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Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*

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Abstract Marine organisms are exposed to increasingly acidic oceans, as a result of equilibration of surface ocean water with rising atmospheric CO₂ concentrations. In this study, we examined the physiological response of *Mytilus edulis* from the Baltic Sea, grown for 2 months at 4 seawater pCO₂ levels (39, 113, 243 and 405 Pa/385, 1,120, 2,400 and 4,000 μatm). Shell and somatic growth, calcification, oxygen consumption and NH₄⁺ excretion rates were measured in order to test the hypothesis whether exposure to elevated seawater pCO₂ is causally related to metabolic depression. During the experimental period, mussel shell mass and shell-free dry mass (SFDM) increased at least by a factor of two and three, respectively. However, shell length and shell mass growth decreased linearly with increasing pCO₂ by 6–20 and 10–34%, while SFDM growth was not significantly affected by hypercapnia. We observed a parabolic change in routine metabolic rates with increasing pCO₂ and the highest rates (+60%) at 243 Pa. NH₄⁺ excretion rose linearly with increasing pCO₂. Decreased O:N ratios at the highest seawater pCO₂ indicate enhanced protein metabolism which may contribute to intracellular pH regulation. We suggest that reduced shell growth under severe acidification is not caused by (global) metabolic depression but is potentially due to synergistic effects of increased cellular energy demand and nitrogen loss.

Introduction

Equilibration of surface ocean waters with increasing atmospheric CO₂ concentrations is causing an increase in seawater pCO₂ (hypercapnia) and a shift in the marine carbonate system towards lower pH values and lower carbonate (CO₃²⁻) ion concentrations (Cao and Caldeira 2008). While ocean acidification is a global phenomenon, seasonal effects, such as upwelling of hypoxic and hypercapnic waters, are superimposed onto slowly progressing acidification in many coastal habitats, e.g. along the Pacific coastline and in the Western Baltic Sea (Feely et al. 2008). High seawater pCO₂ values of >200 Pa (100 Pa = 987 μatm) can be encountered in Kiel Fjord surface waters during summer and autumn (Thomsen et al. 2010). Additionally, brackish habitats such as the Baltic Sea exhibit lower [CO₃²⁻] and, consequently, lower calcium carbonate saturation states (Ω) when compared to the open ocean. Baltic seawater is often undersaturated with respect to aragonite even at low seawater pCO₂. Both low Ω and seasonal acidification events are conditions that might be potentially adverse for shell growth and calcification of mussels (Fabry et al. 2008). Nevertheless, blue mussels (*Mytilus edulis*) can be found in high densities in Kiel Fjord (Enderlein and Wahl 2004), and juvenile settlement of this species occurs when highest seawater pCO₂ values are encountered in summer (Thomsen et al. 2010). This implies a certain resistance of *M. edulis* to acidified seawater.

Previous work with the mussel *M. galloprovincialis* has simultaneously determined growth and respiration and revealed a depression of both parameters in response to long-term (13 weeks) hypercapnic stress of about 500 Pa (Michaelidis et al. 2005). The reduction in physiological performance was concluded to be causally related to an uncompensated extracellular acidosis

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(Michaelidis et al. 2005). During short-term exposure, calcification in *M. edulis* has been found to decrease linearly with increasing $p\text{CO}_2$, and net shell dissolution has been observed at levels above 180 Pa (Gazeau et al. 2007).

It has been hypothesized that hypercapnia elicits a reduction in aerobic metabolism in marine organisms (metabolic depression) as a result of acid–base disturbances (Pörtner et al. 2004). Whereas intracellular pH (pHi) decreases in marine metazoans are typically compensated rapidly, extracellular pH (pHe) remains uncompensated in some species and has been hypothesized to be the trigger for metabolic depression (Pörtner et al. 1998). Several studies, performed under a broad range of seawater $p\text{CO}_2$ (100–1,000 Pa), seem to confirm the reductions of oxygen consumption under elevated $p\text{CO}_2$ in different invertebrate taxa (Langenbuch and Pörtner 2002; Michaelidis et al. 2005; Rosa and Seibel 2008). However, unchanged or even increased oxygen consumption has been observed in some species as well (Gutowska et al. 2008; Wood et al. 2008; Melzner et al. 2009; Munday et al. 2009; Comeau et al. 2010). Studies which investigated calcification under elevated $p\text{CO}_2$ obtained similar heterogeneous responses; reduced, conserved, or even increased calcification rates have been measured in different phyla (Michaelidis et al. 2005; Berge et al. 2006; Gutowska et al. 2008; Wood et al. 2008; Ries et al. 2009; Comeau et al. 2010). Maintained (and increased) rates of calcification under hypercapnia may be causally related to partial or full pHe compensation, as the pHe regulatory response results in an accumulation of extracellular bicarbonate. This might alter the transport kinetics of HCO_3^- across calcifying epithelia or directly increase Ω at the site of calcification. Increased calcification has so far only been observed in species that perform strong pHe regulatory responses (cephalopods: Gutowska et al. 2008, 2009, decapod crustaceans: Ries et al. 2009, fish: Checkley et al. 2009). In contrast, *M. edulis* does not actively elevate extracellular $[\text{HCO}_3^-]$ above seawater $[\text{HCO}_3^-]$ and does not compensate pHe (Thomsen et al. 2010).

