Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*

Basile Michaelidis[1],*, Christos Ouzounis[1], Andreas Paleras[1], Hans O. Pörtner[2]

[1]Laboratory of Animal Physiology, Department of Zoology, Faculty of Science, School of Biology, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece

[2]Alfred-Wegener-Institut für Polar-und Meeresforschung, Ökophysiologie und Ökotoxikologie, Postfach 120161, 27515 Bremerhaven, Germany

ABSTRACT: In the context of future scenarios of progressive accumulation of anthropogenic CO_2 in marine surface waters, the present study addresses the effects of long-term hypercapnia on a Mediterranean bivalve, *Mytilus galloprovincialis*. Sea-water pH was lowered to a value of 7.3 by equilibration with elevated CO_2 levels. This is close to the maximum pH drop expected in marine surface waters during atmospheric CO_2 accumulation. Intra- and extracellular acid–base parameters as well as changes in metabolic rate and growth were studied under both normocapnia and hypercapnia. Long-term hypercapnia caused a permanent reduction in haemolymph pH. To limit the degree of acidosis, mussels increased haemolymph bicarbonate levels, which are derived mainly from the dissolution of shell $CaCO_3$. Intracellular pH in various tissues was at least partly compensated; no deviation from control values occurred during long-term measurements in whole soft-body tissues. The rate of oxygen consumption fell significantly, indicating a lower metabolic rate. In line with previous reports, a close correlation became evident between the reduction in extracellular pH and the reduction in metabolic rate of mussels during hypercapnia. Analysis of frequency histograms of growth rate revealed that hypercapnia caused a slowing of growth, possibly related to the reduction in metabolic rate and the dissolution of shell $CaCO_3$ as a result of extracellular acidosis. In addition, increased nitrogen excretion by hypercapnic mussels indicates the net degradation of protein, thereby contributing to growth reduction. The results obtained in the present study strongly indicate that a reduction in sea-water pH to 7.3 may be fatal for the mussels. They also confirm previous observations that a reduction in sea-water pH below 7.5 is harmful for shelled molluscs.

KEY WORDS: Hypercapnia · Ocean CO_2 accumulation · Marine bivalve · Acid–base balance · Growth rate · Oxygen consumption · Metabolic depression

INTRODUCTION

Global warming and its impact on ecosystems has stimulated considerable scientific interest. It is widely believed that the current increase in atmospheric carbon dioxide (CO_2) (by 31 % in relation to pre-industrial levels; Houghton et al. 2001) is the main cause of global warming. Since CO_2 rapidly equilibrates with the surface ocean, increased CO_2 levels in the atmosphere would cause an increase in sea-water pCO_2 (partial pressure of CO_2) with a concomitant decrease in sea-water pH. An estimated reduction in sea water down to pH 7.3 is expected within the next 300 yr (Caldeira & Wilckett 2003). Such a reduction in sea-water pH at elevated pCO_2 might impact on physiological processes in marine organisms. Moreover, in the light of potential future scenarios of deep-sea CO_2 injections as a means of controlling atmospheric CO_2 levels (Lackner 2003), it also becomes essential to understand the impact of CO_2 accumulation on the physiology of marine organisms. A recent report on the environmental impacts of ocean disposal of CO_2 shows

that marine organisms can only temporarily tolerate any pH reduction below 7.0, and, if feasible, a level of water pH below 7.5 should be avoided (Ormerod & Angel 1996).

Elevated environmental pCO_2 values cause CO_2 to enter the organisms and their body fluids and tissues by diffusion. This occurs especially in those aquatic animals that lack well-developed circulatory and ventilatory systems and depend solely on a favourable tissue-to-environment gradient for CO_2 excretion. Elevated extra- and intracellular pCO_2 values result in decreased extra- and intracellular pH levels. Physiological responses of marine animals from shallow waters to intra- and extracellular hypercapnia have been well characterized, as CO_2 exposure has been instrumental in physiological studies of acid–base regulation (Roos & Boron 1981, Somero 1985, Cameron 1986, 1989, Truchot 1987, Heisler 1989, Walsh & Milligan 1989).

Three main mechanisms are involved in the regulation of acid–base balance: (1) metabolic production and consumption of protons, (2) buffering of intra- and extracellular compartments, and (3) active proton-equivalent ion transport. Buffering of extra- and intracellular fluids is mainly achieved by an increase in the level of bicarbonates, which are formed by passive buffering, by net proton release, and, in bivalves and other calcified animals, by the dissolution of $CaCO_3$ exoskeletons (Lindinger et al. 1984). While the latter mechanism can facilitate the compensation of a temporary acid–base disturbance, long-term exposure to elevated CO_2 levels might be fatal if such levels are sufficient to hamper shell formation. However, the feedback of CO_2-induced disturbances in acid–base equilibria on processes other than calcification as, for example, growth has only rarely been addressed.

Growth is a physiological process, which might be significantly decelerated under long-term exposure of marine animals to increased CO_2 levels. Recent work has shown a close linkage between pH conditions in the field and clam growth rates (Ringwood & Keppler 2002). Moreover, Shirayama (2002) demonstrated that even very moderate increases in CO_2 of 200 ppm above present levels caused a reduction in growth rate and survival of echinoderms and gastropods, indicating that long-term CO_2 changes may be affecting growth of calcifying organisms. Moreover, metabolic depression is, in many cases, an adaptation strategy put in place to survive hypercapnia (Guppy & Withers 1999). A limitation in energy turnover might lead to a reduction in protein synthesis, which would by definition restrict both growth and reproduction. Low pH levels can have a significant reducing effect on protein synthesis in aquatic animals (Kwast & Hand 1996, Reid et al. 1997, Langenbuch & Pörtner 2002).

To our knowledge, very few studies addressed the impacts of long-term exposure of marine animals, especially bivalves, to moderately elevated CO_2 levels. The present paper aspires to contribute to such investigations, by reporting analyses of possibly interdependent changes in acid–base balance, oxygen consumption, nitrogen excretion and growth rate in the Mediterranean bivalve *Mytilus galloprovincialis*. Since, as reported above, a sea-water pH below 7.5 might have critical effects, all mussels were kept under hypercapnic conditions set to cause a sea-water pH of 7.3. The pH value chosen is close to the maximum pH drop expected in marine surface waters during business-as-usual scenarios of anthropogenic CO_2 production and release (Caldeira & Wickett 2003).

