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Tolerance of juvenile *Mytilus galloprovincialis* to experimental seawater acidification

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ABSTRACT: Coastal ocean acidification is expected to interfere with the physiology of marine bivalves. In this work, the effects of acidification on the physiology of juvenile mussels *Mytilus galloprovincialis* were tested by means of controlled CO_2 perturbation experiments. The carbonate chemistry of natural (control) seawater was manipulated by injecting CO_2 to attain 2 reduced pH levels: −0.3 and −0.6 pH units as compared with the control seawater. After 78 d of exposure, we found that the absorption efficiency and ammonium excretion rate of juveniles were inversely related to pH. Significant differences among treatments were not observed in clearance, ingestion and respiration rates. Coherently, the maximal scope for growth and tissue dry weight were observed in mussels exposed to the pH reduction ΔpH = −0.6, suggesting that *M. galloprovincialis* could be tolerant to CO_2 acidification, at least in the highly alkaline coastal waters of Ria Formosa (SW Portugal).

KEY WORDS: Ocean acidification · Blue mussels · Feeding behaviour · Physiological energetics · Absorption bivalves · Metabolism

INTRODUCTION

The dissolution of anthropogenic CO_2 in the oceans has dramatically altered the inorganic carbon chemistry of seawater by reducing the saturation of CO_3^{2-} and the pH (Feely et al. 2004). The decline of CO_3^{2-} concentration has detrimental effects on marine calcifiers (Orr et al. 2005, Gazeau et al. 2007, Miller et al. 2009), whereas changes in pCO_2 and pH are expected to affect the physiology of all marine life (Fabry et al. 2008, Melzner et al. 2009).

Coastal zones are considered highly productive hotspots, contributing >90% to the marine living resources currently harvested (Pauly et al. 2002). These nearshore areas are characterized by a large spatial and temporal variability in the carbonate chemistry as compared with the open ocean. Oceanic (e.g. upwelling) and continental (e.g. river discharge) processes may lead to a coastal zone with low oxygen waters that are supersaturated with CO_2 and have reduced CO_3^{2-} and pH (Salisbury et al. 2008, Koch & Gobler 2009). On the other hand, the alkalinity of continental waters may be a major contributor to the CO_3^{2-} concentration in the coastal zone. Naturally elevated alkalinity may prevent or delay $CaCO_3$ under-saturation in coastal waters under future ocean acidification scenarios (Fernández-Reiriz et al. 2011, Range et al. 2011).

Our current understanding of the effect of future ocean acidification on the physiological and ecological fitness of marine organisms is incomplete. While growing empirical evidence from CO_2 perturbation experiments suggests that several taxa might react quite sensitively to ocean acidification (e.g. Michae-

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lidis et al. 2005), others seem to be surprisingly tolerant (e.g. Gutowska et al. 2008). Naturally acidified habitats allow to investigate organisms and communities under high pCO_2 conditions in general (e.g. Hall-Spencer et al. 2008, Fabricius et al. 2011) and mussels in particular (Thomsen et al. 2010, Rodolfo-Metalpa et al. 2011). Multi-generation experiments can also contribute important evidence about the sensitivity and adaptation potential of a given species. Considering the scarcity of that type of study, an alternative approach is to look at indicators for animal performance during long-term CO_2 perturbation experiments (Melzner et al 2009).

Bivalves dominate the macrofauna of many estuaries and coastal embayments. Understanding their physiological behaviour is crucial for determining their productivity and energy flows. Changes in environmental variables can affect physiological processes in bivalves, modifying their influence on the ecosystem. These effects need to be evaluated in an integrated way, given the important role these organisms play in terms of ecological structure and their value as economic resources for fisheries and aquaculture in many coastal areas. The potential of significant ecological and economic consequences arising from the effects of ocean acidification on bivalves and the need for further research on commercially important species has been explicitly recognized (Kleypas et al. 2006, Fabry et al. 2008, Cooley & Doney 2009).