Responses of mussel metabolism towards various short-term abiotic stressors, such as rapid salinity change, hypercapnia, or toxin exposure, have been the focus of several studies (Lindinger et al. 1984; Tedengren and Kautsky 1986). Generally, mussels respond with a decreased O:N ratio, as a result of either decreased metabolism, increased NH_4^+ excretion rates or a combination of both (Lindinger et al. 1984; Tedengren and Kautsky 1987; Michaelidis et al. 2005). In general, O:N ratios are considered a common indicator for the proportion of the three metabolic substrates, carbohydrates, lipids and proteins, used in energy metabolism (Mayzaud and Conover 1988). Although absolute values vary for different species, regions and seasons, lower O:N ratios indicate a higher fraction of protein metabolism,

whereas a higher ratio indicates an elevated turnover of carbohydrates and lipids (Mayzaud and Conover 1988).

This study was performed in order to estimate the long-term effects of elevated seawater $p\text{CO}_2$ levels on the parameters of shell and somatic growth as well as aerobic metabolism in Baltic blue mussels (*Mytilus edulis*). Specifically, we tested the hypothesis whether elevated $p\text{CO}_2$ elicits metabolic depression at realistic levels of seawater acidification. For this purpose, we investigated shell length and mass growth, somatic growth, respiration and NH_4^+ excretion rates of mussels following a 2-month acclimation period at 4 different seawater $p\text{CO}_2$ levels under optimized feeding conditions.

Materials and methods

Animal collection and experimental set-up

Subtidal *Mytilus edulis* individuals were sampled from Kiel Fjord (54°19.8'N; 10°9.0'E) at a water temperature of 10.6°C on 26 October 2009. Thirteen equally sized mussels were randomly placed into each of 16 experimental aquaria. Morphometric data were measured for a subsample of mussels ($n = 12$). Mean shell length and shell mass at the start of the experimental incubation were 15 ± 0.2 mm and 109 ± 19 mg, respectively. Total fresh mass at the beginning of the incubation period was 1.6 ± 0.04 g aquarium⁻¹. During the acclimation phase, low mortality was observed, in total six mussels died (3% mortality) 1 at 38 Pa, 2 at 113 Pa and 3 at 243 Pa.

Experimental procedure

The long-term acclimation of the mussels was performed in a flow-through seawater set-up. Mussels were incubated in 16 aquaria (volume = 16 l) that were supplied with filtered and UV-radiated seawater from a single header tank. Various seawater $p\text{CO}_2$ in the experimental aquaria (4 replicate aquaria per treatment) were achieved by aeration with CO_2 -enriched air; $p\text{CO}_2$ levels were 39, 113, 243 and 405 Pa (380, 1,120, 2,400 and 4,000 μatm). Flow rates from a header tank into the experimental aquaria were adjusted to 50 ml min⁻¹ aquarium⁻¹. Seawater perfusing the experimental aquaria contained a *Rhodomonas* suspension enabling continuous food supply. *Rhodomonas* were cultured in Provasolis medium II according to Ismar et al. (2008) in 0.2- μm -filtered N (0.55 mmol l⁻¹) and P (0.036 mmol l⁻¹)-enriched seawater. Algae concentration was monitored at weekly intervals using a Coulter Counter (Z2, Beckman CoulterTM). Cell densities were $5,955 \pm 3,889$ cells ml⁻¹ in the inflowing water and $2,330 \pm 482$ cells ml⁻¹ in the outflow of the

aquaria. In all aquaria, algae concentration never fell below $1,500 \text{ cells ml}^{-1}$. The mussels were simultaneously cultured in the 4 CO_2 treatments for 8 weeks between October and December 2009.

Measurements of physiological rates were performed on subsequent days from December 16 to 20, and additional NH_4^+ excretion rates were determined on December 10. Following experimentation, mussel morphometric parameters were assessed. Mussel shell length was measured with an accuracy of 0.1 mm. Shell mass and shell-free dry mass (SFDM) were measured using a balance with an accuracy of 0.1 mg after mussels were dried in an oven at 80°C for 24 h.

Water chemistry parameter monitoring

Water pH_{NBS} , salinity and temperature were monitored daily in the set-up during the entire incubation period using a WTW 340i pH-analyzer and a WTW SenTix 81-electrode for pH and WTW cond 315i salinometer and a WTW TETRACON 325 probe for salinity and temperature. Water parameters measured during the growth period are displayed in Table 1. pH_{NBS} values were stable and differed between pCO_2 treatments over the entire experimental duration. During the 8-week incubation period, water temperature decreased in the aquaria due to winter cooling of the Fjord water, from 9.7 to ca. 8°C , whereas salinity was less variable at values around $18.1 \pm 1.0 \text{ g kg}^{-1}$.

Total alkalinity (A_T) and total dissolved inorganic carbon (C_T) were analysed in water samples taken from the experimental aquaria during the growth phase and in the respiration set-up using VINDTA and SOMMA autoanalyzers (Dickson et al. 2007). Measured values were corrected for instrument shift using DICKSON seawater standard as reference material (Dickson et al. 2003). Carbonate system parameters were calculated from measured A_T and C_T values using CO2SYS software (Lewis and Wallace 1998). Dissociation constants K_1 and K_2 were chosen according to Mehrbach et al. (1973) as refitted by Dickson and Millero (1987) and KHSO_4 dissociation constant after Dickson (1990).

