 °C under normocapnia. However, derived from succinate accumulation anaerobic metabolic rate remained lower under hypercapnia even when Tc was exceeded (Fig. 3), which may imply that an anaerobic energy turnover is suppressed by moderately elevated CO2. Mantle pH was maintained under hypercapnia at moderate temperatures. The strong decrease in pH was already seen at 25 °C compared to 28 °C under normocapnia in accordance with the respective onset of anaerobiosis supporting a downward shift in Tc by around 3 °C under hypercapnia. This may reflect a reduction of energy-dependent processes under combined hypercapnia and warming and may be useful to withstand temperature extremes. However, tolerating the exceedance of Tp is only possible in the short-term but will result in death during longer exposure periods.

An ecologically more important thermal limit is the pejus temperature as it may define the distribution range of the population according to OCLTT (see “Introduction” section). Our findings on oxygen and acid–base status of haemolymph provide evidence that the Tp is also shifted downwards by CO2 as reported for other marine ectotherms (e.g. Metzger et al. 2007; Walther et al. 2009; Dissanayake and Ishimatsu 2011; Schalkhauser et al. 2012; Schiffer et al. 2014; for review see Pörtner 2012). Haemolymph PO2 was initially slightly increased under hypercapnia but decreased progressively above 19 °C a pattern that only occurred above 22 °C under normocapnia. Accordingly, a significant increase in haemolymph PCO2 during warming under hypercapnia was found at 19 °C rather than at 22 °C as under normocapnia. These results indicate a thermal limitation in aerobic performance at lower temperatures and a downward shift of the Tp in parallel to Tc by around 3 °C under hypercapnia. Thus, ocean acidification projected for the end of the century (~700–1100 µatm; Meeth et al. 2007) has the potential to reduce the thermal envelope as well as ecologically relevant thermal limits of sub-Arctic M. edulis.

Hypercapnic exposure caused a decrease in haemolymph pH (pHe) when compared to normocapnia. This reduction was similar across the warming protocol, which induced a general decrease in pHe. Upon warming this lowered pHe reaches a specific threshold at lower temperatures, which might hamper the further rise in MO2 (see “Discussion” section) but can be compensated for by CO2 stimulation. In hypercapnic mussels pHe fell indeed below 7.4 (the suggested pH threshold under normocapnia) at a clearly lower temperature and decreased progressively thereafter, but had no implications on MO2 rise. This is in line with the findings in the North Sea population (Zittier et al. 2015) and emphasizes the stimulating effect of moderate hypercapnia on metabolism (see above).

The temperature-induced extracellular acidosis remained uncompensated throughout the warming protocol until a sudden and significant [HCO3−] accumulation was found at the final temperature of 28 °C, when Tc was already surpassed. The interpretation of bicarbonate accumulation in response to temperature extremes in M. edulis must remain speculative at present. The drop in pHe in hypercapnic mussels might indicate bicarbonate release from intracellular sources (c.f. Table 4) beyond the acidosis caused by anaerobic metabolism. Under normocapnia a trend towards increasing haemolymph [HCO3−] concentration was indicated when
TC was reached. Mussels from the North Sea might have responded similarly. Normocapnic mussels experienced a significant increase and hypercapnic mussels a slight increase in haemolymph [HCO₃⁻] when TC was reached (Zitter et al. 2015). However, data under further warming and on pH status are not available for this population. The authors assumed that residual acid–base regulation capacity may be used as a last defence under normocapnia, which was not possible anymore under hypercapnia in the warmth. The foregoing discussion provides evidence for a higher sensitivity to thermal stress and hypercapnia in sub-Arctic mussels than in temperate ones. Nevertheless, in the more sensitive White Sea mussels bicarbonate was significantly increased at hypercapnia at extreme temperatures. It needs to be clarified in further studies where the bicarbonate comes from and whether this is an additional sign of stress rather than a regulatory response.

**Conclusion**

In conclusion, the thermal tolerance thresholds found in this study are in line with those defined by the OCLTT concept. In line with the general picture (e.g. Pörtner et al. 2009, 2012; Peck et al. 2014) the thermal window of the sub-Arctic White Sea population is shifted to lower temperatures when compared to the temperate North Sea population. In contrast to the North Sea population, CO₂ exposure caused enhanced thermal sensitivity in White Sea mussels indicated by an earlier increase in haemolymph PCO₂ as well as an earlier onset of anaerobic conditions visible in the accumulation of succinate and the accompanying drop of intracellular pH, and possibly, cellular bicarbonate release. Obviously, tolerance to thermal stress and hypercapnia in Mytilus sp. (e.g. Gosling 1992) is constrained at high latitudes as a result of cold adaptation, possibly making this population vulnerable to future climate change. Studies of long-term CO₂ exposure in Mytilus species (Berger et al. 2001; Michaelidis et al. 2005; Beasley et al. 2008; Thomsen and Melzner 2010; Thomsen et al. 2010) indicate a reduction in whole organism performance capacity by CO₂ suggesting that longer exposure periods may exacerbate vulnerability. The larger degree of projected warming of the White Sea area until 2100 by itself (+6°C vs. +3°C in the North Sea, IPCC 2007) but especially in combination with the projected rise in oceanic CO₂ levels (~1000 µatm, IPCC 2007, 2013) may induce physiological stress with implications for the fitness and associated distribution range of this blue mussel population.

**Acknowledgements**

This work is a contribution to the European Project on Ocean Acidification (EPOCA) which received funding from the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no 211384. EPOCA is endorsed by the International Programmes IMBER, LOICZ and SOLAS. This project was also supported by the Helmholtz Graduate School for Polar and Marine Research (POLMAR) and a Research Programme of RAS “Exploratory Basic Research for Development of the Russian Arctic Zone”, AAAA-A17-117021300219-7. The authors would like to thank Rolf Wittig for NMR support and Stephan Frickenzaehn for statistical support.

**References**

Dissernayake A, Ishimaru A (2011) Synergistic effects of elevated CO₂ and temperature on the metabolic scope and activity in a shallow-water coastal decapod (Metapenaeus Joyneri;


IPCC (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 University Press, Cambridge and New York

IPCC (2013) Climate change 2013: the physical science basis contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge and New York


User N, Kuccheva I, Primakov I, Martyanova D (2013) Every species is good in its season: do the shifts in the annual temperature dynamics affect the photoinhibition of the zooplankton species in the White Sea? Hydrobiologia 706:11–33


4 Discussion

The aim of the present thesis was to provide insights on the combined effects of temperature rise and ocean acidification on the physiological performance of calcifying marine invertebrates, the decapod crustacean *Hyas araneus* and the bivalve mollusc *Mytilus edulis* with a focus on populations from high latitudes that may be particularly vulnerable to climate change. Organisms adapted to cold and thermally stable environments like polar areas or the deep-sea exhibit several functional constrains with the result of a high sensitivity to environmental change (Sommer et al. 1997, van Dijk et al. 1999, Peck and Conway 2000, Pörtner 2001, Sokolova and Pörtner 2003, Pörtner et al. 2009, Peck et al. 2014). In view of ongoing climate change such species may be under greatest threat. It is hypothesized that cold-adapted animals show narrower thermal windows and higher temperature sensitivities than species and populations from temperate regions. In general they exhibit low metabolic rates allowing for low energy intake. Energy savings may be enabled by low acid-base regulation capacities, which, in turn, may be a disadvantage when exposed to pH changes. A decrease in pH of body fluids affects oxygen supply, metabolism, protein function and other biological key processes. Acid-base regulation is crucial to ensure cellular function and thus whole animal performance and survival, yet it comes at an energetic cost that has to be covered. Therefore it is hypothesised to be a key process in defining resilience to CO₂. Accordingly, Publication 1 addressed the question to what extent Arctic *Hyas araneus* is able to compensate for acid-base disturbances under different realistic CO₂ scenarios and temperature regimes and how these conditions affect activity performance. Publication 2 and 3 elaborated the physiological characteristics of thermal tolerance and the impact of hypercapnia of two geographically distinct populations of *M. edulis*, from the temperate North Sea and the subarctic White Sea, respectively. In this chapter, the main results of the three publications will be summarised and discussed in an integrated way with regard to potential implications of projected future ocean conditions on the ecological performance of different species at the population level. The following section (4.1) will focus on the physiological performance of Arctic *H. araneus* in the context of climate change. The subsequent section (4.2) will elaborate the climate-related response of different *Mytilus* populations along a latitudinal cline.
4.1 Effect of CO₂ and temperature rise on the performance capacity of *Hyas araneus*