MATERIALS AND METHODS

Animals. Juvenile and adult specimens of *Mytilus galloprovincialis* used for the purposes of the present study were collected in the area of Halastra in the Thermaikos Gulf, Greece. Juveniles were held in aquaria, each containing 200 l of aerated recirculating natural sea water, for 5 d prior to experimentation. Water temperature was controlled at 18 ± 0.5°C and salinity at 32 ± 3.5‰. Adults were kept in aquaria under the same conditions for 2 wk prior to experimentation. Sea-water pH was about 8.05 ± 0.02.

Studies of mussels growth rate under normoxia and normoxic hypercapnia. To study the growth rate of mussels, juveniles ranging from 5 to 25 mm body length were used. Mussels were divided in 2 groups, which had about the same distribution of size frequency, and were put in 2 similar tanks (A and B), each containing 200 l of aerated recirculating sea water. Tank A was used as a control, while hypercapnia was imposed in Tank B. Gaseous CO_2 provided from a CO_2 cylinder was injected into the sea water through a magnetic valve connected to a pH monitor and a pH electrode exposed to the sea water in Tank B. The pH meter was set to a value of 7.3, and pH was controlled at ± 0.05 pH units. Sea water was continuously air bubbled through a separate line connected to an air pump, and pO_2 in the sea water was monitored by a Radiometer E5046 pO_2 electrode. pCO_2 and the level of bicarbonate in the sea water were determined as described below. Conditions under which normoxic and hypercapnic juveniles and adult mussels were kept are summarized in Table 1.

The juvenile mussels were kept under normoxia and normoxic hypercapnia for 3 mo and were fed an algal paste consisting of *Isochrysis* sp. and *Chlorela* sp. All mussels were fed a ration of 30 mg total particular matter (TPM) l^{-1} 4 times d^{-1} every 2 d.

Table 1. Physicochemical conditions of sea water where mussels were kept under normoxia and normoxic hypercapnia

Conditions	Normoxia	Normoxic hypercapnia
pH	8.05 ± 0.02	7.3 ± 0.03
pCO_2 (mm Hg)	0.82 ± 0.09	3.82 ± 0.53
HCO_3^- (mmol l^{-1})	2.34 ± 0.13	2.89 ± 0.38
pO_2 (mm Hg)	>145	>145
Ca^{2+} (mmol l^{-1})	17.45 ± 0.09	17.89 ± 0.054

Mussel growth was recorded at regular time intervals in juveniles. During each sampling occasion a sample of 100 mussels was taken at random from the tanks and their shell growth as well as their total body weight was measured. For the analysis of shell growth, shell length (L), shell width (W) and shell height (H) were measured. From each of the above samples a subsample of about 30 to 40 mussels was used for weight determinations. The mussels were dried on filter paper and weighed. The shells were separated from the body, dried on filter paper to remove excess water, and weighed to the nearest 0.001 g. Dry weights were recorded after drying to constant weight in an oven at 70°C.

Size–frequency histograms were constructed for each sampling using shell length (SL) as the most reliable morphometric parameter for shell growth. The class interval used for the construction of the histograms was 5 mm and was determined by Goulden's method (in Cancela da fonseca 1965).

Oxygen consumption and ammonia excretion under normoxia and normoxic hypercapnia. The rate of oxygen consumption was determined for juvenile and adult mussels under normocapnia and hypercapnia in a closed respirometer system. Changes in pO_2 were monitored by a Radiometer E5046 pO_2 electrode fixed in a respiration chamber of 200 ml. All determinations were performed at 18°C. Rates of oxygen consumption were determined for adults exposed to hypercapnia for 0, 5, 10 and 20 h. Before each determination of oxygen consumption, mussels were scrubbed clean and were left for 1 h in the chamber filled with normoxic, normocapnic filtered (0.2 μm membrane) sea water (pH 8.05) or hypercapnic filtered sea water (pH 7.3). Then the system was closed and changes in pO_2 were monitored for 1 h. Water samples were taken at the beginning and at the end of this period and were analysed for ammonia as described by Bergmeyer et al. (1986). For juvenile mussels, oxygen consumption was determined after exposure to hypercapnic conditions for 0, 15, 30 and 90 d. The procedure for oxygen consumption was the same as described for adult mussels. Comparisons were carried out with corresponding controls (normoxic mussels) sampled at the same time intervals as the hypercapnic specimens.

Acid–base parameters. Determination of acid–base parameters was performed in growing juveniles and in adults kept under the same conditions of normoxia and hypercapnia. In the growing mussels intracellular pH (pH_i) only was determined (see below) in the whole soft body, since it was very difficult to collect haemolymph and to separate tissues after dissection. In control and hypercapnic adults levels of extracellular pH (pH_e), pCO_2, bicarbonate ($[HCO_3^-]_e$) as well as pH_i of mantle, gills, foot, posterior adductor muscle (PAM) and anterior byssus retractor muscle (ABRM) were determined after 0, 0.5, 1, 2, 4 and 8 d of hypercapnia.

Samples of haemolymph were collected by pericardiac puncture according to Fyhn & Costlow (1975) and immediately analyzed for pH_e and total CO_2 (C_{CO_2}) as described below. The remaining haemolymph samples were frozen and kept in liquid nitrogen until extracellular calcium levels $[Ca]_e$ were determined. Tissues were excised, blotted dry and freeze-clamped and kept in liquid nitrogen until used for the determination of pH_i.

