

Benthic interactions in a high CO₂-world

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Summary

The Baltic Sea is a physically diverse habitat with a generally low species diversity. The blue mussel *Mytilus edulis* is widely distributed in the benthic macrozoobenthos of the Western Baltic, the main predators of which are the common sea star *Asterias rubens* and the shore crab *Carcinus maenas*. Any environmental stress influencing the predator-prey interactions between these species has the potential to shape the entire ecosystem. The current increase in atmospheric pCO₂ causes a concurrent increase in the acidification of seawater and can thus pose such an environmental stress. In coastal habitats and specifically the Baltic Sea, the decrease of seawater pH can be much more pronounced than in the open ocean.

In order to estimate possible interaction shifts in the macrozoobenthos under conditions of seawater acidification, this work investigates the effect of an increase in water pCO₂ on the predators *A. rubens* and *C. maenas* and their consumption of *M. edulis*.

The results of three different own studies show an impact of increased seawater pCO₂ around 3500 µatm on growth and mussel consumption in adult sea stars and a seawater pCO₂ of around 1200 µatm to impact growth, mussel consumption, scope for growth and righting response of juvenile *A. rubens*. Mussel sizes consumed, metabolism, NH₄⁺-excretion and calcification were, however, not impacted by an increase in seawater acidification and coelomic pH not regulated by means of active bicarbonate accumulation. Crabs were impacted in metabolism, NH₄⁺-excretion, O:N-ratio and metabolic energy loss at a seawater pCO₂ of about 3500 µatm. Mussel consumption was only impacted at a pCO₂ of 3500 µatm, over intermediate time spans (10 weeks), but not over a longer (six month) time span. Hemolymph pH was regulated to remain at control levels by active bicarbonate accumulation at intermediate (10 week) time spans over all levels of seawater acidification, while hemolymph pH followed the non-bicarbonate buffer line at the intermediate (around 1200 µatm) level and was only regulated at the high (around 3500 µatm) treatment level over the long (six month) time span. Moulting intervals, growth, mussel sizes consumed, carapace thickness, stability, dry weight and calcification were not influenced by seawater acidification

These results indicate a change in feeding pressure on the blue mussel *M. edulis* under future high levels of seawater acidification. Further, *A. rubens* appears stronger impacted by seawater acidification than *C. maenas* and juvenile *A. rubens* even stronger than adult specimen.

I conclude that seawater acidification has the potential to reshape the benthic ecosystem of the Western Baltic. This work therefore helps to understand ecosystem responses to environmental stress and contributes to making predictions on future species distributions in the Baltic Sea.

Zusammenfassung

Die Ostsee ist ein physikalisch diverses Habitat mit einer allgemein geringen Artenvielfalt. Die Miesmuschel *Mytilus edulis* ist im benthischen Makrozoobenthos der westlichen Ostsee weit verbreitet. Ihre Hauptprädatoren sind der gemeine Seestern *Asterias rubens* und die Strandkrabbe *Carcinus maenas*. Jede Form von Umweltstress, der die Interaktionen zwischen diesen Arten beeinflussen kann, hat das Potential das gesamte Ökosystem zu verändern. Der aktuelle Anstieg in atmosphärischem $p\text{CO}_2$ verursacht eine gleichzeitige Ansäuerung des Seewassers und kann somit einen solchen Umweltstress darstellen. In Küstengebieten und insbesondere in der Ostsee kann die Abnahme in Seewasser pH noch stärker ausfallen als im offenen Ozean.

Um mögliche Verschiebungen in den Interaktionen im Makrozoobenthos unter Seewasserversauerung zu machen, untersucht diese Arbeit den Effekt eines Anstiegs in $p\text{CO}_2$ auf die Prädatoren *A. rubens* und *C. maenas* und deren Fraß von *M. edulis*.

Die Resultate aus drei verschiedenen eigenen Studien zeigen einen Einfluss von erhöhtem Seewasser $p\text{CO}_2$ von ca. 3500 μatm auf Wachstum und Muschelfraß von adulten Seesternen und eine Beeinflussung von Wachstum, Muschelfraß, Energieverhältnissen und Umdrehgeschwindigkeit ab ca. 1200 μatm bei juvenilen *A. rubens*. Gefressene Muschelgrößen, Metabolismus, NH_4^+ -Exkretion und Kalzifizierung wurden jedoch nicht beeinflusst und der Coelom-pH wurde nicht durch aktive Akkumulation von Bikarbonat reguliert. Die Krebse wurden in Metabolismus, NH_4^+ -Exkretion, O:N-Verhältnis und metabolischem Energieverlust von einem Seewasser $p\text{CO}_2$ von ca. 3500 μatm beeinflusst. Muschelfraß änderte sich bei einem $p\text{CO}_2$ von 3500 μatm nur bei einer mittleren experimentellen Länge von zehn Wochen und nicht bei einer längeren Phase von sechs Monaten. Der HämolympH-pH wurde durch aktive Akkumulation von Bikarbonat nur über die mittlere (zehn Wochen) Experimentalzeit bei allen $p\text{CO}_2$ -Werten auf reguliert, während er über die Sechsmontatsphase nur bei ca. 3500 μatm auf Kontrollniveau gehalten wurde, bei ca. 1200 μatm der nicht-Bikarbonaten Pufferlinie folgte. Häutungsintervalle, Wachstum, gefressene Muschelgrößen, Carapaxdicke, -stabilität, -trockengewicht und -kalzifizierung wurden nicht von der Seewasserversauerung beeinflusst.

Diese Resultate indizieren eine Veränderung des Fraßdruckes auf die Miesmuschel *M. edulis* unter zukünftig hohen Werten des Seewasser $p\text{CO}_2$ s. Darüber hinaus scheint *A. rubens* stärker durch diesen Umweltstress beeinflusst zu sein, als *C. maenas* und juvenile *A. rubens* stärker als adulte Exemplare.

Ich schließe aus den Ergebnissen, dass Seewasserversauerung das Potential hat, das benthische Ökosystem der westlichen Ostsee zu verändern. Diese Arbeit hilft somit, die Auswirkungen von Umweltstress auf Ökosysteme zu verstehen und trägt dazu bei, Voraussagen über zukünftige Artenverteilungen in der Ostsee zu machen.

Publications and contribution

Parts of this thesis are submitted to and accepted for publication in peer-reviewed journals as followed:

Chapter 1:

Not so sour times for benthic predators - The influence of seawater acidification on growth, feeding behaviour and acid-base status of *Asterias rubens* and *Carcinus maenas*

Appelhans Y. S., Thomsen J., Pansch C., Melzner F., Wahl M.

Accepted for publication in Marine Ecology - Progress Series

I designed and conducted the majority of the experiment. J. Thomsen designed and conducted the investigation of the acid-base balance in crabs and sea stars. I wrote the manuscript with the parts on the methods and results on acid-base balance added by J. Thomsen. All co-authors helped with advice on experimental design and revision of the manuscript.

Chapter 2:

Young and sour - the influence of long-term acclimation to seawater acidification on growth, food consumption, metabolism and calcification of juvenile sea stars (*Asterias rubens*)

Appelhans Y. S.*, Thomsen J.*, Opitz S., Melzner F., Wahl M.

*Both authors have contributed equally to the study and are both considered first authors

Submitted to Marine Ecology - Progress Series

I designed both experiments, conducted experiment 1 and wrote the manuscript together with J. Thomsen. Experiment 2 was conducted by S. Opitz. All co-authors helped with advice on experimental design and revision of the manuscript.

Chapter 3:

Sour crabs - The effect of long-term seawater acidification on growth and moulting, food intake, metabolism, acid-base balance and calcification in the shore crab *Carcinus maenas*

Appelhans Y. S., Trübenbach K., Rosa R., Melzner F., Wahl M.

In prep. for publication

I designed and conducted the majority of the experiment, K. Trübenbach designed and conducted the investigation of the acid-base balance, the metabolism and the NH_4^+ -excretion of the crabs. I wrote the manuscript with the parts on the methods on acid-base balance, the metabolism and the NH_4^+ -excretion added by K. Trübenbach. All co-authors helped with advice on experimental design and revision of the manuscript.

Further publications, which are not part of this thesis:

Stressed, but not defenceless: no obvious influence of irradiation levels on antifeeding and antifouling defences of tropical macroalgae

Appelhans Y. S., Lenz M., Medeiros H. E., da Gama B. A. P., Pereira R. C., Wahl M. (2010)
Marine Biology 157:1151-1159

Effects of elevated seawater pCO₂ on gene expression patterns in the gills of the green crab, *Carcinus maenas*.

Fehsenfeld S., Kiko R., Appelhans Y. S., Towle D. W., Zimmer M., Melzner F. (2011)
BMC Genomics 12: 488

Impacts of ocean warming and acidification on the larval development of the barnacle *Amphibalanus improvisus*

Pansch C., Nasrolahi A., Appelhans Y. S., Wahl M. (2012)
Journal of Experimental Marine Biology and Ecology 420-421: 48-55

General introduction

The Baltic Sea and its physical properties

The Baltic Sea is a physically diverse habitat with a pronounced salinity gradient from west to east (see Fig. 1). The influx of high salinity North Sea water and the simultaneous input of fresh water from riverine origin, combined with an increase of surface water temperature in summer, cause a strong stratification throughout the Baltic Sea, which is especially pronounced in summer months (Dietrich 1950, Matthäus 1977). In winter, generally a simple stratification can be found, with a permanent pycnocline dividing the cold salt-poor surface water from the saltier, warmer deep water. In spring, the increasing temperatures cause a second pycnocline in 20-30 m depth, separating the warmer surface from the cold intermediate water. In the shallower coastal regions of the Western Baltic, stratification alternates with homogeneous phases in winter, while the shallow regions of the Central Baltic do not show any winter stratification (Matthäus 1996). Summer stratification also occurs in shallower regions.

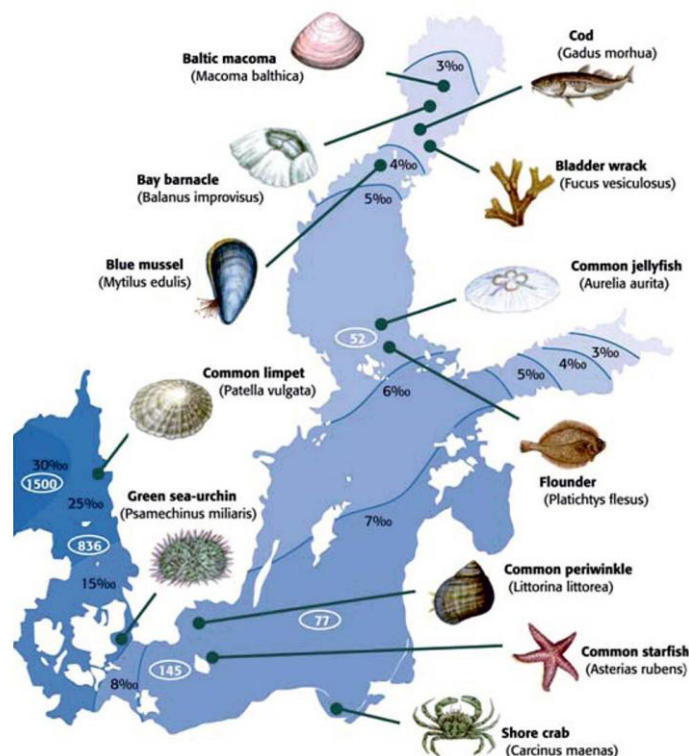


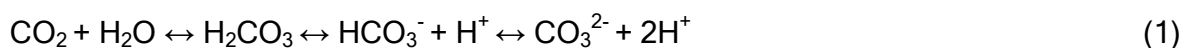
Fig. 1 Salinity across the Baltic Sea and species distribution limits (species occurrence east of limit). Figure by B-O Jansson, Stockholm Marine Research Centre, Stockholm University as published in BACC author team (2008).

The pronounced stratification, especially in deeper regions such as the Bornholm Basin, the Gdansk Basin, the Western and the Eastern Gotland Basin, leads to the occurrence of oxygen minimum zones in the bottom waters, where no photosynthesis takes place and degradation of organic matter causes a depletion of O₂ and production of CO₂ (e.g. Nehring 1996). Since the exchange with the atmosphere is limited by stratification, the bottom waters in these regions exhibit low levels of pO₂ and high levels of pCO₂, until an influx of salt-rich North Sea water renews the water in these regions. The bottom waters low in pO₂ and high in pCO₂ also reach shallower regions through seicheing of deep waters into shallower regions, such as Kiel Bight (Erhardt & Wenck 1984). The occurrence of oxygen minimum zones and thus regions of high pCO₂ is promoted by an increase in eutrophication, such as has been observed over the past decades (e.g. BACC author team 2008).

It has been predicted that with progressing climate change not only the seawater temperature will increase and the salinity decrease in the Western Baltic (BACC author team 2008), but also that larger increases and fluctuations in the seawater pH will occur by the end of the century due to an increase in atmospheric pCO₂ (Melzner *et al. accepted*).

Seawater acidification

The atmospheric pCO₂ has constantly increased over the last decades and centuries, due to the exhaust from burning fossil fuels. Since the beginning of the industrial revolution values have increased from 285 (Petit *et al.* 1999) to currently about 397 ppm (retrieved in June 2012 from <http://co2now.org>, data from Mauna Loa Observatory: NOAA-ESRL). When atmospheric CO₂ enters the seawater via diffusion, carbonic acid (H₂CO₃) is formed, which is not stable and further dissociates into a proton (H⁺) and bicarbonate (HCO₃⁻, equation 1). These excess protons cause the seawater to acidify (e.g. Zeebe & Wolf-Gladrow 2001). The carbonate-ions (CO₃²⁻) present in seawater can buffer this process to a certain extent, by reacting with excess H⁺ to HCO₃⁻.



Nevertheless, an increase in protons steadily occurs. The surface water pH in the open ocean has already dropped by 0.1 scale points, compared to pre-industrial values (Sabine *et al.* 2004). A further 0.3 - 0.5 scale point drop has been predicted for the open ocean by the end of the century, if atmospheric pCO₂ values of about 1000 ppm are reached (Caldeira & Wickett 2005).

The situation in coastal waters differs from that of the open ocean, since variations in pCO₂ and thus pH show a higher variability than in the open ocean (Wootton *et al.* 2008, Hofmann

et al. 2010, Yu *et al.* 2011). In open coasts this is mostly due to the occasional upwelling of CO₂-rich bottom water (e.g. Feely *et al.* 2008). In estuaries and embayments also factors, such as the input of reactive nitrogen and sulphur from fossil fuel combustion and agriculture (Doney *et al.* 2007) and of acidic riverine water (Salisbury *et al.* 2008) leading to a reduction of coastal water alkalinity, as well as the oxidation of organic matter from land (Gattuso *et al.* 1998) have an influence on the variability of acidification (Hofmann *et al.* 2010).

The situation in the Baltic Sea further differs from the predicted acidification of the open ocean. Thomsen *et al.* (2010) have shown seasonal surface pCO₂ values in Kiel Fjord to reach 2300 µatm, i.e. six times the atmospheric pCO₂, when stratification is highest and occasional upwelling of CO₂-rich bottom waters occurs. The reduced salinity in this region with its accompanying low levels of CO₃²⁻ and a thus lower buffering capacity are thought to further be the cause of these high values (Beldowski *et al.* 2010). A recent study has calculated the level of Baltic Sea surface water to reach values of 4000 µatm seasonally by the end of the century, if atmospheric pCO₂ reaches the predicted 1000 µatm (Melzner *et al. accepted*). For the benthic regions, these high values may prevail over even longer time periods. Acidification is thus an environmental stress that may impact benthic organisms of the Baltic Sea stronger than pelagic organisms of the open ocean, while they may yet already be partly adapted to conditions of low seawater pH. Other abiotic (decrease in salinity, increase in temperature) and biotic (predation, competition) stressors can further interact with the predicted seawater acidification and cause shifts in the ecosystem.

The impact of seawater acidification on different species, ecosystems and life stages

Seawater acidification is known to have a varying impact on different phyla and species (e.g. Ries *et al.* 2009, Hendriks *et al.* 2010, Kroeker *et al.* 2010). Considering the high solubility of CaCO₃ in acid, specifically calcifying organisms should be affected by increases in seawater pCO₂ (e.g. Fabry 2008). Meta-analyses of laboratory experiments have indeed shown calcifying species to be most vulnerable to acidification (Hendriks *et al.* 2010, Kroeker *et al.* 2010). Nevertheless, calcification was not in all cases reduced under conditions of acidification, but instead increased in some organisms (Wood *et al.* 2008, Ries *et al.* 2009, Gutowska *et al.* 2010).

Studies on locally restricted acidified ecosystems (mean pH reductions from 8.2 to 7.4 and from 8.1 to 7.8, respectively, though high fluctuations were noted) naturally caused by volcanic vents, have further shown an absence of some, especially calcifying organisms from highly acidified sites, while other organisms, such as macroalgae and seagrasses, increased in biomass (Hall-Spencer *et al.* 2008, Fabricius *et al.* 2011). The same studies demonstrated a general loss in biodiversity with increasing levels of seawater pCO₂, although it has to be

noted that these areas are so small that adaptation towards acidification is not likely to occur, due to lateral gene flow. Also a long-term study over eight years on a North-American coastal habitat revealed that while the mean pH significantly declined over this time period (0.045 scale points per year), the abundance of calcifying, specifically molluscan, species decreased, while fleshy algae and acorn barnacles became more predominant (Wootton *et al.* 2008). A similar pattern has been reported for a mesocosm experiment by Hale *et al.* (2011). The authors demonstrated a reduction in overall species richness with declining levels of pH (up to 6.7, ca. 2000-3000 μatm) and overall fewer molluscs.

But not only different species are affected to a different extent, different life stages of the same species are as well. It is generally assumed that earlier life stages are more vulnerable to acidification than adult individuals (e.g. Ross *et al.* 2011). Few studies, however, have directly compared effects of seawater acidification on different life stages in the same organism (Kurihara & Ishimatsu 2008, McDonald *et al.* 2009).

Predator-prey interactions and environmental stress

Environmental stress is thought to have a strong influence on biotic interactions. Menge and Sutherland (1987) suggest a complex model, according to which mobile organisms (such as most predators) are more strongly affected by environmental stress than sessile organisms. This is supposed to lead to a decrease in food-web complexity with increasing environmental stress.

The observed effects of seawater acidification on ecosystems may thus not only be due to direct effects on single species, but may also be a cause of shifts in species interactions. Latter have mostly been investigated on the prey side and mostly in fish. A study by Dixson *et al.* (2010) has demonstrated an inability of larvae in the settling stage of the marine fish *Amphiprion percula* exposed to a water pCO_2 of 1000 μatm to detect olfactory cues of their predators. Similarly, Ferrari *et al.* (2011) have shown differences in the predatory avoidance of four different species of reef fish with up to 95% loss of response to predation risk and a five- to seven-fold increase in CO_2 -related mortality due to predation in the fish *Pomacentrus chrysurus*. A study on the gastropod *Littorina littorea* conducted at very low levels of pH 6.45 has shown an absence of inducible defences through shell thickening (Bibby *et al.* 2007).

The effects of seawater acidification on the predator side have rarely been investigated, although a few studies have observed reduced feeding rates of clams and sea urchins under conditions of acidification with a minimal pH between 6.0 and 7.19 (Bamber 1987, Siikavuopio *et al.* 2007, Stumpp *et al.* 2012).

Other environmental stressors, such as temperature (e.g. Sanford 1999, Dahlhoff *et al.* 2001) and salinity stress (Witman & Grange 1998) are known to have an influence on predation in

marine rocky intertidal and subtidal ecosystems. Since organisms in the Western Baltic are already exposed to stressful environmental conditions, due to the low salinity in this area, and diversity in harsh environments is known to be low, with only highly resistant species being successful under such conditions (e.g. Menge & Sutherland 1987), additional environmental stress could amplify these effects.

Predator-prey interactions in the macrozoobenthos of the Western Baltic

The species diversity in the Baltic Sea is reduced along this salinity gradient with the lowest number of species occurring at a salinity of 5-8 (Remane 1955, Bonsdorff 2006). In the Western Baltic, the macrozoobenthos is dominated by a community of molluscan species and their predators (e.g. Rumohr 1996). The hard-bottom here is almost entirely covered by the blue mussel *Mytilus edulis*, especially if not controlled by predators (e.g. Reusch & Chapman 1997, Wahl 2001, Enderlein & Wahl 2004).

The sea star *Asterias rubens* and the shore crab *Carcinus maenas* are two of the main predators inhabiting the macrozoobenthos of the Western Baltic. They both consume similar organisms: in the Western Baltic their diet consists of blue mussels to a large extent (e.g. Sommer *et al.* 1999, Enderlein *et al.* 2003). Prey in general is usually not the limiting factor in this environment, mostly due to the high mussel recruitment rates (Reusch & Chapman 1997, Wahl 2001, Enderlein & Wahl 2004). Enderlein and Wahl (2004), however, have shown the species diversity in Kiel Bight to increase in the presence of the predators *A. rubens*, *C. maenas* and *L. littorea*, mostly by controlling blue mussel dominance.

The blue mussel *M. edulis* itself is known to act as an important ecosystem engineer. In the Western Baltic it is one of the main trophic links in benthic-pelagic coupling, since they consume high amounts of phytoplankton, being highly efficient filter-feeders (e.g. Kautsky 1981, Kautsky & Evans 1987). They further significantly increase biodeposition of C, N and P and regenerate large amounts of the N and P demands for pelagic primary production (Kautsky & Evans 1987). Moreover, they act as substratum and shelter for a number of species, increasing biodiversity within the mussel beds (e.g. Norling & Kautsky 2008).

Environmental stressors causing an influence on the interaction between *M. edulis* and its predators thus have the potential of changing the entire ecosystem.

The sea star *A. rubens* can consume Baltic blue mussels up to 48 mm (Sommer *et al.* 1999) by pulling the mussel shells apart and everting its stomach, then releasing digestive enzymes into the mussels and devouring the mussel flesh extra-corporally. Feeding rates and mussel sizes consumed would thus be influenced, if the muscle strength of the mussels was influenced by seawater acidification. It has been reported that although calcification rates

even increase under increased seawater acidification (pH up to 6.8) in the brittlestar *Amphiura filiformes*, the amount of muscle tissue decreases (Wood *et al.* 2008). If a similar effect occurred in blue mussels, this could cause effects on the interaction with its predator *A. rubens*. Likewise, any effect on sea star size, skeleton rigidity, activity, muscle strength or digestive capacities will have consequences for the removal of blue mussels from the system.

Carcinus maenas can consume mussels up to a size of ca. 50 mm (Enderlein *et al.* 2003), the shell of which it crushes with its chelae and mandibles to pick out the edible flesh. Thus any effect of acidification on the shell stability of the mussels is likely to influence consumption rates and mussel sizes consumed by the crabs. It is known that calcification in *M. edulis* is influenced by short-term (2 h) exposure to seawater acidification of up to 2000 μatm (Gazeau *et al.* 2007) and that a reduction in salinity to values below 12 salinity units leads to less stable mussel shells and an increase of mussel size classes consumed by *C. maenas* (Kossak 2006). It is thus possible that similar effects can be observed when mussels are exposed to seawater acidification. As with the sea stars, any effect on size, cuticle stability, activity, claw muscle strength or digestive capacities of the crabs will, in turn, also influence interactions between crabs and blue mussels.

An overview regarding possible factors influencing interactions between adult *M. edulis* and their main predators in the Western Baltic, *A. rubens* and *C. maenas*, is displayed in Fig. 2.

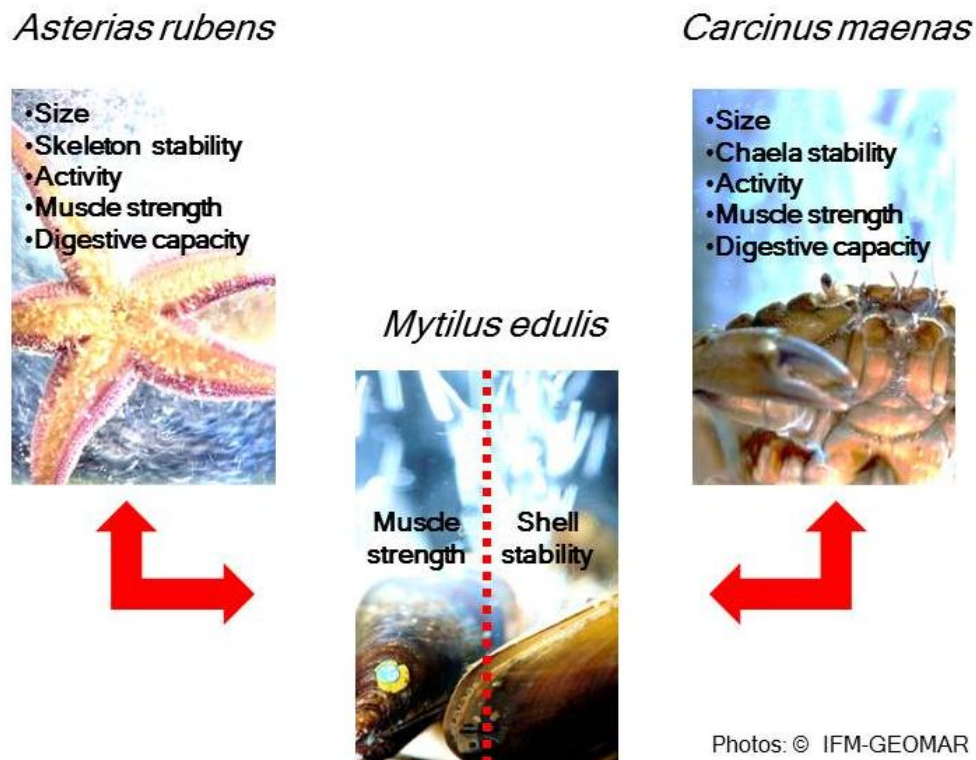


Fig. 2 Factors influencing the interactions between the blue mussel *M. edulis* and its predators *A. rubens* and *C. maenas*

Study organisms

The sea star *A. rubens* can make up more than one tenth of the benthic invertebrate biomass in Kiel Fjord at certain times of the season (Nauen 1979). Baltic specimen have their distribution limits at a salinity of ca. 8 (Binyon 1961). They have been reported to occur not further east than around the Darss Sill (Nauen 1979 Fig. 2) and are usually not found further east than Kiel Bight (Wahl *pers. comm.*). Reproduction and larval development, however, probably only occurs up to a salinity of 15 (Casties *in prep.*, Nauen 1979).

Echinoderms in general are thought to be particularly vulnerable towards seawater acidification (e.g. Raven *et al.* 2005), due to a number of different reasons. One aspect concerns their unique endoskeleton, the stereom. It consists of the highly soluble Mg-calcite in large parts (Nauen & Böhm 1979). This form of calcium carbonate is even more soluble than aragonite and seawater is thus, when the water is acidified, undersaturated towards this ion long before saturation horizons of aragonite and calcite are affected (Andersson *et al.* 2008). Moreover, echinoderms do not regulate ions in their extracellular fluid to a large extent (Binyon 1962) and they generally have low metabolisms (Nauen 1979). Both these traits may cause a greater sensitivity towards seawater acidification than expected for more active organisms with larger capacity to regulate their extracellular ions (Melzner *et al.* 2009).

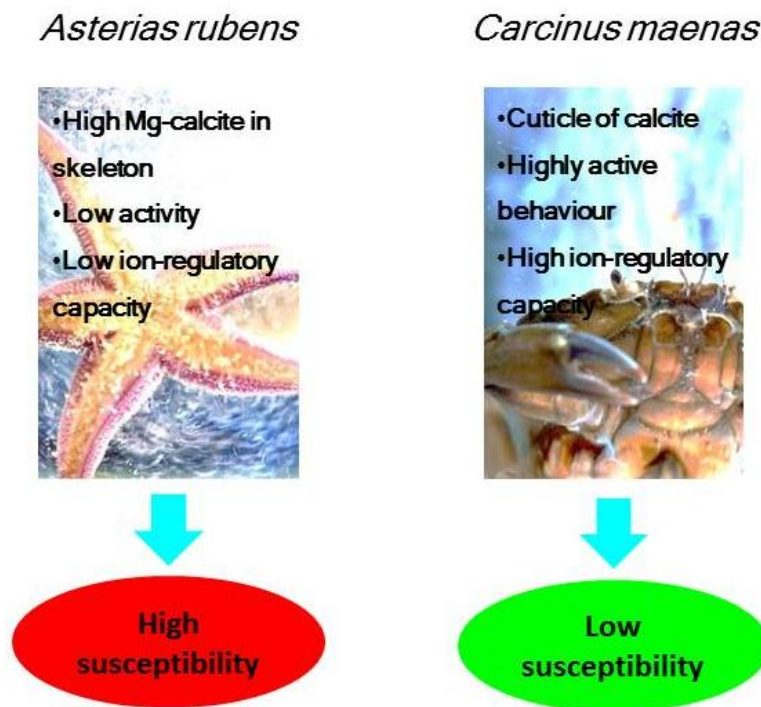
The shore crab *C. maenas* belongs to the most abundant species in the macrozoobenthos of Kiel Fjord (Anger 1975). It has been reported to equally tolerate salinities of ca. 8, but can be found further east than *A. rubens*, up to the Bornholm basin in the Baltic Sea (Bonsdorff 2006).