In general, decapod crustaceans are seen as good acid-base regulators able to compensate for environmentally induced internal acidosis within a few hours or days (Truchot 1975, DeFur et al. 1983, Whiteley et al. 2001, Pane and Barry 2007, Spicer et al. 2007, Small et al. 2010, Dissanayake and Ishimatsu 2011, Rastrick et al. 2014). Yet most studies have been conducted with temperate shallow-water species within their moderate habitat temperatures leaving the situation in cold-adapted species unknown. A first insight from a deep-sea crab revealed a weak ability for haemolymph acid-base regulation under hypercapnia in comparison to a shallow-water counterpart, which was attributed to its cold-stenothermal adaptation (Pane and Barry 2007). While the shallow living crab fully compensated the hypercapnia induced extracellular acidosis by substantial bicarbonate accumulation, the deep-sea crab accumulated only a small quantity of bicarbonate and extracellular pH remained almost uncompensated. Even though the low ability for acid-base regulation was detected in the very short time after exposure to an extremely high CO₂ level (8,000 µatm for 24 hrs) an acid-base imbalance was also observed in Arctic *Hyas araneus* after mid-term exposure to realistic future hypercapnic levels (1,120 and 3,000 µatm for 12 days at 1°C; present study). In fact, *H. araneus* is able to regulate its internal milieu by significantly accumulating bicarbonate under increasing CO₂ level but still failed to restore haemolymph pH. These results confirm a low capacity for acid-base regulation of the Arctic population. Respiration rate and thus energy intake of Artic *H. araneus* is reduced by the low water temperature when compared to populations and species from warmer waters (Camus et al. 2002) leading to the low capacity for active regulation identified in the present study. Warm-acclimation to 4°C (spring condition vs. 1°C winter condition) did not stimulate acid-base regulation but exacerbated the impact of CO₂ resulting in a significantly stronger decrease in haemolymph pH and potential restrictions in active bicarbonate accumulation as levels did not increase further (Fig. 11). The intensified impact of CO₂ due to a +3°C increase in acclimation temperature suggests a high sensitivity to environmental change that may indicate adjustments to life in the cold in the Artic population. This is supported by findings in *H. araneus* larvae from different latitudes suggesting enhanced cold adaptation and sensitivity to environmental change in the Arctic population compared to boreal ones (Walther et al. 2010, Schiffer et al. 2014).

Longer times of exposure might allow animals to fully acclimate to new environmental conditions but can also reveal harmful effects not visible during shorter exposure times. In Artic *H. araneus* long-term hypercapnic exposure (10 weeks, 5°C) resulted in an intensified
DISCUSSION

Fig. 11 Haemolymph pH (A, B) and bicarbonate content (C, D) of Arctic *Hyas araneus* after short-term (12 days, present study) and long-term exposure (10 weeks, Schiffer 2013) to different CO2 concentrations and temperatures. * = significantly different from animals under control CO2 levels at the same temperature, ° = significantly different between 1°C- and 4°C-acclimated animals. Data are given in mean ± SE, n = 4-8.

extracellular acidosis along with constraints in bicarbonate accumulation (Schiffer 2013, Harms et al. 2014; see Fig. 11). After mid-term exposure under 3,000 µatm (present study) haemolymph pH was reduced by 0.3 units while after long-term exposure the same pH reduction was already found under 1,900 µatm along with much lower bicarbonate concentrations in the haemolymph. Covering increased energetic costs to compensate for hypercapnic acidosis may not be possible in the long-term resulting in a (progressive) lowering of the regulation capacity during extended exposures revealing the inability to acclimate. These results indicate that even moderate hypercapnia (<2,000 µatm) may lead to impairments in the long-term and that the Arctic population might not be able to adapt to future hypercapnic conditions. This assumption is
supported by the few available long-term studies (several months) of decapod crustaceans under realistic oceanic CO₂ scenarios (≤ 2,000 µatm) revealing negative effects on growth, immune response, reproduction and even survival (Kurihara et al. 2008, Hernroth et al. 2012, Long et al. 2013).

Furthermore, negative carry-over effects from the embryonic phase to the consecutive larval stages and between larval stages were found under hypercapnia in the Arctic population (Walther et al. 2010, Schiffer et al. 2014). Without adaptive mechanisms, persistent acid-base disturbances impair cellular functioning that will weaken whole animals performance and fitness over time. Food availability is an important factor influencing performance of crustacean and food supply may play a determining role in resilience to ocean acidification (Hartnoll 2001, Pansch et al. 2014, Ramajo et al. 2016).

In ectothermic animals such as crustaceans, temperature affects the capacity of activity. In the cold they show a reduced capacity, slow faster during forced activity, and activity per se even induces higher energetic costs than in temperate waters (McLeese and Wilder 1958, Barlow and Kerr 1969, Rebach 1974, Burke 1979, Young et al. 2006, Barnes and Peck 2008). A progressive decline in activity occurs towards the edges of the thermal tolerance window until it finally ceases (Bennett 1990, Davenport 1997, Hopkin et al. 2006 Brattstrom 1968, Wittmann et al. 2012). Results from an Antarctic crustacean indicate that the loss of motor activity at sublethal extreme temperatures in cold-adapted animals may predominantly be caused by a neuronal breakdown (Clark et al. 2016) likely indicating Tₐ. Further, it has been reported that severe hypercapnic exposure of decapod crustaceans led to disturbances of energy-demanding activities including behaviour (De la Haye et al. 2011, Dissanayake and Ishimatsu 2011). Also, hypercapnic exposure resulted in a narrowing of the thermal tolerance window by a downward shift of the upper critical temperature, which has also been demonstrated for the North Sea population of H. araneus (Metzger et al. 2007, Walther et al. 2009). Thus, hypercapnia is proposed to compromise aerobic scope and associated performance capacity in ectotherms especially towards the edge of the thermal window (q.v. Pörtner and Farrell 2008, Rosa and Seibel 2008).

In Arctic H. araneus neither the rise in acclimation temperature (4°C vs. 1°C) nor moderate hypercapnic exposure and associated disturbances in acid-base balance affected the rate of activity (Fig. 12). Moderate hypercapnia did not lead to any oxygen and associated activity limitation within the ecological temperature range of the arctic population (for a detailed discussion see Publication 1). However, this conclusion relates to short-term exposure and, considering the exacerbated acid-base disturbances (see above), future hypercapnic levels might
have the potential to initiate restrictions in aerobic scope and performance capacity in the long-term.

**Fig. 12** Righting response prior to and after additional heat exposure (12 °C) of A) cold-(1°C) and B) warm (4°C)-acclimated *H. araneus* at different CO₂ concentrations. * = significantly different from animals under control CO₂ levels at the same temperature, + = significantly different between acclimation temperature and heat stress at the same CO₂ concentration, ° = significantly different between cold- and warm-acclimated animals. Data are given in mean ± SE, n = 6.

Acute heat stress (12°C, 15 min) had no effect on the righting activity of all crabs incubated at control CO₂ but led to significant slowing when both stressors were combined and acted synergistically (Fig. 12). The combination of heat and hypercapnic exposure caused a functional limitation in both groups but to different degrees. In cold-acclimated crabs the impact was much stronger and resulted in a drastic slowing of the righting response (from ~60 s at 1°C to ~190 s at 12°C) under medium (1,120 µatm) and high (3,000 µatm) CO₂ and some individuals even stopped responding. Warm-acclimated crabs under these conditions became significantly slower (from ~40 s at 4°C to ~80 s at 12°C) but were still relatively active throughout the righting trials and no differences between CO₂ treatments were found. The integrated stress of two righting trials and heat exposure affected the gas and acid-base status of haemolymph only slightly (for a detailed description see Publication 1). A general decline of haemolymph PO₂ occurred, likely reflecting an increase in oxygen demand during activity and temperature rise (Publication 1, Fig. 3). The decline was strongest in all crabs exposed to the highest CO₂ level providing evidence for restrictions in aerobic scope, either caused by an increased energy demand or by
DISCUSSION

exacerbated capacity constraints, when hypercapnia is combined with warming.

Lactate accumulated in all crabs exposed to the combined stressors implying a contribution of anaerobiosis to cover the energetic needs in all treatments. However, this effect was inverse between the two acclimation groups (Fig. 13). In cold-acclimated crabs lactate accumulation followed the respective activity level with the highest content under normocapnia and the lowest under the highest CO$_2$ level when crabs slowed drastically. Warm-acclimated crabs showed an opposing trend. Although significant slowing occurred under medium and high CO$_2$, all warm-acclimated crabs showed similar activity levels after heat stress and lactate accumulated with rising CO$_2$ level. As for haemolymph $\text{PO}_2$, these results suggest higher energetic costs for activity or exacerbated capacity constraints when hypercapnia is combined with acute warming.

![Fig. 13 Lactate content of the haemolymph prior to and after the additional stress of combined activity and heat exposure (12 °C) for A) cold-(1°C) and B) warm (4°C)-acclimated $H. araneus$ at different CO$_2$ concentrations. * = significantly different from animals under control CO$_2$ levels at the same temperature, + = significantly different between acclimation temperature and heat stress at the same CO$_2$ concentration, ° = significantly different between cold- and warm-acclimated animals. The dashed line gives the detection limit of 6 µM. Data are given in mean ± SE, n = 5-6.](image)

The observed differences between the two acclimation groups are possibly due to the stronger 11°C temperature rise for cold- compared to the 8°C rise for warm-acclimated crabs. This indicates that a relatively small change in acclimation temperature alters the response to temperature extremes, emphasizing the high thermal sensitivity of this population, which is exacerbated in synergism with hypercapnia. Combining the present results and literature data, we assume that CO$_2$ exposure has shifted the upper critical temperature downwards illustrated by a
limitation in functional scope, which indicates restrictions in aerobic scope. Accordingly, crabs acclimated to 1°C reached the edge of their performance window and were, thus, close to their upper critical temperature when acute heat exposure to 12°C was combined with CO₂ levels of 1,120 µatm and higher. Warm-acclimation to 4°C may have shifted the thermal limits upwards so that acute exposure to 12°C under elevated CO₂ levels impair the animals’ performance but, due to their comparatively high activity level, had not reached their critical limits yet. Furthermore, the drastic slowing without signs of fatigue (continuous slowing; see Publication 1) in cold-acclimated crabs under heat stress combined with CO₂ levels of 1,120 µatm and higher also provides evidence for rising dysfunctions in neuronal control as indicated in a cold-adapted amphipod crustacean under heat stress (Clark et al. 2016). It has been demonstrated that hypercapnia can impair ecologically-relevant behaviour in crabs, such as decision-making and predator avoidance and defence, indicating shifts in neurological functions by elevated CO₂ (Bibby et al. 2007, Dixson et al. 2010, de la Haye et al. 2011), yet the underlying mechanisms remain unexplored. Such disturbances are likely to result in adverse effects on the population level and need to be examined for better predictions of climate-induced impacts on ecosystems.