Scientific research on the effects of seawater acidification on bivalves has been increasing rapidly in recent years. Most previous studies have focused on growth and calcification of the shell (Berge et al. 2006, Gazeau et al. 2007, Miller et al. 2009, Gazeau et al. 2010, Thomsen & Melzner 2010, Range et al. 2011), feeding behaviour, reproduction and metabolism (Michaelidis et al. 2005, Beesley et al. 2008, Saphoerster 2008). Fernández-Reiriz et al. (2011) studied the physiological energetics of juvenile clams *Ruditapes decussates* in high pCO_2 conditions and observed reduced ingestion combined with increased excretion, which is generally associated with a reduced energy input and will likely contribute to a slower growth of the clams in future acidification scenarios. There are, however, no previous studies examining the scope for growth (SFG) of bivalves under conditions of increased pCO_2. Anestis et al. (2010) studied the response of physiological parameters of *Mytilus galloprovincialis* to increasing seawater temperature, according to current scenarios of climatic change. They found that the SFG values became negative at temperatures higher than 24°C, probably associated with a significant reduction in the clearance rate. Other recent studies have shown that current and future increases in pCO_2 may deplete or alter the composition of shellfish populations in coastal ecosystems (Bibby et al. 2008, Kurihara 2008, Miller et al. 2009, Talmage & Gobler 2009).

We target the mussel *Mytilus galloprovincialis*. This species is distributed worldwide dominates the extensive cultures of the Galician rías (NW Spain). The experiments were conducted on juvenile mussels, which represent an important life stage for shellfish populations, as reductions in the growth and survival of seed have the potential to translate into declines of adult populations (Arnold 2008). We focused on analyzing the effects of seawater acidification caused by increasing concentrations of pCO_2, as predicted by current scenarios of climate change. Three levels of pH were tested: a natural (control) level and 2 levels of reduced pH, by −0.3 and −0.6 pH units, relative to the control seawater. The response of the mussels was measured after 78 d of exposure to the 3 contrasting conditions, in terms of their key physiological parameters: in particular (1) clearance and ingestion rate; (2) absorption efficiency; (3) oxygen consumption; (4) ammonia excretion; (5) oxygen to nitrogen (O:N) ratio and (6) scope for growth (SFG).

MATERIALS AND METHODS

Biological material

Juvenile *Mytilus galloprovincialis* were obtained from a mussel raft in the Ría de Ares-Betanzos (43° 23′ 23.01″ N, 8° 17′ 27.29″ W). Between 2007 and 2010, the physicochemical characteristics of seawater at this site were monitored with weekly frequency (n = 174 observations). The ranges of recorded values were 30.46 to 35.46 for salinity, 11.08 to 22.35°C for temperature, 81 to 149% for dissolved oxygen saturation and 7.7 to 8.4 for pH. At the beginning of the experiment, the mussels were approximately 6 mo old, ranging from 10 to 15 mm shell length, 6.9 ± 0.7 mg dry weight (100°C, 24 h) and 23.2 ± 0.4% ash (450°C, 24 h).

pH exposure system

The rearing system was installed in an experimental bivalve hatchery in Tavira, Portugal (37° 7′ 17.73″ N, 7° 37′ 12.19″ W), operated by the National Institute of Biological Resources (INRB, I.P./L-IPIMAR). Seawater was pumped from the Ría Formosa lagoon, passed through a sand filter and aer-

ated for 2 to 3 d before entering the rearing system, to ensure adequate oxygenation and stable pH during the exposure. Three levels of pH were tested: natural seawater (control; ΔpH = 0) and 2 levels of reduced pH (ΔpH = −0.3 and ΔpH = −0.6 units as compared with the control). Each level of pH had a separate 250 l header tank and pump, supplying 3 replicate 15 l plastic exposure tanks. The seawater supply to the exposure tanks was manually regulated using PVC valves. A flow-through system was used to minimize any interference from the metabolic waste products of the mussels. Excess water overflowed so that, on average, the volume in each tank was renewed 8 times per day.

Carbonate chemistry of seawater was manipulated in the reduced pH treatments (ΔpH = −0.3 and ΔpH = −0.6 units) by diffusing pure CO_2 in closed reactors (Aqua Medic reactor 1000) installed before the exposure tanks. The gas flux from the pure CO_2 tanks to the reactors was controlled through a pH-stat system (Aqua Medic AT Control) by opening or closing a solenoid valve when the pH readings in the exposure tanks deviated from the predetermined set-points by ±0.1 pH units.