In contrast to echinoderms, brachyuran crabs have been assumed fairly resistant towards seawater acidification (e.g. Melzner *et al.* 2009). The calcified structures of the cuticle consist of the fairly stable calcite (e.g. Boßelmann *et al.* 2007) and their highly active life-style and ion-regulating capacity contribute to their low sensitivity to acidification. (Melzner *et al.* 2009). It has, however, been hypothesised (Melzner *et al.* 2009, Gutowska *et al.* 2010) and affirmed (Ries *et al.* 2009) that some crabs may respond to seawater acidification with an increase in calcification rates. This is thought to result from the active accumulation of HCO_3^- in the hemolymph, when regulating the extracellular pH (pHe, Melzner *et al.* 2009). Such an increase in calcification may also have a negative impact on the organisms. An increase in cuttlebone density in the cephalopod *Sepia officinalis* caused by acidification could potentially negatively influence buoyancy (Gutowska *et al.* 2010) and also an increase in cuticle density in crabs could have negative consequences e.g. for the mobility of latter.

The blue mussel *M. edulis* covers nearly 100 % of all available hard substratum in Kiel Fjord, where not controlled by predators (Reusch & Chapman 1997, Laudien & Wahl 1999). In

contrast to its predators *A. rubens* and *C. maenas*, it can tolerate a salinity of only 4.5 units and can thus be found up to the gulf of Finland (Reimer & Harms-Ringdahl 2001, Westerbom *et al.* 2002).

The effects of seawater acidification on blue mussels has been studied before (Berge *et al.* 2006, Bibby *et al.* 2008, Hiebenthal 2009, Thomsen *et al.* 2010, Thomsen & Melzner 2010), but so far no study has investigated direct effects of acidification on the predator-prey relationships.



Photos: © IFM-GEOMAR

Fig. 3 Prediction of the susceptibility and factors influencing vulnerability in the benthic predators *A. rubens* and *C. maenas* towards seawater acidification

Objective and hypotheses tested

In order to gain insight into the possible impact of seawater acidification on the benthic community of the Western Baltic, the focus of the conducted studies lay on investigating the effects of an increased $p\text{CO}_2$ on the two main predators of adult blue mussels in this region, the common sea star *A. rubens* and the shore crab *C. maenas*.

The hypotheses I tested in the studies conducted in context of this thesis were:

1. The interactions between *M. edulis* and its predators will change with an increase in seawater acidification via a change in consumption rates.

Since I assumed seawater acidification to have an impact on at least one parameter relevant for the interactions between the blue mussels and their predators on prey and/or predator side (see Fig.2), I expected the feeding pressure to change in either direction (i.e. more or less consumption of blue mussels by either predator).

2. The shore crab *C. maenas* is less vulnerable to increases in seawater acidification than the common sea star *A. rubens*.

The shore crab with its calcite cuticle (Boßelmann *et al.* 2007), ion-regulatory capacities (Seck 1958) and highly active behaviour (Wallace 1972) appears better adapted towards changes in seawater acidification than the sea star with its high portion of Mg-calcite, its mostly non-ion-regulatory physiology (Binyon 1962) and its less active behaviour (see Fig. 3). Although an increase in seawater pCO₂ may lead to over-calcification in crabs (Melzner *et al.* 2009, Ries *et al.* 2009, Gutowska *et al.* 2010) possibly with negative effects on the organisms, I thus assumed the crabs to be less affected by an increase in seawater acidification than the sea stars.

3. Juvenile *A. rubens* are more susceptible to changes in seawater acidification than adults of the same species.

Since earlier life stages are in general suspected to be impacted by seawater acidification than later life stages (e.g. Ross *et al.* 2011), I assumed the same to hold true for the juvenile vs. the adult life stage of *A. rubens*.

These hypotheses were tested in three different studies, which are described in the following chapters 1-3.

Chapter 1 investigates the effect of seawater acidification on direct interactions between adult *A. rubens* and *M. edulis* and adult *C. maenas* and *M. edulis*, as well as effects on growth of *A. rubens* and *M. edulis* and the acid-base balance of the adult predators.

The study in chapter 2 deals with the effects of increased seawater pCO₂ on growth, mussel consumption, metabolism and calcification on juvenile *A. rubens*.

Chapter 3 studies the growth and moulting of *C. maenas* under conditions of seawater acidification, alongside with the parameters mussel consumption, metabolism, acid-base balance and calcification to complement the study of chapter 1.

Chapter 1

Not so sour times for benthic predators - the influence of seawater acidification on growth, feeding behaviour and acid-base status of *Asterias rubens* and *Carcinus maenas*

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Abstract

The impact of seawater acidification on calcifying organisms varies at the species level. If it differs between predator and prey in strength and/or sign, trophic interactions may be altered. In this study we investigated whether the quantity or size spectrum of prey consumed, the acid-base status or growth of two predatory species, the common sea star *Asterias rubens* and the shore crab *Carcinus maenas*, are impacted by medium-term exposure to three different seawater $p\text{CO}_2$ -levels of 650, 1250 and 3500 μatm (66, 127 and 356 Pa). We exposed both, the predators and their prey -the blue mussel *Mytilus edulis*- over a time span of 10 weeks and subsequently performed feeding experiments. Intermediate acidification levels had no significant effect on growth or consumption in either predator species. The highest acidification level reduced feeding and growth rates in sea stars by 56%, while in crabs a 41% decrease in consumption rates of mussels could be demonstrated over the 10 week experimental period, but not in the following shorter feeding assays. Since only few crabs moulted in the experiment, acidification effects on crab growth could not be investigated. Active extracellular pH compensation by means of bicarbonate accumulation was observed in *C. maenas*, whereas coelomic fluid pH in *A. rubens* remained uncompensated. Acidification did not provoke a measurable shift in prey size preferred by either predator. Mussels exposed to elevated $p\text{CO}_2$ were preferred by previously not treated *A. rubens*, but not *C. maenas*. The observed effects on species interactions were weak even at the high acidification levels expected for marginal marine habitats like the Baltic Sea. Our results indicate that when stress effects are similar (and weak) on interacting species, biotic interactions may remain unaffected.

Introduction

The brackish Western Baltic Sea is a relatively species poor environment. Two of the main benthic predators in the western Baltic are the common sea star *Asterias rubens* and the shore crab *Carcinus maenas*. A large amount of their prey consists of the highly abundant blue mussel *Mytilus edulis*. Regionally, the bivalve can cover 95-100% of all suitable substrate, especially if predators are absent (e.g. Kautsky & van der Maarel 1990, Reusch & Chapman 1997). Adult crabs and sea stars, however, consume mussels only to the threshold size of about 48 mm shell length, beyond which size refuge is obtained (Sommer *et al.* 1999, Enderlein *et al.* 2003, Laudien & Wahl 2004). The protective effect could be due to large size, strong adductor muscles and/or more robust shells. Any factor prolonging the time a mussel spends in sub-threshold size properties, diminishes its chances for survival. Reduced growth can lead to shifts in trophic interactions with increasing overall predation impact on the mussel population (e.g. Enderlein & Wahl 2004). In addition, when changing environmental conditions affect the feeding rates and/or prey size range of sea stars and crabs, shifts in trophic interactions with community-wide consequences may be expected.

The predicted ocean acidification (IPCC 2007) is one of the environmental shifts with this potential. Seawater acidification at a global and long term scale is caused by the anthropogenic increase of atmospheric CO₂ which, at smaller spatial and temporal scales, may be overridden by biological and biogeochemical processes (Thomsen *et al.* 2010). Until the end of the century, oceanic pH decreases of 0.3-0.4 units (equivalent to atmospheric CO₂- levels of 650 µatm and above) can be expected (Feely *et al.* 2004, Caldeira & Wickett 2005). Due to the unique structure of the Baltic Sea with its generally low salinity but strong stratification and pronounced oxygen-minimum zones occurring in the summer below the pycnocline, pCO₂-levels here can be naturally high, especially in benthic regions. The surface water pCO₂ in this region today occasionally exceeds values of 2300 µatm and is thus higher than expected for the open ocean within the next 300 years (Thomsen *et al.* 2010). Recent model calculations indicate that with increasing atmospheric CO₂-levels, Baltic surface pCO₂ will rise over proportionally. Thus, with a doubling of atmospheric concentrations from currently 385 to future 761 µatm, surface water pCO₂ is expected to seasonally reach peak values of more than 4000 µatm in the study area (Thomsen *et al.* 2010). Moreover, the pH in this region is expected to fluctuate more than under open ocean conditions, since CO₂ effects are not buffered as strongly, mainly due to the lower concentration of HCO₃⁻ and thus lower total alkalinity (*A_T*) (Beldowski *et al.* 2010). These properties (low salinity and thus low *A_T*) are found in many estuarine and coastal systems. Research to date has focused mainly on pelagic open ocean conditions and may be of little relevance to benthic organisms (compare values in Beldowski *et al.* 2010).

It has traditionally been assumed that seawater acidification will most severely impact calcifying organisms (e.g. Fabry 2008). In fact, several recent studies have shown that acidified conditions may impact (in one direction or other) the early development (Kurihara *et al.* 2004, Kurihara & Shirayama 2004, Kurihara *et al.* 2007, Dupont *et al.* 2008, Ellis *et al.* 2009), growth and calcification (Michaelidis *et al.* 2005, Gazeau *et al.* 2007, Kurihara *et al.* 2008, Wood *et al.* 2008, Gooding *et al.* 2009, Thomsen *et al.* 2010) and diverse physiological traits (Michaelidis *et al.* 2005, Metzger *et al.* 2007, Miles *et al.* 2007, Thomsen *et al.* 2010, Thomsen & Melzner 2010) of calcifying echinoderms, crustaceans and bivalves.

How strongly acidification impacts calcifiers varies at the species level (Hall-Spencer *et al.* 2008, Melzner *et al.* 2009, Gutowska *et al.* 2010, Hendriks *et al.* 2010, Kroeker *et al.* 2010, Whiteley 2011). This in part is due to variable combinations of calcite, aragonite and organic matrix in the respective skeletons. While the exoskeleton of crustaceans consists solely of the relatively stable calcite, the shells of mussels such as *M. edulis* is composed partly of calcite and partly of the more soluble aragonite. The skeleton of echinoderms consists of Mg-calcite, a form of carbonate even more soluble than aragonite (Andersson *et al.* 2008). Moreover, as suggested by Melzner *et al.* (2009), highly active organisms (such as crabs) due to their high ion-regulatory and extracellular fluid buffering capacity may be less sensitive to seawater acidification than less active groups (e.g. sea stars and mussels). Also, species from groups that compensate their extracellular fluid pH by active accumulation of HCO_3^- , such as cephalopods (Gutowska *et al.* 2008) or teleost fish (Checkley *et al.* 2009), hypercalcify when being chronically exposed to acidified seawater. This is thought to be mostly due to the excess amount of accumulated HCO_3^- , which could lead to an increase in calcification via a change in ionic driving forces across calcifying epithelia (Gutowska *et al.* 2010). Finally, the amount of organic material covering of the outer skeleton layer may influence the sensitivity towards acidification in the different groups (e.g. Tunnicliffe *et al.* 2009).

The feeding modes of the two predator species is determined by different properties which may react differently to acidification. *Carcinus maenas* crushes the shells to consume the flesh, whereas *A. rubens* slowly pulls the shells apart and digests the soft body in its everted stomach. Thus, with increasing mussel size several features relevant to one or both consumers change. Consumption by crabs is reduced by a reduced handleability and a larger stability of the molluscan shell. Starfish feeding is reduced by an increased strength of the adductor muscle i.e. the amount of myofibrils in smooth muscles and energy available to prolong the catch phase (e.g. Twarog 1967), during which mussels keep their shells closed with minimal energy investment. A study on the brittlestar *Amphiura filiformis* by Wood *et al.* (2008) proposed that not only carbonate structures but also the quantity of muscle tissue may be influenced by acidification. If the amount of smooth muscle fibres in the adductor muscles of bivalves is reduced, or if for any reason the catch mechanism is disturbed, sea star feeding

should be facilitated - unless their muscles, too, are weakened. If calcification of mussel shells is reduced and shells become less stable, crabs may be able to consume a larger quantity and larger sizes of mussels per unit time, again unless they themselves are impacted by acidification.

The aim of this study was to test whether seawater acidification influences the susceptibility of predation by *A. rubens* and *C. maenas* on the blue mussel *M. edulis*, the main biotic controlling factors in the Western Baltic of latter. We tried to differentiate between the influence of acidification on predators and those on prey. We hypothesised that 1. if interacting species are differently affected by acidification, their trophic interaction should shift and 2. acidification on the consumer side should affect sea stars (non-compensating) more than crabs (compensating). So far, very few studies have focused on the predator-prey interaction between organisms under acidification conditions (Bibby *et al.* 2007, Gooding *et al.* 2009) although such indirect effects of stress may be substantially stronger than their direct impact (Wahl 2008).

Materials and methods

Sampling

All organisms investigated were sampled in Kiel Fjord (Western Baltic Sea) via dredging or manual collection in April 2009. The size spectra of selected test animals at the start of the experiment were: *Asterias rubens* 15.5 ± 3.5 g wet weight (WW) and 4.5 ± 0.5 cm arm length, *Carcinus maenas* 4.5 ± 0.5 cm carapace width, *Mytilus edulis* (three classes) 2.00 ± 0.15 cm, 3.00 ± 0.15 cm and 4.00 ± 0.15 cm shell length.

Set-up

The experimental units (EUs) consisted of 2 l plastic aquaria, connected to a flow-through system of seawater from Kiel Fjord. The water was pre-treated in three interconnected header-tanks via bubbling with pressurized air, into which different concentrations of CO₂ (ambient conditions, 1120 μ atm and 4000 μ atm) were mixed. Water from the header-tanks then streamed into the EUs at a flow rate of 5-6 l h⁻¹ and was additionally bubbled with above mentioned CO₂-air mixtures. In this way we established three different treatment levels (Table 1). Due to the naturally acidified conditions of water in the Kiel Fjord (see above), also lowest treatment levels (with a mean of 650 μ atm, see Table 1) were substantially higher than the currently around 380 μ atm CO₂ prevailing in the atmosphere.

To each single EU we added either one crab, one sea star or 20 mussels of all three size classes (60 mussels in total per EU). The three treatment levels were replicated 10 times for each prey or consumer species, resulting in a setup of 90 EUs. In each EU containing

mussels, three random mussels of each size class were individually marked to allow for growth monitoring. *Asterias rubens* were fed *ad libitum* with mussels of 3.50 ± 0.35 cm size and *C. maenas* also *ad libitum* with mussels of 2.50 ± 0.35 cm. The mussels were fed with 10 ml of suspended plankton food (DT's Live Marine Phytoplankton Premium Blend) per EU three times a week. During feeding the flow-through of the mussel EUs was stopped for 2 h. The experimental period lasted for 10 weeks.

The carbonate system was determined via the analysis of C_T (coulometric, SOMMA System autoanalyser) and A_T (potentiometric titration, VINDTA autoanalyser) at the beginning, intermediate phase and end of the experiment in each three representative EUs per respective treatment level and species (nine per measurement day). Calculations of the carbonate system speciation were performed using the CO2SYS macro for low salinities (modified by Körtzinger after Pierrot *et al.* 2006). The dissociation constants K_1 and K_2 were chosen according to Roy *et al.* (1993). Additionally, the pH and the salinity in the EUs was measured weekly with a hand-held pH-meter (WTW 340i pH-analyser, WTW SenTix 81 measuring chain - NBS scale) and salinometer (WTW cond 315i, WTW TETRACON 325 measuring chain). Concentration of NH_4^+ was determined in each 3 EUs per pCO_2 -level at the end of the experimental period using a JBL NH_4^+ aquarium kit.

Growth

Biomass increase (WW) of the sea stars was quantified weekly, weighing each individual three times after softly blotting it dry with a paper towel. Mussel growth (increase in shell length) was documented as shell length increase between the beginning and the end of the experimental period. Crab growth could not be monitored, since only one crab moulted during the experimental period.

Feeding Assays

The consumption of mussels by sea stars and crabs during the experimental period was monitored weekly. After the 10 weeks of treatment at different pCO_2 levels, we performed three different feeding assays (see Fig. 1 for overview). In feeding assay I, predators were starved for one day before being offered three mussel individuals from each of the three different size classes (i.e. nine mussels) simultaneously, with consumers and prey stemming from identical experimental conditions. Number and size class of mussels consumed was monitored daily over six days.

The predators were then again starved for one day, after which feeding assay II was performed, which was identical to assay I, except that the prey was not pre-treated but collected from Kiel Fjord the day before. These mussels served as external references in order to assess which proportion of the effects observed in assay I were due to the predators

rather than on the prey. Consumption in feeding assay II was monitored daily over six days, as before.

In feeding assay III consumers collected from Kiel Fjord (10 sea stars and 10 crabs left to starve for three days) were offered three prey individuals of the medium size class (3.00 ± 0.15 cm) stemming from each of the three treatment levels (i.e. nine mussels). In feeding assay III the predators served as external references, in order to estimate which proportion of the effects observed in assay I were attributable to the treated mussels. Monitoring of feeding assay III took place over six days as well, but only the data obtained during day 1 were analysed, when one of the predators had consumed all three mussels of one treatment level. All feeding assays took place under the respective CO_2 -conditions the predators were previously exposed to.

Acid-base Status

Following the feeding assays, extracellular acid-base status of the consumer species was assessed, in order to determine regulative capacities of the species. *Asterias rubens* was starved for seven days, then the tip of an arm was cut and the coelomic fluid was collected in a cap. Crabs were starved for three days, then hemolymph was drawn bubble-free with a syringe from the infrabranchial sinus without contact to air. pH was measured using a microelectrode (WTW Mic-D and WTW pH 340i), and C_T using a CO_2 analyser (Corning 965). $p\text{CO}_2$ and HCO_3^- concentrations of the body fluids were calculated from measured pH and C_T with the rearranged forms of the Henderson-Hasselbalch-equation:

$$p\text{CO}_2 = C_T \times \left(10^{\frac{\text{pH} - \text{p}K'_1}{\alpha_{\text{CO}_2}}} \times \alpha_{\text{CO}_2} + \alpha_{\text{CO}_2} \right)^{-1}$$

$$[\text{HCO}_3^-] = 10^{\frac{\text{pH} - \text{p}K'_1}{\alpha_{\text{CO}_2}}} \times \alpha_{\text{CO}_2} \times p\text{CO}_2$$

For *C. maenas*, α_{CO_2} , (CO_2 solubility) and $\text{p}K'_1$, the first apparent dissociation constant, were calculated from Truchot (1986) for ambient salinity and temperature. The α_{CO_2} added up to 0.000375 Pa and the $\text{p}K'_1$ to 6.065. In *A. rubens*, α_{CO_2} was calculated from Weiss (1974). $\text{p}K'_1$ was calculated from linear correlation of $\text{p}K$ and pH ($\text{p}K = -0.1331\text{pH} + 7.2481$, $r^2 = 0.3047$) determined *in-vitro* for coelomic fluid at given experimental salinity and temperature. For this purpose, 600 μl samples of body fluid pooled from 10 animals were equilibrated with known $p\text{CO}_2$ levels of 560, 1400, 4000, and 6000 μatm for 1 h. Afterwards pH and DIC of the samples were determined as described above. $\text{p}K'_1$ was calculated using the equation:

$$\text{p}K'_1 = \text{pH} - \log \left(\frac{C_T}{p\text{CO}_2 \times \alpha_{\text{CO}_2}} - 1 \right)$$

The α_{CO_2} used for calculation was 0.0003888 Pa and the $\text{p}K'_1$ were at 6.18 ± 0.01 for the 650 μatm level, at 6.19 ± 0.01 for the 1250 μatm level and at 6.21 ± 0.01 for the 3500 μatm level.

The non-bicarbonate buffer line (NBB) was determined *in-vitro* by correlation of measured pH and calculated HCO_3^- concentration in equilibrated body fluid samples.

Mussel Properties

The mussels not used in the feeding assays were frozen at -20°C after the experimental period. 10 mussels of each size class and treatment level were later defrosted in order to determine the dry soft tissue weight of the mussels at the respective treatment level and correlate it to consumption rates. The others were used to determine adductor muscle dry weight, shell weight and maximum breaking resistance of shells. Pilot assays had shown that a freezing/thawing treatment did not affect detectable differences in shell stability.

In order to determine adductor muscle dry weight, shell weight and maximum breaking resistance of shells, three mussels of each size class per EU were defrosted and opened with a scalpel. The adductor muscles were dissected, dried for 24 h at 80°C until constant weight was reached and weighed individually. The shells were dried with a paper towel, left to surface-dry at room temperature for 2 h and then weighed individually. The breaking resistance was determined with a TAXT2i texture analyzer (Stable Micro Systems, 25-1 measuring cell), using a cylinder of 2 mm diameter and a speed of 1.00 mm s^{-1} , measuring the maximum force necessary for breaking the shell. For these measurements the shells were positioned opening downwards, the rim of the shell on a neoprene sheet to ensure homogeneous distribution of forces and the cylinder position above the highest point of the shell. Both, the left and the right shell half were broken and the higher value of the two was used for analysis.

Statistical Analyses

Total mussel consumption of the predators during the experimental time, the total mussel consumption in feeding experiment I and II, the growth of all three species, as well as the adductor muscle weight, the shell weight and the maximal breaking resistance of the mussels were analysed using a one-way ANOVA, followed by Tukey's HSD post-hoc analysis. The size range of mussels consumed in feeding experiment I and II was compared using a repeated measures ANOVA (following Yun *et al.* 2007). Data were tested for normality using the Shapiro-Wilks-W test before further statistical analysis. If the data were non-normally distributed, the Box-Cox procedure identified the simplest transformation to achieve normality. Percentage data were arcsine transformed. Data were tested for homogeneity of variances using Levene's test. If normality and homoscedasticity could not be achieved, we used parametric tests, but lowered the α -level to 0.01 (following Wakefield & Murray 1998). The data of feeding experiment III were analysed using resampling and a Monte Carlo simulation with 100 permutations (compare e.g. Rohde *et al.* 2004). Data of coelomic fluid and hemolymph acid-base status were plotted in pH-bicarbonate (Davenport) diagrams.

Results

Experimental Conditions

The mean $p\text{CO}_2$ in the EUs at the three treatment levels was 654.2 ± 54.0 SE (650), 1245.7 ± 85.8 SE (1250) and 3466.9 ± 155.6 SE (3500) μatm (rounded values in parentheses are equivalent to 66, 127 and 356 Pa), respectively, during the course of the experimental period (Table 1). Sea water $[\text{NH}_4^+]$ was below 0.2 mg l^{-1} in all cases.

None of the sea stars died in the course of the experiment. One crab individual of each treatment level died during the 10 week experimental period. One crab of the highest treatment level moulted nine weeks into the experiment. Four (650 μatm), three (1250 μatm) and five (3500 μatm) of the six female crabs per treatment level spawned in week 4 or 5 of the experimental period.

Growth

Growth of *Asterias rubens* over the 10 week experimental phase was significantly lower at the high (3500 μatm) as compared to intermediate $p\text{CO}_2$ (1250 μatm) concentrations. Growth did not differ between the 650 and 1250 and between the 650 and 3500 μatm treatments (Fig. 2a, Table 2). Growth of *Mytilus edulis* (Fig. 2b, Table 2) was generally very low and not significantly affected by acidification. Growth of crabs was not assessed.

Consumption

Feeding of *A. rubens* during the experimental phase differed significantly between the $p\text{CO}_2$ treatment levels with highest consumption at the intermediate (1250 μatm) and lowest at the highest (3500 μatm) treatment level (Fig. 3a, Table 2). The same pattern was found in the total consumption by sea stars in feeding assay I, in which pre-treated mussels were fed to the pre-treated sea stars (Fig. 3b, Table 2), as well as in feeding assay II, in which untreated mussels were offered to the pre-treated *A. rubens* (Fig. 3c, Table 2). The prey-size preference exhibited by sea stars was not significantly affected by acidification neither in feeding assay I (Fig. 5a, Table 2) nor in feeding assay II (Fig. 5b, Table 2). In feeding assay III, in which the pre-treated mussels were offered to untreated sea stars, significantly more mussels treated at 3500 μatm were consumed within 24 h than mussels pre-treated with lower $p\text{CO}_2$ levels (Fig. 3d).

In contrast to the sea stars, significant acidification effects on *Carcinus maenas* feeding were detected in the experimental phase only (Fig. 4a, Table 2). Here, consumption was significantly lower at 3500 μatm than at the other levels. Acidification had no measurable effect on the trophic interaction between identically pre-treated mussels and crabs (assay I, Fig. 4b, Table 2), on the feeding behaviour of crabs (assay II, Fig. 4c, Table 2), or on the susceptibility of mussels to crab predation (assay III, Fig. 4d). Similar as for the sea stars, the

differently treated crabs did not differ in prey size preference in assay I (Fig. 6a, Table 2) or II (Fig. 6b, Table 2).

Acid-base Status

The coelomic fluid pH (pHe) of *A. rubens* decreased with increasing seawater $p\text{CO}_2$ along the non-bicarbonate buffer (NBB) line (Fig. 7a, Table 2). We did not observe extracellular HCO_3^- accumulation which deviates from solely passive increment, as presented in the pH-bicarbonate (Davenport) diagram. In contrast, hemolymph pH of *C. maenas* was maintained at control pH levels of 7.82, independently of the $p\text{CO}_2$ treatment level, which resulted from active elevation of $[\text{HCO}_3^-]$ from 7.9 to 10.4 and 11.7 mM, respectively (Fig. 7b, Table 2).

Mussel Properties

The dry mass of the adductor muscles (Fig. 8a, Table 2) and the shell mass (Fig. 8b, Table 2) of *Mytilus edulis* did not significantly differ among the treatment levels, although a trend towards a lower shell mass with increasing seawater $p\text{CO}_2$ was observed. The mean maximum breaking resistance of mussel shells was significantly lowered by approx. 20% at the highest level of 3500 μatm (Fig. 8c, Table 2).

Discussion

Acidification effects on species interactions were weak at most even for the high acidification levels expected for marginal marine habitats like the Baltic Sea. When there were direct effects on the prey (e.g. increasingly fragile shell) concurrent effects on the predators (e.g. lower consumption, slower growth) tended to neutralize any shifts in trophic interactions.

Even though the seawater was undersaturated with aragonite in all treatment levels and with calcite in the two higher $p\text{CO}_2$ -levels, both predator organisms were able to survive under acidified conditions. These observations are in line with recent acidification studies on crabs and echinoderms (Dupont & Thorndyke 2008, Wood *et al.* 2008, Gooding *et al.* 2009, Dupont *et al.* 2010a, Dupont *et al.* 2010b, Whiteley 2011). Furthermore, no significant responses on feeding rate and/or growth were observed under moderate (1250 μatm) seawater acidification scenarios.

Asterias rubens

Asterias rubens was only affected in feeding and growth at the highest chosen CO_2 -level (3500 μatm), whereas intermediate acidification (1250 μatm) even seemed to slightly enhance growth and consumption. A natural pre-adaptation to acidified conditions could explain this pattern. Since the benthic habitat of sea stars, especially in eutrophic coastal

zones, is often an area of high decomposition, CO₂-levels in these regions are often substantially higher than in the pelagic environment (Feely *et al.* 2008, Melzner *et al.* 2009, Thomsen *et al.* 2010). Mean pH in 10m depth in Kiel Fjord was 7.7 (SD 0.2, corresponding to a pCO₂ of ca 1400 µatm) between May and October 2009 (Wahl *et al.* 2010) and probably periodically lower at the sediment-water interface (see also Thomsen *et al.* 2010). Consistent with our findings, Gooding *et al.* (2009) report that the sea star *Pisaster ochraceus* grew significantly better and tended to consume more mussels under intermediate acidification levels (780 µatm) - which, however, were closer to our natural “low” than to our intermediate level. Also, larvae and juveniles of the sea star *Crossaster paporus* showed faster growth and development under a pH of 7.7 than under a pH of 8.1 (Dupont *et al.* 2010a). Our findings indicate that moderate acidification (up to at least 1250 µatm) does not stress *A. rubens* enough to significantly reduce feeding rates, although abiotic stress in general can lead to reduced feeding rates in asteroids. Thus, strong osmotic stress in the sea star *Luidia clathrata* (abrupt salinity reduction from 28 to 17) has been shown to lead to depressed feeding rates over 30 days (Forcucci & Lawrence 1986).