It is well described that (forced) activity in aquatic and terrestrial decapod crustaceans causes a strong rise in haemolymph $P_{CO₂}$ and lactate content associated with a strong depression in pH (Phillips et al. 1977, Burke 1979, D. McDonald et al. 1979, McMahon et al. 1979, Smatresk et al. 1979, Booth et al. 1982, Booth, McMahon, et al. 1984, Wheatly et al. 1986). Bursts of activity are predominantly or at least partially covered by anaerobiosis independent of oxygen availability. These insights relate exclusively to temperate shallow-water specimens and contrast the pattern found in Arctic *H. araneus*. In the Arctic population, two righting trials and acute heat exposure had only slight effects on haemolymph status under normocapnia and changes primarily occurred under additional CO₂ exposure. Under hypercapnia haemolymph $P_{CO₂}$ increased in cold-acclimated crabs, where the rate of activity was low, but decreased in the more active warm-acclimated crabs. It was suggested that the decrease in $P_{CO₂}$ is caused by the higher activity level and potentially related increase in ventilation and/or blood circulation resulting in a higher gas exchange (Publication 1, Fig. 4). Changes in haemolymph pH after activity and heat stress were negligible in cold-acclimated crabs but resulted in a significant pH rise in warm-acclimated crabs under hypercapnia. In fact, a rise in haemolymph lactate concentration (albeit not significant) was found in all crabs after activity and heat stress, however, all measured lactate levels remained far below the ones found in the studies cited above, also below those under resting conditions. The minimal quantities of lactate detected in the present study imply that forced activity in Arctic *H. araneus* is predominantly covered aerobically, even if the scope for
activity is largely reduced, contrasting the situation in temperate species. The low amount of lactate might explain why activity did not result in an acidosis but not the revealed alkalosis. The rise in haemolymph pH may have involved the above-mentioned higher CO₂ release and potential degradation of phosphagen phospho-L-arginine, a mechanism of anaerobic energy production that leads to metabolic alkalization in the absence of glycolysis (Pörtner 1987).

The only two studies available on haemolymph lactate levels of crustacean from cold-stenothermal environments were obtained from an undisturbed polar crab and a deep-sea crab under the effect of elevated CO₂, both revealed concentrations well below those of their counterparts from temperate shallow-water environments (Whiteley et al. 1997, Pane and Barry 2007). On the basis of the few available studies, it appears that the low quantity of lactate formed in undisturbed animals as well as after stress exposure seems to be characteristic for crustaceans living in the cold. Further studies should be performed to verify this hypothesis and to clarify if the low ability for anaerobiosis might be an indicator for cold adaptation in decapod crustacean. The assumption is supported by studies on acute thermal tolerance of various Antarctic species, which revealed a reduced anaerobic ability in cold-adapted fish and several invertebrates (Crockett and Sidell 1990, Clark et al. 2016).

In conclusion, the present results demonstrated that the synergistic effects of ocean acidification and warming reduced whole animal performance of Arctic H. araneus likely mediated by an involvement of acid-base disturbances. In line with the OCLTT concept, exposure to temperature extremes caused restrictions in the scope of performance in all crabs, which was intensified by rising CO₂ levels. A relatively small change in acclimation temperature altered the response of Artic H. araneus to environmental challenges (CO₂, temperature extremes) especially when they act synergistically. The present results emphasize a high thermal sensitivity of this population and suggest permanent cold adaptation associated with enhanced vulnerability to environmental changes. Hence, future ocean conditions may cause reduced organismal performance of Arctic H. araneus resulting in potential adverse effects for the population’s fitness with consequences at the ecosystem level.

4.2 Thermal tolerance and the impact of CO₂ on Mytilus edulis populations

The following discussion (4.2.1) will elaborated the physiological characteristics of thermal tolerance under normocapnia as well as hypercapnia of two geographically distinct populations of M. edulis, from the temperate North Sea (54°N, Publication 2) and the subarctic White Sea
(66°N, Publication 3). The following section (4.2.2) will focus on common phenomena in the warming response and how they are impacted by CO$_2$. Subsequently, latitudinal differences, and thus the differences between mussel populations from the North Sea and White Sea, are outlined (4.2.3). In addition, unpublished material on the environmental stress response in *M. galloprovincialis* from the Mediterranean Sea (41°N) will be included (in section 4.2.2 and 4.2.3). The information of this closely related species is implemented to gain insight into the general response of *Mytilus* species to climate change and to identify potential differences in the thermal response across a climate gradient.

4.2.1 Thermal tolerance and the impact of CO$_2$ on temperate and subarctic *M. edulis*

**North Sea**

In normocapnic *M. edulis* from the North Sea acute warming (10° - 31°C) led to an exponentially increasing oxygen consumption rate (MO$_2$) until a limitation set in. The pattern deviated from the exponential relationship at a calculated breakpoint temperature of 25.2°C (Fig. 14; Publication 2, Fig. 2A). Heart rates rose progressively upon warming and stagnated above 25°C indicating that the capacity limit of cardiac performance coincided with the beginning constraints in MO$_2$ (Fig. 15; Publication 2, Fig. 5A, B). These findings demonstrate the onset of a limitation in oxygen supply capacity and thus aerobic performance above 25°C characterizing the pejus temperature (T$_p$, onset of falling aerobic scope) according to the concept of oxygen- and capacity-limited thermal tolerance (OCLTT; see section 1.1). A putative T$_p$ of around 25°C in North Sea *M. edulis* was confirmed by previous studies, which showed a beginning limitation in several performance parameters such as ventilation and filtration rate at this temperature (Bayne 1973, Widdows 1973a, 1973b, Jansen et al. 2007). Hence, in normocapnic *M. edulis* the breakpoint temperature of MO$_2$ can act as an indicator for T$_p$.

In the current study, analyses of haemolymph acid-base status indicated that temperature-induced changes in extracellular pH (pHe) were involved in setting the pejus limit. While other haemolymph parameters (i.e. PO$_2$, PCO$_2$, [HCO$_3^-$]; Publication 2, Fig. 4) showed no clear picture, acute warming induced a progressive decrease in pHe with a drastic drop between 22° and 25°C that may have influenced the subsequent course of respiration and heart rate. As extracellular acidosis can hamper metabolic rates (e.g. Reipschläger and Pörtner 1996, Michaelidis et al. 2005b) the observed limitation in MO$_2$ likely involved metabolic depression induced by a decreased pHe (see Publication 2).
DISCUSSION

Fig. 14 Oxygen consumption rate (MO$_2$) in blue mussels, *M. edulis* from the North Sea under 390 µatm (filled circles) and 1,120 µatm CO$_2$ (open circles) during acute warming (3°C/night). Data are fitted by a sigmoidal model and vertical lines indicate the breakpoint temperature, when a limitation in MO$_2$ rise occurs (for details see Publication 2 and section 2.2.6). For better viewing symbols were shifted to the left (filled circles) or right (open circles). The number of animals is given in parentheses if below 5. * = significantly different from the respective data at 10°C. Data are given in mean ± SD.

According to the OCLTT concept, the critical temperature limit (T$_c$) is defined by the onset of anaerobic energy production that occurs when the oxygen supply to tissues is insufficient to cover the temperature-induced rise in energy demand. In the present study, the main anaerobic metabolite of bivalve mitochondria, succinate, accumulated (in mantle tissue) during the final temperature rise from 28° to 31°C (Fig. 15). The transition to anaerobiosis was accompanied by a falling heart rate indicating the breakdown of the circulatory system as recently described for scallops (Xing et al. 2016). Hence, the T$_c$ of normocapnic North Sea *M. edulis* was reached above 28°C. In addition, about half of the animals (4 out of 7) showed a drop in MO$_2$ at the final temperature, in line with a lower performance capacity to cope with temperature extremes.

All these indications suggest insufficient oxygen delivery to mitochondria that might also be reflected by a falling haemolymph PO$_2$ as shown for various marine taxa (Frederich and Pörtner 2000, Peck et al. 2002, Lannig et al. 2004, 2008, Giomi and Pörtner 2013). In fact, *M. edulis* exhibited a decreasing trend in PO$_2$ with warming. However, values showed high inter-individual variability and the total reduction of the mean was ~25% resulting in a final level of
~85 Torr (Publication 2, Fig. 4A). For comparison, in the Antarctic clam *Laternula elliptica* haemolymph $PO_2$ decreased slightly with warming until it fell sharply when $T_c$ was reached resulting in a total reduction of >70% and a final value of ~20 Torr, which occurred concomitant with a rapid decline in $MO_2$ and heart rate (Peck et al. 2002). In the study by Peck et al. (2002) haemolymph was withdrawn directly from the ventricle, while in the present study it was sampled from the posterior adductor muscle. Here blood flows through open sinuses, which may disguise cellular oxygen deprivation (cf. Booth, McDonald, et al. 1984, Walsh et al. 1984, Gosling 2003). However, haemolymph $PCO_2$ of *M. edulis* rose substantially when $T_c$ was reached (Publication 2, Fig. 4B). This increase is likely caused by internal acidosis and reduced gas exchange in line with the observed restrictions in respiratory and cardiac functions. Therefore, haemolymph $PCO_2$ may be more suitable to characterize thermal limits than $PO_2$. This is also supported by our findings in the populations of the White Sea (Publication 3) and the Mediterranean Sea (unpublished material; see below).