Determination of extracellular pH, C_{CO_2}, pCO_2, pO_2 and Ca^{2+} in haemolymph and water: The pH of the haemolymph (pH_e) was measured at 18°C using a Radiometer glass capillary pH electrode (G299A A, BMS Mk2) calibrated with Radiometer precision buffers. C_{CO_2} was determined according to the method of Cameron (1971) using a Radiometer pCO_2 electrode (E5037) calibrated with known concentrations of $NaHCO_3$. Haemolymph pCO_2 was calculated by the modified version of the Henderson–Hasselbalch equation:

$$pCO_2 = C_{CO_2}/(10^{pH-pK} \times \alpha CO_2 + \alpha CO_2)$$

where αCO_2 is the solubility of CO_2. Values for the solubility and the apparent dissociation constant under physiological conditions were calculated according to Heisler (1986). The levels of bicarbonate in the haemolymph ($[HCO_3^-]_e$) were calculated from the C_{CO_2} according to the equation:

$$[HCO_3^-]_e = C_{CO_2} - (\alpha CO_2 \times pCO_2)$$

The level of Ca^{2+} in the haemolymph was determined by atomic absorption, as described by Jokumsen & Fyhn (1982).

pO_2 in the haemolymph was determined by means of a thermostatted (18°C) Radiometer O_2 electrode connected to a Radiometer acid–base analyzer. The pO_2 electrode was calibrated at 18°C with Radiometer precision buffers.

Similar procedures were followed in order to determine acid–base parameters of sea water.

Determination of intracellular pH (pH$_i$): The pH$_i$ of tissues was determined by the homogenate method developed by Pörtner et al. (1990). Tissue samples were ground under liquid nitrogen, and 100 mg of tissue powder was added to an Eppendorf vial containing 200 µl ice-cold medium (160 mmol l^{-1} potassium fluoride, 1 mmol l^{-1} nitrilotriacetic acid, pH 7.4). After completely filling the vial with medium, the mixture was briefly stirred with a needle, in order to release air bubbles, then mixed on a Vortex mixer, and finally centrifuged for 30 s. Within 3 min after thawing of the tissue powder in the medium, the pH of the supernatant was measured using a Radiometer glass capillary pH electrode (G299A). This value approximates pH$_i$ with an experimental error of less than 0.01 pH units (Pörtner et al. 1990).

Determination of extracellular (haemolymph) buffer values: The non-bicarbonate buffer value ($\beta_{NB} = -\Delta[HCO_3^-]_e / \Delta pH_e$) of haemolymph was determined by CO_2 equilibration at 18°C in an intermittently rotating cuvette (tonometer model 273, Instrumentation Laboratory), which was flushed with humidified CO_2 gas mixtures (between 0.2 and 1%) in air at 18°C. The samples of haemolymph were tonometered for 25 min for full equilibration and were subsequently analyzed for pH and C_{CO_2} as described above.

Statistical analyses. Changes over time were tested for significance at the 5% level by using 1-way analysis of variance (ANOVA) and by performing the Bonferroni post-hoc test for group comparisons. Values are presented as means ±SE throughout.

RESULTS

The effect of hypercapnia on shell growth is shown in size–frequency histograms (Fig. 1). Control shell length increased progressively and ranged between 25 and 40 mm within 90 d, with a maximum frequency between 25 and 30 mm. A similar pattern of shell length growth was observed in hypercapnic mussels. However, shell growth was slower in hypercapnic mussels and, after a period of 90 d, their shell length ranged between 10 and 35 mm. In the latter case, mussels ranging between 20 and 25 mm were predominant. The plot derived from the means of shell-length and fresh-weight increments for both controls and hypercapnic mussels indicates a lower rate of shell growth in the latter (Fig. 2). Moreover, the relationship between dry weight and shell length reflected an exponential regular growth rate in both controls and
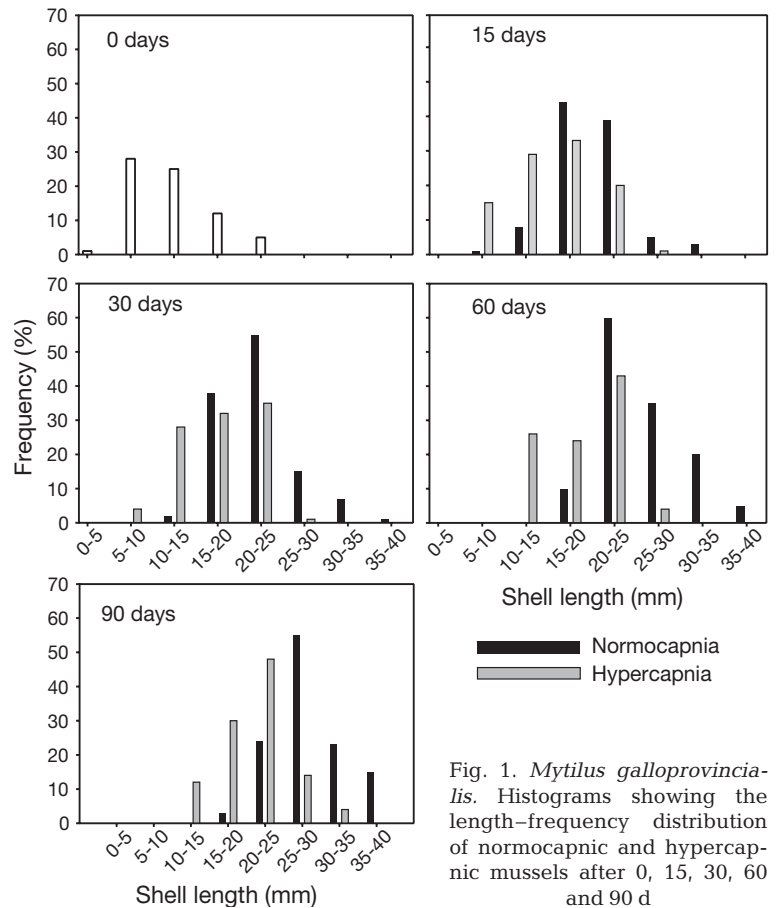