Sea star tolerance was reduced at very high levels of acidification, as reflected in reduced growth and consumption rates. The underlying causality is not clear. However, recent studies have demonstrated that acidification can lead to energy budget re-allocations that result in a reduction of scope for growth in larval and adult echinoderms (Stumpp 2011). Excess energy might be required for maintenance of intracellular pH when extracellular pH is decreased (e.g. Michaelidis *et al.* 2005, Thomsen & Melzner 2010), thus limiting the amount of energy that can be invested into digestion, assimilation and growth. In addition, the low extracellular pH could impact the energy available for calcification. However, energetic trade-offs are mostly relevant under energy-limited conditions which we tried to avoid in our experimental set-up. It is also possible that decreased feeding rates were connected to a reduced functionality of digestive enzymes - if stomach pH was affected by seawater acidification, or if more energy had to be invested into stomach pH regulation (which we did not assess). The stomach pH of adult asteroids has been shown to be between 7.3 and 7.5 and an optimum of enzymatic activity was found at a similar pH (Irving 1926). The change in extracellular and environmental pH in our study might have caused a decrease in enzyme activity.

Carcinus maenas

As *A. rubens*, the shore crab *Carcinus maenas* is not influenced in feeding by moderate (1250 µatm) seawater acidification. At the highest pCO₂, a significant reduction in feeding was observed during the comparatively long period of the experiment, but not in the subsequent feeding assays. Since only one of the crabs moulted during the entire study period, we cannot be certain whether or not growth of the crabs is affected by acidification. However, a relative insensitivity of decapod crustaceans towards short-term acidification

stress has been demonstrated (e.g. Spicer *et al.* 2007) and has been explained by their high regulatory capacity. On the other hand, Kurihara *et al.* (2008) found significantly lower moulting frequencies and growth of the shrimp *Palaemon pacificus* when exposed to CO₂-levels of 1900 μ atm over a longer time period of 30 weeks. Our results clearly show that the crabs -in contrast to sea stars- can compensate pHe when facing substantial pH shifts in their environment. A compensation of respiratory acidosis caused by hypercapnia has been demonstrated before and its mechanisms explained by accumulation of HCO₃⁻ in its hemolymph (Truchot 1979). While short term pHe regulation in crabs is probably necessary for efficient hemocyanin mediated blood oxygen transport (e.g. Lallier & Truchot 1989), it cannot be excluded that in the long run the excess amount of energy invested in pHe regulation negatively impacts energy allocation to other processes, such as reproduction, growth or even feeding processes (digestion, prey handling). Again, this would mainly apply for energy-limited conditions.

Only one of the crabs moulted during the experimental period in our study. This long intermoult period is not unusual for the crab size used in our experiments and the prevailing water temperature (Adelung 1971, Klein Breteler 1975, Hartnoll 2001). Monitoring growth rates and moulting intervals over a longer time period (6-12 months) may therefore be necessary to test whether acidification impacts energy allocation into somatic growth in *C. maenas* and whether calcification effects increase with increasing time exposure to high p CO₂ seawater and the concomitant increases in hemolymph [HCO₃⁻].

In the previously mentioned study by Kurihara *et al.* (2008) no difference in feeding rates were found between shrimp treated at ambient conditions, 1000 and 1900 μ atm. However, previous studies have shown the handling time of mussels by *C. maenas* to be extended and thus the consumption rates reduced when crabs were kept under other stressful conditions, such as hypoxia (Brante & Hughes 2001). Melzner *et al.* (2009) proposed that organisms with high and highly fluctuating metabolic rates (thus, high fluctuations in extracellular fluid p CO₂) should, on average, cope better with increases in environmental p CO₂. This concept, however, is based on the assumption that uncompensated extracellular pH leads to metabolic depression (Pörtner *et al.* 2004), which does not seem to hold true in all cases. In our study, sea stars of the intermediate treatment suffered from respiratory acidosis, but mussel flesh consumption tended to increase. Also a few other studies on organisms of different phyla show that metabolic rates may remain constant or even increase under conditions of acidification in organisms that do not compensate extracellular pH (Beniash *et al.* 2010, Comeau *et al.* 2010, Lannig *et al.* 2010, Thomsen & Melzner 2010). The crabs in our experiments, although completely compensating pHe, demonstrated decreased food consumption during the experimental period at 3500 μ atm, indicating that impacts of elevated seawater p CO₂ are not exclusively related to extracellular acid-base status.

The influence of the prey organism

The fact that the consumption of mussels pre-treated under highest acidification levels by (not pre-treated) sea stars suggested that the adductor muscle strength of the mussels might have been weakened. In a study by Wood *et al.* (2008) the authors proposed that the change in Ca^{2+} to arm mass ratio in regenerated arms of brittle stars indicates higher levels of calcification, but less muscle tissue biomass when grown under acidified conditions (pH of 7.7, 7.3 and 6.8). In the present study, no differences in posterior adductor muscle mass were detected between treatment levels. Possibly, rather than degradation of muscle, acidification affected the catch phase of the smooth muscle by an as yet unknown cause. Surprisingly, despite a significant decrease in breaking resistance of the shell, mussels did not become more susceptible to crab predation under high acidification levels. Since the consumption efficacy of the crabs was not impacted by acidification, as verified by offering reference prey in feeding assay II, the decrease in shell stability was either too small to enhance vulnerability, or was compensated for by other, as yet unknown factors. It has previously been suggested that in addition to shell stability (Kossak 2006), the handleability of a mussel is a crucial factor limiting crab consumption (Enderlein *et al.* 2003).

Although it is possible that our results concerning mussel performance are slightly biased by generally low growth rates, which might have been caused by food quality and quantity (Melzner *et al.* 2011), our results suggest the influence on the size and amount of mussels consumed to be more dependent on recent history of the predators than their prey.

Conclusions

All three species show a shift in certain traits when exposed to high levels of acidification. Mussel shells become more brittle, sea stars (which cannot compensate pHe) grow slower, and both sea stars and crabs (the latter capable of pHe compensation) feed less under strong acidification. Interestingly, the enhanced vulnerability of mussels seems to be neutralised by the decreased consumption of the predators under high acidification. These results illustrate that differential stress effects on interacting species may not only enhance but also buffer community level effects. The overall weak effects of substantial acidification on the species studied here corroborates the expectation of enhanced tolerance towards hypercapnic hypoxia in the low-salinity and periodically hypoxic Baltic (Thomsen *et al.* (2010).

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Tables

Table 1: Measurements and calculations of the water carbonate system for the different treatment levels. Water $p\text{CO}_2$, Ω_{calcite} and $\Omega_{\text{aragonite}}$ were calculated using measured C_T and A_T values, as well as temperature and salinity values of the respective EUs on the day of measuring, with the CO2SYS macro for low salinities (Pierrot *et al.* 2006). Values are means \pm SE.

$p\text{CO}_2$ of pressurized air [μatm]	Measurements in set-up					Calculations		
	C_T [μmol kgSW^{-1}]	A_T [μmol kgSW^{-1}]	pH (NBS scale)	temperature [$^{\circ}\text{C}$]	salinity	$p\text{CO}_2$ [μatm]	Ω_{calcite}	$\Omega_{\text{aragonite}}$
Ambient (>387)	1999.2 ± 18.0	2046.4 \pm 18.6	8.06 \pm 0.005	12.9 \pm 0.07	14.8 \pm 0.13	654.2 \pm 54.0	1.64 \pm 0.17	0.96 \pm 0.10
1120	2076.2 ± 20.3	2051.60 ± 20.7	7.84 \pm 0.006			1245.7 ± 85.8	0.91 \pm 0.10	0.53 \pm 0.06
4000	2208.8 ± 24.0	2060.54 ± 19.5	7.36 \pm 0.008			3466.9 ± 155.6	0.34 \pm 0.03	0.20 \pm 0.02

Table 2: ANOVA results. Analyses performed with different response variables and the dependent factor $p\text{CO}_2$ measured during the acclimatisation phase and the subsequent feeding assays (FA) and three different $p\text{CO}_2$ treatments. For non-parametric data, the α -level was adjusted to 0.01. Statistically significant results are underscored.

	α	df	MS	F	p	Partial η^2
<i>Asterias rubens</i>						
<u>Increase in biomass</u>	0.05	2	8563.71	3.80	<u>0.04</u>	0.22
<u>Consumption during experiment</u>	0.05	2	375.23	6.37	<u>0.01</u>	0.32
<u>FA I, total consumption</u>	0.05	2	67340.00	4.13	<u>0.03</u>	0.23
<u>FA II, total consumption</u>	0.01	2	208276.00	4.95	<u>0.01</u>	0.27
FA I, consumption per mussel size class	0.01	4	1.47	1.98	0.11	0.13
FA II, consumption per mussel size class	0.01	4	0.34	0.68	0.61	0.05
<u>pHe</u>	0.01	2	0.20	40.67	<u>< 0.01</u>	0.75
<u>HCO₃⁻e</u>	0.01	2	0.07	3.08	0.06	0.19
<i>Carcinus meanas</i>						
Increase in biomass	0.01	2	786.81	0.85	0.44	0.07
<u>Consumption during experiment</u>	0.05	2	1911.26	3.93	<u>0.03</u>	0.25
FA I, total consumption	0.01	2	9505.00	0.31	0.73	0.03
FA II, total consumption	0.01	2	57996.00	0.42	0.66	0.03
FA I, consumption per mussel size class	0.01	4	1.04	2.38	0.06	0.17
FA II, consumption per mussel size class	0.01	4	0.73	1.33	0.27	0.10
pHe	0.01	2	< 0.01	0.12	0.88	0.01
<u>HCO₃⁻e</u>	0.01	2	34.00	5.67	<u>0.01</u>	0.35
<i>Mytilus edulis</i>						
Increase in shell length	0.01	2	0.32	1.33	0.28	0.09
Adductor muscle weight	0.01	2	< 0.01	0.14	0.87	0.01
Shell weight	0.01	2	4.10	1.12	0.34	0.08
<u>Breaking resistance</u>	0.05	2	< 0.01	6.70	<u>< 0.01</u>	0.33

Figure legends

Fig. 1: Schematic overview over conducted feeding assays. Capital letters indicating mussel size. In feeding assay I, II and III, “S” marks small mussels of 2.200 ± 0.15 cm, “M” mussels of 3.00 ± 0.15 cm and “L” mussels of 4.00 ± 0.15 cm size. During the experimental period *Asterias rubens* were fed with mussels of 3.50 ± 0.35 cm and *C. maenas* with mussels of 2.50 ± 0.35 cm length.

Fig. 2: Mean growth during 10 weeks of experiment of a) *Asterias rubens* and b) *Mytilus edulis* over the different treatment levels. Vertical bars denote \pm 95% CI. Results from one-way ANOVAs followed by post-hoc testing (Tukey’s HSD). Here and in the following graphs “F” denotes the test statistic, “p” the probability value. Groups with identical letters do not differ at a) $p \leq 0.05$, b) and c) $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 3: Mean mussel consumption of *Asterias rubens* a) during experiment, b) in feeding assay I (treated sea stars, treated mussels), c) in feeding assay II (treated sea stars, untreated mussels) and c) in feeding assay III (untreated sea stars, treated mussels) over treatment levels. Vertical bars denote \pm 95% CI. Results in a), b) and c) from one-way ANOVAs followed by post-hoc testing (Tukey’s HSD). Groups with identical letters do not differ at a) and b) $p \leq 0.05$ and c) $p \leq 0.01$. Results in d) from re-sampling and Monte Carlo simulation. Statistically significant p-values are underscored.

Fig. 4: Mean mussel consumption of *Carcinus maenas* a) during experiment, b) in feeding experiment I (treated crabs, treated mussels), c) in feeding experiment II (treated crabs, untreated mussels) and c) in feeding experiment III (untreated crabs, treated mussels) over treatment levels. Vertical bars denote \pm 95% CI. Results in a), b) and c) from one-way ANOVAs followed by post-hoc testing (Tukey’s HSD). Groups with identical letters do not differ at a) $p \leq 0.05$, b) and c) $p \leq 0.01$. Results in d) from re-sampling and Monte Carlo simulation. Statistically significant p-values are underscored.

Fig. 5: Mean consumption of differently sized mussels by *Asterias rubens* in a) feeding experiment I (treated sea stars, treated mussels) and b) feeding experiment II (treated sea stars, untreated mussels) over treatment levels. Vertical bars denote \pm 95% CI. Results from repeated-measures ANOVAs.

Fig. 6: Mean consumption of differently sized mussels by *Carcinus maenas* in a) feeding experiment I (treated crabs, treated mussels) and b) feeding experiment II (treated crabs,

untreated mussels) over treatment levels. Vertical bars denote $\pm 95\%$ CI. Results from repeated-measures ANOVAs.

Fig. 7: pH-bicarbonate (Davenport diagrams) of a) coelomic fluid of *Asterias rubens* and b) hemolymph of *Carcinus maenas* at the different treatment levels. Vertical and horizontal bars denote SD. Isopleths display $p\text{CO}_2$. Dotted line indicates non-bicarbonate buffer line (NBB). Statistical results from one-way ANOVAs. Groups with identical letters do not differ in a) extracellular pH (pHe) and b) extracellular HCO_3^- (HCO_3^-e) at $p \leq 0.01$.

Fig. 8: a) Mean adductor muscle dry weight per shell length, b) mean shell weight per shell length and c) mean maximal breaking resistance of the mussel shell of *Mytilus edulis* after the experimental period. Vertical bars denote $\pm 95\%$ CI. Results from one-way ANOVAs followed by post-hoc testing (Tukey's HSD). Groups with identical letters do not differ at a) and b) $p \leq 0.01$ and c) $p \leq 0.05$. Statistically significant p-values are underscored. Values with a subscript T are derived from transformed data.