Another characteristic of the critical limit in normocapnic *M. edulis* was the sudden rise in haemolymph bicarbonate. During the temperature-induced progressive extracellular acidosis, $[HCO_3^-]$ content remained relatively constant until a sudden and significant accumulation occurred above 28°C (Publication 2, Fig. 4D; Fig. 21). This may indicate that reaching $T_c$ provokes the use of residual acid-base regulation capacity as a last effort in defence (but see section 4.2.2).

In general, the short-term (14 days) moderate CO$_2$ exposure (1,120 µatm) had little impact on physiological measures and thermal limits of North Sea mussels, but modulated their response to warming (for a detailed discussion of the results see Publication 2). At the control temperature (10°C), CO$_2$ exposure caused an extracellular acidosis that was partially compensated for by significant $[HCO_3^-]$ accumulation resulting in a somewhat lower pH$_e$ than under normocapnia. Nevertheless, $MO_2$, heart rate, and anaerobic metabolism of CO$_2$-exposed mussels remained close to control values suggesting that the basal metabolic turnover as well as the cardiac activity of resting blue mussels at control temperature are unaffected by moderate hypercapnia, at least in the short-term.

Both upper thermal limits, pejus and critical, of North Sea *M. edulis* appear to be unaffected by moderate CO$_2$ exposure. As seen under normocapnia, warming of hypercapnic mussels caused a significant drop in pH$_e$ during the temperature rise from 22° to 25°C followed by a limitation in cardiac performance (Fig. 15). This indicates a similar $T_p$ of around 25°C for both CO$_2$-groups. The transition to anaerobiosis, determining $T_C$, occurred above 28°C accompanied by a sudden rise in haemolymph $PCO_2$ regardless of CO$_2$ exposure. In contrast to normocapnia,
the rise in haemolymph $[\text{HCO}_3^-]$ when reaching $T_C$ is distinctly lower under hypercapnia. Apparently, the residual acid-base regulation capacity at extreme temperatures is not available for CO$_2$-exposed mussels suggesting a higher energy demand when warming is combined with hypercapnia. Furthermore, CO$_2$ exposure delayed the onset of limitation in MO$_2$ rise resulting in a breakpoint temperature of 28.8°C (vs. 25.2°C under control CO$_2$, Fig. 14). The breakpoint under hypercapnia is close to the $T_C$ whereas the one under normocapnia indicated the $T_P$ and likely involved metabolic depression. Consequently, mild hypercapnia stimulated metabolic rate and thereby prevented metabolic depression in North Sea $M. edulis$ (see Publication 2). The increase in energy demand under hypercapnia, in turn, might have contributed to lower the acid-base regulation capacity at critical temperatures. If so, these findings could indicate a potential shift in $T_C$ caused by CO$_2$ exposure, which might be too small to be resolved by the chosen experimental temperature steps.

The blue mussel population from the subtidal zone around Helgoland naturally experience only low variabilities in CO$_2$, as the concentration in seawater of this region is relative stable throughout the year with maxima of approx. 400 µatm (Thomas et al. 2007). The mean sea surface temperature during summer is 18°C with extreme temperatures up to 21°C (Wiltshire and Manly 2004, Federal Maritime and Hydrographic Agency (BSH) 2016). Hence, present day environmental conditions during summer are in the optimum range of tolerance of North Sea $M. edulis$. According to the putative thermal limits ($T_P \sim 25°C$ and $T_C > 28°C$ under both 390 µatm and 1,120 µatm CO$_2$), projected environmental changes for 2100 under business as usual in the North Sea (warming by +3°C at CO$_2$ levels of ~700 µatm (IPCC 2007) might not exceed their pejus limit, and hence might not (yet) present a challenge for this blue mussel population.

Fig. 15 Selected physiological measures reflecting thermal thresholds in $M. edulis$ from the North Sea (see next page). Heart rate (A), haemolymph pH (B), and succinate content in mantle tissue (C) under 390 µatm (filled circles) and 1,120 µatm CO$_2$ (open circles) during acute warming (3°C/night). The shaded areas indicate the upper pejus ($T_P$, blue) and critical ($T_C$, violet) temperatures. For better viewing symbols were shifted to the left (filled circles) or right (open circles). The number of animals is given in parentheses if below 4. * = significantly different from the respective data at 10°C. Data are given in mean ± SD.
Fig. 15

A

- Heart Rate [bpm]
- Haemolymph pH

B

- Temperature [°C]
- Haemolymph pH

C

- Temperature [°C]
- Succinate [a.u.]

Legend:
- 390 µatm
- 1120 µatm

Note: (1), (2), (3) indicate data points and significance levels.
White Sea

In normocapnic White Sea *M. edulis* 5 out of 8 mussels showed a progressive rise in MO\textsubscript{2} throughout warming (10° - 28°C) while the others showed an initial increase and then developed a progressive decline above 22°C (see Publication 3, Fig. 1A). The distinct inter-individual differences in the response to rising temperatures are striking since they were neither mirrored in any other parameter measured nor described for other *Mytilus* populations, including one from the subarctic (Widdows 1973b, 1976, Jansen et al. 2007, Thyrring et al. 2014, Publication 2, and unpublished material). In fact, in comparable subarctic and high Arctic populations from Greenland all individuals developed a decline in MO\textsubscript{2} beyond 21°C (Thyrrin et al. 2015) similar to those White Sea individuals showing restrictions in MO\textsubscript{2}. A high variability in the response of individuals was mainly found near critical limits, which likely reflect differences in individual fitness and in the ability to withstand the thermal challenge. However, the observed decline in MO\textsubscript{2} occurred well before the T\textsubscript{c} when anaerobic energy production set in. Considering the other measured parameters, the suppression of the metabolic rate in some animals may be the response to surpassing the pejus temperature of the entire population; accordingly, alterations in haemolymph gas and acid-base status emerged simultaneously in all individuals. All normocapnic White Sea mussels showed a significant rise in haemolymph PCO\textsubscript{2} above 19°C followed by a decline in haemolymph PO\textsubscript{2} above 22°C (Publication 3, Fig. 4). The latter suggests a reduction in oxygen availability to tissues. The substantial rise in PCO\textsubscript{2} can be related to insufficient gas exchange resulting either from distinct elevated or marked depressed metabolic rates. Further, warming induced a lowering of pH at unchanged bicarbonate level in haemolymph (see below). Altogether, the haemolymph alterations indicate an emerging respiratory acidosis and restrictions of aerobic scope induced by thermal stress. These findings imply the onset of a limitation in aerobic performance in all individuals characterizing a pejus limit of >19°C for the entire population.

It is striking that the decline in MO\textsubscript{2} seen in some specimens occurred directly after the distinct alterations in haemolymph status. Using the average MO\textsubscript{2} course of White Sea mussels allows a calculation of breakpoints (see section 2.2.5 and Publication 3) and, moreover, provides an insight in the population response. The resulting breakpoint temperature is 20.5°C (Fig. 16), which is close to the putative T\textsubscript{p} reflected by the changes in haemolymph status. Hence, the breakpoint in MO\textsubscript{2} of the White Sea population can act as an indicator for the upper T\textsubscript{p} under normocapnia, even if it may not be visible in each specimen. The observed difference in oxygen consumption beyond T\textsubscript{p} might therefore reflect different capacities of individuals to cope with temperature stress, potentially emphasizing differences in individual fitness. However, the
causation of the inter-individual differences remains cryptic, and further studies are necessary for clarification.

Succinate of normocapnic White Sea mussels started to accumulate (in mantle tissue) above 25°C defining the $T_C$ (Fig. 17). Studies on intracellular pH (pHi) of ectothermic species from different taxonomic groups revealed that, independent of potential extracellular changes, intracellular pH is maintained during warming until an extreme drop occurs, which is interpreted to reflect $T_C$ (Sommer et al. 1997, van Dijk et al. 1999, Mark et al. 2002, Melzner et al. 2006b, Morley et al. 2009). In White Sea mussels, the pHi remained almost constant throughout the warming trial until an abrupt drop by 0.4 pH units occurred above 25°C (Fig. 17). The breakdown, thus, occurred concomitantly to the onset of anaerobiosis. The results emphasize the proposed assumption of a correlation between $T_C$ and the drop of pHi in marine ectotherms.