Fig. 1. *Mytilus galloprovincialis.* Histograms showing the length–frequency distribution of normocapnic and hypercapnic mussels after 0, 15, 30, 60 and 90 d
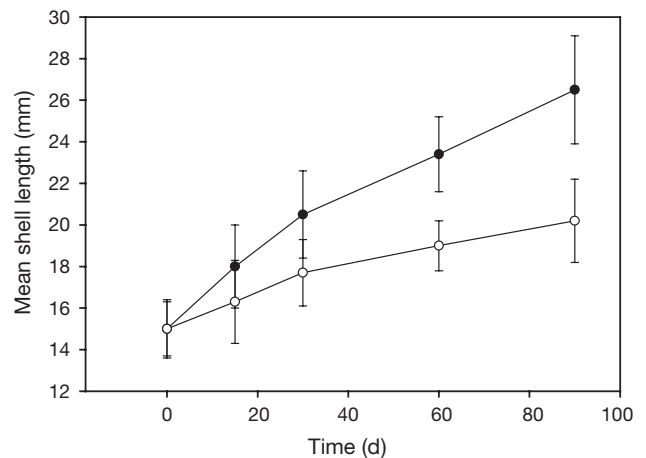

Fig. 2. *Mytilus galloprovincialis.* Mean shell lengths of normocapnic (●) and hypercapnic (○) mussels

hypercapnic mussels. The dry-weight–shell-length relationship was not statistically different between controls and hypercapnic mussels (Fig. 3). Similarly, the relationships between other morphometric characters, such as dry weight and shell width or dry weight and shell height, showed no statistical differences between the 2 groups of mussels (data not shown). This
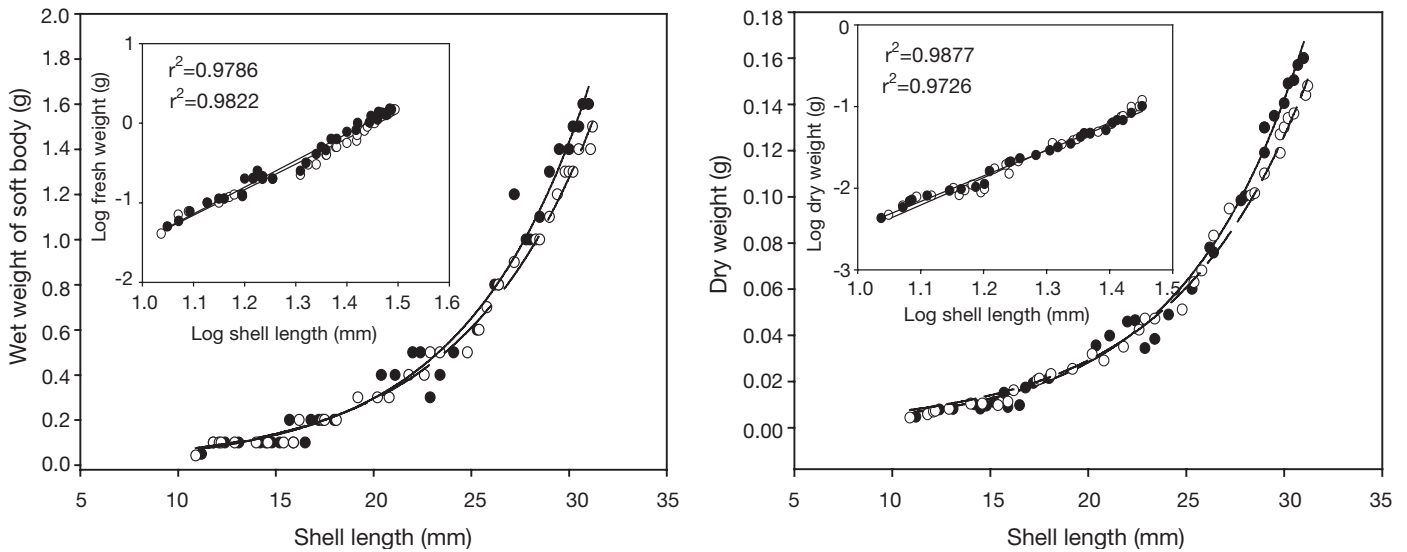
Fig. 3. *Mytilus galloprovincialis*. Relationships of the morphometric characteristics weight (fresh and dry) and shell length in growing mussels during normocapnia (solid line — ●) and hypercapnia (dashed line — ○)

comparison suggests that reduced shell growth goes hand in hand with decreased growth of soft-body tissues under hypercapnia.

The time course of changes in extracellular acid–base status in adult mussels is depicted in Fig. 4. Control extracellular acid–base parameters at time 0 (before the onset of hypercapnia) were pH = 7.55 ± 0.02, pCO_2 = 1.15 ± 0.21 mm Hg and $[HCO_3^-]$ = 1.62 ± 0.12 mmol l^{-1}. During the first 2 d of hypercapnia, pH_e decreased from 7.55 ± 0.02 to 7.36 ± 0.05 and remained more or less constant thereafter (Fig. 4a). Haemolymph pCO_2 increased from 1.15 ± 0.21 to 3.92 ± 0.20 mm Hg within the first 4 d. After 8 d it reached a value of 4.35 ± 0.30 mm Hg (Fig. 4b). The pattern of changes in $[HCO_3^-]_e$ was similar to those of pCO_2 in haemolymph. $[HCO_3^-]_e$ increased almost linearly within the first 4 d (from 1.62 ± 0.12 to 3.45 ± 0.16 mmol l^{-1}). After 8 d it reached a value of 4.01 ± 0.20 mmol l^{-1} (Fig. 4c). Within the first 4 d the level of haemolymph [Ca] increased from 17.45 ± 2.13 to 26.05 ± 1.50 mmol l^{-1}. After 8 d it found to be 27.45 ± 2.55 mmol l^{-1} (Fig. 4d). pO_2 was determined to be 52.03 ± 6.89 mm Hg in the haemolymph of controls. Hypercapnia did not cause any change in the level of pO_2.