The exposure started on 21 December 2009, when the 9 tanks were stocked with 200 juvenile mussels each. The pH of the acidified treatments was gradually reduced to the target values over the following week. The mussels were fed with a 1:1 mixture of 2 microalgae strains, Tahitian *Isochrysis aff. galbana* (T-ISO) and *Chaetoceros calcitrans*, supplied in continuous flow to each tank by a peristaltic pump (ISMATEC MPC Process) to maintain a concentration of 19 to 20 cells μl^{-1}, equivalent to a total particulate matter load of 3.84 mg l^{-1} with 60% organic content. The seawater used for the preparation of the diet was filtered through a cartridge filter system with an effective pore size of 1 µm and pre-treated with ultraviolet light. The diet was maintained in an aerated tank to generate a homogeneous mixture and prevent sedimentation. All the physiological experiments were done with the same seawater supply and diet used during the exposure, thus ensuring that the relevant environmental conditions (temperature, salinity, pH and food supply) were maintained. Feeding was stopped 24 h before starting the physiological measurements.

Monitoring of the physical-chemical variables of seawater

Temperature, salinity and pH of seawater were continuously monitored in the exposure tanks during the 78 d of the experiment using dedicated electrodes and the data-logger function of the controller. Automatic readings were validated against regular manual determinations with a calibrated YSI 556 multi-probe. The pH electrodes were standardized against Tris seawater buffers (ionic strength of 0.7 M) and readings were expressed in the total scale (pH_T). Salinity readings were calibrated with an AutoSal salinometer using IAPSO standard seawater.

Water samples were collected on 8 February 2010. Dissolved oxygen was determined by the Winkler method. Determinations of total alkalinity (TA) were done by automatic titration with HCl past the endpoint of pH 4.5. Dissolved inorganic carbon (DIC), partial pressure of CO_2 in seawater (pCO_2) and the $CaCO_3$ saturation state for calcite (Ωcal) and aragonite (Ωara) were calculated from *in situ* temperature, salinity, pH and TA, according to the procedures described by Range et al. (2011).

Physiological experiments

The feeding and digestive behaviour and metabolic activity of *Mytilus galloprovincialis* were determined at the beginning of the exposure (initial), under natural pH conditions, and after 78 d of exposure (09 March 2010) at each of the 3 levels of pH considered (ΔpH = 0.0; ΔpH = −0.3; and ΔpH = −0.6 units). First physiological measurements (on 21 December 2009) were performed after the individuals had been maintained for 12 h under natural pH conditions. Mortalities during the exposure varied from 5% to 10% and the final shell length ranged from 11.5 to 32 mm. Neither of these variables differed significantly among pH treatments.

After 78 days of exposure, 2 size-classes were distinguished, according to the modal size class (small: 19 to 21 mm and large: 22 to 29 mm shell length). The physiological determinations of clearance, ingestion, respiration and excretion rates were determined using 3 distinct pools of 5 (large) or 10 (small) individuals for each combination of pH level and size class. Individuals in each pool were randomly selected from the same exposure tank. A minimum of 3 ind. from each pool used in the physiological determinations on Days 0 and 78 were subsequently sacrificed for determination of tissue (drying: 100°C, 24 h) and organic dry weight (DW) (combustion: 450°C, 24 h). In all determinations, the physiological rates were also referred to the unit of organic weight (specific rates).

Clearance (CR) and ingestion rates (IR)

The CR was estimated from the reduction in suspended particles concentration, measured as volume of particles ($mm^3\ l^{-1}$), between the water surrounding the individuals and the outflow of the experimental chamber following Filgueira et al. (2006). The mussels (see the Physiological experiments section) were placed in a cylindrical chamber of 300 ml with a water inflow in the lower part and water outflow in the upper opposite side The mussels were placed in the chambers in such a way that the input flow was directed to the inhalant aperture and that the exhalant aperture was directed toward the water outflow, thus preventing re-filtration processes. For each pH level, 2 chambers without mussels served as blanks for the calculation of the CR. The ingestion rate (IR) was calculated as the product of CR and food concentration.