Measurements of oxygen consumption and ammonium excretion

Respiration and NH_4^+ excretion rate measurements were performed at 8.3°C in 100-ml plexiglas chambers which were

placed in a 100-l water bath. UV-sterilized and $0.2\text{-}\mu\text{m}$ -filtered seawater was equilibrated with a specific pCO_2 for 24 h before and during the measurements. Four replicate chambers (3 replicates with mussels, one chamber without mussels serving as a bacterial control) were combined in four measuring circuits using gas-tight Tygon tubing (R-3603, Saint-Gobain, France). Each chamber contained 10 randomly chosen mussels from one replicate aquarium. Transfer from aquaria to the chambers lasted less than 1 min, and measurements started after half an hour of acclimation to experimental conditions. During the incubation period in the respiratory chambers, mussel valves remained open.

Oxygen consumption (MO_2)

MO_2 of *M. edulis* was measured using an intermittent-flow system. Water inside the respirometry chambers was continuously circulated using a peristaltic pump (MCP ISM 404, ISMATEC, Switzerland) at a rate of 2 ml s^{-1} . Oxygen concentrations were recorded every 5 min using fibre-optic oxygen sensors (needle-type optodes, Presens, Regensburg, Germany) inserted into the tubing of the measuring circuit via a plastic y-piece. One measuring interval lasted 45 min. Between measuring intervals, chambers were flushed with seawater from the water bath for 3 h using 5 W submersible pumps (Eheim, Deizisau, Germany). The whole incubation lasted for 20 h per pCO_2 treatment, with 6 MO_2 determinations per replicate chamber. NH_4^+ concentrations in the water bath were always below one $\mu\text{mol l}^{-1}$ at the end of an incubation.

For calculation of oxygen consumption rates, the linear decrease in oxygen concentration during measuring intervals between 5 and 35 min was considered; $[\text{O}_2]$ maximally decreased to 65% air saturation (average: 74%, see Fig. 1 for typical respiration traces). The observed decrease in oxygen concentration resulted in an increase in C_T and therefore pCO_2 in the closed chambers. Depending on oxygen consumption rates in the different pCO_2 treatments, seawater pCO_2 in the chambers increased by 4–18 Pa (<10% of the initial pCO_2) during incubations. The results of 6 separate runs per chamber measured during the whole incubation interval were averaged. Over the course of 20-h incubation, consumption rates slightly decreased in the last runs at all pCO_2 levels. Molar oxygen consumption rates (MO_2) are expressed as $\mu\text{mol O}_2 \text{ g}^{-1} \text{ SFDM h}^{-1}$.

Table 1 Carbonate system parameters during 2-month incubation in the four pCO_2 treatments. Values display means and standard deviation

pCO_2 treatment (Pa)	pH_{NBS}	A_T ($\mu\text{mol kg}^{-1}$)	C_T ($\mu\text{mol kg}^{-1}$)	pCO_2 (Pa)	Ω_{ca}	Ω_{ar}
39	8.03 ± 0.04	$2,044.6 \pm 52.4$	$1,968.9 \pm 21.7$	47.8 ± 5.3	1.86 ± 0.20	1.11 ± 0.12
113	7.70 ± 0.04	$2,040.7 \pm 23.4$	$2,031.1 \pm 27.3$	92.2 ± 6.9	1.02 ± 0.06	0.61 ± 0.04
243	7.38 ± 0.06	$2,051.0 \pm 29.2$	$2,124.1 \pm 13.9$	204.3 ± 26.6	0.50 ± 0.07	0.30 ± 0.04
405	7.14 ± 0.08	$2,044.3 \pm 22.2$	$2,213.9 \pm 26.7$	377.8 ± 24.1	0.27 ± 0.02	0.16 ± 0.01

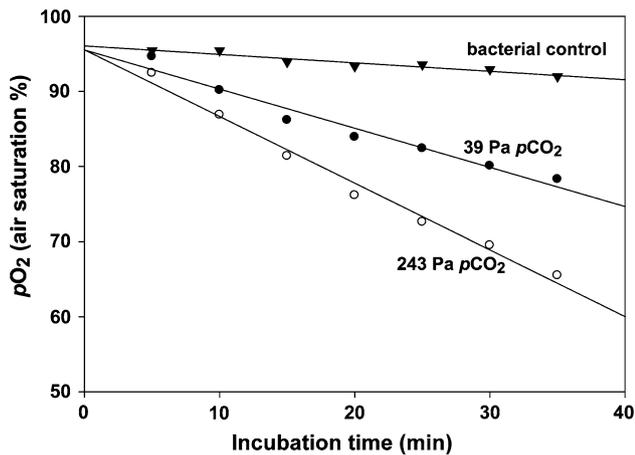


Fig. 1 Representative courses of pO_2 in the respiration chambers during measuring intervals between 5 and 35 min. *Triangles* represent the pO_2 change in a bacterial control. *Black and white dots* represent courses for mussels of the 39 and 243 Pa pCO_2 treatments, respectively

Ammonium excretion

Seawater ammonium (NH_4^+) concentrations were determined prior to and following the respiration trials. An initial 10-ml water sample was taken from every chamber before closing for oxygen consumption measurements, and after 1 h incubation, a second sample was taken. Additionally, excretion rates were assessed in a stop-flow experiment during the growth period 1 week earlier (December 10). Seawater flow-through was stopped, and mussels were incubated in closed aquaria. Following 8.5 h of incubation, 10 ml of water samples was taken from each replicate aquarium.