Fig. 1

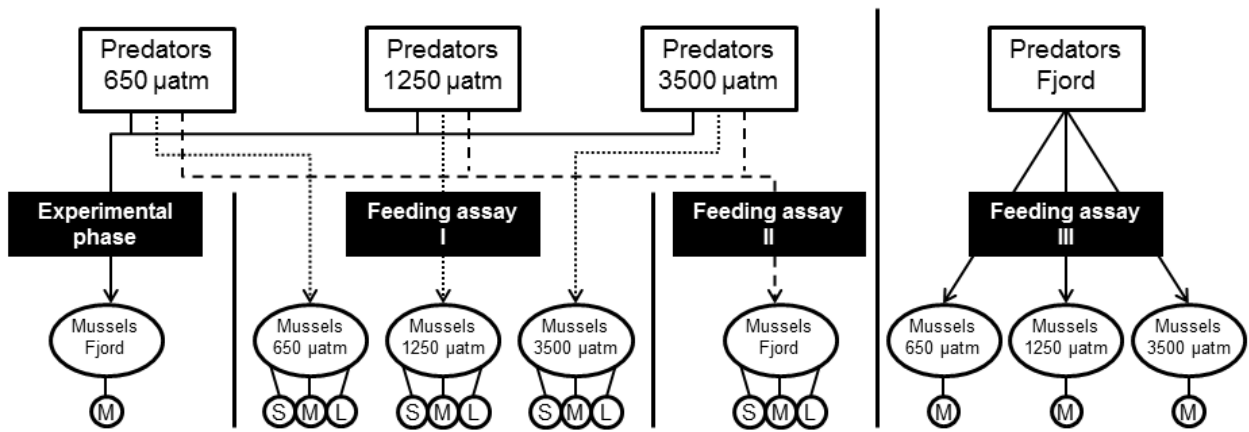


Fig. 2

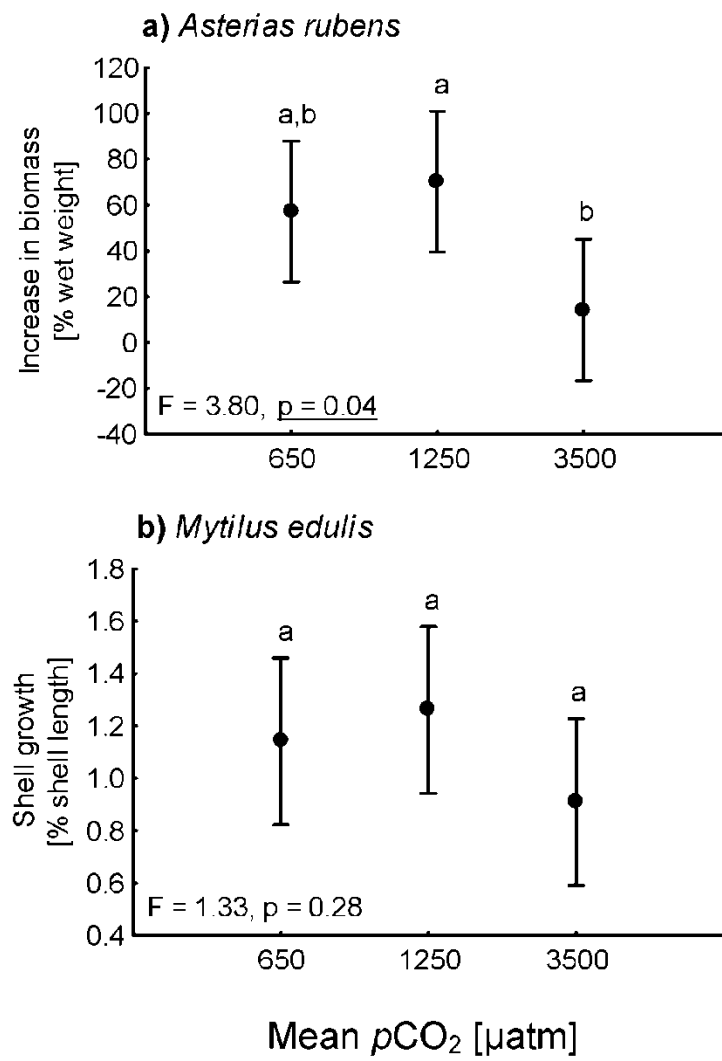


Fig. 3

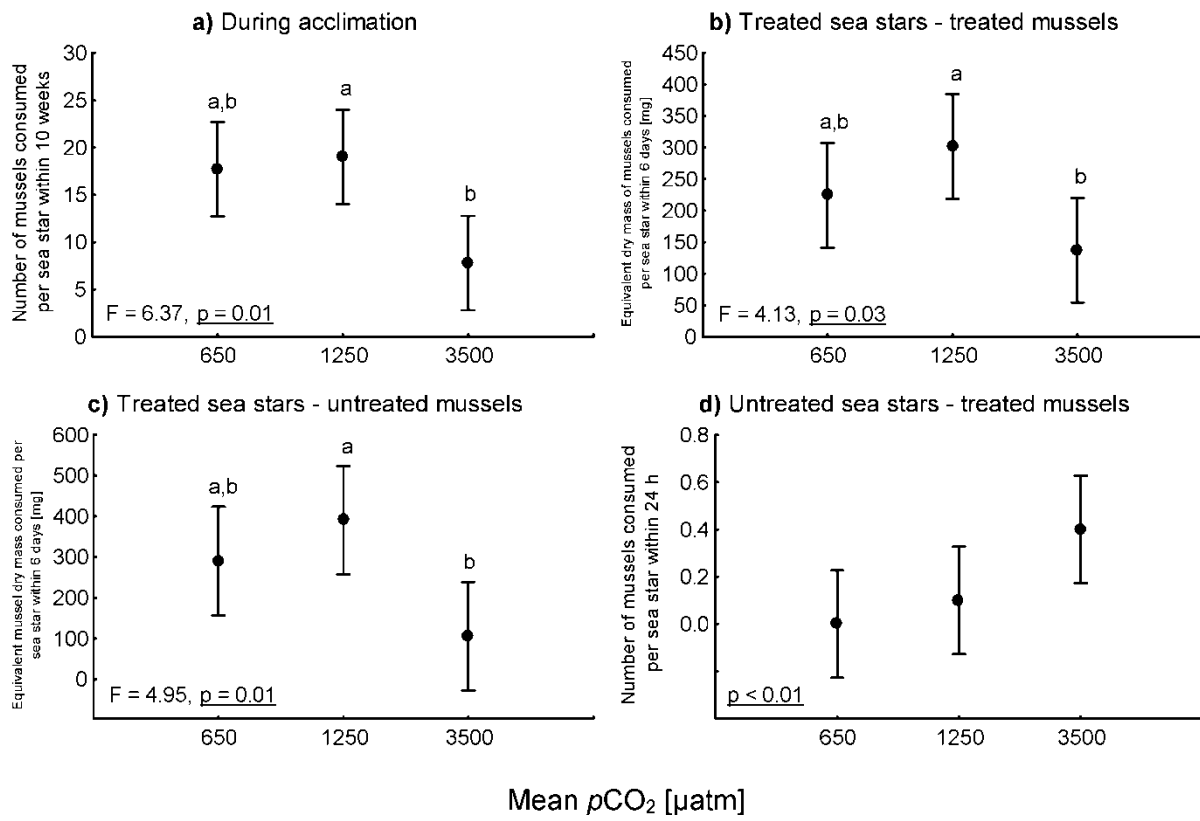


Fig. 4

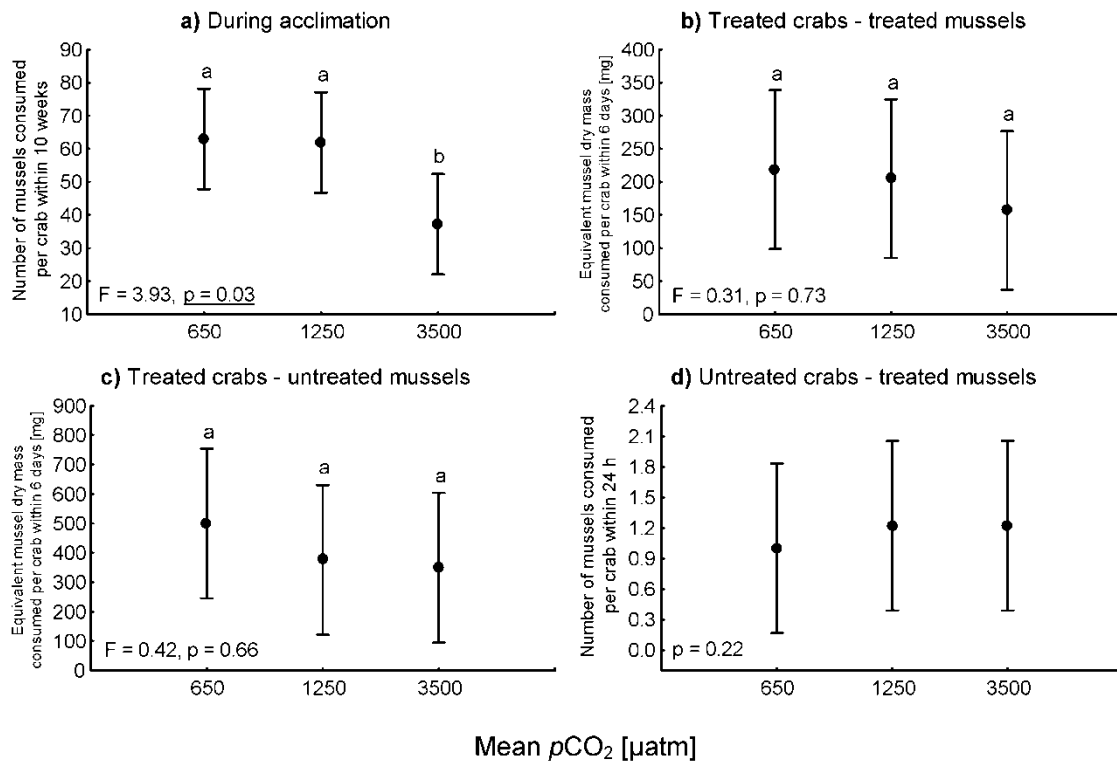


Fig. 5

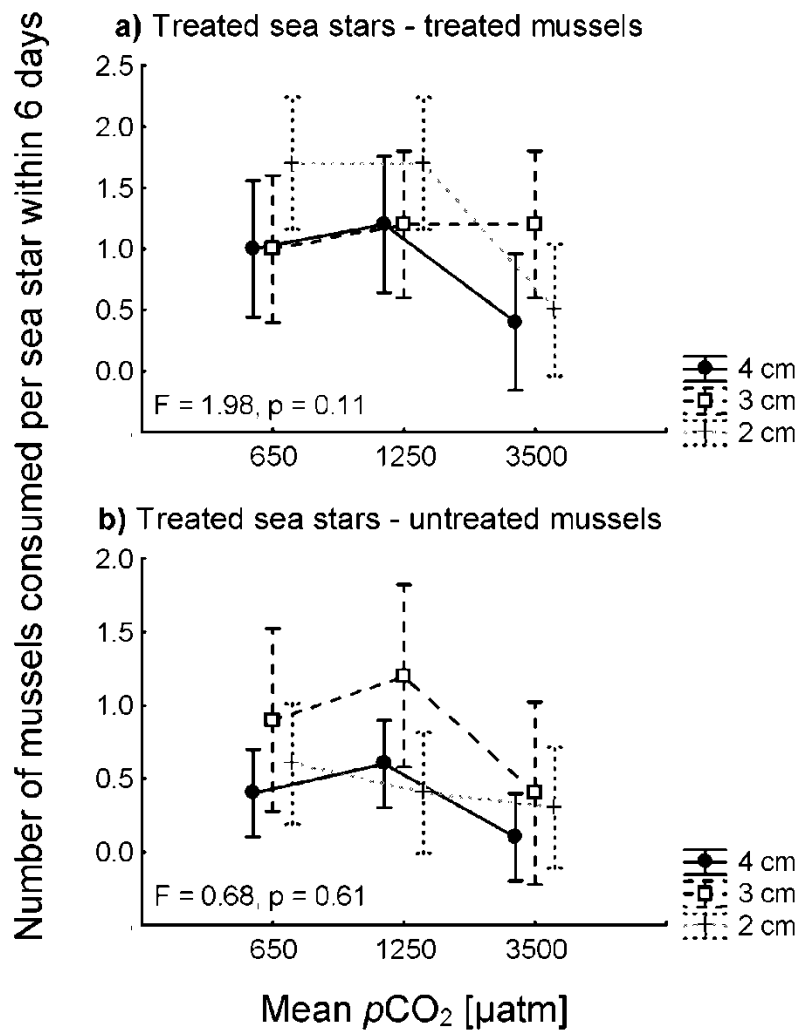


Fig. 6

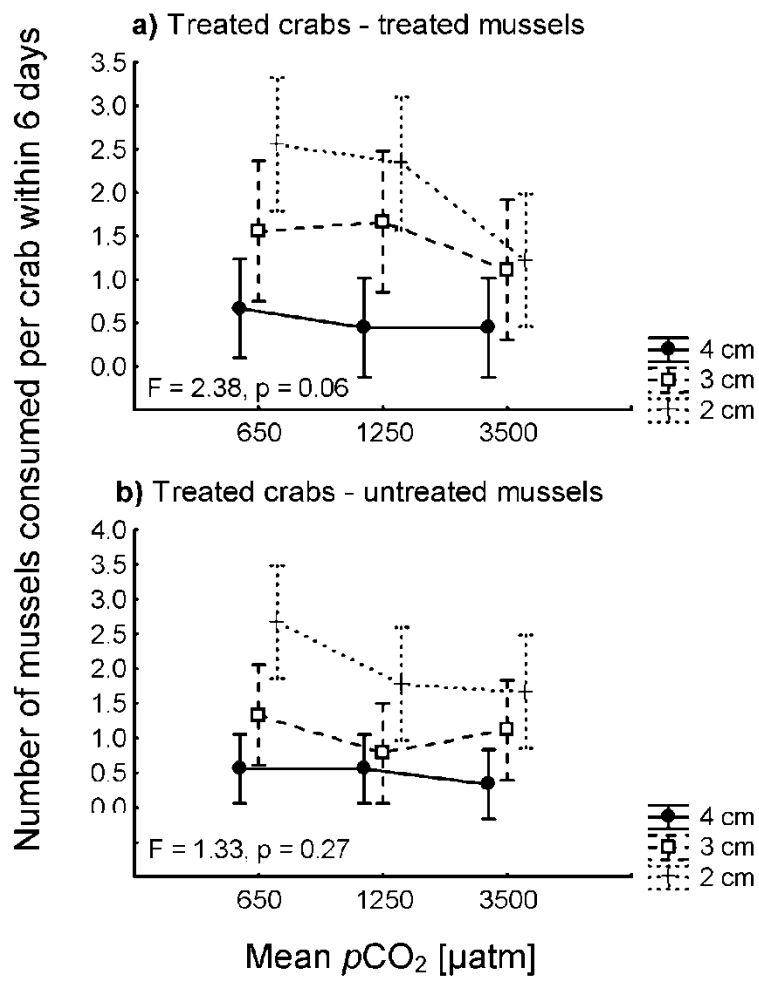


Fig. 7

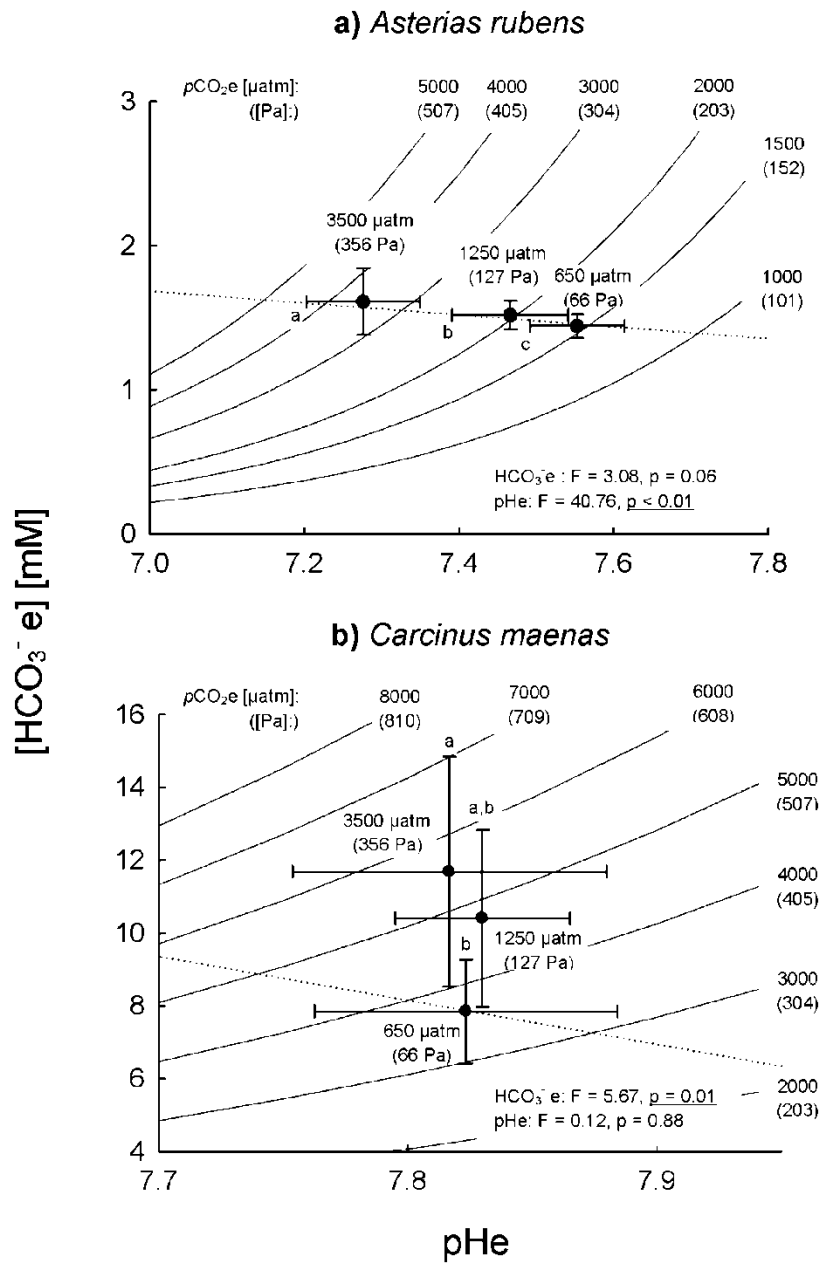
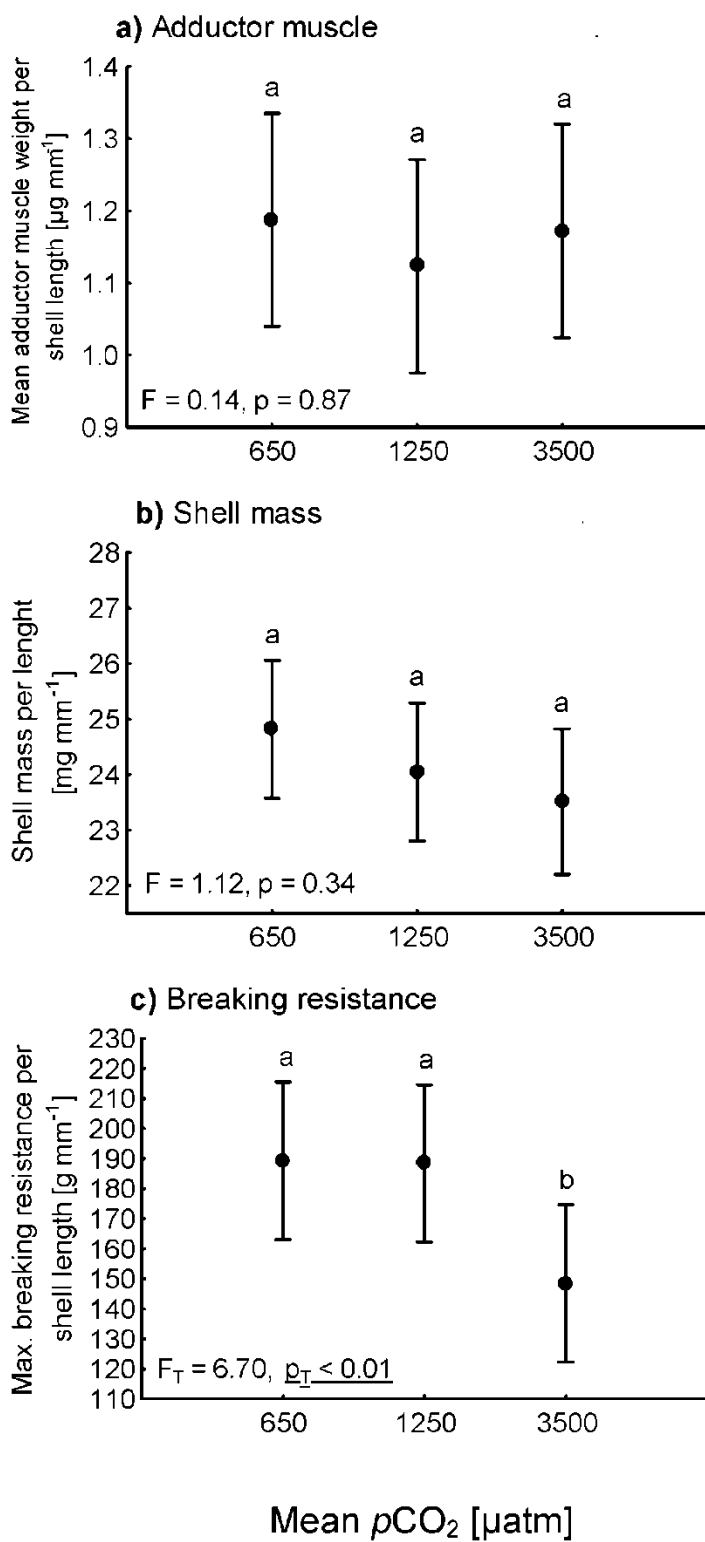


Fig. 8



Chapter 2

Young and sour - the influence of long-term acclimation to seawater acidification on growth, food consumption, metabolism and calcification of juvenile sea stars (*Asterias rubens*)

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Abstract

Ocean acidification is expected to affect the growth and calcification of benthic invertebrates, particularly of early life stages. We acclimated juvenile *A. rubens* from Kiel Fjord (western Baltic Sea) to three different seawater pCO₂ (650-3500 µatm) in a long- (37 week) and a short-term (6 week) experiment. In both experiments, survival was not affected by elevated pCO₂, but growth rates decreased with increasing pCO₂ (up to five-fold differences), also at levels which have previously been shown not to affect adult specimen. The same held true for feeding rates (up to nine times lower than at control levels), while aerobic metabolism and NH₄⁺-excretion were not significantly affected. As a consequence, scope for growth was reduced in the high-pCO₂ groups. Calcification was not impaired by elevated pCO₂. We conclude that juvenile sea stars are more strongly affected by seawater acidification than adults with regard to growth and food consumption rates, but not in their survival or ability to calcify. Future studies need to test, whether reduced scope for growth also impacts reproductive investment and success and whether combined effects of acidification and temperature impact fitness of this ecologically important species.

Introduction

Different life stages of the same organism are often differently affected by environmental stress. This holds especially true for benthic invertebrates with pelagic larval stages (Pechenik 1999). Although only few studies have compared responses in these different ontogenetic stages, juveniles are commonly assumed less sensitive than larvae, but more sensitive than adults of the same species (Bamber 1987, Lagadic *et al.* 1994).

One of the effects likely to cause disturbances in marine communities within the next decades is the progressing acidification of seawater (e.g. Fabry 2008). Its impact does not only vary among species (e.g. Kroeker *et al.* 2010), but also among life stages of the same species (review by Ross *et al.* 2011, Pansch *et al.* 2012, Dupont *et al.* *in press*). In addition, carry-over effects from one generation to the next have been reported (Parker *et al.* 2012).

Seawater acidification driven by increasing atmospheric CO₂ concentrations has been shown to gradually increase in the open ocean (e.g. Caldeira & Wickett 2003, Doney *et al.* 2009). By the end of the century, a reduction in seawater pH by about 0.4 units is expected (Caldeira & Wickett 2005). At the same time, coastal and estuarine habitats exhibit a variability in pCO₂ and pH which may approach or even exceed the predicted shift of these variables in the open ocean over the next decades (Wootton *et al.* 2008, Hofmann *et al.* 2010, Yu *et al.* 2011). The Western Baltic Sea is an example of such a habitat with fluctuating levels of pCO₂.

During summer and autumn, the strong stratification of the water column in the Baltic inhibits gas exchange between bottom water and atmosphere, leading to a low pO₂ and high pCO₂ below the pycnocline. Occasional upwelling of these water masses can abruptly and drastically elevate surface pCO₂ (Thomsen *et al.* 2010). Future increases of atmospheric CO₂-concentrations will amplify pCO₂ in such seasonally hypoxic habitats (Melzner *et al.* *accepted*). Organisms from the Western Baltic have been shown to be particularly robust to changes in seawater pCO₂ (Thomsen *et al.* 2010, Thomsen & Melzner 2010, Franke & Clemmesen 2011, Pansch *et al.* 2012, Appelhans *et al.* *accepted*). This robustness may result from adaptation to the fluctuating levels of acidification typical for their habitat as hypothesised for other ecosystems with fluctuating abiotic conditions, such as tidal pools (Moulin *et al.* 2011).

Echinoderms have long been assumed to belong to the groups of organisms most likely to be negatively affected by ocean acidification (e.g. Raven *et al.* 2005). This was thought to be not only due to the fact that their skeleton consists of the fairly soluble Mg-calcite to a large extent (e.g. Andersson *et al.* 2008). Further, many echinoderms are weak ion-regulators with comparatively low metabolic rates, a trait that may render them more vulnerable to acidification than more active, extracellular pH-regulating species (Melzner *et al.* 2009). In contrast, a review by Dupont *et al.* (2010b) has found echinoderms to be relatively robust to moderate seawater acidification at pH-variations up to 0.4 scale points compared to control

values. The authors note that these findings of apparent robustness might have been caused by the short duration of most studies, by ignoring ecological feedbacks and by the lack of identifying possible bottlenecks in ontogeny. Early life stages of various echinoderm species have been shown to react relatively sensitive to seawater acidification (Shirayama & Thornton 2005, Siikavuopio *et al.* 2007, Dupont *et al.* 2008, Brennand *et al.* 2010, O'Donnell *et al.* 2010, Martin *et al.* 2011, Stumpp *et al.* 2011a, Stumpp *et al.* 2011b, Yu *et al.* 2011, Stumpp *et al.* 2012, Dupont *et al. in press*).

Adults of the sea star *Asterias rubens* from Kiel Fjord have been shown to be affected by very high pCO₂ (3500 µatm) with respect to growth and food consumption in a 10 week experimental trial (Appelhans *et al. accepted*). Moderate increases in acidification (pCO₂ of 780 µatm and pH 7.7, respectively) have been shown to slightly increase growth in juveniles of the sea star *Pisaster ochraceus* (Gooding *et al.* 2009) and in larvae and juveniles of the sea star *Crossaster papposus* (Dupont *et al.* 2010a). Arm regeneration, righting response and growth of the sea star *Luidia clathrata* were not impacted by a pCO₂ of 780 µatm (Schram *et al.* 2011) and metabolism was not significantly changed by a pH of 7.8 (pCO₂ of about 750 µatm) in the sea star *Parvulastra exigua* (McElroy *et al.* 2012). In contrast, adult *A. rubens* were found to be immune suppressed when exposed to a pH of 7.7 (pCO₂ of ca. 1000 µatm) for six months (Hernroth *et al.* 2011). Larvae of *A. rubens* have been reported to develop slower when exposed to a pH of 7.9 - 7.7 and larvae from adults, which had been maintained at pH 7.7 were characterized by enhanced mortality, abnormal development and inability to feed (Dupont & Thorndyke 2008).

Asterias rubens belongs to the ecologically most important predators of the blue mussel *Mytilus edulis* in the Western Baltic. Its mean annual biomass constitutes up to 12 % of the entire biomass of benthic macro invertebrates in Kiel Bight (Nauen 1979). Therefore, any impact on growth and consumption of sea stars can have large consequences for the entire ecosystem. Since, however, the genus *Asterias* is also widely distributed in coastal areas of the North Sea and the western Atlantic in general (Vevers 1949, Nauen 1979), impacts by environmental stress on this predator may influence ecosystems at a large scale.

This study investigates the effects of seawater acidification on the juvenile life stage of the sea star *A. rubens* in a short- and a long-term acclimation experiment. It thereby closes the gap between previous investigations that studied effects of acidification on larval (Dupont & Thorndyke 2008) and adult (Hernroth *et al.* 2011, Appelhans *et al. accepted*) life stages of this species.

Materials and methods

The study consisted of a long-term experiment with an experimental phase of 39 weeks (exp. 1) performed from September 2009 until June 2010 and a short-term experiment with an experimental phase of six weeks (exp. 2) performed from October until December 2010.

Organism collection

For exp. 1, *Asterias rubens* were sampled on August 31st 2009 in Kiel Fjord (Western Baltic Sea) via manual collection. The sea stars were selected to be of a size of 9.5 ± 1 mm in outer diameter (longest distance of arm tips) and weighed 68.5 ± 17.1 mg wet weight (WW). The sea stars for exp. 2 were sampled from October 4th to 7th 2010 in Kiel Fjord and selected to be 21.5 ± 3.5 mm and weighed 447.78 ± 129.30 mg WW.

Specimens were stored in a large tank with aerated flow-through water from the fjord for two days (exp. 1) and ten days (exp. 2) prior to placing them individually in the experimental units (EUs).

Experimental incubations

The general set-up of both experiments follows the one described in Appelhans *et al.* (*accepted*). Briefly, we established a flow-through of sea water ($5\text{-}6 \text{ l h}^{-1}$) from the fjord into three inter-connected header-tanks, then the EUs, which consisted of 2 l plastic aquaria. Three different treatment levels were achieved by bubbling pressurized air with ambient, 1120 and 4000 μatm $p\text{CO}_2$ into header-tanks and EUs.

Each treatment level was replicated 15 (exp. 1) or 18 (exp. 2) times. One specimen of the lowest treatment level (651 μatm) and one of the highest treatment level (3484 μatm) were lost (washed down the drain) while cleaning of the EUs during the course of exp. 1.

Carbonate system monitoring

In both experiments, pH_{NBS} was measured weekly using a WTW pH meter and a Sentix 81 electrode. Temperature and salinity were also measured on a weekly basis (WTW Cond 340i and a TetraCon 325 electrode). We calculated carbonate system parameters from water samples taken monthly (exp.1) or weekly (exp.2) from the EUs. Water samples were analysed for C_T and pH_{total} directly after each sampling. C_T was measured using an AIRICA system (Marianda, Kiel, Germany) via a LI-COR 7000 infra-red $\text{CO}_2/\text{H}_2\text{O}$ analyser.

Measured C_T values were corrected using Dickson seawater standard as reference material (Dickson *et al.* 2003). pH_{total} was determined in a water bath (21.6°C) using a Metrohm 6.0262.100 electrode calibrated with Tris/AMP seawater buffers mixed for a salinity of 15. Carbonate system parameters were calculated from measured values using CO2SYS

(Pierrot *et al.* 2006). The values for K1, K2, and KHSO₄ constants were chosen according to Roy *et al.* (1993). See Table 1 for values.

Growth

In exp. 1 we monitored the length (diameter) and biomass increase (wet weight) of the sea stars every 6th week and at the end of the 39 week period. In exp. 2, weekly measurements of the two parameters were taken.

Feeding

During both experimental periods, we fed the sea stars *ad libitum* with living *Mytilus edulis* from their original habitat. The mussel size fed was adjusted to sea star size at the time of feeding. In exp. 1, mussel size was chosen to be equal to, in exp. 2 chosen to be 10.0 ± 1.0 mm smaller than the total sea star diameter, since larger *A. rubens* are known to consume mussels up to roughly their own body size (Sommer *et al.* 1999).

In exp. 2, consumption of mussels by sea stars was monitored weekly. Five mussels of each fed size class were frozen and soft tissue (somatic) dry mass was determined. These values were then used to calculate total soft tissue dry mass of mussels consumed by each sea star. In exp. 1, a feeding assay was performed after the experimental period. Mussels of the size classes of 10.0 (± 1.0), 20.0 (±1.0), 30.0 (±1.5) mm and 40.0 (±1.5) mm were fed *ad libitum* and the amount of mussels consumed counted. The sea stars were left to feed for 27 days in total. Each 10 mussels of each size class were analysed for soft tissue dry weight prior to the feeding experiment in order to calculate total amount of soft tissue consumed by each sea star.

In order to be able to compare weight corrected feeding levels in exp. 2, we had to take into account the weight differences between experimental animals from the different treatment levels. Thus, in order to avoid weight dependent bias, only consumption rates of 12 sea stars per treatment level, which did not significantly differ in weight between each other (selected by weight deviation from the overall mean weight 0.9 ± 0.6 g), were used for comparison. We then calculated mean weight corrected consumption rates (wccr)

$$\text{wccr} = \frac{\text{mussel dry weight consumed week}^{-1}}{\text{sea star wet weight in the respective week}} \quad (1)$$

Righting response

In exp. 1, righting responses were determined after the experimental period, in exp. 2 after three and after six weeks. All individuals were placed on their aboral side and the time needed for them to turn around and place all arms back on the aquarium floor was measured three times in exp. 1 and once in exp. 2. Mean values were determined and corrected for biomass using a size factor. This size factor was established by measuring the righting

response of 25 differently sized *A. rubens* (wet weights covering all wet weights found in sea stars from the experiment) which were collected directly from the Fjord. The thus determined natural relationship between biomass and righting response:

$$\text{righting [s needed to turn around]} = 99.95 * \text{biomass [g wet weight]} + 9.94 \quad (2)$$

$(r^2 = 0.81, p < 0.0001)$

The righting response was then given as corrected righting response (crr):

$$\text{crr} = \log \frac{\text{measured righting response}}{\text{righting response of non-treated sea star of equal size}} \quad (3)$$

Aerobic metabolism

In exp. 2, respiration rates of the sea stars were determined in closed 250 ml glass respirometers, which were kept at a constant temperature of 13° C in a water bath. Respirometer chambers were filled with 0.2 µm filtered seawater equilibrated to treatment pCO₂ prior to addition of one sea star. A magnetic stirrer was used to gently mix the water body within the respirometer. Decreasing oxygen concentrations of the water were recorded using O₂-sensitive dye spots glued onto the inner side of the respirometer and an optic fibre connected to an Oxy-4-mini instrument (Presens, Regensburg, Germany). Two-point calibration was performed with air saturated water for 100% and using 100 mg Na₂SO₄ per 10 ml of water for the 0% point calibration, according to the manufacturer's instructions. Measurements lasted, depending on animal size, for 1.5-3 h. Oxygen concentration never fell below 90% air saturation. Following the respiration measurement, the animal was removed from the respirometer. Two duplicate water samples were taken for determination of NH₄⁺ excretion rates prior to and at the end of the incubation period. Subsequently, the small water deficit (10 ml) was replenished with filtered water and the respirometer was closed again to measure bacterial background respiration for a period of 30 minutes, in which the bacterial control decreased by about 1 % air saturation. For calculation of oxygen consumption, the linear decrease of the O₂-concentration was considered for further analysis and bacterial respiration was subtracted. In two cases with measurements of very small sea stars, bacterial respiration exceeded total respiration. In order not to bias the outcome towards high values by deleting these apparent outliers, the lowest negative value in O₂-consumption (net production) was estimated the system error and added on to all end-values. Sea star respiration is known to vary non-linearly with body mass (e.g. Nauen 1979). In order to account for size-dependent differences in respiration rates and size differences between treatment levels, only respiration rates of the 12 sea stars per treatment level, which did not significantly differ in weight, were used for comparison.

NH₄⁺ excretion

In exp. 2, NH₄⁺ excretion rates were calculated from the difference of NH₄⁺ concentration measured in the water before and following the respiration measurement. Two 10 ml water samples were taken from the pre-equilibrated water before it was filled into the respirometers. A second duplicate was taken from the respirometer following the respiration measurement. Measurements of NH₄⁺ concentration were performed according to Holmes *et al.* (1999) by addition of 2.5 ml reagent containing orthophthaldialdehyde, Na₂SO₃ and Na₂B₄O₇·10H₂O. Samples were measured using a Kontron SFM25 fluorometer (Kontron Instruments, Everett, USA) at an excitation and emission wavelength of 360 and 422 nm, respectively after 2 h of incubation. The O:N ratio was calculated by dividing the molar oxygen consumption rate by the molar NH₄⁺ excretion rate. As with the metabolic rate determinations, only data of the same 12 equally sized sea stars per treatment level were analysed.

Scope for growth (SfG)

The energy available for growth processes (scope for growth, SfG) was calculated for the last two weeks of the experimental phase of exp. 2 according to the formula:

$$\text{SfG} = \text{E} - \text{R} - \text{U} \quad (4)$$

With E = Energy uptake by feeding, R = Energy loss by respiration and U = Energy loss by ammonium excretion

Energy uptake by consumption of mussel dry mass (18.85 Joules mg⁻¹) was calculated according to Brey *et al.* (1988), Energy lost by respiration (0.45 Joules per μmol O₂) and through excretion (0.347 Joules per μmol excreted NH₄⁺) were taken from Gnaiger (1983) and (Elliott & Davison 1975), respectively.

Calcification

Eleven sea stars per treatment level of exp. 1 and all 18 of exp. 2 were stored in a freezer at -20° C for two days and then dried in a dry oven at 80° C for a minimum of 24 h. Subsequently, the dried sea stars were placed in a muffle furnace at 500° C for 24 h. Calcification rates were determined by calculation of the ratio of ash weight to sea star dry weight. Since pilot-studies showed that there are no significant size-dependent differences in the degree of calcification under natural conditions in sea stars from Kiel Fjord ($r^2 = 0.10$, $p = 0.09$), values were not corrected for sea star biomass (see supplementary material for graph).

Statistical analyses

Total size increment of sea stars during both experimental phases as well as total and weight corrected food consumption in the experimental phase of exp. 2 and the feeding assay of exp. 1, as well as the corrected righting responses, the respiration and excretion levels, the scope for growth, energy efficiency and the total calcification were analysed using a one-way

ANOVA, followed by Tukey's HSD post-hoc analysis. The size range of mussels consumed in the feeding assay of exp. 1 was compared using repeated measures ANOVA as was the size increment over time and the food consumption (including weight corrected consumption) over time. Significant differences between treatment levels were then identified optically by comparison of the overlap of confidence intervals. All data were tested for normality using the Shapiro-Wilks-W test before further statistical analysis. If the data were non-normally distributed, the Box-Cox procedure identified the simplest transformation to achieve normality. Percentage data were first arcsine square root transformed before attempting other transformation. Data used for one-way ANOVA were tested for homogeneity of variances using Levene's test. If data used for repeated measures ANOVA did not meet requirements of sphericity (Mauchly's test for sphericity), p-values were corrected using the Greenhouse-Geisser correction. If normality and homoscedasticity (sphericity) could not be achieved, we used parametric tests, but lowered the α -level to 0.01 (see e.g. Appelhans *et al.* 2010).

If missing data values resulted in an unbalanced design, random values of other treatment levels were deleted to achieve balance.

Results

Mortality and growth

One of 15 sea stars exposed to the highest treatment level (3484 μatm) died during the long-term experiment (exp. 1). No mortality was observed during exp. 2.

During the 39 week experimental phase in exp. 1 ($p < 0.0001$, Fig. 1a, Table 2), mean increase in biomass was five-fold higher in the lowest (651 μatm) treatment level of exp. 1 than in the highest (3484 μatm), two-fold higher in the highest than in the intermediate (1155 μatm) level and two-fold higher in the intermediate than in the highest level. In exp. 2, growth under the lowest (690 μatm) treatment was three-fold higher than under the highest (3128 μatm) treatment ($p < 0.001$, Fig. 1b, Table 2).

In exp. 1, biomass in the lowest (651 μatm) and the highest (3484 μatm) treatment level started differing significantly from week 12 onwards, in the lowest and the intermediate (1155 μatm) level from week 18 and in the intermediate and highest level from week 39 ($p < 0.0001$, Fig. 1c, Table 2). In exp. 2, significant differences between the lowest (690 μatm) and highest (3128 μatm) level were observed from week four onwards ($p < 0.0001$, Fig. 1d, Table 2). Mean biomass was always lowest in sea stars of the highest (3484 and 3128 μatm), and increased in the intermediate (1155 and 1102 μatm) and in the lowest (651 and 690 μatm) pCO_2 -treatments of the two experiments.

Feeding

The mean consumption level under the lowest (651 μatm) treatment was four times higher than under the intermediate (1155 μatm) treatment and nine times higher than under the highest (3484 μatm) treatment level in the feeding assay of exp. 1 ($p < 0.0001$, Fig. 2a, Table 2). The overall mean consumption level over the six-week experimental period of experiment 2 differed significantly only between the lowest (690 μatm) and the highest (3128 μatm) treatment level ($p < 0.01$, Fig. 2b, Table 2). Here the difference between the two was two-fold. Interactions between mussel sizes consumed and treatment level of sea stars in the feeding assay of exp. 1 were not significant ($p = 0.12$, Fig. 2c, Table 2). Nevertheless, almost only sea stars acclimated to intermediate (1155 μatm) and lowest (651 μatm) treatment levels consumed mussels of the 2 cm size class and only mussels treated at lowest CO_2 -levels consumed mussels of the 3 cm and the 4 cm size class.

The consumption level of mussels over time in exp. 2 followed a less consistent pattern than the growth ($p < 0.01$, Fig. 2d, Table 2), with significant differences between treatment levels changing weekly.

The mean weight corrected consumption rates in exp. 2 showed a non-significant pattern of lower consumption per g sea star wet weight at intermediate (1102 μatm) and the highest treatment level (3128 μatm) compared to the lowest (690 μatm) level ($p = 0.38$, Fig. 3, Table 2).

Righting response

Although there was a general trend of the righting response (time needed for sea stars to turn from aboral to oral side) to be faster in the lowest (651 and 690 μatm , respectively) treatment levels compared to intermediate (1155 and 1102 μatm) and high (3484 and 3128 μatm) levels, this response proved to be highly variable (Fig. 4, Table 2). The difference in the righting response among extreme acidification levels was only statistically significant in exp. 2 three weeks into the experimental phase ($p = 0.001$, Fig. 4b, Table 2). Here, the size corrected righting response was five-fold lower (shorter time taken to turn around) in the lowest (690 μatm) treatment level than in the highest (3128 μatm) treatment level.

Metabolism, NH_4^+ excretion, O:N-ratio, scope for growth and energy balance

There were no significant differences in respiration ($p = 0.56$, Fig. 5a, Table 2) and NH_4^+ excretion rate ($p = 0.48$, Fig. 5b, Table 2) between the twelve equally-sized sea stars per treatment level in exp. 2. Consequently, the O:N-ratio did not differ significantly between the different treatment levels ($p = 0.97$, Fig. 5c, Table 2).