Absorption efficiency

Absorption efficiency was estimated by determining the organic and inorganic content of the food and the faeces following the method of Conover (1966). Representative samples of the diet were collected during the experiments and the absorption efficiency was calculated for a given pool of mussels by collecting the faeces in each experimental chamber. Samples of food and faeces were filtered through pre-combusted, pre-weighed Whatman GF/C membranes. Filters were rinsed with isotonic ammonium formate, dried to a constant weight at 80°C, and then weighed and combusted at 450°C for 3 h. The filters were weighed again to estimate the organic and inorganic fraction contained in the food and faeces.

Respiration rate (V_{O2})

Respiration rates were determined by incubating the mussels in sealed 100 ml Erlenmeyer flasks containing seawater at each pH studied (see the Physiological experiments section). Temperature was maintained during the determinations by immersing the flasks in an isothermal bath. Two Erlenmeyer without animals were used as a control for each treatment. The mussels were left undisturbed until most of them had their valves opened, or at least for 45 to 60 min. Subsequently, oxygen measurements were started using a manual probe (HACH HQ40). The depletion of oxygen in the chamber, due to respiration by the mussels, was recorded for 30 to 60 min, depending on the size class. The measurements were stopped before the oxygen concentration dropped below 30%, relative to control Erlenmeyer without mussels. Respiration rates were calculated from the difference in concentration between the chambers with and without animals.

Ammonia excretion rate ($V_{NH4\text{-}N}$)

Ammonia excretion rate was determined after placing the mussels in open Erlenmeyer flasks with 250 ml of filtered seawater (0.2 µm Millipore membranes) at each pH studied (see the Physiological experiments section). Temperature was maintained during the determinations by immersing the flasks in an isothermal bath. Two Erlenmeyer without mussels were used as a control for each treatment. After 90 min, water samples were collected from each flask and frozen to −20°C until analysis using the phenol-hypochlorite method (Solorzano 1969). Ammonia excretion rates were calculated from the difference in ammonia concentration between the chambers with and without animals. The ratio of oxygen consumed to nitrogen excreted (O:N) was computed by atomic equivalents, according to Widdows (1985).

Scope for growth (SFG)

SFG is defined as the fraction of the absorbed energy available for somatic or gametogenic growth once metabolic requirements have been met (Widdows 1985). Accordingly, SFG was computed following the equation for energetic balance developed by Winberg (1960) and Ivlev (1966),

$$SFG = I - Fae - M = AR - M \quad (1)$$

where I is the ingested energy, Fae is the energy loss in the faeces, M summarizes the metabolic expenditure plus the energy loss due to excretion and AR is the absorbed energy, computed as the product of ingestion rate and absorption efficiency (Labarta et al. 1997).

The following energy conversion factors were used, as described by Bayne et al. (1985):

1 mg POM = 23.5 J
1 ml O_2 = 20.36 J
1 µg NH_4-N = 0.0249 J

Statistical analysis

The effects of pH and size on the variation of physiological rates (clearance and ingestion rates, ab-

sorption efficiency and metabolism) and dry weight were compared using 2-factor analyses of variance (ANOVA). Homogeneity of variance was confirmed by means of Bartlett's tests. When appropriate, multiple comparisons for means were carried out on significant main effects and interactions using Tukey's tests (Snedecor & Cochran 1980, Zar 1984). Differences between means were considered statistically significant for $p < 0.05$.

RESULTS

Physical-chemical characteristics of seawater

On average, the planned differences in pH between control and acidified treatments were achieved (Table 1). During the last 5 wk of exposure, a decreasing trend was apparent in the reduced pH treatments (Fig. 1), which might have been caused by slight drift in the reference electrodes. Salinity values consistently decreased during the exposure (Fig. 1), from 30 to 26. Seawater temperature varied between 15 and 20°C, while TA ranged from 3542 to 3554 µmol kg^{-1}. Dissolved oxygen in the exposure tanks consistently exceeded 96%.