The changes in acid–base variables in the haemolymph of *Mytilus galloprovincialis* during exposure to hypercapnic conditions are depicted in a pH_e–$[HCO_3^-]_e$ diagram (Fig. 5) in relation to the non-bicarbonate buffer line, β_{NB} (solid line), for haemolymph. The β_{NB} of the haemolymph was determined to be 0.65 ± 0.1 Slykes. The diagram shows that after an initial phase (0.5 to 1 d) of uncompensated respiratory acidosis, an accumulation of bicarbonate more or less fully compensated for the rise in pCO_2 and prevented a further decrement in pH_e.

The effect of hypercapnia on pH_i is shown in Fig. 6. In control adult mussels, the pH_i was found to be 7.17 ± 0.04 in the mantle,
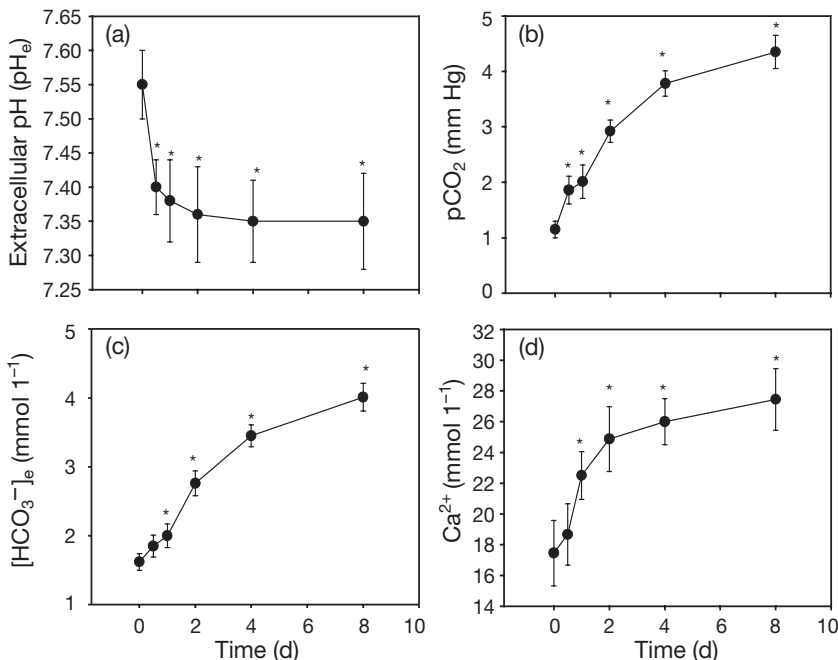

Fig. 4. *Mytilus galloprovincialis*. Acid–base parameters and Ca^{2+} levels in the haemolymph of hypercapnic adult mussels. N = 15 determinations on separate preparations from different animals. Asterisks indicate the values significantly different from the control value (0 d)
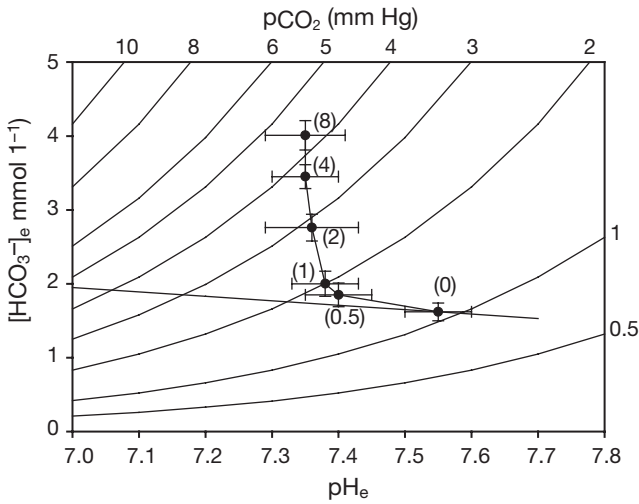
Fig. 5. *Mytilus galloprovincialis*. Changes in extracellular (haemolymph) acid–base status of adult mussels during hypercapnia presented in a $pH_e/[HCO_3^-]_e$ diagram and in relation to the buffer line (solid line measured *in vitro*). The numbers in parentheses indicate the days of hypercapnia
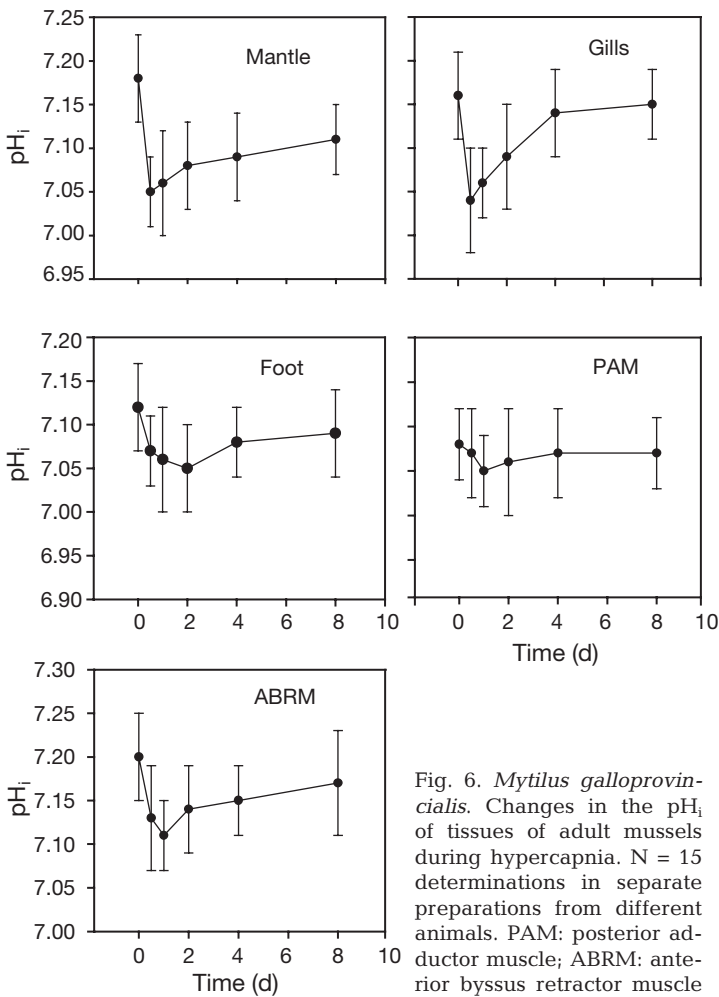