The scope for growth of the sea stars acclimated to lowest treatment levels (690 μatm) was significantly higher (four-fold difference) than at highest (3128 μatm) treatment levels ($p < 0.01$, Fig. 6a, Table 2).

Calcification

Calcification (fraction of dry weight inorganic matter) did not significantly differ between the different treatments in exp. 1 ($p = 0.99$, Fig. 7a, Table 2) and in exp. 2 ($p = 0.62$, Fig. 7b, Table 2).

Discussion

Effects on the species level

Our study shows that the parameters potentially influenced by seawater acidification in juvenile *Asterias rubens* are primarily food consumption rates and growth rates. As a consequence of lowered food consumption and unchanged metabolic rates, SfG decreased in the higher pCO₂-treatments. In contrast to our previous findings in adults (Appelhans *et al. accepted*), intermediate levels of acidification (ca. 1000 μ atm) affected consumption and growth in juveniles. No effects were visible on respiration, NH₄⁺-excretion, O:N-ratio and calcification. Only one sea star died over the course of the experimental period. Thus, juvenile sea stars seem to be able to survive, grow and calcify even under high levels of acidification for extended time periods albeit with reduced consumption and growth rate. Calcification rates are only reduced as a consequence of lowered overall growth.

The vulnerability of juveniles

Our data support the findings of recent studies that early life stages are more sensitive to environmental stressors such as elevated pCO₂ than adults. Drastic reductions of survival were obtained in brittle star larvae (Dupont *et al.* 2008), however this dramatic response does not seem to be a general pattern in echinoderms. Most species exhibit delayed growth at the larval stage, with no effects on survival (Yun *et al.* 2007, Brennand *et al.* 2010, O'Donnell *et al.* 2010, Martin *et al.* 2011, Stumpp *et al.* 2011a, Stumpp *et al.* 2012). Similar results were obtained for juvenile sea urchins, which exhibited lower feeding rates at elevated pCO₂ (Siikavuopio *et al.* 2007, Stumpp *et al.* 2012). This decrease in feeding rate also resulted in reduced growth, reproductive investment and calcification rates (Siikavuopio *et al.* 2007, Stumpp *et al.* 2012). In contrast, juvenile sea stars of the genus *Pisaster ochraceus* increased calcification at moderately enhanced levels of acidification (Gooding *et al.* 2009). These and the results of the current study thus show that bottlenecks in ontogeny may vary on the species level and have to be identified individually.

Reduced consumption leads to reduced growth

Here we show that reduced feeding under conditions of acidification is the reason for the observed reduced growth rates. Lower consumption at higher $p\text{CO}_2$ lead to the observed lower SfG and thus to the reduction in growth.

The mechanistic cause for reduced feeding under conditions of hypercapnia, which has been demonstrated for different species before (Bamber 1987, Siikavuopio *et al.* 2007, Stumpp *et al.* 2012), has to be further investigated. Reduced feeding rates might result either from lower foraging activity, an impairment of the feeding and digestion process or from re-arrangement in energy budget allocation. However, the measured righting response revealed only small effects on the overall activity which suggests that also foraging activity remains unchanged.

One reason for the reduced feeding rates might be the reduction of seawater pH and the accompanied uncompensated acidosis of the coelomic fluid under elevated $p\text{CO}_2$ (Appelhans *et al. accepted*). In sea stars, digestive enzymes, such proteases and amylases, are produced in the pyloric caeca which are surrounded by the coelomic fluid (Peng & Williams 1973, Williams 1975). These enzymes are either excreted into the stomach for the extracellular digestion or remain in the caeca, where intracellular digestion and storage of nutrients takes place (Holzman *et al.* 1985). A lowering of the pH at the site of extracellular digestion may result in lower activities of proteases, such as trypsin-like enzymes with alkaline pH optima (Winter & Neurath 1970, Adelung 1971, Peng & Williams 1973). In contrast, storage and mobilization of energy stored as lipids in the pyloric caeca might not be affected, as pH optima for e.g. triacylglycerol lipase activity are much lower (pH 6-7 Oudejans *et al.* 1983).

Another reason for reduced feeding might be a shift of the cellular energy budget towards higher acid-base and ion regulatory demand observed under elevated $p\text{CO}_2$, may lead to reductions of energetically costly processes such as feeding (Deigweier *et al.* 2010, McGaw & Twitchit 2012). The feeding process, which includes e.g. mussel opening and digestion, is highly energy demanding (specific dynamic action, SDA). Especially in sea stars with low basal metabolic rates, this process has a pronounced impact on the overall energy budget as the metabolic rates increase more than two-fold compared to control rates (Vahl 1984, Hughes *et al.* 2011, McGaw & Twitchit 2012). However, the opening of prey such as mussels contributes by only about 3.5 % to the total SDA (McGaw & Twitchit 2012).

Maintenance of aerobic metabolism

It has previously been suggested that uncompensated extracellular pH (pHe) under elevated CO_2 can lead to metabolic depression which could then cause growth reductions under elevated $p\text{CO}_2$ (Pörtner *et al.* 2004). Michaelidis *et al.* (2005) observed reductions of aerobic metabolism in *M. galloprovincialis* under highly elevated $p\text{CO}_2$ (5000 μatm) when extracellular pH was reduced. However, at moderately elevated $p\text{CO}_2$ levels (1000-3000

μatm) molluscs (Beniash *et al.* 2010, Comeau *et al.* 2010, Lannig *et al.* 2010, Thomsen & Melzner 2010) and echinoderms (Wood *et al.* 2008, Stumpp *et al.* 2012) exhibited constant or even elevated metabolic rates. Similarly, the sea stars in this study maintained metabolic rates at similar levels in all treatments, although pHe is known to decrease at elevated pCO₂ (Hernroth *et al.* 2011, Appelhans *et al. accepted*). Therefore, metabolic depression elicited by reduced pHe does not play a role under pCO₂ levels relevant for ocean acidification in *A. rubens*. At the same time, food uptake was drastically reduced, which resulted in a reduction of SfG. Similar reductions have been observed for larval and adult stages of sea urchins (Stumpp *et al.* 2011b, Stumpp *et al.* 2012). Therefore growth reductions under elevated pCO₂ are not related to the general down-regulation of metabolism (metabolic depression Michaelidis *et al.* 2005), but result from reduced food uptake or higher metabolic turnover (Thomsen & Melzner 2010, Stumpp *et al.* 2011a, Stumpp *et al.* 2011b, Stumpp *et al.* 2012). In contrast to other invertebrates (Michaelidis *et al.* 2007, Thomsen & Melzner 2010, Stumpp *et al.* 2012), the metabolism of *A. rubens* does not appear to shift to higher protein turnover under acidification stress, as would be indicated by higher NH₄⁺ excretion and lowered O:N-ratios. This is most probably related to the fact that carnivorous sea stars solely feed on nitrogen-rich prey.

Skeletal requirements

The mesodermal skeleton of asterioids comprises two different parts: the external spines on the body surface and the endoskeleton, which plays an important structural role. *Asterias rubens* from the Baltic are known to be less calcified than populations from the North Sea (48 vs. 57 %, Kowalski 1954). This results from the less calcified surface of Baltic specimens. At the same time, sea stars “lock” their endoskeleton to cause a mechanistic rigidity when pulling mussel shells apart (Christensen 1957, Norberg & Tedengren 1995). Therefore, the ambulacral arches play an important role in the feeding process of sea stars (Eylers 1976). Hence, the mechanics of feeding requires certain skeletal structures which cannot be reduced even in the Baltic specimens.

Moreover, the endoskeleton is not in direct contact with the coelomic fluid, but is separated from it through an electron dense, organic matrix coat (Märkel *et al.* 1989). In echinoderms, calcification occurs in an extracellular, but separated syncytial compartments (intrasyncytial vacuoles, Decker *et al.* 1987, Dubois & Chen 1989). Crystal growth proceeds in a fluid which most probably differs from the conditions encountered in the coelomic fluid e.g. in saturation state of CaCO₃ (Dubois & Chen 1989). Therefore, low pH and CaCO₃ undersaturation in the body fluid may only have limited effects on the calcification process even under elevated seawater pCO₂ (Stumpp *et al.* 2012, Appelhans *et al. accepted*).

Consequently, calcification does not seem to be the process which is primarily affected by elevated pCO₂ but is only secondarily reduced due to overall growth reductions.

Consequences on the ecosystem level

Seawater acidification has previously been proven to have the potential to change ecosystem structures (Hall-Spencer *et al.* 2008, Hale *et al.* 2011). Our study indicates that the overall feeding pressure of sea stars on mussels in the Western Baltic can be expected to decrease with an increase in seawater acidification - if the sea stars do not adapt. Since *A. rubens* is known to be among the predators to mainly control distribution and abundance of the dominant filter feeder of the Western Baltic, the blue mussel *Mytilus edulis* (Enderlein & Wahl 2004), a decrease in feeding pressure by *A. rubens* may cause community shifts with ensuing changes in ecosystem services such as water clearance and pelago-benthic coupling.

It has been shown that adaptation over time (Form & Riebesell 2012), a variation of effects over generations (Sunday *et al.* 2011) and carry-over effects from one generation to the next (Dupont & Thorndyke 2008, Dupont *et al. in press*) are possible. Nevertheless, slower growth of juvenile *A. rubens* may lead to retarded reproduction with corresponding demographic consequences. Since very few predators prey on *A. rubens* in the Baltic Sea (Nauen 1979), retarded growth will not enhance mortality (by consumers) and will have predominantly bottom-down effects.

Conclusions

We conclude that *A. rubens* from the Baltic Sea is affected more strongly by seawater acidification than previously thought. Nevertheless, survival even under heavily acidified conditions seems to be the rule and some individuals exhibit high growth rates even under highly elevated pCO₂ and development into adult stages. Selection pressure on the population may thus lead to a tolerance of this species to high pCO₂. Future studies should investigate possible carry-over effects over generations and exact mechanisms of reduced feeding.

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Tables

Table 1: Measurements and calculations of the water carbonate system for the different treatment levels in exp. 1 and exp. 2. Water $p\text{CO}_2$, A_T , Ω_{calcite} and $\Omega_{\text{aragonite}}$ were calculated using measured C_T and pH values, as well as temperature and salinity values of the respective EUs on the day of measuring, with the CO2SYS macro for low salinities (Pierrot *et al.* 2006). Values are means \pm SD.

	Experiment 1			Experiment 2			
	Ambient	1120	4000	Ambient	1120	4000	
$p\text{CO}_2$ of pressurised air [μatm]							
Measured	C_T [$\mu\text{mol kg}_{\text{seawater}}^{-1}$]	2041.30 \pm 131.57	2079.30 \pm 106.24	2172 \pm 137.96	1967.37 \pm 55.06	2030.88 \pm 61.18	2195.73 \pm 64.52
	pH (total scale)	7.85 \pm 0.07	7.64 \pm 0.12	7.17 \pm 0.05	7.86 \pm 0.05	7.72 \pm 0.13	7.24 \pm 0.04
	Mean temperature [$^{\circ}\text{C}$]	9.00 \pm 3.94	9.03 \pm 3.94	9.00 \pm 3.94	13.37 \pm 0.44	13.33 \pm 0.41	13.34 \pm 0.38
	Mean salinity	16.99 \pm 2.16	16.61 \pm 2.18	16.98 \pm 2.15	16.66 \pm 1.23	16.66 \pm 1.25	16.65 \pm 1.67
Calculated	$p\text{CO}_2$ [μatm]	650.69 \pm 153.63	1155.26 \pm 385.33	3483.86 \pm 429.93	689.56 \pm 79.81	1101.57 \pm 387.58	3128.39 \pm 217.29
	A_T [$\mu\text{mol kg}_{\text{seawater}}^{-1}$]	2075.09 \pm 140.80	2059.87 \pm 107.15	2029.91 \pm 111.97	2015.29 \pm 59.48	2048.68 \pm 68.15	2079.28 \pm 67.64
	$\Omega_{\text{aragonite}}$	0.85 \pm 0.23	0.52 \pm 0.20	0.16 \pm 0.02	0.94 \pm 0.11	0.72 \pm 0.30	0.24 \pm 0.03
	Ω_{calcite}	1.44 \pm 0.37	0.88 \pm 0.33	0.27 \pm 0.04	1.58 \pm 0.19	1.22 \pm 0.49	0.41 \pm 0.05

Table 2: ANOVA results. Analyses performed with different response variables and the dependent factor pCO₂ (three levels). For non-parametric data, the α -level was adjusted to 0.01. Statistically significant results are underscored.

	α	df	MS	F	p	Partial η^2
<i>Long-term experiment (exp. 1)</i>						
<u>Increase in biomass - total</u>	0.01	2	0.68	17.60	<u>< 0.0001</u>	0.50
<u>Increase in biomass - over time (interactions)</u>	0.01	14	23800000	15.06	<u>< 0.0001</u>	0.46
Righting response	0.05	2	0.05	1.43	0.25	0.07
<u>Mussel consumption - total</u>	0.01	2	232.14	18.00	<u>< 0.0001</u>	0.50
Mussel consumption - per size class (interactions)	0.01	6	1.55	1.75	0.12	0.12
Calcification	0.05	2	0.40	0.02	0.99	< 0.01
<i>Short-term experiment (exp. 2)</i>						
<u>Increase in biomass - total</u>	0.01	2	36.37	8.08	<u>< 0.0001</u>	0.24
<u>Increase in biomass - over time (interactions)</u>	0.01	12	0.19	6.19	<u>< 0.0001</u>	0.20
<u>Righting response week 3</u>	0.01	2	0.28	7.48	<u>0.001</u>	0.23
Righting response week 6	0.01	2	0.31	4.01	0.02	0.14
<u>Mussel consumption - total</u>	0.01	2	9.68	6.54	<u>< 0.01</u>	0.20
<u>Mussel consumption - over time (interactions)</u>	0.01	10	295.94	2.66	<u>< 0.01</u>	0.09
Weight corrected mussel consumption	0.01	2	1.00	1.03	0.37	0.05
Respiration	0.05	2	1.24	0.58	0.56	0.03
NH ₄ ⁺ Excretion	0.05	2	0.64	0.75	0.48	0.04
O:N ratio	0.05	2	0.98	0.03	0.97	< 0.01
<u>Scope for growth</u>	0.01	2	88253.00	6.60	<u>< 0.01</u>	0.23
Calcification	0.01	2	120.70	0.48	0.62	0.01

Figures

Fig. 1: Mean growth of sea stars over a), c) 39 weeks and b), d) six weeks of experimental period at the different treatment levels. Vertical bars denote \pm 95% CI. a) and b): results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). Raw data is displayed to allow comparison of individual growth of single sea stars across treatment levels. c) and d): results from repeated measures ANOVA testing for interactions. Here and in the following graphs "F" denotes the test statistic, "p" the probability value. Groups with different lower-case letters significantly differ at $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 2: Mean consumption of mussels by sea stars during a), c) feeding assay following exp. 1 and b), d) six-week experimental period of exp. 2. Vertical bars denote \pm 95% CI. a) and b): results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). c) and d): results from repeated measures ANOVA testing for interactions. Groups with different lower-case letters significantly differ at $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 3: Mean weight corrected consumption rates in exp. 2 during six weeks of experimental phase at the different treatment levels. Vertical bars denote \pm 95% CI. Results from one-way ANOVA.

Fig. 4: Mean weight corrected righting response levels after a) 39 weeks, b) three weeks and c) six weeks of experimental phase at the different treatment levels. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). Groups with different lower-case letters significantly differ at a) $p \leq 0.05$, b) and c) $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 5: Mean rates of a) respiration, b) NH_4^+ -excretion and c) O:N-ratio at the different treatment levels after six weeks of experimental period in exp. 2. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). α -level at $p \leq 0.05$. F_T and p_T values were derived from transformed data.

Fig. 6: Mean scope for growth (SfG) of the sea stars during the last two weeks of the six-week experimental period of exp. 2 over the different treatment levels. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). Groups with different lower-case letters significantly differ at $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 7: Mean calcification levels after a) 39 weeks experimental period plus 27 days feeding assay and b) six weeks of experimental period over the different treatment levels. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). α -level at a) $p \leq 0.05$ and b) $p \leq 0.01$.