The critical limit was further characterized by a (second) significant rise in haemolymph $PCO_2$, which likely indicates that the functional capability of the respiratory and cardiovascular system was exceeded. In contrast to these sudden and non-linear changes in $PCO_2$, haemolymph pH decreased progressively throughout the warming trial (Publication 3, Fig. 3). Hence, thermal limits could not be observed in the pHe pattern. Despite the progressive extracellular acid-base disturbance, haemolymph [$HCO_3^-$] content remained relatively low even at high temperatures providing no indication for a comprehensive internal regulation. In fact, bicarbonate was slightly elevated at 28°C, which may indicate the onset of a regulatory effort when $T_C$ was reached but further temperature steps (or longer exposure times) would be necessary to confirm this trend. In turn, intracellular [$HCO_3^-$] concentration dropped along with a breakdown of pHi that might indicate the release of bicarbonate into the extracellular fluid (see below, further discussion in the context of CO$_2$ exposure).
**Fig. 16** Oxygen consumption rate (MO$_2$) in blue mussels, *M. edulis* from the White Sea under 390 µatm (filled circles) and 1,120 µatm CO$_2$ (open circles) during acute warming (3°C/night). Data are fitted by a sigmoidal model and vertical lines indicate the breakpoint temperature, when a limitation in MO$_2$ rise occurs (for details see Publication 2 and section 2.2.6). * = significantly different from the respective data at 10°C. Data are given in mean ± SD, n = 5-8.

**Fig. 17** Selected physiological measures reflecting thermal thresholds in *M. edulis* from the White Sea (see next page). Haemolymph PCO$_2$ (A), intracellular pH (B), and succinate content in mantle tissue (C) under 390 µatm (filled circles) and 1,120 µatm CO$_2$ (open circles) during acute warming (3°C/night). The shaded areas indicate the upper pejus (T$_p$, blue) and critical (T$_c$, violet) temperatures and their shifts under CO$_2$-exposure. For better viewing symbols were shifted to the left (filled circles) or right (open circles). * = significantly different from the respective data at 10°C, † = significantly different between control and CO$_2$-exposed mussels at the respective temperature. * = significantly different from the respective data at 10°C. Data are given in mean ± SD.
Intracellular pH

Fig. 17
Hypercapnic exposure (1,120 µatm) of White Sea mussels had no effect on MO$_2$ at the control temperature but altered the MO$_2$ course during warming. In contrast to normocapnia, all hypercapnic mussels displayed a consistent response to warming with the typical exponential course of rising MO$_2$ until deviation. The limitation occurred at a breakpoint temperature of 25.8°C (vs. 20.5°C under control CO$_2$; Fig. 16). The breakpoint under hypercapnia was, thus, shifted towards higher temperatures. Moreover, it coincided with the onset of succinate accumulation in hypercapnic mussels, which was already elevated at 25°C (Fig. 17). Hence, the breakpoint in MO$_2$ under hypercapnia acts as an indicator of T$_C$ instead of T$_P$ as indicated under normocapnia. The accumulation of succinate began at 25°C rather than 28°C under normocapnia, which implies a downward shift of T$_C$ due to CO$_2$ exposure. The pH$i$ analysis provides further evidence for a lowering of T$_C$ under hypercapnia. The pattern of pH$i$ changes during warming was similar under control and elevated CO$_2$ conditions. However, the sudden and distinct intracellular acidosis developed at 25°C under hypercapnia, and at 28°C under normocapnia, concomitant to the alterations in succinate levels (Fig. 17). Again, the presented results support the above-mentioned assumption that the incidence of a pH$i$ collapse represents the T$_C$.

In contrast to intracellular pH, extracellular pH was reduced by CO$_2$ exposure and also remained lower under hypercapnia than under normocapnia during the temperature-induced pH decrease (Publication 3, Fig. 3A). Other measured haemolymph parameters were not affected by CO$_2$ exposure alone but disturbances occurred during the additional thermal challenge. When hypercapnia was combined with warming alterations in haemolymph gas status occurred at lower temperatures indicating a higher thermal sensitivity of hypercapnic White Sea mussels. Haemolymph P$_{CO2}$ was already significantly increased above 16°C followed by a decrease in P$_{O2}$ from 19°C onwards (vs. 19°C and 22°C, respectively, at normocapnia) giving evidence for a downward shift of the pejus temperature by CO$_2$. Even though haemolymph pH was reduced by CO$_2$, bicarbonate content was similar to normocapnic levels during the warming trial. However, a stronger rise was detected at 28°C resulting in a significantly higher [HCO$_3^-$] level under hypercapnia than normocapnia (Publication 3, Fig. 3; Fig. 21). As thermal limits were lowered by CO$_2$ exposure the strong rise in haemolymph [HCO$_3^-$] occurred when the T$_C$ of hypercapnic mussels was already exceeded. Upon reaching the T$_C$, intracellular [HCO$_3^-$] concentration dropped significantly in combination with the collapse of pH$i$ (Publication 3, Tab. 4). Thus, the critical pH$i$ might have stimulated extracellular bicarbonate accumulation, which was detected at the subsequent temperature, as a protective mechanism. Another possibility is that the drop of intracellular [HCO$_3^-$] might indicate a release into the extracellular space thereby elevating the

92
haemolymph level, which could be a sign of cellular stress (further discussed in section 4.2.2). A bicarbonate release from the tissue into the blood was observed in a cephalopod mollusc during exercise to exhaustion (Pörtner et al. 1996). As the source of bicarbonate in the studied M. edulis is currently uncertain, its relevance and potential benefits to the organisms’ homeostasis remains open and needs to be investigated in further studies.

The quantified upper $T_r$ of $> 19^\circ C$ of normocapnic White Sea mussels is in accordance to the ambient temperatures these animals experience during summer (mean of 15$^\circ C$, maxima of 20$^\circ C$; Sukhotin and Berger 2013, Usov et al. 2013). During summer, seawater $P_{CO_2}$ levels of the White Sea equal average present day values that can fall to preindustrial values of around 280 $\mu$atm during phytoplankton blooms, while in winter $P_{CO_2}$ levels are around 500 $\mu$atm (Yakusbev and Mikhailovsky 1995 and citation therein). Thus, the examined population is naturally exposed to either high temperatures or high $CO_2$ concentrations, but not to a combination thereof. According to climate scenarios, the White Sea will warm by $+6^\circ C$ until 2100 (IPCC 2007), which in itself may exceed the pejus limit of this population. Especially in combination with predicted $CO_2$ levels of around 1,000 $\mu$atm (IPCC 2007, 2013), which may enhance vulnerability to high temperatures, future environmental conditions of the White Sea may be a challenge for the populations’ sustainability.

4.2.2 Common features of the climate-related response

In Publication 2 and 3 we were able to demonstrate that thermal thresholds in M. edulis can be characterised in line with the OCLTT concept, which also holds true for the closely related M. galloprovincialis (unpublished data). The critical limit is set by insufficient oxygen supply to tissues to cover basal energetic costs at high temperatures resulting in a (partial) transition to anaerobic energy production. The onset of anaerobiosis in the three examined populations was accompanied by a breakdown of the cardiorespiratory system and (determined in White Sea mussels) intracellular acid-base regulation. These physiological characteristics were demonstrated in several ectotherms indicating that these may be universal features of ectothermic organisms (e.g. Walsh et al. 1984, Butler and Day 1993, Sommer et al. 1997). We further demonstrated that disturbances in haemolymph gas and acid-base status (fall in pH and $PO_2$, rise in $PCO_2$) may be associated with setting the pejus limit in blue mussels, supported by our findings in M. galloprovincialis (Fig. 19). The beginning of thermal limitation in aerobic performance is reinforced by subsequent restrictions in respiratory and cardiac functions, in line with the OCLTT concept. Such a response was also found in M. galloprovincialis from the
Mediterranean Sea (Fig. 18 and Fig. 19) indicating that this may be a general feature in *Mytilus* species.

While the general pattern in the response is quite uniform the exact course of haemolymph parameters during warming was slightly different. The alpha stat hypothesis postulates that the pH of extra- and intracellular fluids change with temperature by – 0.017 units per °C to maintain the ionisation state of imidazole groups ensuring the functional integrity of enzymes (Reeves 1972, for a review see Burton 2002). The rates of pH change (extra- and intracellular) observed in the present study (below upper T_C, cf. Pörtner and Bock 2000) are not fully in line with alpha stat regulation in *Mytilus*, an observation in agreement with previous findings in molluscs (Walsh et al. 1984, Abele et al. 1998, Pörtner et al. 1999, Melzner et al. 2006b). Nonetheless, all three populations revealed a decreasing extracellular pH with warming, albeit at different rates (Fig. 21). In the populations from the highest and lowest latitudes (White Sea and

**Fig. 18** Oxygen consumption rate (MO_2) in blue mussels, *M. galloprovincialis* from the Mediterranean Sea under 390 µatm (filled circles) and 1,120 µatm CO_2 (open circles) during acute warming (3°C/night). Data are fitted by a sigmoidal model and vertical lines indicate the breakpoint temperature, when a limitation in MO_2 rise occurs. Grey symbols indicate mean values of MO_2 at the respective CO_2 level after a maximum was reached, which are excluded from the calculation of the sigmoidal model (for details see section 2.2.6). The number of animals is given in parentheses if below 5. * = significantly different from the respective data at 10°C. Data are given in mean ± SD.
Mediterranean Sea) pHe decreased progressively upon warming, while in temperate North Sea mussels a significant drop in pHe indicated the transition to the pejus range. In the White Sea population the T_P was rather indicated by a sudden and significant rise in $PCO_2$. A rise in $PCO_2$ around the respective T_P was present in all three examined populations, albeit it was most pronounced and thus statistically significant in White Sea animals.