Dry weight and physiological parameters

The DW and physiological parameters determined for the juvenile mussels before the exposure are shown in Table 2. At the end of the experiments, size and pH were both significantly correlated with the tissue DW (Table 2; 2-way ANOVA, Tukey's test, $p < 0.01$) resulting in the highest DW in the 22 to 29 mm mussel size class in the ΔpH = −0.6 treatment. Clearance, ingestion, metabolic rates and O:N index were significantly related to the mussel size, but not to the pH treatment (Tables 2 & 3; Tukey's test, $p < 0.01$, 2-way ANOVA).

pH was the only significant source of variation (2-way ANOVA) in absorption efficiency (AE) and ammonia excretion rate (V_{NH4-N}) of mussels (Table 3). Similarly to the DW, AE and V_{NH4-N} were inversely related to pH, for both size classes of mussels, with minimal values for the control pH and maximal values for ΔpH = −0.6. There were no significant differences in relation to size ($p > 0.05$) for these variables.

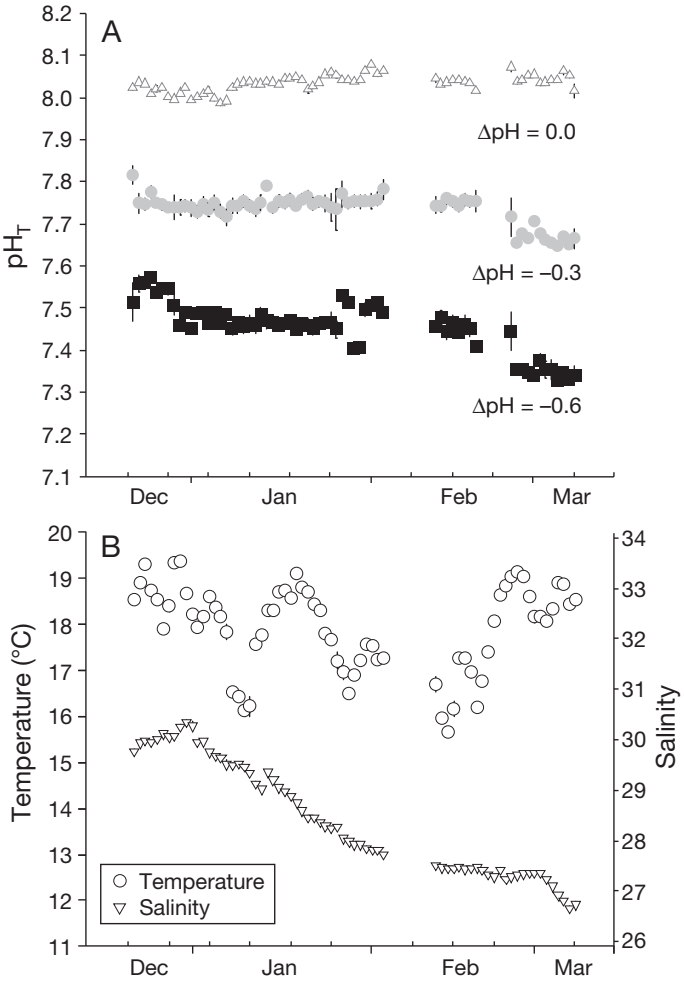

Fig. 1. Daily values (mean ± SE) for (A) pH and (B) temperature and salinity of seawater during the 78 d of exposure

Table 1. Seawater carbonate chemistry variables (mean ± SE, n = 3): pH values (total scale) are the average of automatic records during the exposure; (T = 17.73 ± 0.03°C), salinity (S = 28.79 ± 0.01) and total alkalinity (TA) were measured on 8 February 2010; dissolved inorganic carbon (DIC); partial pressure of CO_2 in seawater (pCO_2) and saturation state for calcite (Ω_{cal}) and aragonite (Ω_{ara}) were calculated from *in situ* temperature, salinity, pH and TA

pH level	pH_T	TA (µmol kg^{-1})	DIC (µmol kg^{-1})	pCO_2 (µatm)	Ω_{cal}	Ω_{ara}
ΔpH = 0.0	8.03 ± 0.01	3549 ± 2	3303 ± 3	963 ± 14	5.53 ± 0.07	3.58 ± 0.05
ΔpH = −0.3	7.74 ± 0.00	3542 ± 2	3452 ± 2	1989 ± 19	3.03 ± 0.00	1.96 ± 0.00
ΔpH = −0.6	7.48 ± 0.01	3554 ± 16	3584 ± 18	3790 ± 55	1.72 ± 0.03	1.11 ± 0.02

Scope for growth (SFG)

The 2-way ANOVA indicated that pH, size and the interaction of both factors all had a significant effect on SFG. The values of SFG were inversely related to pH, for both mussel size classes, with maximal values for ΔpH = −0.6. There were significant differences in relation to size (Tukey's test, $p < 0.01$) for this variable.