Fig. 1

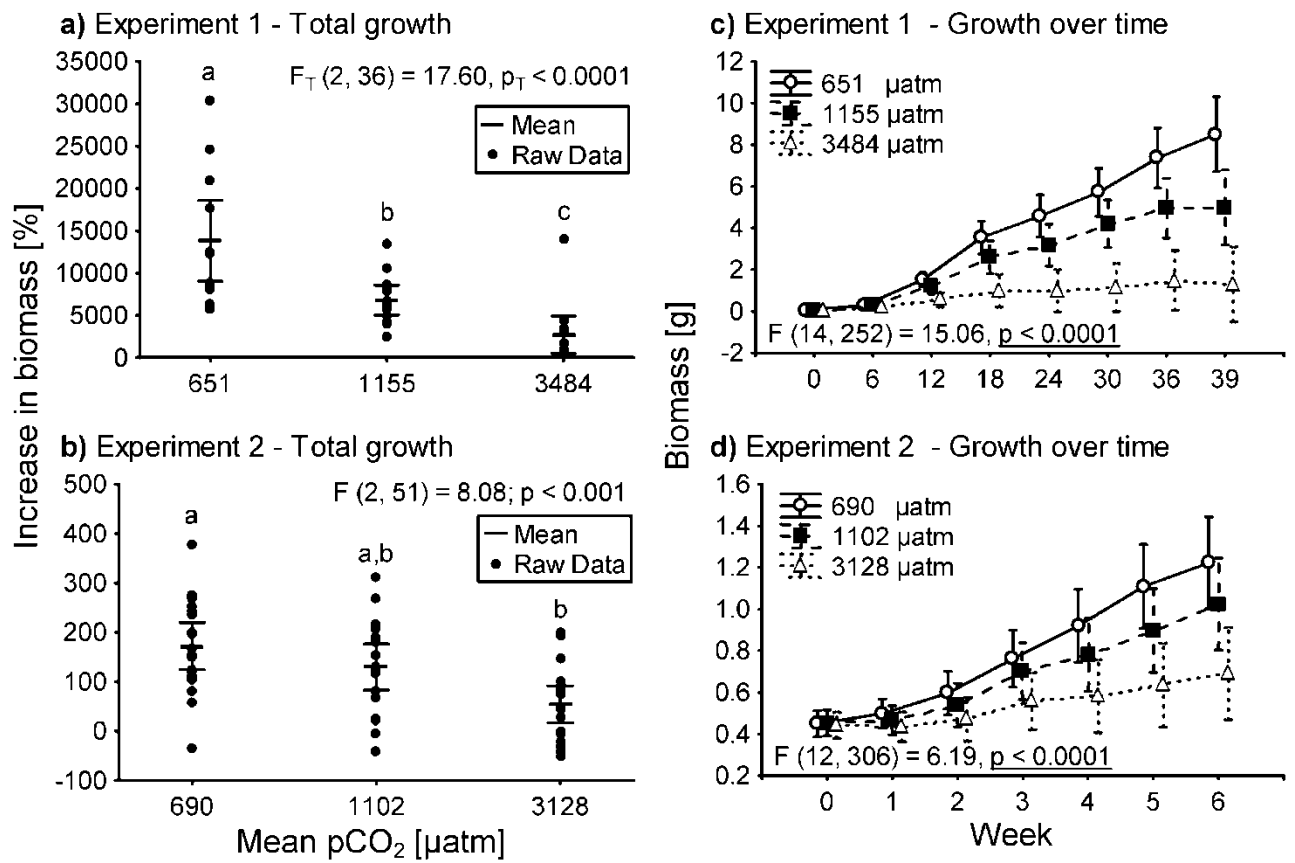


Fig. 2

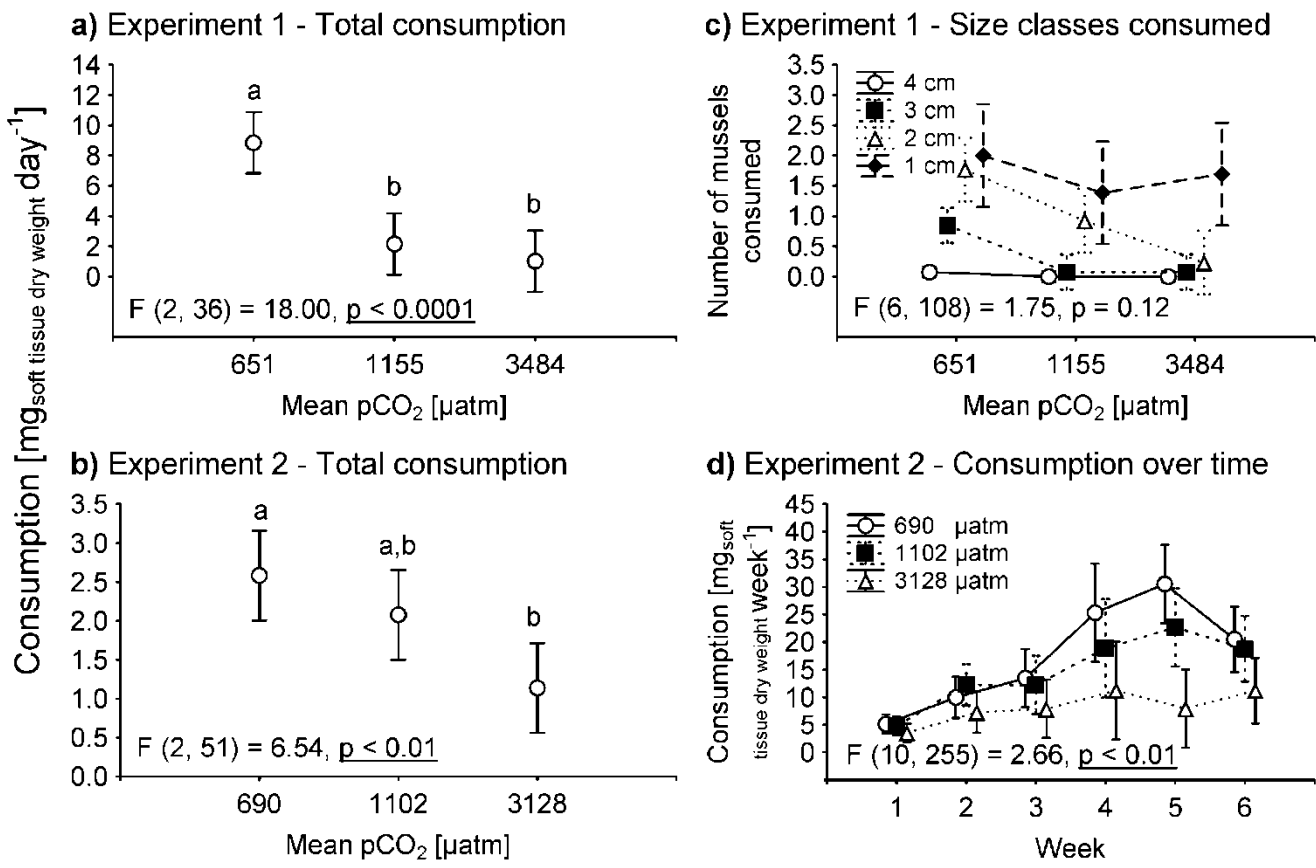


Fig. 3

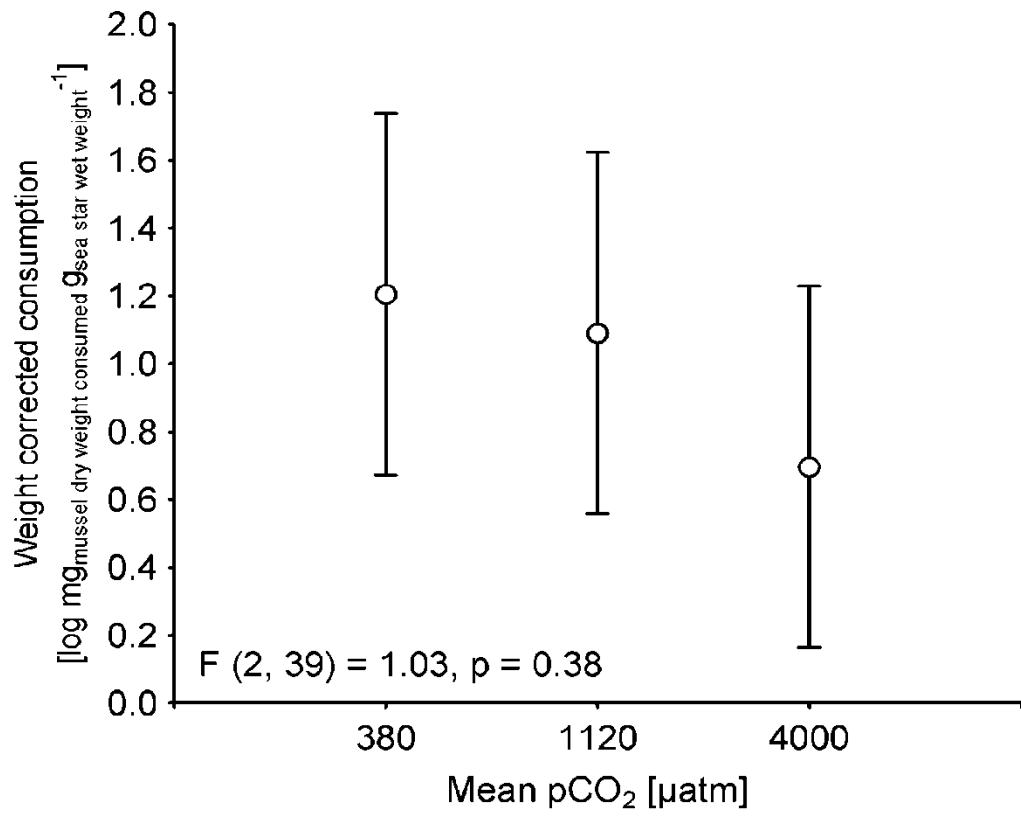


Fig. 4

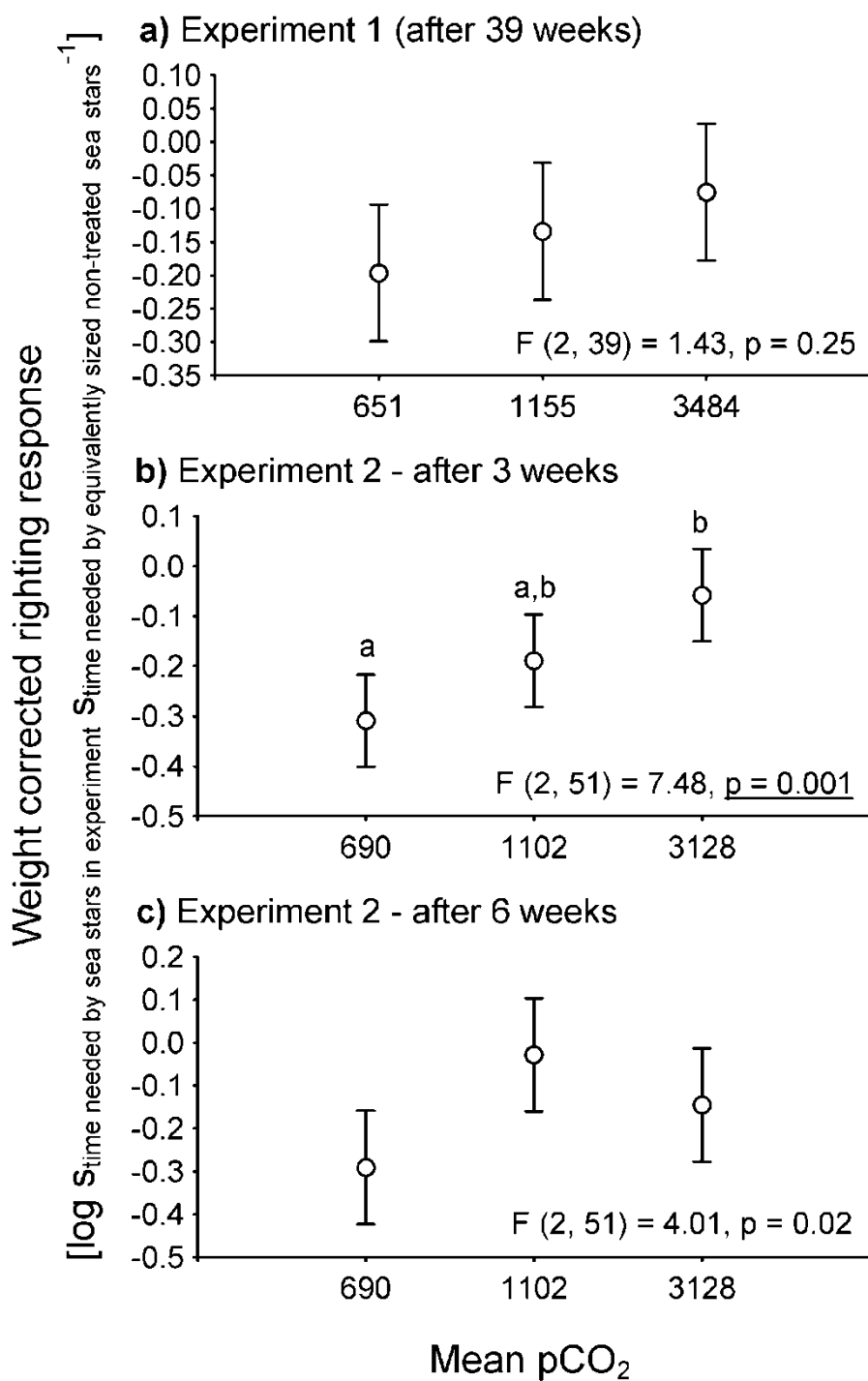


Fig. 5

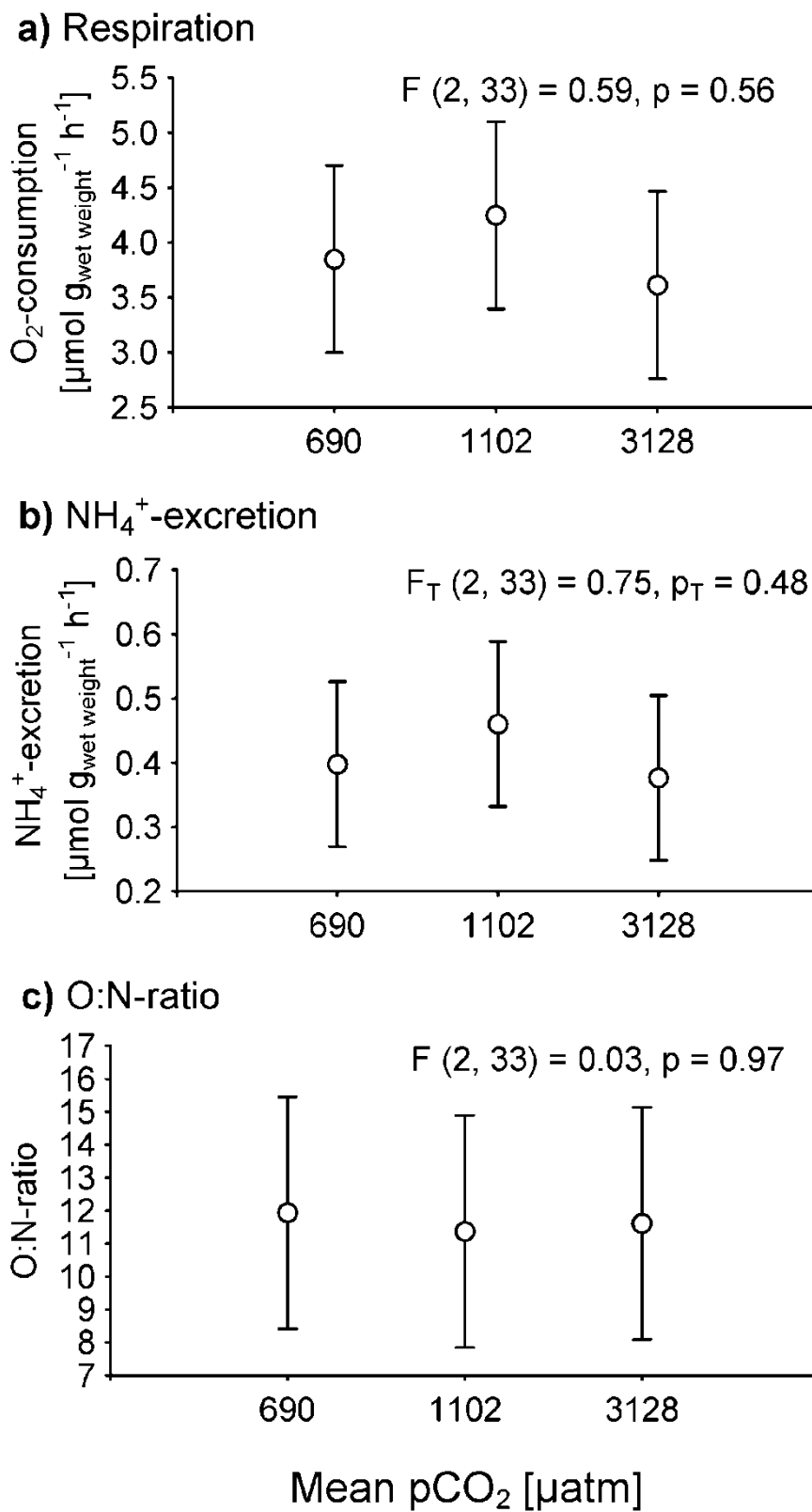


Fig. 6

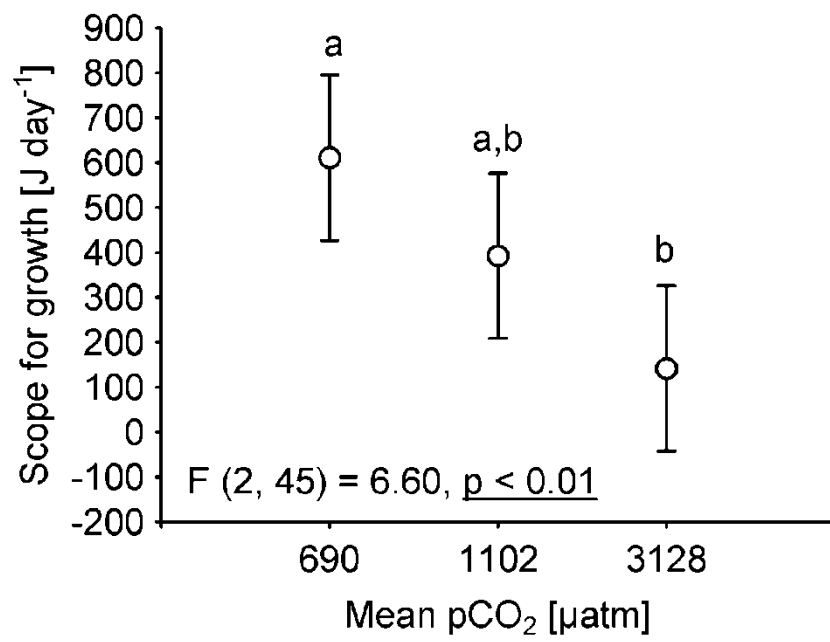
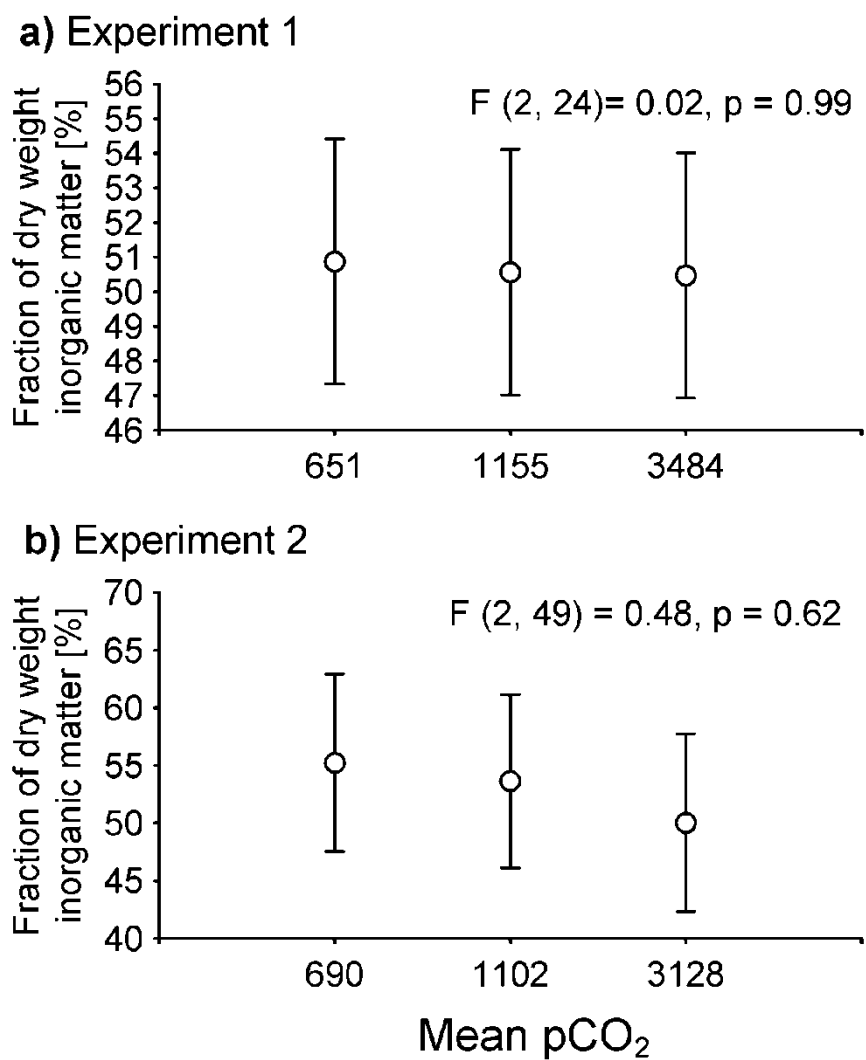
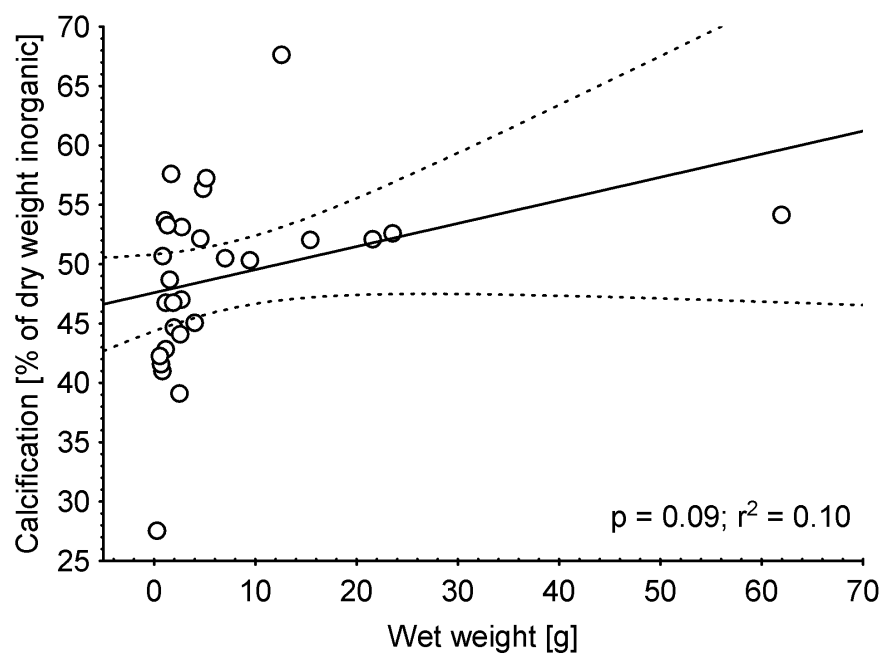


Fig. 7





Supplementary Fig. 1: The relationship between sea star wet weight and their calcification. Specimen collected in Kiel Fjord. Results from linear regression analysis. Dotted lines denote the ± 0.95 confidence interval.

Chapter 3

Sour crabs - The effect of long-term seawater acidification on growth and moulting, food intake, metabolism, acid-base balance and calcification in the shore crab *Carcinus maenas*

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Abstract

Although crustaceans are generally considered particularly resistant to the threat of seawater acidification at short- and medium time spans, knowledge regarding their biological responses in a long-term scenario is scarce. Here, we investigated the effect of long-term environmental hypercapnia (six month incubation at a pCO₂ of 883, 1268 and 3326 µatm) on growth, food intake, metabolism, acid-base status and calcification of the shore crab *Carcinus maenas*.

While growth, moulting intervals, food intake and calcification were not affected by acidification, metabolism, NH₄⁺-excretion, O:N-ratio, metabolic energy loss and acid-base balance were significantly influenced at the highest CO₂-level. Extracellular pH (pHe) was only regulated at the highest pCO₂ (3326 µatm). We assume that the two-fold rise of O₂-consumption and three-fold rise in NH₄⁺-excretion in the highest pCO₂-treatment were a consequence of the increased costs associated with acid-base regulation. Thus, we argue that such physiological responses will cause negative effects on both growth and reproductive investment over longer time spans.

Introduction

The shore crab *Carcinus maenas* is a generalist decapod crustacean capable of coping with large fluctuations in temperature, salinity and oxygen concentration (Wolf and Sandee 1971; Taylor et al. 1977; Nagaraj 1993). The species occurs in many coastal areas - originally inhabiting European shores - and has spread around the world as an invasive species (e.g. Le Roux et al. 1990; Thresher et al. 2003). Within the Baltic Sea, *C. maenas* is one of the main predators of dominant blue mussels (e.g. Adelung and Dries 1982) and plays an important role in structuring coastal communities (Enderlein and Wahl 2004). Due to its potential for adaptation to a wide variety of environmental conditions, the investigation of its ability to deal with an increase in seawater acidification promises to reveal possible mechanisms for resilience towards this form of environmental stress.

The acidification of seawater is gradually increasing as atmospheric pCO₂-levels rise (e.g. Caldeira and Wickett 2003). A drop in pH of 0.1 units is already measurable in the open ocean today (Sabine et al. 2004). Coastal habitats, however, naturally underlie much greater fluctuations in pH-level, due to the input of fresh water and upwelling of CO₂-rich water, among other factors (Wootton et al. 2008; Hofmann et al. 2010; Yu et al. 2011). Kiel Fjord in the Western Baltic is a coastal habitat in which such strong fluctuations in acidification are observed seasonally, also due to its reduced buffering capacity. Values of 2300 µatm pCO₂ have been measured (Thomsen et al. 2010) and by the end of the century these may exceed 3000-4000 µatm in the summer months (Melzner et al. *accepted*).

Decapod crustaceans seem to be fairly resistant to seawater acidification. This is considered due to their largely active behaviour and capacity for ionic regulation (Melzner et al. 2009). Moreover, their exoskeleton consists of calcite, a form of CaCO₃ fairly stable towards dissolution (e.g. Boßelmann et al. 2007). A recent review by Whiteley (2011) has identified large differences on the species level, but has confirmed the overall robustness of this phylum. However, efficient acid-base regulation might, on the other hand, be responsible for hypercalcification observed in efficient pHe regulating species (e.g. Checkley et al. 2009; Ries et al. 2009; Gutowska et al. 2010).

Previous studies with *C. maenas* have shown that prey consumption (over three months) was significantly affected at pCO₂ of 3500 µatm and that the crabs actively compensated extracellular hemolymph pH (pHe) at 1250 and 3500 µatm (Appelhans et al. *accepted*). It has also been demonstrated that this species was moderately affected at the gene expression level during brief (one week) acclimation to a pCO₂ of 4000 µatm (Fehsenfeld et al. 2011) and to be influenced in claw muscle length, while mortality, consumption of periwinkles, cuticle thickness and breaking resistance of the claw were not impacted by a reduction of seawater pH to 7.7 (Landes and Zimmer 2012). Additionally, a study conducted at very low pH (6.4)

has reported a reduced cuticle thickness and a high mortality of crabs when attempting to moult (Adelung 1971).

The aim of the present study was to investigate a comprehensive set of biological responses to long-term environmental hypercapnia (six months) in *C. maenas*, namely: i) moulting and growth, mortality and egg production; ii) food intake; iii) oxygen consumption and ammonium excretion rates; iv) extracellular acid-base status; v) and calcification processes.

Materials and methods

Sampling of organisms

Specimen of *Carcinus maenas* were collected in Kiel Fjord, Western Baltic, in August 2011 via dredging and manual collection while scuba diving. Their size range varied between 25.0 and 37.5 cm carapace width and they weighed around 8.87 ± 4.88 g wet weight (WW). Crabs were distinguished according to gender and the same number of males and females were used in each treatment level.

Set-up

Experimental units (EUs) were 4.5 l plastic aquaria, spray-painted black on four of the five sides to avoid visual contact between the crabs. A Plexiglas lid was placed on top of the aquaria. Glass beads of 0.4 - 0.8 mm diameter (ASIKOS blasting abrasive) were soaked in running seawater for 24 h and then added to the aquaria as a substrate to allow crabs to bury themselves. PVC-pipes were placed on the bottom of the units to serve as shelter for the crabs. Since *C. maenas* is known to decrease moulting intervals with shorter light availability (Adelung 1971), we established a light regime with a 9:15 h light:darkness cycle. The aquaria were supplied with water from Kiel Fjord in a flow-through fashion and the water was pre-treated by bubbling pressurised air with three different CO₂-concentrations (ambient, 1120 µatm, 4000 µatm) in three inter-connected header tanks. The water then ran into the EUs at 5-6 l h⁻¹, where it was additionally bubbled with the above mentioned air-CO₂ mixtures. Due to the naturally acidified conditions of water in the Kiel Fjord (already discussed above), the lowest treatment level was substantially higher (mean of 883 µatm, Table 1) than that of water in equilibrium with the atmosphere (around 390 µatm CO₂). When temperatures in the aquaria reached temperatures below 10° C (due to natural temperature fluctuations in the Fjord), the water was pre-heated in a 100 l tank to 15° C before being pumped into the header tanks. Each pCO₂ treatment was replicated 16 times, i.e., 16 crabs (eight per gender) were individually placed in separate EUs for each pCO₂ treatment.

Due to malfunction of heaters, water temperatures in the EUs reached up to 31° C for ca. 12 h on December 24th 2011. Three crabs died during this episode. Since the over-heating, however, occurred long before the end of the experimental period in March 2012, the time

frame in which the crabs were exposed to stress was relatively short and identical for all treatment levels, we assume no influence on the outcome of our study. The experimental period lasted 25 weeks.

Carbonate system determination

pHNBS was measured weekly using a WTW pH meter and a Sentix 81 electrode. Temperature and salinity were also measured on a weekly basis with a WTW Cond 340i and a TetraCon 325 electrode. We calculated carbonate system parameters from water samples taken weekly from two EUs per each treatment level. Water samples were analysed for total dissolved inorganic carbon (C_T) and pH_{total} directly after each sampling. C_T was measured using an AIRICA system (Marianda, Kiel, Germany) via a LI-COR 7000 infra-red CO_2/H_2O analyser.

Measured C_T values were corrected for instrument offset using a correction factor derived from the deviation of measured and given values of Dickson seawater standard as reference material (Dickson et al. 2003). pH_{total} was determined using Tris/AMP seawater buffers mixed for a salinity of 15 in a water bath (21.6° C). Carbonate system parameters were calculated from measured values using CO2sys for experimental conditions (Pierrot et al. 2006). The values for K_1 , K_2 , and $KHSO_4$ constants were chosen according to Roy et al. (1993). All the parameters of the carbonate system are shown in Table 1.

Moulting and growth, mortality and egg production

Carapace width was measured in the beginning of the experiment and seven days after every moult, i.e., when the carapace was hardened enough for measurement. The WW was determined in the beginning and at the end of the experiment.

Crabs were monitored daily for moulting and egg production.

Food intake

During the experimental period, crabs were fed *ad libitum* with mussels of equal sizes (2.00 to 2.25 ± 0.25 cm). We monitored consumption (number of consumed mussels) on a weekly basis, from week 4 onwards. After the experimental period, a feeding assay was conducted and mussels of six size classes (4.0, 3.5, 3.0, 2.5, 2.0 and 1.5 cm each ± 0.1 cm) were fed to the crabs *ad libitum* for seven days (consumed size classes were replaced) and consumption monitored over this time frame.

Twenty mussels of each size class were frozen and soft tissue (somatic) dry mass was determined. These values were then used to calculate total soft tissue dry mass of mussels consumed by each crab.

Oxygen consumption, ammonium excretion, O:N-ratio and metabolic energy loss

After the feeding assays, routine oxygen consumption rates were quantified ($n = 5$ per CO_2 treatment chosen according to moulting frequency and size) via an intermittent-flow system in starved *C. maenas* (3-4 days). Three 1 l plexiglas chambers (one empty, bacterial control) were placed in a 200 l water bath connected to a further 200 l reservoir via a submersible pump (10 W, Eheim, Deizisau, Germany) to prevent ammonium accumulation ($< 1 \mu\text{mol l}^{-1}$). Reservoir tanks and respiration chambers were sterilized with 70% EtOH prior to measurements and crabs were rinsed with filtered ($3 \mu\text{m}$), UV-sterilized seawater to avoid respiratory activity by bacteria. Respiratory runs were conducted at 16°C and controlled by immersion heaters in the reservoir tanks.

Respiration chambers were flushed via a submersible pump (5W, Eheim, Deizisau, Germany) with filtered ($0.3 \mu\text{m}$), UV-sterilized and equilibrated (air/ CO_2 mixtures: 38.7, 11.2 and 400 Pa, respectively) seawater prior (> 45 min, acclimation period) and between (> 15 min) to the measuring cycles (three cycles per run). During incubation periods (20 minutes each) aeration of chambers was interrupted.

Oxygen consumption in respiration chambers was determined in separate measuring circuits using gas-tight Tygon tubing (R-3603, Saint-Gobain, France) and fibre-optic oxygen sensors (needle-type optodes, Presens, Regensburg, Germany) connected to an oxymeter (4-channel, PreSens). Optodes were calibrated with saturated air and $100 \text{ mg Na}_2\text{SO}_4$ per 10 ml water for 100% and 0% oxygen saturation, respectively, according to manufacturer's instructions. Water within the measuring circuits continuously circulated via a peristaltic pump (Watson Marlow 505 S, tubes 2.79 mm) with a flow rate of 30 ml min^{-1} and oxygen saturation was recorded every 15 s via optodes inserted into the gas-tight tubings via plastic y-pieces. During the incubation period, the respiratory chambers were covered with a lid to prevent stress and movement of crabs. Molar oxygen consumption rates (MO_2) were calculated from linear declines of oxygen saturation values (average decrease 90%, maximum 84%) and are expressed as $\mu\text{mol O}_2 \text{ g}^{-1} \text{ WW h}^{-1}$.

Ammonium excretion rates ($\text{NH}_4^+_{\text{ex}}$) were determined from NH_4^+ concentration measurements prior to and following incubation of *Carcinus maenas* for respiration measurements (after last cycle). Before and after closing the respiration chambers, a 10 ml seawater sample was removed and 2.5 ml of reagent containing orthophthaldialdehyde, sodium sulphite and sodium borate was added (Holmes et al. 1999). Samples were then incubated for 2 h at room temperature in the dark until fluorescence was determined at an excitation and emission wavelength of 360 and 422 nm, respectively (Kontron SFM25 fluorometer). Additionally, a separate glass chamber was incubated without crabs to determine background readings of filtered seawater and ammonium excretion of bacterial controls. Ammonia (NH_3) was not measured as NH_3 concentrations at pH values of 8 to 7.1 are negligible (0.2-2% of total

ammonium/ammonia, Körner et al. 2001). Ammonium excretion rates are expressed as $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{WW h}^{-1}$.

The atomic ratio of oxygen uptake and excreted nitrogen was calculated from MO_2 and NH_4^+ ex via following equation:

$$\text{O:N} = 2 \text{MO}_2 (\text{NH}_4^+ \text{ex})^{-1} \quad (1)$$

The aerobic energy loss was calculated using a mean oxycaloric equivalent of $0.44 \text{ J } \mu\text{mol O}_2^{-1}$ representing a mixed protein-dominated catabolism of hydrocarbons, lipids and proteins as metabolic substrates (Lauff and Wood 1996). Calculations were performed without consideration of potential changes in the fraction of the three substrates to total metabolism in the different pCO_2 treatments. Energy loss by NH_4^+ excretion was calculated using an energy value of $0.347 \text{ J } \mu\text{mol}^{-1} \text{NH}_4^+$ according to Elliott and Davison (1975). Total metabolic energy loss (ENET, $\text{J g}^{-1} \text{WW h}^{-1}$) was calculated from aerobic energy metabolism and nitrogen excretion:

$$\text{ENET} = \text{MO}_2 * 0.44 \text{ J} + \text{NH}_4^+ \text{ex} * 0.347 \text{ J} \quad (2)$$

Extracellular acid-base status

Crabs were caught within max. three seconds, immediately chilled on ice to reduce stress and stop further biological activity. After 120 s, hemolymph (HL) was withdrawn from the infrabranial sinus (last leg) via a gas-tight Hamilton syringe. Subsequently, extracellular pH (pHe) and total dissolved inorganic carbon (C_T) values were assessed and the remaining hemolymph immediately frozen (liquid N_2) for further analysis. pHe ($150 \mu\text{L}$ HL) was quantified using a microelectrode (WTW Mic-D) and a WTW pHi 340 pH meter (precision ± 0.01 units) that was calibrated with Radiometer precision buffers 7 and 10 (S11M44, S11M007). Extracellular pH measurements were performed in a water bath at 16°C to maintain seawater/blood temperatures. Total dissolved inorganic carbon was determined in duplicates ($100 \mu\text{l}$ each) via a Corning 965 carbon dioxide analyser (precision $\pm 0.1 \text{ mmol l}^{-1}$; Olympic Analytical Service, England) that was calibrated by generating a sodium bicarbonate (Fluka, Germany) standard curve with a fresh dilution series of 20, 10, 5, 2.5 and 1.25 mM bicarbonate in distilled water.

Carbonate system speciation (i.e. pCO_2 , $[\text{HCO}_3^-]$) within the HL of *C. maenas* ($n = 5$ per CO_2 treatment chosen according to moulting frequency and size, same specimen as for the analysis of metabolism and NH_4^+ -excretion) was calculated from pHe and C_T measurements according to the Henderson-Hasselbalch equation

$$\text{pCO}_2 = \text{C}_T (\alpha (10 (\text{pH} - \text{pK}'1) + 1))^{-1} \quad (3)$$

$$[\text{HCO}_3^-]_e = \text{C}_T - (\alpha \text{pCO}_2), \quad (4)$$

where α is the solubility coefficient and $\text{pK}'1$ the dissociation constant of carbonic acid in blood samples of *Carcinus maenas* (Truchot 1975) at the prevailing experimental salinity (18 g kg^{-1}) and temperature (16°C).

A non-bicarbonate buffer line (NBB) was constructed from mean protein and bicarbonate concentrations and mean pHe values of post-branchial hemolymph samples of control animals. The negative slope of the NBB line, $-\Delta([\text{HCO}_3^-] + [\text{CO}_3^{2-}]) \Delta\text{pH}^{-1}$, defined as β_{NBB} , is typically proportional to the extracellular protein concentration and was calculated according to Truchot (1976). Protein concentrations were determined spectrophotometrically (at 595 nm, Shimadzu, UV-1800) using Bradford reagent (Sigma).

A pH-bicarbonate (Davenport) diagram was constructed from mean pHe and bicarbonate values and its calculated NBB line and isopleths.

Thickness, breaking resistance and calcification of carapace

The analysis of carapace thickness, breaking resistance and calcification was performed in individuals that moulted at least once under treatment conditions and whose last moult was more than 14 d prior to carapace removal ($n = 6$). This is the known time frame for the recalcification process to take place (Robertson 1960).

We determined the thickness of the carapaces in the exact same spot of each individual, using a micrometer screw (Etalon) with a precision of 0.01 mm. The breaking resistance of the carapace was measured using a TAXT2i texture analyser (Stable Micro Systems, 25-1 measuring cell), using a pointed needle of 2 mm diameter. For the breaking process, a neoprene ring of 3 mm thickness and 20 mm outside and 16 mm inside diameter was glued to a the cut surface of a 18 mm PVC-pipe and placed under the carapace. The needle was then lowered to the exact same spot (middle point of transverse groove) of each carapace at 1.00 mm s^{-1} and the force (g) measured needed to punch a hole into the material.

The level of calcification was determined by drying the carapaces in a dry oven at $80 \text{ }^\circ\text{C}$ for 24 h, weighing and then placing them in the muffle furnace at $500 \text{ }^\circ\text{C}$ for 24 h. The calcification level was then defined as the percentage share of inorganic matter (ash weight) of the dry weight.