Ammonium concentrations were determined according to Holmes et al. (1999). 2.5 ml of a reagent were added to the water samples that contains orthophthaldialdehyde, sodium sulphite and sodium borate. After 2-h incubation, samples were measured using a Kontron SFM25 fluorometer at an excitation and emission wavelength of 360 and 422 nm, respectively. Ammonia (NH_3) was not measured since its concentration is negligible (Körner et al. 2001). At seawater pH values of 8–7.1, NH_3 concentrations are in the range of 0.2–2% of total $[NH_3 + NH_4^+]$. Ammonium excretion rates ($NH_4^+_{ex}$) are expressed as $\mu\text{mol } NH_4^+ \text{ g}^{-1} \text{ SFDM h}^{-1}$.

Calculation of O:N ratio and metabolic energy loss

O:N ratio

The atomic ratio of oxygen uptake and excreted nitrogen was calculated from MO_2 and $NH_4^+_{ex}$.

$$O : N = MO_2 NH_4^+_{ex}^{-1}$$

Metabolic energy loss

Aerobic energy loss was calculated using a mean oxyca-
loric equivalent of $0.44 \text{ J } \mu\text{mol } O_2^{-1}$ representing a mixed, but 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 $0.347 \text{ J } \mu\text{mol}^{-1} NH_4^+$ according to Elliott and Davison (1975). Total metabolic energy loss (E_{NET} , $\text{J g}^{-1} \text{ SFDM h}^{-1}$) was calculated from aerobic energy metabolism and nitrogen excretion:

$$E_{NET} = MO_2 \times 0.44\text{J} + NH_4^+_{ex} \times 0.347\text{J}$$

Statistics

Data were analysed by quadratic or linear regression using SIGMA PLOT 10. Data sets were analysed for normality and Cook's distance. ANCOVA (STATISTICA 8) was performed to compare NH_4^+ excretion rates obtained by the two different methods. All graphically represented values are means of pooled sub-replicates (from 4 replicated aquaria of each treatment). The error in the equations of regression equations is the standard error of the mean.

Results

M. edulis were continuously fed with a *Rhodomonas* suspension which enabled high growth. Mean shell length of control mussels increased from 15.0 ± 0.2 to 22.9 ± 0.3 mm, thus by about 7.9 ± 0.3 mm (Fig. 2a). Mussels from higher pCO_2 treatments displayed reduced growth. With increasing pCO_2 , shell length decreased linearly. Shell length increment of 113- and 405-Pa-treated mussels was reduced to 7.4 ± 0.9 and 6.3 ± 0.3 mm, respectively. This corresponds to shell length growth reductions of 6–20% compared to control mussels.

Similar results were obtained for *M. edulis* shell mass growth. Mussels from all treatments accreted shell mass. From an initial mass of 109 ± 19 mg, 319 ± 6.6 mg was reached in the 39 Pa treatment, corresponding to an increase of 290% (Fig. 2b). Similar to shell length, shell mass increment decreased linearly with increasing pCO_2 . Final shell mass ranged from 297 ± 21.5 to 243.7 ± 15.5 mg between the 113 and 405 Pa treatments. Thus, shell mass growth rates were reduced by 10–36% with elevated pCO_2 . SFDM increased from 17.8 ± 5.5 mg to mean values of 56–60.3 mg with no significant differences between pCO_2 groups (Fig. 2c). Thus, somatic growth of