Fig. 6. *Mytilus galloprovincialis*. Changes in the pH_i of tissues of adult mussels during hypercapnia. N = 15 determinations in separate preparations from different animals. PAM: posterior adductor muscle; ABRM: anterior byssus retractor muscle

7.16 ± 0.05 in the gills, 7.12 ± 0.05 in the foot, 7.07 ± 0.04 in the posterior adductor muscle (PAM) and 7.20 ± 0.04 in the anterior byssus retractor muscle (ABRM). The pattern of pH_i change was similar in all tissues during hypercapnia. pH_i declined within the first day and recovered to close to control levels thereafter. It must be pointed out, however, that the decline of pH_i was more evident in mantle and gills than in the muscular tissues, likely due to higher buffer values in the muscle tissues. With respect to pH_i measured in the whole soft body of juvenile mussels, the results indicate that the initial pH disturbance remained fully compensated and that hypercapnia did not cause significant pH_i fluctuations within 90 d (Fig. 7).

The results obtained by closed-system respirometry revealed that exposure to hypercapnia caused a significant reduction in oxygen consumption by adult *Mytilus galloprocincialis* (Fig. 8a) and in growing juveniles (Fig. 9). The rate of oxygen consumption was reduced to 35% of control levels after 20 h of exposure to hypercapnic conditions in adults and to 65% in juveniles. In contrast, the rate of ammonia excretion into sea water increased by 60% (from 0.71 to 1.12 mEq l^{-1}) within the first 5 h of hypercapnia in adults (Fig. 8b). Thereafter it remained constantly elevated above the value seen during 20 h of exposure to control conditions (Fig. 8b).

DISCUSSION

Similarly to data obtained in other marine bivalves, hypercapnia caused a marked respiratory acidosis in extracellular fluids, resulting in a permanent reduc-
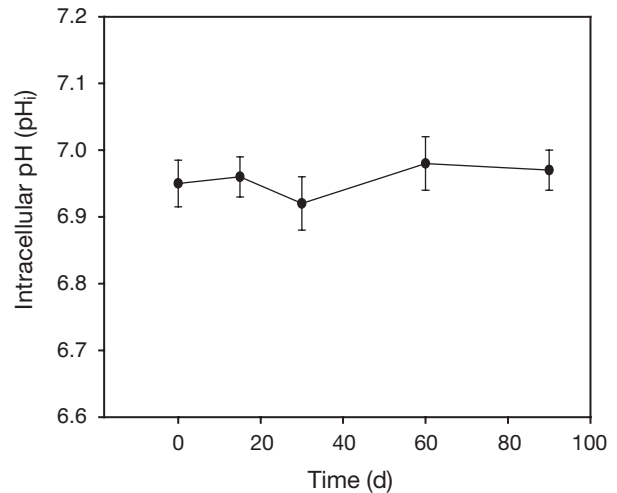

Fig. 7. *Mytilus galloprovincialis*. Changes in the pH_i of the soft body of growing mussels after exposure to hypercapnia for 0, 15, 30, 60 and 90 d. N = 15 determinations on separate preparations from different animals
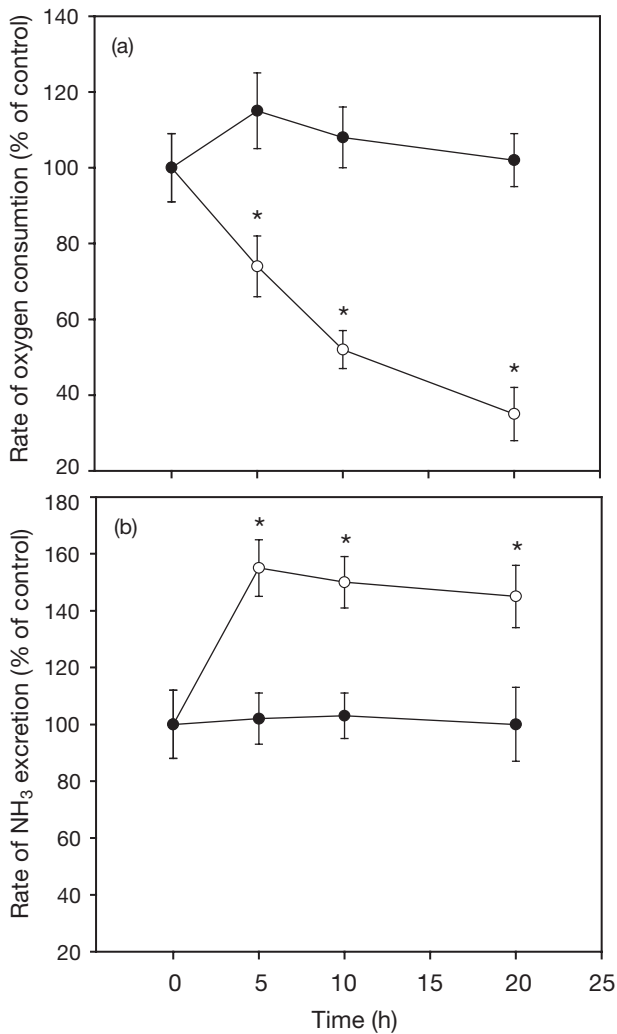
Fig. 8. *Mytilus galloprovincialis*. Changes in (a) oxygen consumption (○) and (b) ammonia release (○) of adult mussels after exposure to hypercapnia for 0, 5, 10 and 20 h. N = 10 determinations in separate preparations from different animals. Asterisks indicate the values significantly different from the control value (●)