DISCUSSION

Current knowledge about the ecophysiological effects of seawater acidification on different taxonomic groups is limited (Melzner et al. 2009). Pörtner et al. (2004), Fabry et al. (2008) and Pörtner (2008) emphasized that we are only beginning to see the patterns that define tolerance versus sensitivity to future ocean acidification scenarios. Furthermore, Miller et al. (2009) suggested that the biological responses to acidification, especially for calcifying biota, will be species-specific and much more variable and complex than previously reported.

Our study shows that the levels of seawater acidification tested (pH reduced by 0.3 and 0.6 units, relative to the natural pH levels of Ría Formosa lagoon) had no effect on feeding (clearance and ingestion rates) and metabolic rates (V_{O2}) of juvenile *Mytilus galloprovincialis*. In contrast, the pH reductions tested increased AE, ammonia excretion ($V_{NH4\text{-}N}$) and organic tissue weight (see Table 2). The physiological energetics values measured in the mussel seed before the experiments were similar to those observed in previous studies (Babarro et al. 2000a, b).

Saphoerster (2008) reported inhibition of filtration activity for the blue mussel *Mytilus edulis* at elevated concentrations of CO_2 (4000 ppm), corresponding to a seawater pH of 7.2 and severe $CaCO_3$ under saturation. In our previous study with *Ruditapes decussatus* (Fernández-Reiriz et al. 2011), similar manipulations of seawater carbonate chemistry produced deleterious effects on feeding behaviour (clearance and ingestion rates). Furthermore, the most extreme pH reduction (ΔpH = −0.7) also caused a decrease in metabolic rates and an increase in ammonia excretion. That study also showed a loss of organic tissue weight with decreasing pH.

Other studies have shown deleterious effects of CO_2-induced acidification on bivalve molluscs. Bamber (1990), Michaelidis et al. (2005) and Gazeau et al. (2010), among others, recorded suppression of feeding activity and growth, depressed metabolism, increased N excretion and loss of tissue weight for marine bivalves exposed to reduced seawater pH. It should be noted, however, that significant effects were only observed at pH values < 7 or at pCO_2 levels which are beyond the worst CO_2 emission scenarios.

Table 2. *Mytilus galloprovincialis.* Physiological parameters (mean ± SD) of mussel juveniles. No. ind.: total number of individuals used in each physiological determination; CR: clearance rate; IR: ingestion rate; AE: absorption efficiency; V_{O2}: respiration rate; $V_{NH4\text{-}N}$: ammonia excretion rate; O:N: ratio of oxygen consumed to nitrogen excreted; SFG: scope for growth; OW: organic weight. Means within the same columns with different superscript letters are significantly different ($p < 0.05$). Three distinct pools of 5 (large) or 10 (small) individuals were used for each combination of pH level and size-class; each pool was composed of randomly selected individuals (within each size class) from 1 of the 3 replicate acclimation tanks for each level of pH. The decrease and increase values (%) in dry weight, AE and $V_{NH4\text{-}N}$ of mussels exposed to ΔpH −0.6 and ΔpH −0.3 versus pH control are given in brackets; the 2 values are for each of the 2 size classes (smallest and largest group respectively). Dates are given as dd.mo.yr