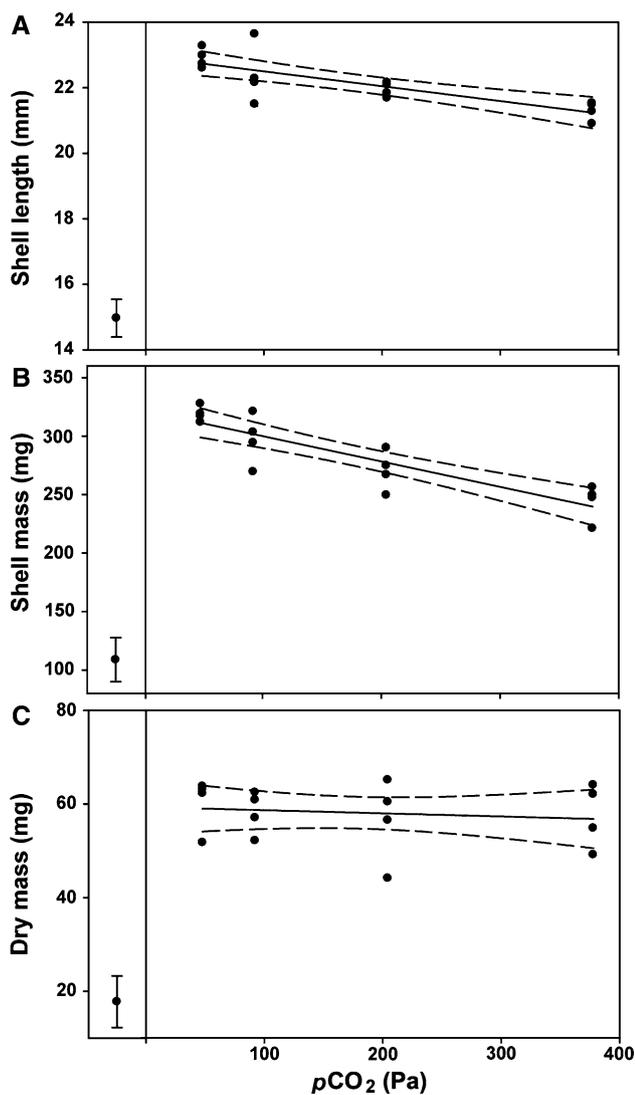


Fig. 2 Shell length (a), shell mass (b) and shell-free dry mass (SFDM)(c) of *M. edulis* grown at 4 different $p\text{CO}_2$ treatments for 2 months. Each dot represents the mean of the mussels from a replicate aquarium. *Black lines* give linear function of shell length and shell mass. *Long dashed lines* are 95% confidence intervals. The single point left of the vertical line displays the initial shell length, shell mass and SFDM. **a** Shell length = $-0.0045(\pm 0.001) p\text{CO}_2 + 22.9544(\pm 0.2106)$, $R^2 = 0.6177$, $F = 22.6244$, $P < 0.01$; **b** Shell mass = $-0.2170(\pm 0.032) p\text{CO}_2 + 321.7090(\pm 7.01)$, $R^2 = 0.7532$, $F = 46.7735$, $P < 0.01$; **c** SFDM = $-0.0067(\pm 0.0124) p\text{CO}_2 + 59.3475(\pm 2.748)$, $R^2 = 0.0203$, $F = 0.2906$, $P = 0.598$; $N = 16$

M. edulis was not significantly reduced by elevated seawater $p\text{CO}_2$.

Effects of long-term hypercapnia on oxygen consumption of *M. edulis* are shown in Fig. 3a: mussels raised at a control $p\text{CO}_2$ of 39 Pa displayed mean oxygen consumption rates of $19.8 \pm 1.7 \mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$. Increasing $p\text{CO}_2$ led to a change in respiration rates which followed a

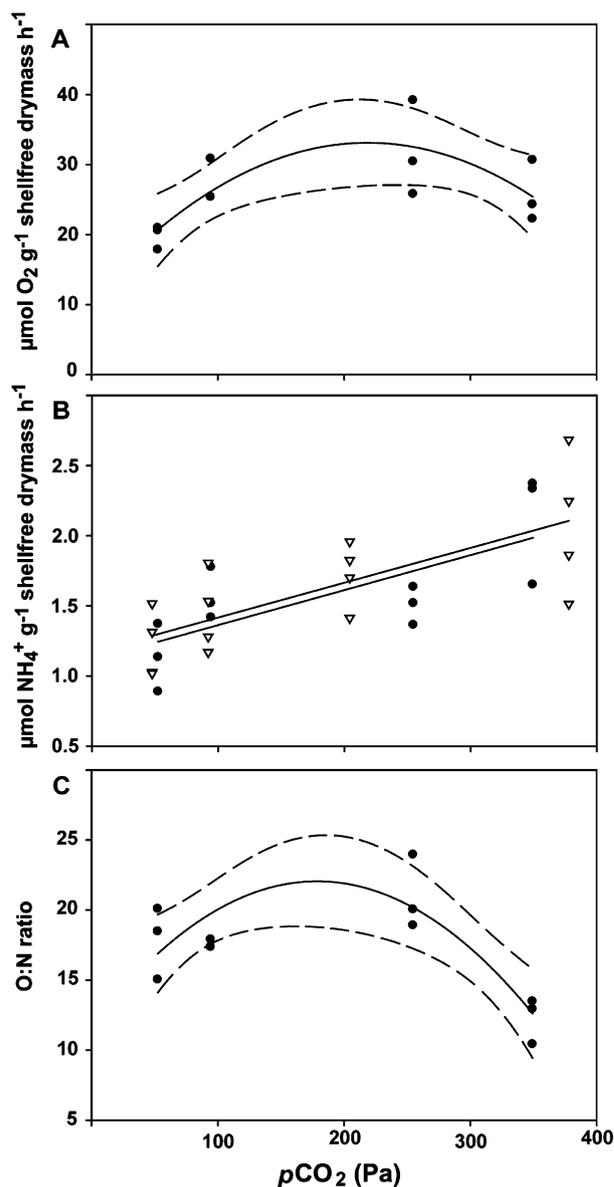


Fig. 3 Oxygen consumption (a), NH_4^+ excretion (b) and O:N ratio (c) of *M. edulis* as a function of 4 different $p\text{CO}_2$ levels. **a** Dots display means of 6 repeated measurements of 10 mussels from every replicate aquarium. *Long dashed lines* give 95% confidence intervals. $-0.0005(\pm 0.0002) \times p\text{CO}_2^2 + 0.1971(\pm 0.0656) \times p\text{CO}_2 + 11.5505(\pm 4.7267)$, $R^2 = 0.458$, $F = 5.2253$, $P < 0.05$, $N = 11$; **b** Dots represent measurements of ammonium excretion in the respiration trial. $\text{NH}_4^+ = 0.0025(\pm 0.0007) p\text{CO}_2 + 1.113(\pm 0.1668)$, $R^2 = 0.4788$, $F = 11.1054$, $P < 0.01$, $N = 12$. Triangles represent ammonium excretion in the stop-flow experiment $0.0025(\pm 0.0006) p\text{CO}_2 + 1.1681(\pm 0.1361)$, $R^2 = 0.5041$, $F = 16.2457$, $P < 0.01$, $N = 16$; **c** The ratio was calculated from measured molar oxygen consumption and ammonium excretion rates. *Long dashed lines* give the 95% confidence interval. $\text{O:N} = -0.0003(\pm 0.00009) \times p\text{CO}_2^2 + 0.1159(\pm 0.0352) \times p\text{CO}_2 + 11.6953(\pm 2.533)$, $R^2 = 0.6089$, $F = 8.7858$, $P < 0.01$, $N = 11$

quadratic function (see figure caption for regression). Measured respiration rates peaked in the 243 Pa treatment. A further elevation in $p\text{CO}_2$ to 405 Pa led to decreased

oxygen uptake, but still slightly elevated MO_2 when compared to the control condition.