Statistical analyses

Total size increment of crabs over the experimental phase, the length of the moulting intervals, the total and weight corrected consumption during the experimental phase and the feeding assay, as well as the respiration and excretion levels, the pHe, the bicarbonate levels and the total calcification were analysed using a one-way ANOVA, followed by Tukey's HSD post-hoc analysis. The size range of mussels consumed in the feeding assay was compared using repeated measures ANOVA (see Appelhans et al. *accepted*). All data were tested for normality using the Shapiro-Wilks-W test before further statistical analysis. If the data were non-normally distributed, the Box-Cox procedure identified the simplest transformation to achieve normality. Percentage data were first arcsine square root transformed before attempting other transformation. Data used for one-way ANOVA were tested for homogeneity

of variances using Levene's test. If data used for repeated measures ANOVA did not meet requirements of sphericity (Mauchly's test for sphericity), p-values were corrected using the Greenhouse-Geisser correction. If normality and homoscedasticity (sphericity) could not be achieved, we used parametric tests, but lowered the α -level to 0.01 (see e.g. Appelhans et al. 2010)

If missing data values resulted in an unbalanced design and only one data point of any treatment level was missing, a dummy value of the mean of all other values of this treatment level was calculated and used for analysis. If more than one value was missing, random values of other treatment levels were deleted to achieve balance.

Results

Moulting, growth and reproduction

The level of pCO₂ in the seawater had no measureable influence on size increment in *C. maenas* (Figs. 1a,b; $p > 0.05$; Table 2), weight increase ($p = 0.88$, Fig. 1c, Table 2) or the time intervals between moults (Fig. 1d, $p = 0.32$, Table 2). Each four of the initially eight females per treatment level spawned during the course of the experimental period.

Food intake

Food intake (mg mussel per week) did not differ significantly with pCO₂ level throughout the entire experimental period ($p = 0.87$, Fig. 2a, Table 2). Also during the feeding assay (performed after the long-term exposure), there was no difference in food intake ($p = 0.83$, Fig. 2b, Table 2). In this assay, although there was a general preference for smaller mussels (1.5 cm), mussel sizes consumed (number of mussels) did not differ significantly between the CO₂-treatments ($p = 0.86$, Fig. 2c, Table 2).

Acid-base regulation

The hemolymph pH (pHe) of the intermediate (1268 μ atm) treatment level followed the non-bicarbonate buffer line (NBB) and reached a level of pH 7.75, while the pHe of the highest (3326 μ atm) treatment level was maintained at control pH level of 7.86 through the active elevation of [HCO₃⁻]e from 10.6 mM to 20.1 mM, as can be seen in the pH-bicarbonate (Davenport) diagram (Fig. 3). Thus, there were significant differences between: i) the pHe, the hemolymph HCO₃⁻ and the hemolymph pCO₂ of the crabs treated at 3326 μ atm and 1268 μ atm ($p < 0.001$, Fig. 3, Table 2), and ii) the pHe of the intermediate and the lowest (883 μ atm) treatment levels ($p < 0.001$, Fig. 3, Table 2).

Metabolism, NH₄⁺-excretion, O:N-ratio, Energy loss

Oxygen consumption rates increased significantly (2.2-fold) from the lowest to the highest pCO₂ level treatment ($p < 0.001$, Fig. 4a, Table 2). Similar increasing trends were observed for: i) the NH₄⁺-excretion rates; in this case the difference was around 3-fold ($p < 0.001$, Fig. 4b, Table 2), and ii) metabolic energy loss (from respiration and excretion), with a 2.2-fold variation ($p < 0.001$, Fig. 4d, Table 2). On the other hand, the highest value of the O:N-ratio was attained at the intermediate pCO₂ level ($p = 0.01$, Fig. 4c, Table 2).

Carapace thickness, stability, dry weight, calcification

There were no significant differences between carapace thickness ($p = 0.97$, Fig. 5a, Table 2), carapace stability ($p = 0.21$, Fig. 5b, Table 2), carapace dry weight ($p = 0.32$, Fig. 5c, Table 2) or carapace calcification ($p = 0.11$, Fig. 5d, Table 2) over the different CO₂-levels.

Discussion

Our results show that long-term exposure of *Carcinus maenas* to seawater acidification (up to 3326 µatm) did not influence moulting intervals or size after moulting, weight increase, food intake and the different calcification parameters. Yet, environmental hypercapnia caused acid-base disturbances. Moreover, the metabolic and NH₄⁺-excretion rates were enhanced.

The absence of effects on growth, prey consumption and calcification

Previous studies have shown a negative influence of seawater acidification on growth through a decrease of the moulting frequency of shrimps and penaeid prawns (Wickins 1984; Kurihara 2008). Yet, Ries et al. (2009) have shown that some decapod crustaceans (namely blue crabs and lobsters) increase calcification when exposed to seawater acidification. Although experimentally not assessed, the authors attribute this due to an increase in moulting frequency rather than carapace thickness (Ries pers. comm.). It has been hypothesised that a possible increase in calcification in brachyuran crabs may result from the active accumulation of HCO₃⁻ when compensating hemolymph pHe (Melzner et al. 2009; Gutowska et al. 2010). Here, we did not observe a significant increase in calcification during a long-term exposure time, which is in agreement with the findings obtained by Landes and Zimmer (2012).

Contrary to our previous study (Appelhans et al. *accepted*), where we documented a reduction of mussel consumption over ten weeks at a pCO₂ of 3500 µatm, we did not observe such a trend in this longer study. Such difference may be related to the moulting process (no moulting was observed in the previous study), since it is known that in the days before and after moulting, crabs do not feed, and that the feeding rates are increased when the

exoskeleton has hardened after the moult (Adelung 1971; Klein Breteler 1975). Thus, we argue that the moulting cycle may mask the possible effects of seawater acidification on prey consumption rates.

Acid-base regulation

Previous studies with the velvet swimming crab showed a compensation of acidosis over a medium time span of 30 days at a very low water pH - down to 6.05 (c.a. 60,000 μatm , Spicer et al. 2007) and down to a pH of 6.70 (12,341.23 μatm , Small et al. 2010). Our previous study conducted over an intermediate time interval (ten weeks) also showed acid-base regulation of *C. maenas* at 1250 and 3500 μatm (Appelhans et al. *accepted*). Our present study, however, indicates that this regulation of pHe over a longer time period is not upheld at all treatment levels. It appears that active bicarbonate accumulation over months only occurs when a certain pCO₂ threshold is reached. Since acid-base regulation is energetically costly for marine animals (Pörtner et al. 1998), it seems likely that the advantages of bicarbonate accumulation are outweighed by the associated energetic costs at low and intermediate levels of acidification. Only at high levels, the potential harm of a strongly acidic pHe may make such regulation worthwhile. Although we would have expected only partial compensation at 3326 μatm to pH levels found at 1268 μatm , this is an effect that should be further investigated.

Enhanced metabolism and excretion

Contrary to the present study, where a significant augment in metabolic rates occurred with the increase in pCO₂ levels, previous ones have shown the occurrence of metabolic depression instead (e.g. Small et al. 2010). Although it has been hypothesised that exposure to hypercapnia leads to metabolic depression in marine organisms (Pörtner et al. 2004), other studies with echinoderms (Wood et al. 2008), bivalves (Beniash et al. 2010; Lannig et al. 2010) and pteropods (Comeau et al. 2010; but see Seibel et al. 2012) also showed increased metabolism under increased seawater pCO₂ (1000-3000 μatm). We argue that the active bicarbonate accumulation at high (3326 μatm) levels of seawater pCO₂ enhances the metabolic demands. Moreover, the increase in NH₄⁺-excretion may also be related to the intracellular acid-base regulation. Since the intracellular pH (pHi) can be regulated by protein degradation, leading to formation of NH₃ and extrusion as NH₄⁺ (Boron 2004), it has been speculated that this mechanism might lead to increase in NH₄⁺-excretion (Langenbuch and Pörtner 2004; Michaelidis et al. 2005; Thomsen and Melzner 2010). In fact, protein degradation probably supports HCO₃⁻ production and thereby pHi regulation, as already suggested for sipunculids and the mussel *M. galloprovincialis* (Langenbuch and Pörtner 2004; Michaelidis et al. 2005).

The observed O:N ratios in the control treatment (between 20 and 30) indicate a mixed break-down of protein and non-protein substrates for aerobic energy production. Pure protein metabolism can be expected at a ratio < 20 and lipid-carbohydrate substrates dominate at values > 30 (Shulman et al. 2002). The increase in the O:N ratio (to about 45) at 1268 μatm indicates a switch from a more protein dominated diet to lipid-carbohydrate catabolism, as protein metabolism is energetically less efficient compared to the turnover of carbohydrates and lipids, since it implies a continuous energy loss by enhanced excretion of NH_4^+ (Tedengren and Kaustky 1986; Thomsen and Melzner 2010). This might be linked to less acid-base regulatory activity, especially via NH_4^+ -excretion. At the high pCO_2 treatment, in contrast, the O:N ratio declined compared to control (to around 20) indicating enhanced unfavorable protein catabolism and elevated NH_4^+ excretion rates, while fully compensating pHe.

Conclusions

Our results indicate that the shore crab *C. maenas* is fairly resistant to seawater acidification. Although there are effects on the physiological level, this does not seem to have consequences for growth and consumption rates of carapace properties over a period of six months. Naturally, it cannot be excluded that the observed acidification effects on physiological parameters will cause effects in the long run (years).

We conclude that the shore crab *C. maenas* is able to moult and grow normally under future high CO_2 -levels and that prey consumption levels are upheld over long time periods. Future studies should investigate if this resistance can be sustained over multiple generations and in combination with the predicted near-future warming and decrease in salinity in the Baltic Sea.

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Tables

Table 1: Measurements and calculations of the water carbonate system for the different treatment levels. Water $p\text{CO}_2$, A_T , Ω_{calcite} and $\Omega_{\text{aragonite}}$ were calculated using total dissolved inorganic carbon (C_T) and pH values, as well as temperature and salinity values of the respective EUs on the day of measuring, with the CO2SYS macro for low salinities (Pierrot et al. 2006). Values are means \pm SD.

$p\text{CO}_2$ of pressurised air [μatm]		Ambient	1120	4000
Measured	C_T [$\mu\text{mol kg}_{\text{seawater}}^{-1}$]	1923.16 ± 103.31	1916.14 \pm 324.96	2166.35 ± 124.97
	pH (total scale)	7.76 ± 0.13	7.57 ± 0.11	7.23 ± 0.16
	Mean temperature [$^{\circ}\text{C}$]		13.63 \pm 1.87	
	Mean salinity		15.89 \pm 1.03	
	$p\text{CO}_2$ [μatm]	882.54 ± 292.22	1267.52 ± 176.65	3326.32 ± 1840.67
Calculated	A_T [$\mu\text{mol kg}_{\text{seawater}}^{-1}$]	1947.05 \pm 111.30	1901.91 \pm 330.70	2043.67 \pm 144.25
	$\Omega_{\text{aragonite}}$	0.75 ± 0.22	0.49 ± 0.15	0.25 ± 0.07
	Ω_{calcite}	1.28 ± 0.36	0.84 ± 0.25	0.43 ± 0.12

Table 2: ANOVA results. Analyses performed with different response variables and the dependent factor pCO₂ (three levels). For non-parametric data, the α -level was adjusted to 0.01. Statistically significant results are underscored.

	α	df	MS	F	p	Partial η^2
Size increase - all crabs	0.01	2	115.69	0.19	0.83	0.01
Size increase - twice moulted crabs	0.05	2	6.58E+14	0.31	0.73	0.03
Weight increase - all crabs	0.05	2	12.65	0.13	0.88	0.01
Moulting intervals	0.05	2	6.90E+19	1.21	0.32	0.12
Total consumption - experimental period	0.05	2	1844.00	0.14	0.87	0.01
Total consumption - feeding assay	0.05	2	16.47	0.19	0.83	0.01
Mussel sizes consumed - feeding assay (interactions)	0.01	10	0.55	0.54	0.86	0.03
<u>pHe</u>	0.05	2	0.02	6.90	<u>0.01</u>	0.54
<u>Hemolymph bicarbonate</u>	0.01	2	134.64	18.50	<u>< 0.001</u>	0.76
<u>Hemolymph pCO₂</u>	0.05	2	164761	17.17	<u>< 0.001</u>	0.74
<u>Respiration</u>	0.05	2	12.25	18.42	<u><0.001</u>	0.75
<u>NH₄⁺ Excretion</u>	0.01	2	0.23	19.88	<u><0.001</u>	0.77
<u>O:N ratio</u>	0.01	2	936.07	6.83	<u>0.01</u>	0.53
<u>Metabolic energy loss</u>	0.05	2	2.97	21.36	<u>< 0.001</u>	0.78
Carapace thickness	0.05	2	59.00	0.03	0.97	<0.01
Carapace stability	0.05	2	0.11	1.70	0.21	0.14
Carapace dry weight	0.05	2	11.41	1.22	0.32	0.10
Carapace calcification	0.01	2	32.20	2.49	0.11	0.19

Figures

Fig. 1: Effect of long-term environmental hypercapnia (883, 1268 and 3326 μatm) on growth parameters of *Carcinus maenas*. a) mean size increase of all crabs used in the experiment; b) mean size increase of the crabs that moulted twice during the experimental phase; c) mean weight increase of all crabs during the experimental period; d) mean moulting intervals of crabs that moulted twice during the experimental period. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). Here and in the following graphs "F" denotes the test statistic, "p" the probability value. F_T and p_T values were derived from transformed data.

Fig. 2: Effect of long-term environmental hypercapnia (883, 1268 and 3326 μatm) on the food intake (mg mussel dw per week) of *Carcinus maenas* during a) the 25 week experimental period, and b) the subsequent feeding assay c) shows the number of mussels consumed, according to size, during the feeding assay. Vertical bars denote \pm 95% CI. a) and b): results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). c): results from repeated measures ANOVA testing for interactions. F_T and p_T values were derived from transformed data.

Fig. 3: A pH-bicarbonate Davenport diagram describing the hemolymph acid-base status of *Carcinus maenas* exposed for 6 months to the different $p\text{CO}_2$ levels (883, 1268 and 3326 μatm). Vertical and horizontal bars denote SD. Isoleths display $p\text{CO}_2$. Dotted line indicates non-bicarbonate buffer line (NBB). Statistical results from one-way ANOVAs. Groups with different lower-case letters differ significantly at a,b $p \leq 0.01$ and x,y $p \leq 0.05$.

Fig. 4: Effect of long-term environmental hypercapnia (883, 1268 and 3326 μatm) on the a) oxygen consumption rates ($\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$), b) NH_4^+ -excretion rates ($\mu\text{mol g}^{-1} \text{ h}^{-1}$), c) O:N-ratio d) metabolic energy loss ($\text{J g}^{-1} \text{ h}^{-1}$) in *Carcinus maenas*. Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). Groups with different lower-case letters significantly differ at a) and d) $p \leq 0.05$, b) and c) $p \leq 0.01$. Statistically significant p-values are underscored.

Fig. 5: Effect of long-term environmental hypercapnia (883, 1268 and 3326 μatm) on *Carcinus maenas*' carapace structure. a) thickness (μm), b) stability (g), c) dry weight, and d) fraction of inorganic dry weight (%). Vertical bars denote \pm 95% CI. Results from one-way ANOVA followed by post-hoc testing (Tukey's HSD). F_T and p_T values were derived from transformed data.

Fig. 1

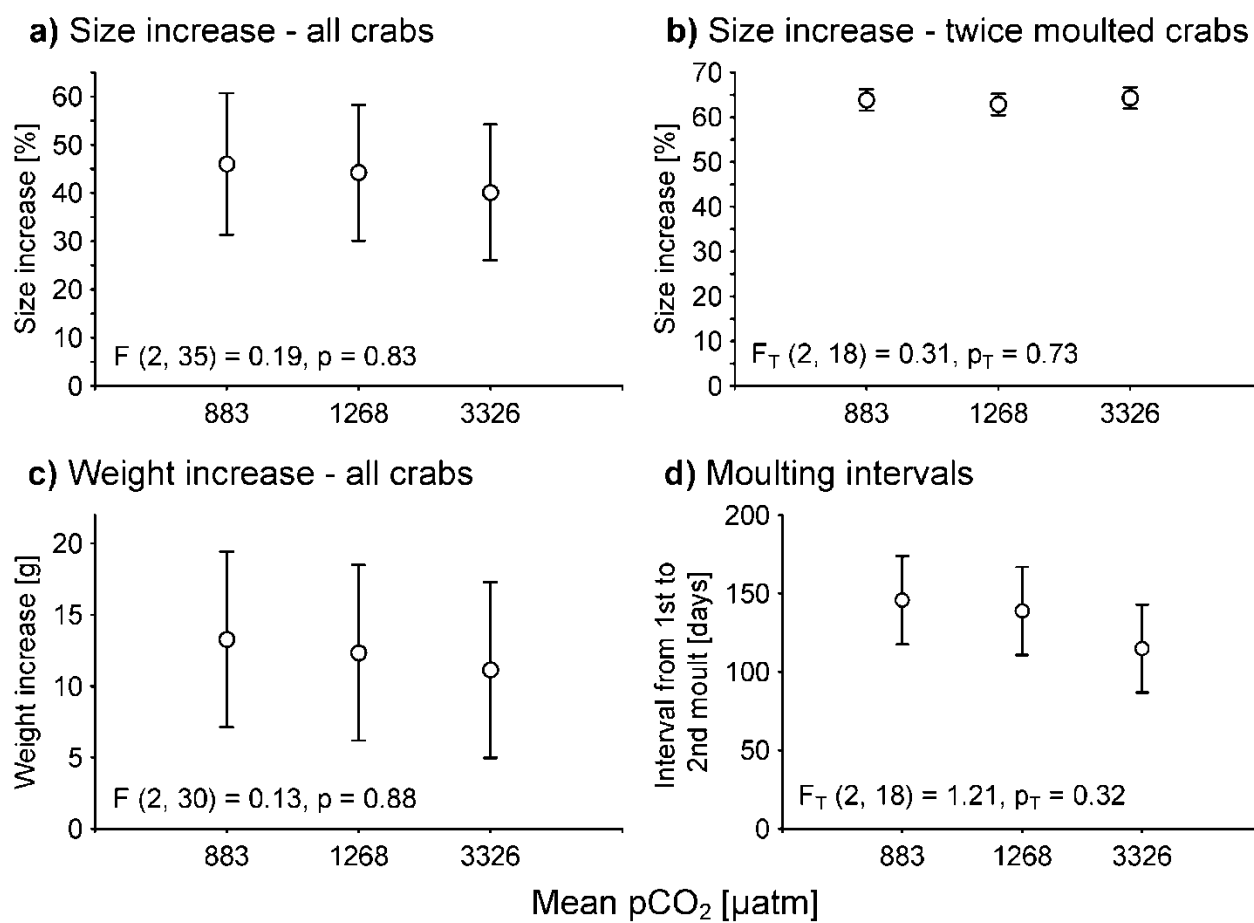
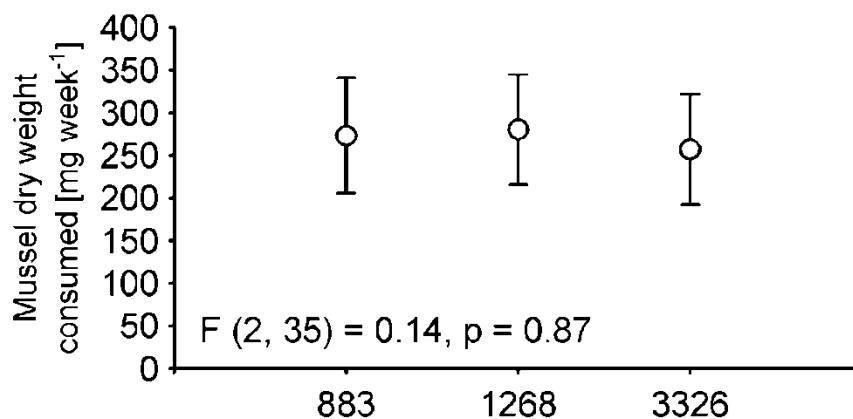
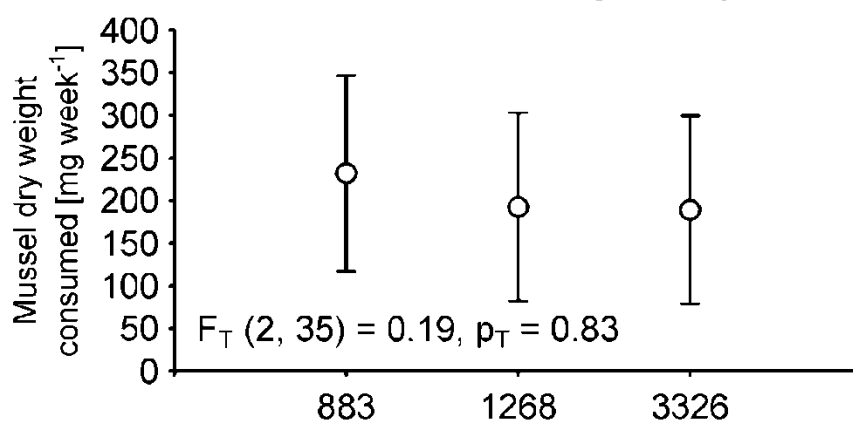