The observed alterations in haemolymph status (fall in pH, rise in $PCO_2$, unchanged [$HCO_3^-$]; for the latter see below) identify a primary respiratory acidosis emerging with the transition to the pejus range of the respective population (see section 4.2.1 and Fig. 21). The respiratory acidosis was accompanied by beginning restrictions in respiration rate, heart rate (determined in North Sea animals only), and a decreasing haemolymph $PO_2$. All these findings indicate restrictions in oxygen availability and consequently a limitation in aerobic performance defining the pejus temperature in accordance with the OCLTT concept.

Mytilus is pre-adapted to life in the intertidal and evolved mechanisms, such as metabolic depression, to support survival time during extreme stress conditions (e.g. Connor and Gracey, 2012). They can, thus, tolerate a high variability in temperature, oxygen availability, and CO2 concentration imposed by seasons and tidal cycles. The temperature-dependent pattern of MO2 in the three examined populations is unexpected as respiration rate under normocapnia already levelled off (likely involving metabolic depression) when reaching the pejus limit. It is striking that each calculated breakpoint temperature of MO2 (indicating metabolic rate transition) fits the putative T_P of the respective population indicated by haemolymph alterations and stagnation in heart rate (see section 4.2.1 and Fig. 18). The MO2 breakpoint temperature can thus serve as an indicator for the upper pejus limit under normocapnia. Judging from the present results under normocapnic seawater conditions, metabolic rate transitions occur when surpassing the upper T_P, as a common response in Mytilus species during acute warming experiments (see Fig. 20).

**Fig. 19** Selected physiological measures indicating thermal thresholds *M. galloprovincialis* from the Mediterranean Sea. Haemolymph $PO_2$ (A), haemolymph pH (B), and succinate content in mantle tissue (C) under 390 µatm (filled circles) and 1,120 µatm CO2 (open circles) during acute warming (3°C/night). The shaded areas indicate the upper pejus (T_P, blue) and critical (T_C, violet) temperatures and the shift of T_C under CO2-exposure. For better viewing symbols were shifted to the left (filled circles) or right (open circles). * = significantly different from the respective data at 10°C, † = significantly different between control and CO2-exposed mussels at the respective temperature. Data are given in mean ± SD, succinate is given in mean ± SE.
**Fig. 19**

**A**
- P.O₂ [Tor]: 10 13 16 19 22 25 34
- Haemolymph pH
- Temperature [°C]
- Succinate [a.u.]
- TC +CO₂
- TP
- 390 µatm
- 1120 µatm

**B**
- Haemolymph pH
- Temperature [°C]
- 40 50 60 70 80 90 100 120

**C**
- Succinate [a.u.]
- Temperature [°C]
- 10 13 16 19 22 25 28 31 34

**Discussion**
This contrasts findings in other ectotherms such as fish, squid, and an intertidal burrowing polychaete, in which restrictions in MO$_2$ occur at critical temperatures (Mark 2001, Melzner 2005, Melzner et al. 2006a, Wittmann et al. 2008). Fish and squid are permanently submerged high power organisms and may not exploit or express any capacity to depress energy demand during environmental change. In contrast, the lugworm *Arenicola marina*, as an inhabitant of the intertidal, has a high tolerance to environmental change and exhibits mechanisms to passively survive transiently harmful conditions. *A. marina* possesses a powerful anaerobic metabolism to compensate for short-term periods of insufficient oxygen supply (Toulmond 1975). The overall metabolic rate of this species is only depressed under prolonged periods of extreme stress conditions to conserve energy and thereby extending survival time. Compared to *Mytilus*, the animals mentioned above (fish, squid and lugworm) exhibit a strong acid-base regulation capacity and a well-developed circulatory system (e.g. Sommer et al. 1997, Pörtner et al. 1998, Melzner 2005). Moreover, these organisms possess respiratory pigments improving oxygen transport, which are lacking in *Mytilus*. Hence, they may utilize metabolic depression under critical stress conditions only (if at all).

The described physiological response patterns to warming in the examined *Mytilus* populations were similar under additional CO$_2$ exposure except for the course in MO$_2$. Moderate hypercapnia (1120 µatm) stimulated the respiration rate, thus preventing a metabolic depression at moderate temperatures in all three examined populations (Fig. 20). Until recently, it was assumed that hypercapnia causes a metabolic depression in *Mytilus* species, however, insights on metabolic depression were mainly discovered under air exposure and severe CO$_2$ treatments (e.g. DeFur et al. 1983, Guppy et al. 1994, Michaelidis et al. 2005). Recent studies using realistic future CO$_2$ scenarios (<4000 µatm) revealed that moderate levels may stimulate the metabolic rate, especially when combined with warming, while higher levels may eventually result in a metabolic depression (Thomsen and Melzner 2010, Ellis 2013), which is in accordance to our data.

The breakpoint temperature of MO$_2$ occurred concomitant to the transition to anaerobiosis and thereby shifted from $T_P$ to $T_C$ under hypercapnia (see section 4.2.1 and Fig. 18). Under hypercapnia the breakpoint temperature is, thus, indicative for $T_C$ instead of $T_P$ as seen under normocapnia. This implies, when moderate warming was combined with hypercapnia mussels remained in a more active state and showed a response pattern similar to more powerful ectotherms under normocapnia, where thermal restrictions in MO$_2$ occur at critical temperatures (see above). Consequently, metabolic rate stimulation by moderate hypercapnic conditions under warming may be a characteristic trait in *Mytilus* species and potentially other bivalves (Lannig et
al. 2010) but was, for example, not seen in decapod crustacean (Small et al. 2010, Schiffer 2013). However, further studies are necessary to elaborate whether the elevation of the basal metabolic rate under combined moderate hypercapnia and warming is beneficial in the long-term, and may indicate a potential adaptation capacity or if the rise in maintenance costs will result in a depletion of energy reserves ultimately reducing animal performance. The latter may cause a reduction in the population’s fitness with consequences for the species abundance and distribution.

**Fig. 20** Schematic picture of the respiration rate (MO$_2$) in *Mytilus* spp. under normocapnia and hypercapnia during acute warming. CO$_2$ exposure stimulated the respiration rate resulting in a further rise beyond $T_P$ while the rate of normocapnic mussels already levelled off likely involving metabolic depression. When $T_C$ is reached (regardless of CO$_2$) a strong reduction in MO$_2$ occurs. Note that $T_P$ and $T_C$ can differ between normocapnic and hypercapnic mussels. For further details see text.

In summary, the breakpoint temperature of MO$_2$ can act as an indicator of $T_P$ under normocapnia but not under hypercapnia as respiratory constrains in the warmth were prevented by moderate CO$_2$ exposure. Consequently, the respiration rate provides useful insights about energy turnover and potential modulation of metabolic transitions but may not be a suitable marker for identifying $T_P$ under acidifying conditions. Alternatively, heart rate may be a suitable marker for the transition to the pejus range under varying CO$_2$ conditions. Heart rate analysis of the present study (North Sea population) revealed a limitation in cardiac performance when exceeding $T_P$ regardless of CO$_2$ treatment. Based on the few data points available at high temperatures, heart beat frequency remained unaffected by moderate hypercapnia. Yet, this was examined in North Sea *M. edulis* and needs to be validated for other populations and species of
DISCUSSION

This genus. Insights from decapod crustacean support this assumption as heart rates levelled off at Tp under varying CO₂ treatments (Walther et al. 2009). In decapod crustacean, CO₂ stimulated the temperature induced rise in heart rate. This feature was not indicated in the present study, but due to limited data at high temperatures the exact CO₂ effect remains unclear.

With falling pHe and rising PCO₂, haemolymph bicarbonate levels remained unchanged upon warming in all three populations. This is in accordance with the common consent that mussels are weak acid-base regulators possessing only a low ability to compensate for acidosis in their haemolymph by bicarbonate ion accumulation (Thomsen and Melzner 2010, Gazeau et al. 2013, 2014, Lewis et al. 2016). Remarkably, haemolymph bicarbonate concentration started to increase in all populations and in each CO₂ treatment only when the critical temperature was reached or exceeded (discussed below). Bicarbonate can derive from shell dissolution, as found under very high CO₂ or after longer exposure times under moderate CO₂ concentrations (Lindinger et al. 1984, Michaelidis et al. 2005, Thomsen et al. 2010). However, CO₂ alone had no impact on bicarbonate levels, which exclusively increased when Tc was reached or even exceeded regardless of the applied CO₂ level (Fig. 21). Moreover, accumulation set in at different temperatures (depending on population and CO₂ treatment), was strongest under normocapnic conditions, and thus far cannot be explained by the measured haemolymph pH or PCO₂ values. Based on this information, the rise in bicarbonate may not (or at least not predominantly) result from passive shell dissolution but is rather caused by physiological mechanisms. Due to the variability observed in the patterns, however, the cause and effect of bicarbonate accumulation in response to temperature extremes in Mytilus species must remain speculative at present. In temperate North Sea M. edulis, where hypercapnia had no significant effect on thermal limits (see Publication 2 and section 4.2.1), haemolymph bicarbonate increased at Tc, with a significantly stronger rise in normocapnic than in hypercapnic mussels. Based on these findings it was assumed that this indicates a regulatory response as a final protection against critical conditions (for a detailed discussion see Publication 2). In subarctic White Sea M. edulis, where CO₂ affected thermal limits, a trend towards rising [HCO₃⁻] at Tc was visible under normocapnia (> 25°C) albeit not significant (see Publication 3 and section 4.2.1). Under hypercapnia, haemolymph [HCO₃⁻] was not elevated at Tc (> 22°C) but significantly increased at the subsequent temperature step. In subarctic mussels, the intracellular acid-base status (of mantle tissue) was analysed additionally. pH collapsed at the Tc of the respective CO₂ treatment accompanied by a drop in intracellular [HCO₃⁻] level, which may indicate a release into the extracellular space causing the rise in the haemolymph level (see Publication 3 and section 4.2.1). This, however, would indicate a functional failure instead of a regulatory response as
indicated in North Sea mussels. Comparable data on intracellular acid-base status from the other examined populations or from the literature are not available. Thus, it cannot be clarified whether the accumulated bicarbonate in haemolymph derived from internal or external sources. In Mediterranean *M. galloprovincialis*, haemolymph [HCO₃⁻] significantly increased at Tᶜ under normocapnia, as seen in North Sea and indicated in White Sea mussels. However, under hypercapnia a trend towards rising levels was indicated only distinctly beyond the putative Tᶜ (>25°C) at the highest temperature applied (34°C). This is particularly surprising as (contrary to the other populations) the pH of Mediterranean mussels started to fall drastically with reaching Tᶜ (Fi. 21) concomitant with an increase in mortality. Hypercapnic mussels had already developed a very severe extracellular (and, most likely, intracellular) acidosis at the highest applied temperature, yet only a small non-significant rise of haemolymph bicarbonate occurred. The lack of a bicarbonate rise in the haemolymph under hypercapnia is obscure as, according to the present findings, an intracellular bicarbonate release due to a physiological breakdown in response to these critical (external and internal) conditions would be expected. The underlying mechanisms of bicarbonate fluxes need to be identified. Further studies need to clarify the origin of bicarbonate, its near absence in hypercapnic mussels despite a severe acidosis, and whether an occurring accumulation is an additional sign of considerable physiological stress rather than a regulatory response.