A linear correlation between sea water pCO_2 and NH_4^+ excretion was found. Mussels placed in the respiration chambers displayed mean NH_4^+ excretion rates of $1.13 \pm 0.24 \mu\text{mol } NH_4^+ \text{ g}^{-1} \text{ h}^{-1}$ in the control treatment (Fig. 3b). When mussels were subjected to higher seawater pCO_2 , NH_4^+ excretion increased to values of 2.12 ± 0.41 in the 405 Pa treatment. Mussel NH_4^+ excretion measured in the stop-flow experiment resulted in similar mean values (control: 1.22 ± 0.24) and an identical slope of NH_4^+ excretion increase with increasing water pCO_2 (ANCOVA, $F_{(1,25)} = 0.2034$, $P = 0.656$).

Oxygen uptake and nitrogen excretion measurements were used to calculate the O:N ratio of the mussels. In the lower pCO_2 treatments, the O:N ratio increased slightly with rising pCO_2 as a result of increasing oxygen uptake and reached a maximum in the 243 Pa treatment (Fig. 3c). In the highest treatment (405 Pa), O:N values decreased due to decreasing respiration rates despite elevated NH_4^+ excretion. The mean O:N values for control mussels were 17.9 ± 2.6 and 21 ± 2.7 at 243 Pa. Mussels from the 405 Pa treatment were characterized by O:N values of 12.3 ± 1.6 .

Calculations of the energy lost by respiration and NH_4^+ excretion per g dry mass and h are displayed in Table 2. Correlated to rising oxygen consumption rates, the amount of energy lost by the experimental animals increased at elevated pCO_2 . Control mussels lost a total of $9.10 \pm 0.82 \text{ J g}^{-1} \text{ SFDM h}^{-1}$ of energy, which slightly increased in the treatments up to 243 Pa and started to decrease at 405 Pa. The linear rise of NH_4^+ excretion at elevated pCO_2 resulted in a rising energy loss from 0.39 ± 0.08 to $0.74 \pm 0.14 \text{ J g}^{-1} \text{ h}^{-1}$ in the 39 and 405 Pa treatments, respectively. Due to the different order of magnitude between the two fluxes, increased nitrogen excretion only had a minor impact on the total energy loss (Table 2). Energy loss by ammonium excretion was calculated to contribute ca. 5% to total energy loss.

Table 2 Metabolic energy loss ($\text{J g}^{-1} \text{ SFDM h}^{-1}$) by *M. edulis* subjected to 4 pCO_2 treatments

pCO_2 treatment (Pa)		39	113	243	405
Energy loss by respiration ($\text{J g}^{-1} \text{ h}^{-1}$)	Mean	8.71	12.36	13.99	11.32
	sd	0.75	1.70	2.99	1.93
Energy loss by NH_4^+ excretion ($\text{J g}^{-1} \text{ h}^{-1}$)	Mean	0.39	0.55	0.52	0.74
	sd	0.08	0.06	0.05	0.14
Total energy loss ($\text{J g}^{-1} \text{ h}^{-1}$)	Mean	9.10	12.92	14.51	12.06
	sd	0.82	1.79	3.04	2.03
Fraction of NH_4^+ excretion of total energy (%)	Mean	4.30	4.29	3.67	6.12
	sd	0.63	0.09	0.42	0.82

Discussion

Long-term acclimation of *Mytilus edulis* from the Western Baltic Sea to elevated seawater pCO_2 resulted in an increase in aerobic metabolic rates during moderate hypercapnia, rather than metabolic depression. As our companion study demonstrated that *M. edulis* does not control pHe when exposed to seawater acidification (Thomsen et al. 2010), it is clear that there is no causal relationship between metabolic depression and extracellular acid–base status under moderate acidification scenarios. Oxygen consumption and NH_4^+ excretion in hypercapnic treatments remained elevated above control rates, whereas shell growth declined during 2 months of incubation under elevated pCO_2 . Our control respiration and NH_4^+ excretion rates were in the range of published results for *Mytilus* spp. (Tedengren et al. 1990; Okumus and Stirling 1994; Michaelidis et al. 2005).