Date	Shell length (mm)	Dry weight (mg)	Ash (%)	No. ind.	CR (ml h^{-1})	CR (specific) (ml (mg OW)$^{-1}$ h^{-1})	IR (µg OW h^{-1})	IR (specific) (µg OW (mg OW)$^{-1}$ h^{-1})
Initial								
21.12.09	10-15	6.9±0.8	23.2±0.4	100	158.1±9.6	11.3±3.2	97.6±8.5	5.6±1.6
Exposure								
ΔpH = −0.6								
09.03.10	19–21	23.6±1.9^{a}	19.0±1.9	30	322.2±40.3^{a}	16.7±4.3^{a}	408.9±32.7^{a}	21.2±5.4^{a}
09.03.10	22–29	45.4±5.9^{b} (+10.8; +24.9)	19.6±1.0	15	841.8±82.9^{b}	23.3±4.0^{b}	1068.2±60.9^{b}	29.5±5.0^{b}
ΔpH = −0.3								
09.03.10	19–21	22.3±2.1^{c}	18.4±0.6	30	330.8±27.4^{a}	18.3±3.9^{a}	357.0±20.5^{a}	19.8±2.8^{a}
09.03.10	22–29	38.8±3.5^{d} (+4.4; +6.7)	21.7±1.0	15	793.2±78.5^{b}	25.7±6.5^{b}	952.7±78.6^{b}	30.8±7.8^{b}
ΔpH = 0.0								
09.03.10	19–21	21.3±2.8^{e}	16.6±0.5	30	325.7±31.4^{a}	18.4±2.8^{a}	367.5±21.5^{a}	20.8±7.6^{a}
09.03.10	22–29	36.3±2.0^{f}	18.5±0.8	15	850.4±65.9^{b}	28.6±4.7^{b}	1055.4±55.7^{b}	35.5±5.9^{b}

The responses of bivalves to ocean acidification are complex and suggest a large degree of variability in their sensitivity to this type of perturbation. Talmage & Gobler (2009) have shown that levels of atmospheric CO_2 similar to those predicted for the 21st century may affect some species of bivalves (e.g. *Mercenaria mercenaria*) more than others (e.g. *Crassostrea virginica*). Berge et al. (2006) have reported larger growth increments for small *Mytilus edulis* reared under conditions of increased pCO_2 and reduced pH (by 0.5 units), but attributed the effect to random variation. Furthermore, unchanged or even increased oxygen consumption has been observed for different marine taxa when exposed to acidification (Gutowska et al. 2008, Melzner et al. 2009, Comeau et al. 2010). The response in terms of calcification has also been showed to be more heterogeneous than previously thought. Reduced, conserved or even increased calcification rates under acidified conditions have been measured for different phyla and life stages (Gazeau et al. 2007, 2010, Gutowska et al. 2008, Wood et al. 2008, Ries et al. 2009, Comeau et al. 2010). In this respect, it is important to consider the remarkable characteristics of the carbonate chemistry of seawater from Ría Formosa lagoon. The TA is within the range observed in inner coastal waters of the southern Iberian Peninsula (Cabeçadas & Oliveira 2005, De la Paz et al. 2007, 2008), which generally exceeds the values reported in previous ocean acidification perturbation experiments (Nisumaa et al. 2010, Range et al. 2011). This naturally elevated TA (around 3550 µmol kg^{-1}) prevented undersaturation of $CaCO_3$, even in the CO_2-acidified treatments, avoiding any significant impacts related to the carbonate supply. Instead, as suggested in a recent study with lobster larvae that reported a similar buffering mechanism (Arnold 2008), the physiological alterations observed here were most likely the result of acidosis or hypercapnia interfering with normal homeostatic function.

Our study showed a significant increment in AE caused by seawater acidification (up to 72% in large mussels in the ΔpH = −0.6 treatment, see Table 2). This pattern may be related to the optimization of certain digestive enzymes (amylase, glucosidase and peptidase) under conditions of reduced pH (Wojtowicz 1972, Areekijseree et al. 2004), which could facilitate nutrient absorption. In agreement with the absorption efficiency (AE) response, the maximal SFG and tissue DW were observed for mussels exposed to the most extreme pH reduction (ΔpH = −0.6).

Intertidal species are usually equipped with compensatory physiological mechanisms that allow them to maintain homeostasis during tidal cycles (Montecinos et al. 2009). During periods of anaerobic metabolism there is a release of inorganic molecules (including $CaCO_3$ from valves) into the pallial cavity of molluscs, in order to maintain the acid-base balance (Chaparro et al. 2009). J. Espinosa (unpubl. data) observed that the range of pH in the pallial fluid of *Mytilus galloprovincialis* under anaerobic conditions is ~6.8 to 7.2, differing substantially from the average pH of seawater (~8.2). This suggests that the pallial fluid can act as an active compartment, which is involved in the physiological response of mussels. According to Pörtner et al. (2004), hypercapnia elicits a reduction in the aerobic metabolism of marine organisms as a result of acid-base disturbances. The metabolic response of mussels to the acidification of the pallial fluid and haemolymph (caused by the permanent acidification of the medium) could, therefore,