Fig. 2

a) Total consumption - experimental period



b) Total consumption - feeding assay



c) Mussel sizes consumed - feeding assay

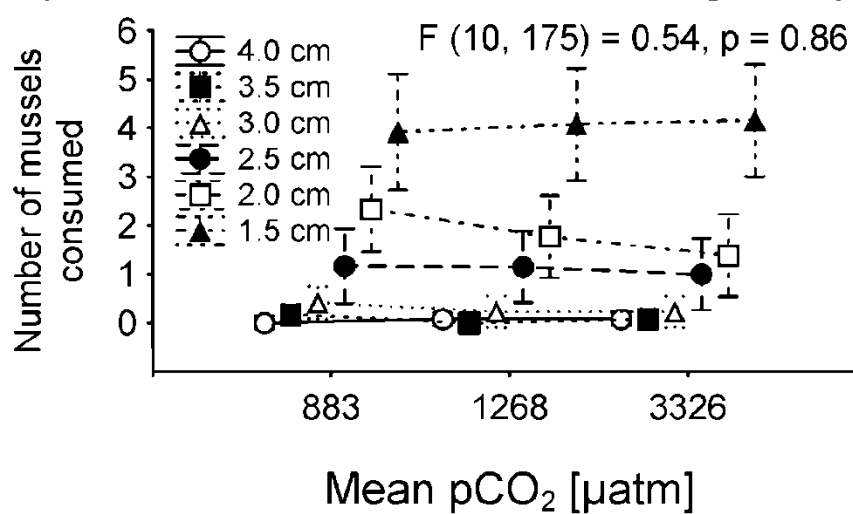


Fig. 3

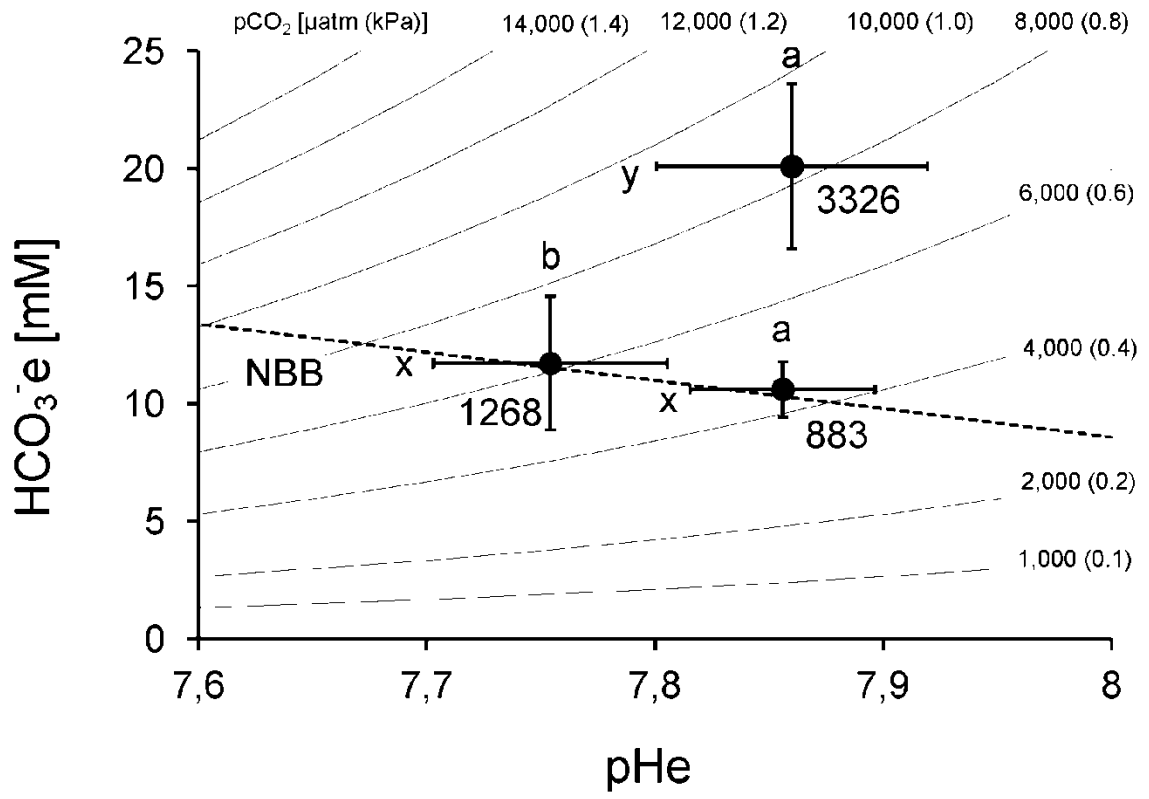


Fig. 4

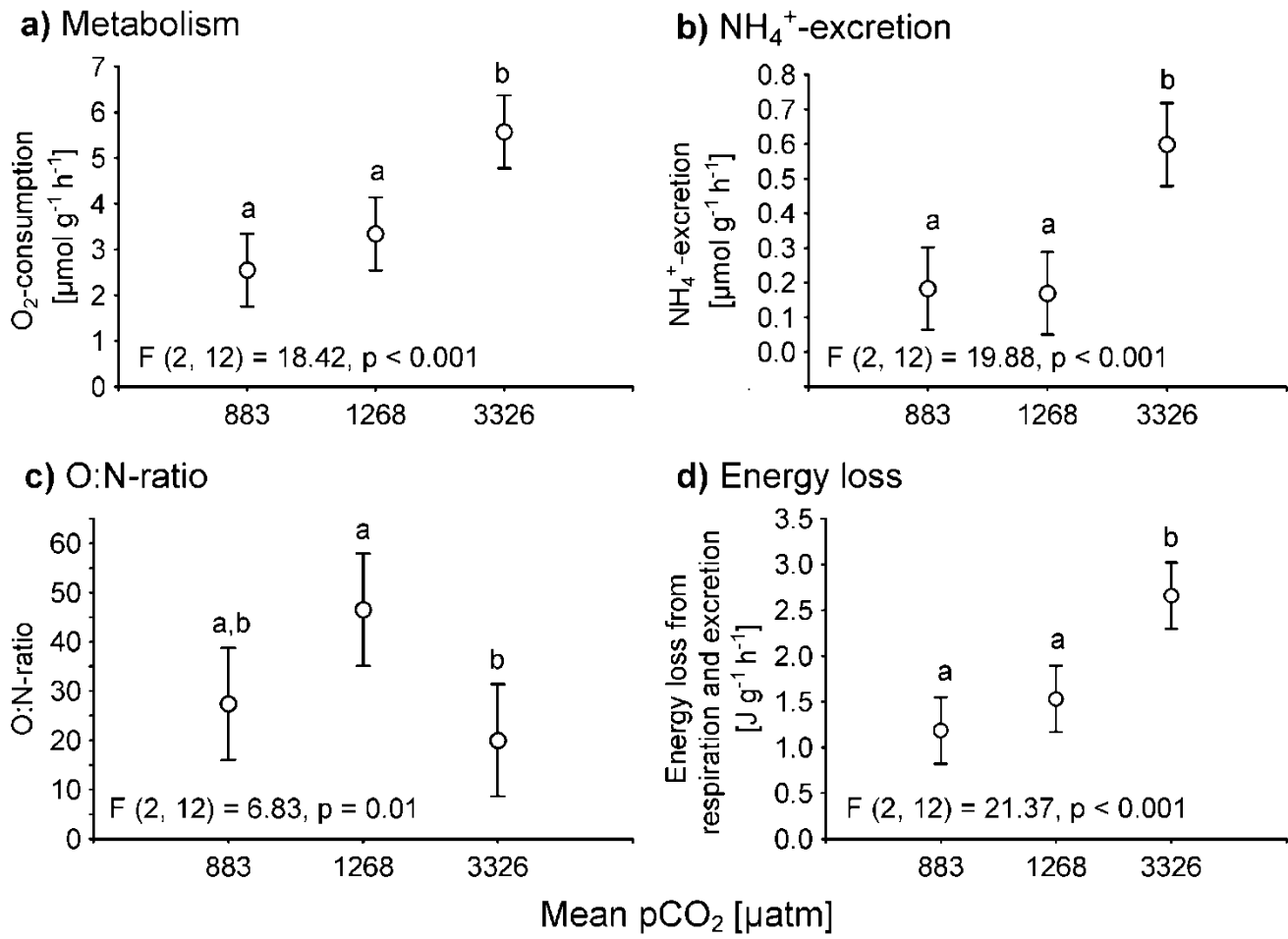
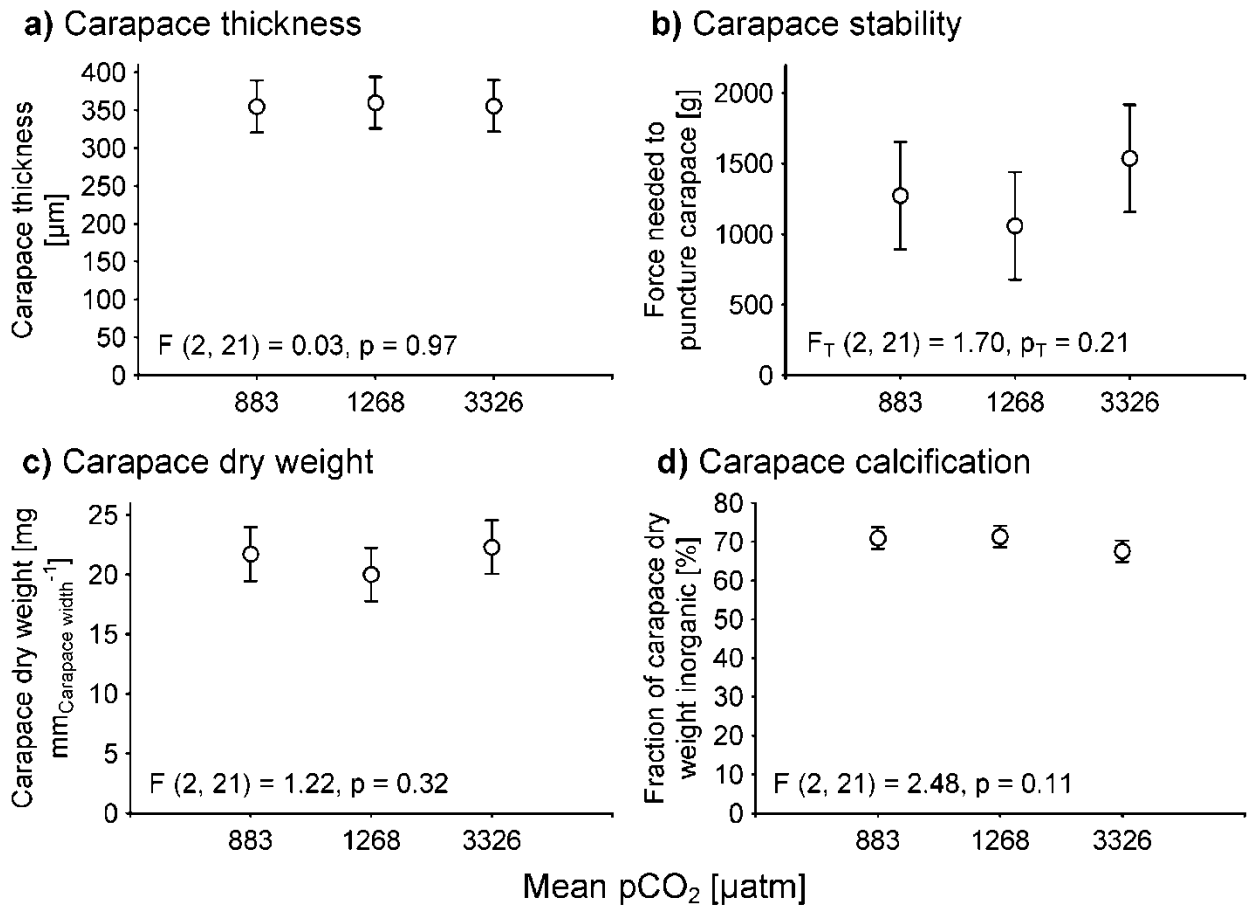


Fig. 5



General discussion

The conducted studies have shown different responses for the different predator species, the common sea star *Asterias rubens* and the shore crab *Carcinus maenas* and for different life stages of the species towards an increase in seawater acidification. A summary of the results is displayed in Table 1.

Table 1 Overview over responses of the two different predatory species *A. rubens* and *C. maenas* towards seawater acidification as tested in the studies described in chapters 1 and 2 (*A. rubens*) and 1 and 3 (*C. maenas*).

Species	Response variable	Direction of effect compared to ambient pCO ₂ at	
		1200 µatm	3500 µatm
<i>A. rubens</i>	Growth - adults	-	↓
	Growth - juveniles	↓	↓
	Mussel consumption - adults	-	↓
	Mussel consumption - juveniles	↓	↓
	Mussel sizes consumed	-	-
	Coelom HCO ₃ ⁻	-	-
	pHe	↓	↓
	Righting response (time)	↑	↑
	Metabolism	-	-
	NH ₄ ⁺ -excretion	-	-
	O:N-ratio	-	-
	Scope for growth	-	↓
	Calcification	-	-
	<i>C. maenas</i>	Moulting intervals	-
Growth		-	-
Mussel consumption - medium term		-	↓
Mussel consumption - long term		-	-
Mussel sizes consumed		-	-
Hemolymph HCO ₃ ⁻ - medium term		-	↑
Hemolymph HCO ₃ ⁻ - long term		-	↑
pHe - medium term		-	-
pHe - long term		↓	↑
Metabolism		-	↑
NH ₄ ⁺ -excretion		-	↑
O:N-ratio		-	↓
Metabolic energy loss		-	↑
Carapace thickness		-	-
Carapace stability		-	-
Carapace dry weight		-	-
Carapace calcification	-	-	

Tested hypotheses

1. The interactions between *Mytilus edulis* and its predators will change with an increase in seawater acidification via a change in consumption rates.

The results of our studies show that the feeding pressure on *M. edulis* indeed changes with increases in seawater pCO₂ (see Fig. 4). This is mostly due to effects of acidification on mussel consumption by sea stars and less on mussel consumption by crabs. Also the reduction in growth of *A. rubens* under conditions of acidification will ultimately lead to a reduction in sea star biomass and thus feeding pressure on the mussels. Our results do not indicate a decrease of prey size over intermediate time spans, which is probably due to a general preference of similar mussel size classes, irrespective of sea star size with only much larger sea stars preferring larger mussel sizes (Sommer *et al.* 1999). Over very long time spans, however, also the prey sizes consumed may be reduced.

Our findings on reduced mussel consumption under conditions of acidification, however, do not necessary indicate reduced consumption of blue mussels under future high atmospheric CO₂-levels. A study on the sea star *Pisaster ochraceus* exposed to increased seawater temperatures (12°C as opposed to 9°C) has shown feeding of this species to be higher at higher temperatures (Sanford 1999). It is thus possible that although the increased seawater pCO₂ levels would lead to reductions in consumption, this effect could be counteracted by an increase of seawater temperatures caused by an increase in atmospheric CO₂. It is thus necessary to investigate seawater acidification alongside with the increase in seawater temperature and also decrease in seawater salinity as predicted for the Baltic Sea (BACC author team 2008) before further predictions about the future feeding pressure on *M. edulis* can be made.

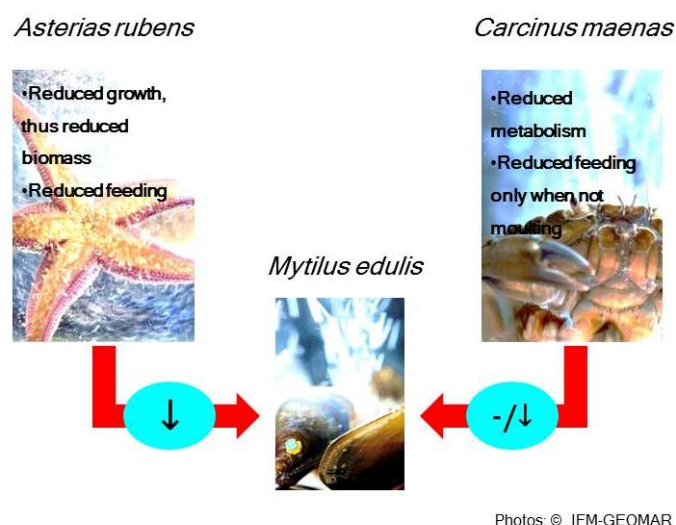


Fig. 4 Observed direction of the change in feeding pressure on the blue mussel *M. edulis* under conditions of seawater acidification and factors investigated in this work influencing it.

2. The shore crab *C. maenas* is less vulnerable to increases in seawater acidification than the common sea star *A. rubens*.

Concerning the parameters investigated, our studies indeed show *C. maenas* to be less vulnerable towards seawater acidification than *A. rubens*. A review on the reactions of echinoderms towards seawater acidification has stated these organisms fairly resistant towards seawater acidification, as well (Dupont *et al.* 2010). Effects, however, have only been included into analysis for pH-variations by max. 0.4 scale points, while pH-values are likely to be lower than these values predicted for the pelagic open ocean under future atmospheric pCO₂-conditions for many coastal benthic habitats of the adult stages of echinoderms (Hofmann *et al.* 2010, Melzner *et al. accepted*). Many echinoderm species have shown negative effects towards seawater acidification (Kurihara *et al.* 2004, Kurihara & Shirayama 2004, Siikavuopio *et al.* 2007, Dupont *et al.* 2008, O'Donnell *et al.* 2010, Stumpp *et al.* 2011a, Stumpp *et al.* 2011b, Stumpp *et al.* 2012, Dupont *et al. in press*), in some cases even at very low increases of pCO₂ of about 200 µatm (Shirayama & Thornton 2005). Our studies demonstrate that especially the food uptake of *A. rubens* is influenced, which leads to reductions in growth. Similar observations have been made for the sea urchin *Strongylocentrotus droebachiensis*, which, too, reduces consumption at pCO₂-levels of 2800 µatm (Stumpp *et al.* 2012). It is possible that these reductions in food uptake with elevated seawater acidification are a general pattern in echinoderms. Echinoderms in general may thus belong to the organisms more strongly affected by future seawater acidification.

A review on crustacean responses to an increase in seawater pCO₂ confirms the general robustness of this phylum towards seawater acidification and identifies the only vulnerable species to be those that are poor iono- and osmoregulators, slow moving and inhabitants of low-energy environments (Whiteley 2011). None of these traits are found in *C. maenas* which has remarkable ion- and osmoregulatory capacities (e.g. Seck 1958), an active mode of life (Klein Breteler 1975) and inhabits the Western Baltic with its high food supply. A recent study on the impact of seawater acidification down to a pH of 7.7 over five months showed no effect on mortality, calcification or consumption of periwinkles (Landes & Zimmer 2012). *Carcinus maenas* from the Baltic Sea thus seem fairly resistant towards seawater acidification.

Since, however, acid-base regulation under conditions of seawater acidification has recently been shown to be linked to sensory and behavioural impairment in fish (Nilsson *et al.* 2012) and we have shown the crabs to be actively regulating their pHe by means of HCO₃²⁻ accumulation, future studies have to investigate how these traits are influenced in *C. maenas* under conditions of increased seawater pCO₂. An impairment of detecting olfactory cues of predators has been shown for reef fish under conditions of seawater acidification (Dixson *et al.* 2010, Ferrari *et al.* 2011). If an increase in seawater pCO₂ would similarly lead to difficulties for crabs to detect olfactory cues of their prey, this would in turn strongly affect *C. maenas*' capability of growth and survival under conditions of acidification.

3. Juvenile *A. rubens* are more susceptible to changes in seawater acidification than adults of the same species.

The data presented in chapter 2 demonstrate that juvenile sea stars are indeed more vulnerable towards increases in seawater pCO₂ than adults. Although comparisons between larval and adult stages in reacting towards environmental stress are abundant in the literature, comparisons between juvenile and adult life stages are scarce. While Hummel *et al.* (1996) found no significant differences in resistance to copper exposure between juvenile and adult life stages of the bivalve *Macoma balthica*, Foster (1971) found young benthic forms of barnacles more sensitive towards environmental stress than adult forms. Different sensitivities in life stages could, however, not be demonstrated for the barnacle *Amphibalanus improvisus* from the Baltic Sea (Pansch *et al.* 2012, *submitted* and *in prep.*).

The results presented in chapter 2 emphasise the necessity of investigating all life stages to identify possible bottlenecks in ontogeny.

Ecological indications of our results

Harley *et al.* (2006) have stated the direct ecological responses to climate change (including seawater acidification) to depend on the relationships between the abiotic environment, processes on the organism level, population dynamics and community structure with ultimate effects on ecological responses, such as distributional shifts, changes in diversity, changes in productivity and microevolutionary change (Fig. 5).

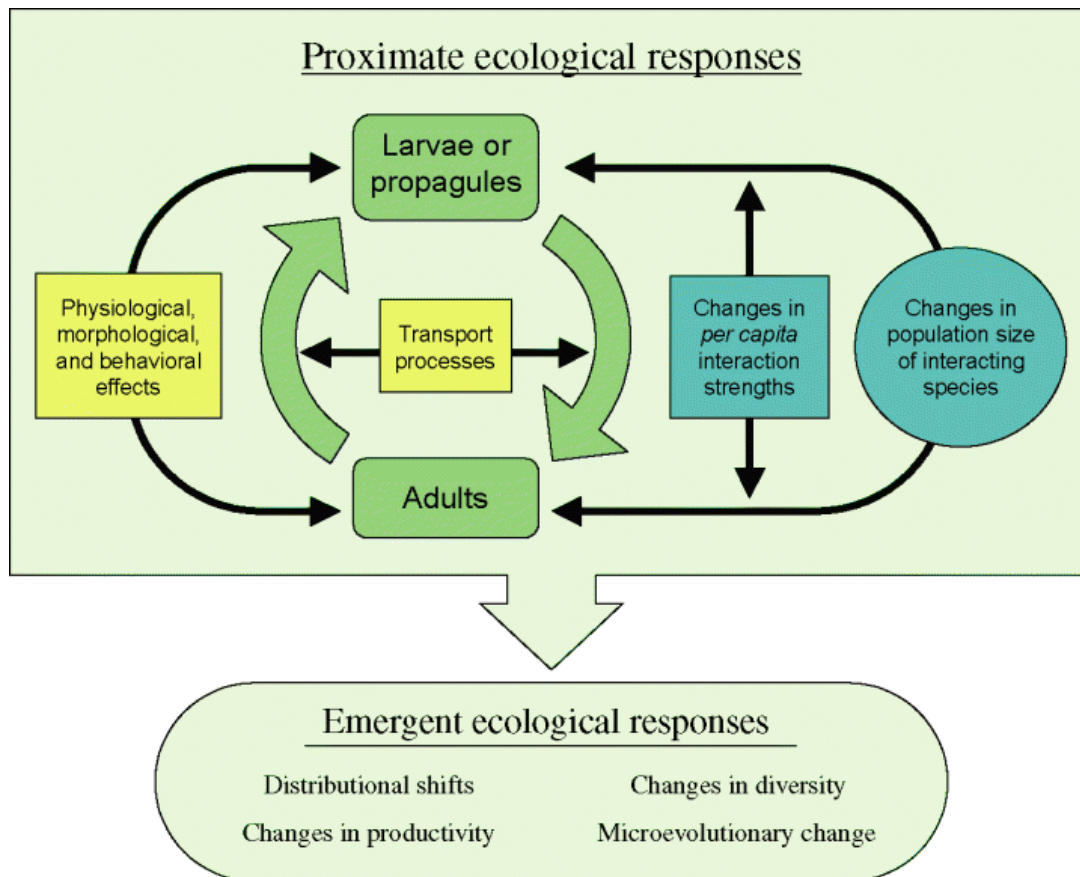


Fig. 5 Proximate ecological responses of climate change (including seawater acidification) on marine organisms and resulting ecological responses. Yellow: direct effects of abiotic changes, green: generic life cycle of a marine organism, blue: community-level effects. Taken from Harley *et al.* (2006).

Applied to the studies described in chapters 1-3, the model by Harley *et al.* (2006) implies possible ecological responses from the proximate effects on physiological, morphological and behavioral effects on adult and juvenile predators and changes on the *per capita* interaction strengths. The responses to seawater acidification of single organisms investigated in this work thus have the potential to strongly change the entire ecosystem of the Western Baltic. This potential is even stronger, if species are not all affected in the same direction and in the same strength (see discussion of chapter 1). It is also important to take into account effects on prey species to be able to make predictions about future ecological responses.

Previous studies have shown the blue mussel *M. edulis* to be impacted in growth at a seawater $p\text{CO}_2$ from about 4000 μatm onwards (Hiebenthal 2009, Thomsen *et al.* 2010), although this effect was also observed at lower levels of $p\text{CO}_2$ (Thomsen & Melzner 2010). Other prey organisms, such as the barnacle *Amphibalanus improvisus* (Pansch *et al.* 2012, *submitted* and *in prep.*) and the periwinkle *Littorina littorea* (Landes & Zimmer 2012) were not affected in growth by seawater acidification ($p\text{CO}_2$ of 4000 μatm and pH of 7.7, respectively), although calcification and shell stability were reduced. None of the three prey species displayed reduced survival rates under increased seawater acidification. Although the results

presented in chapter 1 indicate the effect of seawater acidification on the predators to more strongly determine the predator-prey interactions than the effect on the prey organisms, clearly any effect on the abundance of the prey would, in turn, also cause effects on the ecosystem level. If prey became a limiting factor, intra- and inter-specific competition of predatory species would increase, adding an additional stress on these organisms. Since, however, prey organisms are likely to be abundant in the macrozoobenthos of the Western Baltic also under future high levels of $p\text{CO}_2$, an amplification of effects on the predator species caused by food deficiency are not likely, although mismatch-situations in development could cause this effect. It has been shown the intensity of predation on the bivalve *Macoma balthica* to increase with increases in seawater temperature (Philippart *et al.* 2003). This is considered due to a mismatch in settlement of juveniles and peak of shrimp abundance. Similarly, mismatch-situations could increase the effects of seawater acidification on single species in the benthic habitat of the Western Baltic.

Although ecological responses caused by seawater acidification are likely, adaptation towards environmental stress is always possible. Sunday *et al.* (2011) have simulated evolutionary response to an increase in seawater acidification in larval stages of the sea urchin *Strongylocentrotus franciscanus* and the mussel *Mytilus trossulus* and found adaptation over generations towards seawater acidification possible. Also, multi-generational adaptation to other environmental stressors, such as an increase in seawater temperature is known to exist for tropical reef fish (Donelson *et al.* 2012). Positive carry-over effects have been shown for larvae from the oysters, which reacted with reduced growth, developmental speed and survival to direct exposure towards acidified water of $856 \mu\text{atm } p\text{CO}_2$, whereas when larvae stemmed from adults, which were previously exposed to the same level of acidification, growth and developmental speed even increased compared to larvae from control levels and survival was at equal levels (Parker *et al.* 2012). Further, selective breeding has been shown to allow populations of oysters to adapt to seawater acidification (Parker *et al.* 2011, Parker *et al.* 2012). Form and Riebesell (2012) have observed acclimation of one generation of cold water corals exposed to a seawater $p\text{CO}_2$ of ca. $1000 \mu\text{atm}$ for six months. A similar potential for adaptation over single or multiple generations is likely to be found in other organisms.

Fig. 6 shows an overview of the influences on predator-prey interactions in the macrozoobenthos of the Western Baltic under conditions of seawater acidification.

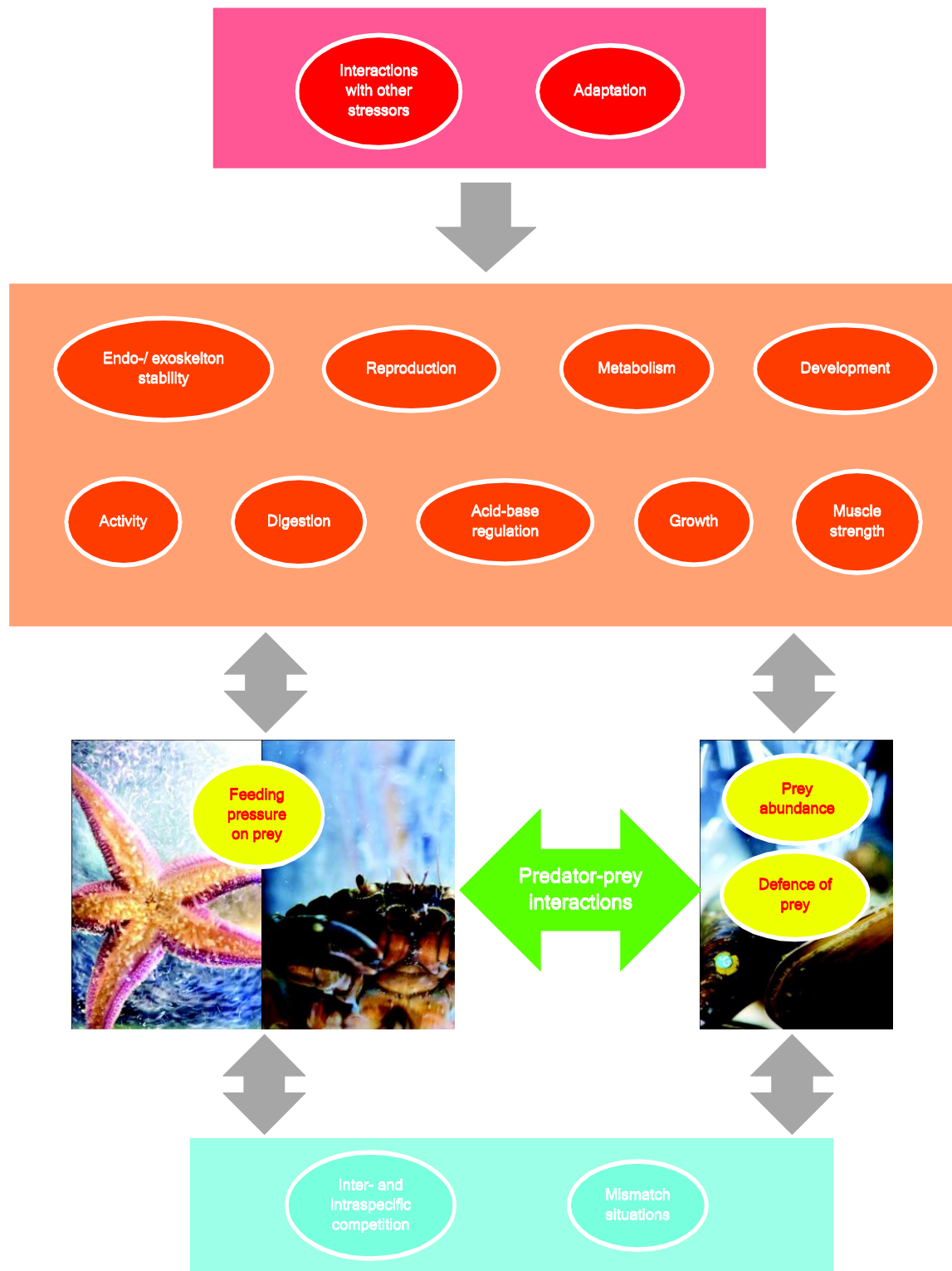


Fig. 6 Predator-prey interactions under conditions of seawater acidification and factors known or likely to be influencing them. Orange: parameters of influence on the species level with red: factors to likely influence these parameters, blue: factors on the ecosystem level.

Conclusion

I conclude that the feeding pressure on *M. edulis* may change with future high levels of seawater pCO₂ with a reduction of mussel consumption by the sea star *A. rubens*, while feeding pressure by the shore crab *C. maenas* is likely to remain largely unchanged. This is mostly due to a reduction in sea star growth and consumption rates. These changes may, in the long run, have effects on ecosystem responses. Nevertheless, adaptation of organisms to acidified conditions is possible.

This work demonstrates that anthropogenically caused seawater acidification can have a significant influence on predator-prey interactions and thus has the potential to re-shape the benthic ecosystem of the Western Baltic.

Outlook

Future studies should investigate the exact mechanisms of reduced feeding observed in many invertebrate species exposed to seawater acidification (Bamber 1987, Siikavuopio *et al.* 2007, Stumpp *et al.* 2012), as was in the sea stars in these studies.

Additionally, other life stages of the investigated predator species should be investigated to specifically identify bottlenecks in ontogeny under future high levels of seawater pCO₂.

To further investigate the effects of climate change on the Baltic ecosystem, multi-factorial experiments should investigate possible effects of the predicted warmer temperatures and lower salinities (BACC author team 2008) alongside with seawater acidification, ideally in multi-year mesocosm experiments.

Also a direct comparison of the interactions between *A. rubens*, *C. maenas* and *M. edulis* stemming from the Baltic Sea with its high fluctuations in seawater pCO₂ and the same species stemming from a habitat with less fluctuations e.g. the North Sea would promise further clarification on possible adaptation mechanisms of marine organisms towards seawater acidification.

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 2007 Studentische Hilfskraft für die ISOS (Integrated School of Ocean Sciences). Entwicklung und Verwirklichung eines Lernmoduls in Biostatistik für die E-learning Plattform der ISOS.

Poster und Vorträge:

Appelhans Y. S., Lenz M. (2008) Partners Wanted: The International Research Program GAME Establishes a Unique Global Network in Marine Benthic Ecology. Poster presentation: Benthic Ecology Meeting 2008, Providence, Rhode Island, U.S.A.

Appelhans Y. S., Lenz M., Medeiros H. E., da Gama B. A. P., Wahl M. (2008) Metabolites for Nothing and Defense for Free? The Influence of Low-Light Stress on Defensive Traits against Herbivory in Four Brazilian Macroalgae. Poster presentation: Benthic Ecology Meeting 2008, Providence, Rhode Island, U.S.A.

Appelhans Y. S., Thomsen J., Pansch C., Melzner F., Wahl M. (2009) Sour times for hungry starfish - Feeding rates of *Asterias rubens* are significantly reduced by seawater acidification. Poster presentation: Training Workshop on the fundamentals of carbon biogeochemistry, Bergen, Norway

Pansch C., Nasrolahi A., Appelhans Y. S., Melzner F., Wahl M. (2009) The neglected bottleneck: Early life stage ecology in times of global change. Cluster Retreat "The Future Ocean", Salzau, Germany

Appelhans Y. S., Pansch C., Melzner F., Wahl M. (2009) Sour times for benthic predators: influences of seawater acidification on the feeding behavior of *Carcinus maenas* and *Asterias rubens*. Oral presentation: 44th European Marine Biology Symposium (EMBS), Liverpool, UK

Pansch C., Nasrolahi A., Appelhans Y. S., Melzner F., Wahl M. (2009) Ocean acidification and warming: consequences for various life stages of the barnacle *Amphibalanus improvisus*. Poster presentation: 44th European Marine Biology Symposium (EMBS), Liverpool, UK

Appelhans Y. S., Thomsen J., Pansch C., Melzner F., Wahl M. (2010) Young and sour - Seawater acidification influences feeding and growth of juvenile sea stars. Poster presentation: 45th European Marine Biology Symposium (EMBS), Edinburgh, UK

Pansch C., Nasrolahi A., Appelhans Y. S., Melzner F., Wahl M. (2010) Possible pre-adaptation in marginal habitats - The barnacle *Amphibalanus improvisus* tolerates high levels of ocean acidification. Oral presentation: 45th European Marine Biology Symposium (EMBS), Edinburgh, UK

Appelhans Y. S., Pansch C., Thomsen J., Nasrolahi A., Melzner F., Wahl M. (2010) Sour life - The influence of seawater acidification on different life stages of benthic organisms from the Baltic Sea. Poster presentation: Joint EPOCA, BIOACID and UKOARP meeting, Bremerhaven, Germany

Erklärung

Hiermit erkläre ich gemäß § 9, dass ich diese Abhandlung, abgesehen von der Beratung durch meinen Betreuer, nach Form und Inhalt selbständig erarbeitet habe und keine anderen als die von mir aufgeführten Quellen und Hilfsmittel verwendet wurden.

Diese Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde weder ganz noch in Teilen an anderer Stelle im Rahmen eines Prüfungsverfahrens eingereicht.

Kiel, Juni 2012

Yasmin Appelhans