Up to a certain seawater pCO_2 , mussels increase metabolic rates (<243 Pa). Beyond this pCO_2 , metabolism starts to decrease but remains above control rates. Similar effects of hypercapnia on respiration rates have already been observed for other marine taxa at comparable pCO_2 levels, such as an ophiuroid echinoderm and tropical teleost fish species (Wood et al. 2008; Munday et al. 2009; Comeau et al. 2010). In contrast, Michaelidis et al. (2005) reported a drop of respiration rates for *Mytilus galloprovincialis* during short- and long-term incubation at a pCO_2 of ca. 500 Pa. These findings are not contradictory to our results since the course of oxygen consumption in our experiment described a downward trend at higher pCO_2 . Thus, it is possible that at $pCO_2 > 405$ Pa, a downregulation of MO_2 may be encountered. During prolonged emersion or in a hypoxic/anoxic environment, extracellular pCO_2 values in bivalve extracellular fluids rise and are coupled with decreases in pO_2 in the seawater encased by the closed shell valves (Famme 1980; Wang and Widdows 1993). Therefore, downregulation of energy demand and aerobic metabolism at very high pCO_2 may result from evolutionary adaptation of mussels to fluctuating pCO_2 in intertidal habitats. During 8-h air exposure at 12°C, mussel haemolymph pH decreased by 0.4 units, and at the same time, haemolymph pCO_2 raises up to 440 Pa (Booth et al. 1984). Highly elevated extracellular pCO_2 could serve as a signal for cellular metabolic depression, a process that prevents fatal thermodynamic imbalances and cell death during short/intermediate-term abiotic stress (Guppy and Withers 1999).

O:N ratios around 20 indicate that amino acid catabolism dominates Baltic Sea *M. edulis* aerobic metabolism. Pure protein metabolism can be expected at a ratio of 3–16, balanced lipid and protein degradation occurs at O:N = 50–60 (Mayzaud and Conover 1988). This is in agreement with the results of studies that compared Baltic and

North Sea *M. edulis* metabolism. These studies revealed lower O:N ratios in Baltic mussels, resulting from increased NH_4^+ excretion as a consequence of osmoregulatory demands (Tedengren and Kautsky 1986; Tedengren et al. 1990). It has been hypothesized that this lowered ratio explains reduced growth rates of Baltic mussels in the field due to enhanced unfavourable protein metabolism. Higher amino acid metabolism is energetically less efficient compared to turnover of carbohydrate and lipid as it implies a continuous energy loss by enhanced excretion of, in the case of mussels, ammonium (Tedengren and Kautsky 1986). Additionally, relatively low O:N ratios observed in this study may result from the high protein content of the diet which enabled high protein metabolism (Kreeger and Langdon 1993; Hatcher et al. 1997). The mussels of this study were fed with *Rhodomonas* sp., which is characterized by a high nitrogen content (Berggreen et al. 1988). However, increasing respiration and NH_4^+ excretion lead to an almost unchanged O:N ratio which indicates no dramatic proportional change in metabolic substrate choice in the treatments up to 243 Pa. Rather, parallel increases in metabolic rate and NH_4^+ excretion indicate an absolute rise of protein metabolism during hypercapnia. Only very high seawater $p\text{CO}_2$ (405 Pa) causes a marked reduction in the O:N ratio which might indicate a larger fraction of protein metabolism in these mussels.

Increased NH_4^+ excretion rates of mussels have been already observed by Lindinger et al. (1984) and Michaelidis et al. (2005) during short-term (20–24 h) incubation at $p\text{CO}_2$ between 500 and 4,000 Pa. Our results confirm these short-term findings for the long-term acclimation process in mussels subjected to elevated $p\text{CO}_2$. The authors of the former study proposed a contribution of the positively charged ion to proton excretion under elevated $p\text{CO}_2$ (Lindinger et al. 1984). Increased metabolic formation of NH_3 by protein breakdown and following extrusion as NH_4^+ can serve as an intracellular pH regulatory mechanism (Boron 2004). Increases in NH_4^+ excretion suggest a significant contribution of this mechanism to proton removal in *M. edulis*, as NH_4^+ excretion almost doubled at the highest $p\text{CO}_2$. This may have the added benefit that protein degradation probably supports HCO_3^- production and thereby pHi regulation as already suggested for sipunculids and the mussel *M. galloprovincialis* (Langenbuch and Pörtner 2002; Michaelidis et al. 2005). Studies that analysed NH_4^+ and CO_2 excretion in fish gill concluded that CO_2 excretion lowers pH in the boundary layer of the gill and thereby facilitates passive NH_3 diffusion (Wright et al. 1989; Wilkie 2002). Hypercapnia might have a similar effect on the boundary layer, thereby enabling higher nitrogen excretion rates. However, since in *Mytilus* spp. extracellular pH decreases during hypercapnia while pHi is maintained, this increases the driving force for H^+

flux into the intracellular space. In fact, high levels of seawater acidification even lead to a reversal of the proton gradient from the extra- to the intracellular space (Michaelidis et al. 2005). Thus, cellular acid extrusion effort needs to be upregulated (Boron 2004; Michaelidis et al. 2005). One possible pathway of NH_4^+ excretion is via primary active transport, e.g. by basolateral Na^+/K^+ -ATPase transporting NH_4^+ (instead of K^+) into epithelial cells (Wilkie 1997; Knepper 2008; Pagliarani et al. 2008). Subsequently, nitrogen extrusion from the intracellular compartment is performed either by diffusion of uncharged NH_3 or as charged NH_4^+ . In the first case, NH_3 is protonated in the seawater boundary layer on the apical side of the cells. This can be a result by either separate H^+ transport via V-type H^+ -ATPase and Na^+/H^+ exchanger or by hydration of CO_2 via carbonic anhydrase (Wilkie 2002; Kaloyianni et al. 2005; Nawata et al. 2007; Knepper 2008). In the latter case, NH_4^+ may be removed by an apical $\text{Na}^+/\text{NH}_4^+$ exchanger as described for marine fish (Wilkie 1997). Irrespective of the mechanism, active transport of base and acid equivalents across the cell membranes needs to be increased in order to achieve pHi compensation when pHe is decreased. This has been already reported for the energy budget in isolated perfused fish gill where energy expenditure for ion regulation rose during hypercapnia (Deigweiher et al. 2009).