The above outlined physiological characteristics in the thermal response under varying CO₂ conditions including the shift of the MO₂ breakpoint from Tᵖ to Tᶜ under CO₂ exposure seems to be a general feature during acute thermal challenges in *Mytilus* species. The occurrence of metabolic depression when entering the pejus range as well as the prevention of this mechanism at Tᵖ under moderate CO₂ exposure should be assessed in more detail in future studies. Further, it should be examined if the observed warming responses with respect to hypercapnia might be a universal feature of sedentary, sluggish animals that possess the ability for metabolic depression.
Fig. 21 Haemolymph partial pressure of oxygen ($P_{CO_2}$, A-C), pH (D-F), and bicarbonate content ([HCO$_3^-$], G-I) in blue mussels, *Mytilus edulis* from the North Sea and White Sea and *Mytilus galloprovincialis* from the Mediterranean Sea under 390 µatm (filled circles) and 1,120 µatm CO$_2$ (open circles) during acute warming (3°C/night). For better viewing symbols were shifted to the left (filled circles) or right (open circles). The number of animals is given in parentheses if below 4. * = significantly different from the respective data at 10°C, † = significantly different between control and CO$_2$-exposed mussels at the respective temperature. Data are given in mean ± SD.

4.2.3 Latitudinal differences in the climate-related response

Beside general features the present study on *Mytilus* populations from distinct geographic regions revealed latitudinal differences in their thermal tolerance and how it is affected by CO$_2$ exposure. In comparison to the temperate North Sea population, thermal stress sets in at lower temperatures in White Sea mussels. Both upper thermal limits pejus and critical were shifted downwards at high latitudes. The reduced heat tolerance is likely the result of evolutionary adaptation to the subarctic climate (see section 4.2.1 and Publication 3). Cold adapted polar
animals exhibit lower functional capacities in several physiological processes resulting in a high sensitivity to environmental stress (Sommer et al. 1997, van Dijk et al. 1999, Peck and Conway 2000, Sokolova and Pörtner 2003, Peck et al. 2014). In accordance, moderate CO$_2$ exposure caused a downward shift of both upper thermal limits $T_p$ and $T_c$ in subarctic White Sea $M. edulis$ demonstrating their thermal tolerance is lowered under acidifying conditions, a phenomenon not seen in the North Sea population. Consequently, tolerance to environmental challenges is constrained in $M. edulis$ at high latitudes.

Judging from the present results, during summer, North Sea mussels experience water temperatures well within their optimum range while White Sea mussels live at ambient water temperatures close to the quantified $T_p$. Projections show that high latitudes will be subject to greater changes in the climate than temperate latitudes (IPCC 2007, 2014). Thus, subarctic $M. edulis$ that are more susceptible to environmental change may be even under a greater threat by future climate conditions than temperate ones. Predicted changes of ocean warming and acidification for 2100 may already exceed the present pejus limit of the subarctic population with potentially negative implications for its ecological performance, which may not be the case for the North Sea population in their habitat.

**Fig. 22** Schematic picture of the upper thermal limits ($T_p$, $T_c$) and associated aerobic performance curve of $Mytilus$ populations along a latitudinal cline. The performance curve shifts to lower temperatures of populations living in colder habitats reflecting adaptation to different climatic regions. Position and width of pejus range can differ between populations along a climate gradient. For further details see text.
According to the general picture (e.g., Pörtner et al. 2009, Pörtner 2010), the lowest $T_C$ was found in the population from the highest latitudes (subarctic White Sea) and the highest $T_C$ in Mediterranean mussels from the southern edge of its geographical range, which is in accordance to the respective thermal regime of their natural habitat. Compared to the temperate North Sea population, thermal tolerance was reduced by a downward shift of both upper thermal limits in the subarctic mussels, while in Mediterranean mussels the enhanced thermal tolerance may result from extending only their passive range of tolerance (shift in $T_C$ but not $T_P$). While North Sea and White Sea mussels show distinct changes in extracellular acid-base parameters when reaching $T_P$, the warming induced alterations are more gradual in Mediterranean Sea mussels and thus not marking $T_P$ as clearly (Fig. 21). From the insights gained for the two *M. edulis* populations, the breakpoint of the MO$_2$ course acts as an indicator for $T_P$ under normocapnia. Based on this conclusion, it appears that the $T_P$ of Mediterranean mussels is similar to the one of the North Sea population or even slightly lower. The putative $T_P$ of Mediterranean mussels is supported by a significantly reduced haemolymph $P_{O_2}$ indicating restrictions in the aerobic performance. While their pejus limit was similar to that of North Sea mussels, the critical limit was shifted towards higher temperatures. It appears that the upper limit of optimal physiological activity in Mediterranean Sea mussels is similar to North Sea mussels but their capacity is enhanced to passively tolerate more extreme temperatures in the short-term. As found for the northern edge population, hypercapnia narrowed the thermal window of the southern edge population, however, by restricting the passive tolerance range solely (lowered $T_C$ at unchanged $T_P$). Other studies on Mediterranean *M. galloprovincialis* demonstrated that temperatures around 24°C elicit thermal stress responses from the molecular to the whole animal level (Anestis et al. 2007, 2010). These studies further showed that when temperatures exceeded 24°C for several days mortality increased drastically (regardless of moderate CO$_2$ exposure). The described onset of the thermal stress response corresponds well with the quantified acute pejus threshold in the present study (MO$_2$ breakpoint at 23.3°C; see Fig. 18). According to these insights, the detrimental thermal effects on the population level set in beyond the upper pejus limit determined in acute temperature experiments on adult animals, which is in line with the conclusions drawn for fish (Pörtner and Knust 2007). Thus, the acute upper pejus limits of adults are applicable to predict natural thermal thresholds at the ecologically relevant population level, as proposed for other ectotherms (Frederich and Pörtner 2000, Mark et al. 2002, Lannig et al. 2004, Pörtner and Knust 2007). However, Mediterranean mussels regularly experience temperatures beyond 24°C within their habitat resulting in increased mortality and indicating that the population is living close to its lethal limits (Anestis et al. 2008, Gazeau et al. 2014). In the
context of future climate change, current warming may be more relevant than ocean acidification for the survival of the Mediterranean population, as previously proposed by Gazeau and colleagues (2014).

In summary, when compared to temperate North Sea mussels, the thermal window of subarctic mussels is shifted to lower temperatures while Mediterranean mussels show an enhanced thermal resilience by extending their passive tolerance range. Hypercapnia enhanced the thermal sensitivity of populations living at the latitudinal edges of their distribution. In the northern edge population, both upper thermal limits were shifted downwards, whereas the thermal window of the southern edge population was narrowed by restricting the passive tolerance range solely. The presented insights indicate evolutionary temperature adaptation of the studied populations according to the environmental conditions in their habitat. This resulted in shifts of their thermal limits and modulated their physiological response to warming and to acidifying conditions. The latitudinal differences between population responses need to be considered in order to understand the consequences of ocean warming and acidification on abundance patterns and food web structure. The results further emphasize that, when projecting species sensitivity to climate change and resulting ecological consequences, populations from several geographically distinct regions should be considered. Conclusions based on a single population may fail to predict potential future ecosystem changes.