Fig. 9. *Mytilus galloprovincialis.*Changes in oxygen consumption of growing juvenile mussels after exposure to hypercapnia (○) for 0, 15, 30 and 90 d. N = 10 determinations in separate preparations from different animals. Asterisks indicate the values significantly different from the control value (●)

tion of pH_e from 7.55 ± 0.02 to 7.36 ± 0.05 (Fig. 4a). Acid–base parameters were found similar in the present study of *Mytilus galloprovincialis* as in those reported for *M. edulis* (Booth et al. 1984). However, Lindinger et al. (1984) reported a pH_e value which was 0.3 lower in *M. edulis* controls, likely due to the stress caused by DMO (5,5-dimethly[^{14}C]oxazoline-2,4-dione) infusion (DMO was used to analyse intracellular pH; Walsh et al. 1984). Similar to findings by Lindinger et al. (1984) in *M. edulis*, respiratory acidosis in *M. galloprovincialis* elicits an accumulation of bicarbonates in extracellular fluids to compensate for the acidosis (Fig. 4c). In contrast to observations in fish, where the extracellular acidosis is frequently fully compensated (Heisler 1986), bicarbonate accumula-
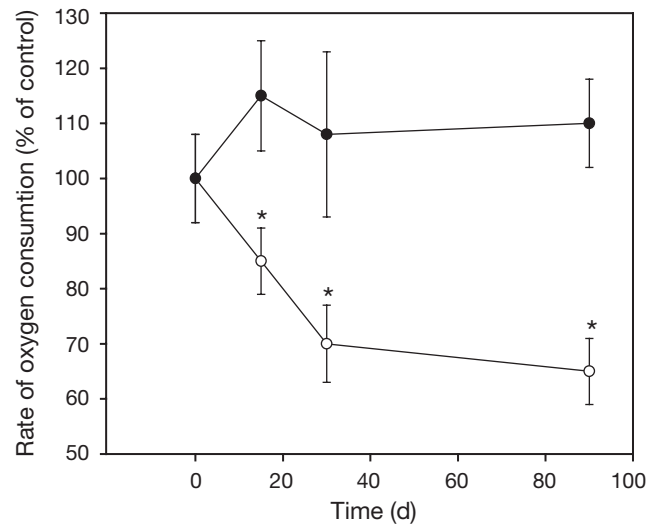
tion in mussel haemolymph occurred at a reduced steady-state value of extracellular pH. This may be a common phenomenon in lower marine invertebrates and was also seen in a sipunculid worm, *Sipunculus nudus* (Pörtner et al. 1998). As reported in the case of *M. edulis* (Lindinger et al. 1984), shell dissolution seems to be the main source of bicarbonates in *M. galloprovincialis*, indicated by the more than equimolar increase in Ca^{2+} levels in extracellular fluids (Fig. 4d).

Although pH_e levels remained reduced throughout hypercapnia (Fig. 4a), pH_i dropped initially but was restored within the first 2 d; thereafter, it was maintained close to control levels in all 4 tissues analysed (Fig. 6). This conclusion is supported by the long-term analysis of pH_i in the whole soft body of juveniles. Complete compensation for the intracellular acidosis was not observed in *Mytilus edulis* during more severe hypercapnia of pCO_2 = 26 mm Hg (Lindinger et al. 1984). Perhaps this observation is not only due to the severity of hypercapnia but also to the limited duration (24 h) of the hypercapnic exposure in the study by Lindinger et al. (1984). According to our results, mussels are able to fully restore pH_i levels when they are exposed to moderate hypercapnia (water pCO_2 = 3.82 ± 0.53 mm Hg and water pH 7.3). The preferential compensation of pH_i at only partially compensated extracellular pH was also observed in the sipunculid worm exposed to long-term hypercapnia (Pörtner et al. 1998). In the sipunculid it appeared that full compensation of the intracellular acidosis depended on extracellular accumulation of bicarbonate. The patterns observed in the present study would be in line with this

conclusion. Previous results also emphasize a dependency of pH_i on the degree of hypercapnia and on pH_e (Zange et al. 1990, Reipschläger & Pörtner 1996). To support pH_i regulation, some animals, including *M. edulis*, are able to shift steady-state levels of bicarbonate to higher values according to the degree of hypercapnia. The final steady-state value of their pH_e results as a function of the acid–base characteristics of the medium (Lindinger et al. 1984, Cameron & Iwama 1989, Heisler 1989).

Although control values of pH_i were successfully re-established and maintained constant during long-term hypercapnia (Fig. 7), elevated CO_2 levels caused a decrease in the rate of oxygen consumption in juvenile and adult mussels (Figs. 8a & 9), clearly indicating a drastic reduction in metabolic rate. A number of studies conducted on several invertebrates have shown that hypercapnia alone is capable of eliciting depressed metabolic rates (Barnhart & McMahon 1988, Barnhart 1989, Rees & Hand 1990). Metabolic suppression is, in many cases, an adaptive strategy for survival under transiently stressful conditions. Low pH levels and hypercapnia can trigger metabolic depression in many organisms (Guppy & Withers 1999), possibly because oxygen and CO_2 levels are often inversely correlated in aquatic habitats (Truchot & Duhamel-Jouve 1980). The fact that pO_2 levels in the haemolymph of adult mussels were maintained during hypercapnia excludes a contribution of hypoxia and indicates not only that mussels remained aerobic during hypercapnia but also that hypercapnia alone elicited metabolic depression. This is similar to recent findings in *Sipunculus nudus* (Pörtner et al. 1998). Metabolic depression is frequently discussed to be associated with a decrease in pH_i, which inhibits activities of many enzymes involved in intermediary metabolism (Somero 1985). Moreover, low pH_i levels favour the transition of some glycolytic enzymes to less-active forms in marine bivalves and consequently support a dormant state of the organism (Brooks & Storey 1997).

In several cases, however, metabolic depression occurs independent of reduced pH_i levels (Hand 1998, Pörtner et al. 1998). The full restoration of pH_i observed during early hypercapnia in adult *Mytilus galloprovincialis* (Fig. 6) and the long-term maintenance of compensated pH_i values in juveniles (Fig. 7) also indicate that the reduction in oxygen consumption is not elicited by low pH_i in the case of *M. galloprovincialis*. The drop in oxygen consumption might rather be associated with low pH_e. In fact, the depressant effect of CO_2 in the above-mentioned sipunculid was correlated with the pH drop in extracellular rather than intracellular fluids (Reipschläger & Pörtner 1996, Pörtner et al. 1998). The pH_e-dependent modification of oxygen consumption rates seems to be partly medi-

ated by inhibition of net proton transport across the cell membrane during acidosis (Reipschläger & Pörtner 1996, Pörtner et al. 2000).