The observed reduction in mussel shell growth rates is in accordance with the results of Michaelidis et al. (2005) and our own findings from a similar experiment performed in summer 2009 (Thomsen et al. 2010). We found significant CO_2 -induced reductions in shell length and shell mass growth at high $p\text{CO}_2$ (405 Pa). However, we did not find significant decreases in SFDM, indicating that somatic growth is not as strongly affected by elevated $p\text{CO}_2$ as the calcification process. The elevated ion regulatory activity necessary to achieve a new steady state for intracellular pH homeostasis may force *M. edulis* to allocate a surplus of energy to cellular ion and pH homeostasis. At elevated $p\text{CO}_2$ levels of 113 and 243 Pa, total energy expenditure in *M. edulis* rose by 42 and 58% above control level. Therefore, decreased shell growth may be a consequence of increased energy demand and, potentially, energy re-allocation during hypercapnia. Mussels shift towards protein/amino acid metabolism as indicated by an increase in NH_4^+ excretion. Increased energy loss cannot be balanced by an increased energy uptake by means of elevated filtration rates. *M. edulis* filtration rates remain on control levels at elevated seawater $p\text{CO}_2$ (Saphörster, Thomsen, Melzner, unpublished). In general, mussel shell growth rate does not only depend on the food energy content but is more closely correlated to the nitrogen/protein content (Kreeger and Langdon 1993; Kreeger et al. 1996). Increased protein turnover and thereby elevated loss of nitrogen as NH_4^+ at

higher $p\text{CO}_2$ cannot be compensated by increased nitrogen uptake by enhanced filtration activity during hypercapnia. Thus, mussel metabolism might be ultimately impaired by an increasing nitrogen limitation. The high growth and protein accretion efficiency of *M. edulis* mainly results from a high recycling rate of protein degradation products (Hawkins 1985; Hawkins et al. 1989; Bayne and Hawkins 1997). Therefore, decreased shell growth rates may in part also be related to increased protein breakdown and nitrogen loss since calcified structures of bivalve shells are enclosed by an organic matrix consisting of proteins and chitin (Matsushiro and Miyashita 2004; Addadi et al. 2006). A reduced capacity to form matrix structures might in turn have adverse effect on the deposition of calcium carbonate crystals. It has been previously described that elevated $p\text{CO}_2$ has an impact on protein turnover in several marine organisms leading to lowered synthesis or even enhanced degradation (Langenbuch and Pörtner 2003; Langenbuch et al. 2006; Wood et al. 2008). On the other hand, it cannot be excluded that the progressively more acidic environment of the extrapallial fluid (c.f. Thomsen et al. 2010), which is in direct contact with the newly formed nacre layers, negatively impacts the energetics of CaCO_3 deposition and/or organic matrix formation, or even the interaction between organic and mineral phase construction. Thus, the observed decreases in shell growth could be partly explained by higher maintenance requirements for cellular homeostasis and a shift towards unfavourable nitrogen cycling causing reduced protein accretion rates. As a consequence, calcification may not necessarily be affected exclusively by lowered $[\text{CO}_3^{2-}]$ in body fluids and ambient seawater but also in a secondary fashion by lowered net protein deposition.

According to our results, the general hypothesis of metabolic depression in mussels might only be valid for very high levels of hypercapnia and does not play a role at seawater $p\text{CO}_2$ values predicted for the most parts of the oceans within the next ca. 50–100 years (Cao and Caldeira 2008). At moderate seawater $p\text{CO}_2$ (<243 Pa), mussels try to compensate unfavourable abiotic conditions such as intracellular acid loads instead of decreasing their activity. Overall, mussel metabolism rises and shell growth rates decrease while somatic growth is preserved under elevated seawater $p\text{CO}_2$. This will be particularly important for the Kiel Fjord mussel population, which is exposed to very high seawater $p\text{CO}_2$ in summer and autumn. Seawater $p\text{CO}_2 > 200$ Pa have been recorded during summer and autumn 2008 as a result of upwelling of hypoxic–hypercapnic bottom waters. Synergistic effects of future ocean acidification and this natural phenomenon can potentially increase seawater $p\text{CO}_2$ to values above 400 Pa, given a doubling in surface $p\text{CO}_2$ by the year 2100 (see Thomsen et al. 2010). Additionally, elevated metabolic rates due to

ocean warming will probably challenge the energy budgets of *M. edulis* even more, leaving less energy for growth of calcified structures. It remains to be established to what degree energy allocation into reproduction will be compromised during exposure to hypercapnia as well. It has been already revealed that mussel reproduction is highly susceptible to environmental stress such as extreme temperatures and elevated $p\text{CO}_2$ (Petes et al. 2007; Whitman Miller et al. 2009; Gazeau et al. 2010). While the capacity of *M. edulis* to calcify at high rates in seawater highly undersaturated with CaCO_3 is astonishing, it is also likely that this feature is costly and may only be possible in eutrophic habitats. Future studies clearly need to address (i) the role of energy supply to maintain high rates of growth and calcification under elevated seawater $p\text{CO}_2$ and (ii) identify the cellular processes that lead to increased metabolic demands. It is likely that the combined action of elevated temperature and high seawater $p\text{CO}_2$ can negatively impact the fitness of *M. edulis* in coastal upwelling habitats in the next decades.

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