Future environmental conditions may be more detrimental for the ecological performance of populations from the latitudinal range edges as they already live close to their tolerance limits. Current climate change may particularly threaten populations from the southern edge of their temperature-dependent distribution that already experience lethal temperatures in their habitat as indicated for *M. galloprovincialis*. This is in line with the globally observed poleward shift in the southern distribution limit of *Mytilus* and other ectothermic species and populations over the last decades (e.g. Holbrook et al. 1997, Wootton et al. 2008, Jones et al. 2009, 2010, Sunday et al. 2011). Our results stress the necessity to consider latitudinal differences in the climate-related response of species and populations when projecting potential future changes in structure and functioning of ecosystems.
5 Conclusion

The present results demonstrated that the synergistic effects of ocean acidification and warming reduced the whole animal performance of Arctic *H. araneus* likely mediated by an involvement of acid-base disturbances. This confirms that this Arctic population has a low capacity for acid-base regulation. Arctic spider crabs were able to buffer their internal milieu via bicarbonate accumulation under hypercapnia but failed to restore haemolymph pH. The induced extracellular acidosis was more severe in warm- (4°C) than in cold-acclimated (1°C) crabs, potentially due to restrictions in bicarbonate acquisition. Our findings emphasize that hypercapnia resulted in a limitation of aerobic scope and, thus, performance, which was exacerbated when combined with warming.

Acclimation temperature, hypercapnic exposure as well as the resulting acid-base disturbances did not affect the activity level of Arctic *H. araneus* at least in the short-term. However, exposure to temperature extremes (12°C) resulted in a reduced scope for performance in all crabs, which was exacerbated by increasing CO$_2$ concentrations. The findings are in line with the OCLTT concept, which postulates that climate-related impacts are greatest at the edges of the geographical range. When heat stress was combined with moderate hypercapnia (>750 µatm) Arctic crabs became functionally limited, seen in a significant decrease of righting activity. This impact was more extreme in cold- than in warm-acclimated crabs, suggesting a lower scope for activity mediated by thermal constrains.

The present results suggest permanent cold adaptation associated with enhanced sensitivity to environmental challenges in the Arctic population as suggested elsewhere (Walther et al. 2009, Schiffer 2013, Harms et al. 2014). Reduced activity capacity has implications for organism performance including reproduction, food supply, foraging and antipredator behaviour with likely adverse ecological consequences.

Acute temperature experiments with *Mytilus* populations along a latitudinal cline (*M. edulis* from the North Sea and the White Sea and *M. galloprovincialis* from the Mediterranean Sea) revealed physiological characteristics of thermal tolerance in line with the OCLTT concept. Accordingly, the pejus limit is set by a beginning constrains in aerobic performance caused by a capacity limitation within the oxygen supply system (ventilation and/or circulation). Our findings highlight a pivotal role of extracellular acidosis in setting the pejus temperature in *Mytilus spp*. The critical limit is reached when oxygen provision to tissues becomes insufficient to cover basal maintenance cost resulting in a partial transition to anaerobiosis. The emerging
CONCLUSION

e xtracellular acidosis may not always be clearly enough expressed. Thus, it may serve as an indicator but not necessarily as a general marker to identify the T_p of *Mytilus* species. Instead heart rate may be a suitable proxy for T_p under varying CO_2 conditions. Yet, this was examined in North Sea *M. edulis* and needs to be validated for other populations and species of this genus. The transition to anaerobiosis coincides with a drastic drop in intracellular pH (examined in White Sea mussels), which thus clearly marked the T_C and may be a reliable proxy of the critical temperature in *Mytilus* species.

The *Mytilus* populations across the examined climate gradient clearly showed latitudinal differences in their response to environmental change. Compared to temperate *M. edulis*, the subarctic population showed a reduced thermal tolerance by a downward shift of both upper thermal limits (pejus and critical), whereas the southern edge population of the closely related *M. galloprovincialis* from the Mediterranean Sea showed enhanced thermal tolerance by extending their passive tolerance range only (shift in T_C).

Hypercapnia reduced the heat tolerance of both the northern and southern edge populations, an impairment not indicated in North Sea mussels. The resulting thermal constraints were more extensive in the subarctic population where both upper T_p and T_C were shifted downwards by CO_2. In contrast, in Mediterranean mussels hypercapnia left the pejus limit unchanged but lowered T_C thereby narrowing the passive range of tolerance. Considering that Mediterranean *M. galloprovincialis* suffer from an enhanced mortality due to current warming (e.g. Ramón et al. 2005), it appears that trends in ocean warming may be more critical for this population than acidification.

Overall, the presented insights provide evidence that populations of eurythermal species living at high latitudes exhibit features of cold adaptation resulting in reduced thermal tolerance and resilience to environmental challenges. Combining the available findings, hypercapnia can reduce heat tolerance of marine ectotherms as reported elsewhere (e.g. Walther et al. 2009, Findlay et al. 2010, Lannig et al. 2010). Further, it appears that populations close to the latitudinal range edges, which live close to their upper thermal limits, are especially sensitive to future climate changes. Reduced organismal performance can impair a population’s fitness, which will contribute to changes in species abundance and distribution with consequences for communities and food web structures. Our results stress that latitudinal differences between populations must be considered when forecasting climate-induced effects on ecosystem structure and functioning.
References


References


Ellis, R. P. (2013). The impact of ocean acidification, increased seawater temperature and a bacterial challenge on the immune response and physiology of the blue mussel, Mytilus edulis.


REFERENCES


Jones, S. J., Lima, F. P., and Wethey, D. S. (2010). Rising environmental temperatures and


REFERENCES


REFERENCES


limitation of thermal tolerance in animals. Naturwissenschaften, 8(4), 137-146. doi:10.1007/s001140100216.


REFERENCES


REFERENCES


List of Abbreviations

a.u. arbitrary units
bpm beats per minute
$CCO_2$ total carbon content
$CO_2$ carbon dioxide
$CO_3^{2-}$ carbonate
$D_2O$ deuterium oxide
$DiC$ dissolved inorganic carbon
$DW$ shell-free dry weight
$H^+$ proton
$HCO_3^-$ bicarbonate
$H_2CO_3$ carbon acid
$IPCC$ Intergovernmental Panel on Climate Change
$K_e$ equilibrium constant
$KF$ potassium fluoride
$KOH$ potassium hydroxide
$MO_2$ oxygen consumption, respiration, metabolic rate
$NTA$ nitrilotriacetic acid
$OCLTT$ oxygen- and capacity-limited thermal tolerance
$P_{atm}$ atmospheric pressure
$PCA$ perchloric acid
$P_{CO_2}$ partial pressure of carbon dioxide
$P_{H_2O}$ temperature-specific water vapour pressure
$pH$ potential of hydrogen, measure of hydrogen ion activity
$pHe$ extracellular (haemolymph) pH
$pHi$ intracellular pH
$pH_{NBS}$ pH in NBS (National Bureau of Standards) scale
$pH_{total}$ pH in total scale
$pK$ apparent dissociation constant
$P_{O_2}$ partial pressure of oxygen
$psu$ practical salinity unit
$Q_{10}$ temperature coefficient
$RCP$ Representative Concentration Pathway
$SMR$ standard metabolic rate
$TA$ total alkalinity
$T_c$ critical temperature
$T_d$ denaturation temperature
$T_p$ pejus temperature
$TSP$ trimethylsilyl propionate
$V_{fl}$ flow rate
$\alpha_{CO_2}$ carbon solubility
$\beta_{O_2}$ oxygen solubility
$\mu_{atm}$ micro atmosphere
Acknowledgement

First of all, I would like to thank Hans Otto Pörtner who made this work possible and supported me the whole time. Thank you for allowing me a lot of room to develop my thesis, for discussions and encouraging me.

Bela H. Buck is sincerely acknowledged for being a member of my dissertation committee, for his advice, and for evaluating my dissertation.

Many thanks go to Christian Bock who substantially supported my work. Thank you for encouraging and inspiring me, for a lot of fun times at work and leisure activities and for believing in me throughout.

I would also like to thank sincerely Ralf Dringen for stepping in at short notice to complete the dissertation committee. [Note: The text was modified due to a change in the committee.]

Special thanks go to Alex Sukhotin who made a unforgettable expedition to the Kartesh station at the White Sea possible. Even so it wasn’t an easy time I have very good memories from that time, I learned a lot, enjoyed life out there in the nature, and met wonderful people. Thank you for all the intense and fruitful discussions and for your sympathetic ear, it is always great to meet and talk to you.

I am very grateful to the Integrative Ecophysiology group for all the cooperation and support. I would like to thank Nils for good times in- and outside the institute; the same goes for Gisela, who inspired me a lot and is always open for serious and funny discussions. Heidi, Kadda, Lena, Kristina and Axinja, Felix thank you for the time shared, the persistence and for cheering me up.

A huge thanks goes to Timo, who taught me so much about technical issues. Thank you for all the good lab-times in the institute and during all our expeditions, for our exquisite dinners and funny rum-evenings.

A very special thanx to Nicole H. for a wonderful friendship, for being there in good times and in bad, and for all the enormous support that can’t be listed here. I couldn’t have done it without you!

Britta G. I’m happy about our long friendship and thank you for being there when needed.

I thank my WG for being a special island in this world and providing me food and shelter, you are truly my home base.

I thank my Family and Rosina for always being there for me, for all the encouraging words and support.
Erklärung gemäß § 6(5) der Promotionsordnung (vom 14. März 2007) der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

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

"Synergistic impacts of ocean acidification and temperature rise on the physiology of marine invertebrates in a latitudinal cline"

1. ohne unerlaubte fremde Hilfe angefertigt habe,
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel angegeben habe und
3. die den benutzen Werken wörtlich oder inhaltlich entnommene Stellen als solche kenntlich gemacht habe

________________________________________
Zora Zittier