Further mechanisms may contribute to metabolic depression at reduced values of pH_e. Similarly to observations in *Mytilus edulis* and *Sipunculus nudus* (Lindinger et al. 1984, Pörtner et al. 1998), hypercapnia stimulated the excretion of ammonia in *M. galloprovincialis* despite falling rates of oxygen consumption (Fig. 8b), resulting in falling O/N ratios. Lindinger et al. (1984) suggested that ammonia excretion may be an important mechanism for acid excretion in *M. edulis*; however, most of the ammonia may be lost by diffusive release of ammonia rather than excretion of ammonium. In any case, increased ammonia release indicates increased use of amino acids or protein as metabolic substrates during long-term hypercapnia. Recent studies of the influence of hypercapnia on amino-acid catabolism conducted in the marine worm *S. nudus* (Langenbuch & Pörtner 2002) indicate a close correlation of the degree of extra- and intracellular acidosis with the rate of amino-acid catabolism and ammonia excretion. The enhanced N catabolism despite low oxygen consumption as seen in the marine worm and in the present study was hypothesized to indicate preferred catabolism of low O/N amino acids such as asparagine, glutamine and their dicarboxylic acids. These changes reflect the net formation of metabolic bicarbonate and may thereby support pH regulation (Langenbuch & Pörtner 2002).

Moreover, it has been shown that low pH can induce a significant reduction in protein synthesis in aquatic animals (Kwast & Hand 1996, Reid et al. 1997). The stimulation of amino-acid catabolism in mussels during exposure to hypercapnic conditions may damage their cellular protein pool and contribute to the drop in somatic growth. The latter would also be involved in the reduction in energy turnover during the dormant state, since protein synthesis is an ATP-consuming process. Moreover, compensation of pH_e by shell bicarbonate during long-term hypercapnia likely slows down shell growth in *Mytilus*. The results obtained in the present study are therefore in line with the hypothesis that environmental hypercapnia is associated with a decrease in growth performance involving both metabolic depression and reduced shell growth. As shown in Fig. 3, the relationship between shell length and dry weight is identical in normocapnic and hypercapnic mussels, and the mathematic relationships between morphometric characteristics did not differ between the 2 groups of mussels. These observations clearly indicate both a slower rate of shell growth and reduced growth of soft body in hypercapnic mussels (Figs. 1 & 2). According to the acid–base status of extra- and intracellular fluids in *M. galloprovincialis*

such a long term delay in mussel growth seems to be attributed to extracellular rather than intracellular acidosis. Similarly to our results Bamber (1990) reported that shell length of *M. edulis* showed a significant positive correlation with pH, notably at pH < 7.5. Moreover, as reported in the same study, the responses of marine bivalves *Ostrea edulis*, *Grassostrea gigas* and *M. edulis* to reduced pH levels in sea water occur by growth suppression, shell dissolution, tissue weight loss and feeding activity suppression. In addition, further reduction of pH in sea water caused significant mortality among marine bivalves. The percent mortality was positively correlated with the length of time that mussels were exposed to acidic sea water (Bamber 1990). Similarly, Yamada & Ikeda (2000) reported that the mortality of zooplankton is directly proportional to exposure time at low sea-water pH. In addition, Hinga (2002) has shown that phytoplankton population dynamics is affected by the changes in the pH of sea water.

Results of other studies of pH effects in bivalve molluscs showed that some deleterious effects set in at pH ≈ 7.5. However, all studies show that marine bivalve molluscs cannot tolerate pH ≤ 7.0. A positive correlation between sea-water pH and growth rate has also been reported for other shallow-living marine organisms (Takeuchi et al. 1997, Ringwood & Keppler 2002, Shirayama 2002).

In conclusion, the results presented here confirm the statement that a sea-water pH < 7.5 (surprisingly close to the lower limits of pH variability in natural sea water) is harmful at least for marine bivalves. Shell dissolution under modest hypercapnia can facilitate the compensation of temporary acid–base disturbances. However, chronic exposure seems to slow down several physiological processes, resulting in a low rate of mussel metabolism and growth. The latter may finally lead to an increase in mussel mortality, an assumption consistent with other data, which emphasize that shallow-living organisms are generally intolerant to hypercapnia (Knutzen 1981). CO_2 effects on marine organisms and their physiology are also relevant in the light of a potential future scenario of atmospheric CO_2 disposal into deep water (Pörtner & Reipschläger 1996, Seidel & Walsh 2003). As reported, such a process will affect marine ecosystems, and it may have large effects depending on the sensitivity of individual species. In line with our present findings, recent investigations of CO_2 effects in a deep-sea echinoid (sea urchin) also indicate that long-term exposure under CO_2 disposal scenarios would be fatal if CO_2 excursions are sufficiently large enough to initiate shell dissolution (Barry et al. 2002). According to a recent overview of the biological impact of elevated sea-water CO_2 concentrations (Pörtner et al. 2004) CO_2 itself as well CO_2-

related changes in pH and bicarbonate levels affect molecular, cellular, tissue and whole organism functions, i.e. functions situated at different organizational levels. Those functions affected at lower levels of organization (molecular, etc.) likely integrate into a hierarchical system of CO_2 effects, where those found at the highest organizational levels appear most sensitive and are co-defined by the integration of molecular functions and vice versa. The reduction in growth rate as observed here may finally result from such an integrated scenario of CO_2 effects. The degree of change observed at water pH 7.3 (growth reduction to 55% and metabolic depression to 65% of normocapnic values) suggests that the observed effects set in much before this pH value is reached and, therefore, much earlier than in the year 2300. A more detailed titration of these effects considering the progressive rise in CO_2 levels as expected during the next decades thus awaits further study.

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