AE (%)	No. ind.	V_{O2} (ml O_2 h^{-1})	V_{O2} (specific) (µl O_2 (mg OW)$^{-1}$ h^{-1})	No. ind.	V_{NH4-N} (µg NH_4-N h^{-1})	V_{NH4-N} (specific) (µg NH_4-N (mg OW)$^{-1}$ h^{-1})	O:N (J h^{-1})	SFG
91.1±0.7	50	0.4±0.1	0.1±0.0	50	27.8±1.7	5.3±0.3	16.6±2.0	0.1±0.0
59.2±1.3^{a}	30	0.1±0.0^{a}	6.4±2.0^{a}	30	20.3±1.5^{a}	1.1±0.1^{a}	7.6±1.2^{a}	2.7±0.2^{a}
60.6±5.4^{a}	15	0.2±0.0^{b}	6.2±0.6^{a}	15	24.0±1.3^{a}	0.7±0.0^{b}	12.0±1.8^{b}	10.1±1.5^{b}
(+56.1; +72.1)					(+17.9; +59.6)			
38.6±2.4^{b}	30	0.1±0.0^{a}	6.9±1.3^{a}	30	19.5±2.8^{a}	1.1±0.2^{a}	7.7±1.9^{a}	0.3±0.1^{c}
41.6±3.0^{b}	15	0.2±0.0^{b}	6.4±0.2^{a}	15	22.3±1.9^{a}	0.7±0.1^{b}	12.9±2.1^{b}	4.3±1.0^{d}
(+1.7; +18.4)					(+13.1; +48.6)			
37.9±4.4^{bc}	30	0.1±0.0^{a}	6.4±2.0^{a}	30	17.2±4.5^{b}	0.8±0.3^{b}	6.3±1.9^{a}	0.4±0.1^{c}
35.2±7.6^{c}	15	0.3±0.0^{b}	6.9±0.8^{a}	15	15.0±3.1^{b}	0.7±0.2^{b}	14.1±2.3^{b}	3.5±0.8^{d}

Table 3. *Mytilus galloprovincialis.* Two-way ANOVA testing the physiological parameters in mussels exposed to different values of pH. O:N: ratio of oxygen consumed to nitrogen excreted; ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Source	Sum of squares	df	Mean square	*F*-ratio
Dry weight				
pH	95.1	2	47.5	4.0*
Size	1374.4	1	1374.4	115.7***
pH × Size	33.7	2	16.8	1.4ns
Error	142.6	12	11.9	
Clearance rate				
pH	7.2×10^3	2	3.6×10^3	1.4ns
Size	1223.9×10^3	1	1223.9×10^3	474.4***
pH × Size	0.5×10^3	2	0.3×10^3	0.1ns
Error	30.9×10^3	12	2.5×10^3	
Ingestion rate organic				
pH	3.6×10^3	2	1.8×10^3	0.3ns
Size	1609.6×10^3	1	1609.6×10^3	319.2***
pH × Size	18.8×10^3	2	9.4×10^3	1.8ns
Error	60.5×10^3	12	5.0×10^3	
Absorption efficiency				
pH	2.0×10^3	2	1.0×10^3	73.2***
Size	0.6	1	0.6	0.0ns
pH × Size	55.2	2	27.6	2.0ns
Error	165.2	12	13.7	
Metabolic rate (V_{O2})				
pH	0.000	2	0.000	0.017ns
Size	0.053	1	0.053	36.242***
pH × Size	0.000	2	0.000	0.162ns
Error	0.018	12	0.001	
Ammonia excretion rate (V_{NH4-N})				
pH	53.8	2	26.9	4.2*
Size	16.1	1	16.1	2.5ns
pH × Size	8.6	2	4.3	0.7ns
Error	77.0	12	0.4	
O:N index				
pH	0.939	2	0.469	0.083ns
Size	152.193	1	152.193	26.979***
pH × Size	9.616	2	4.888	0.852ns
Error	67.694	12	5.641	
Scope for growth (SFG)				
pH	72.325	2	36.163	22.231***
Size	105.173	1	105.173	64.656***
pH × Size	15.583	2	7.792	4.790**
Error	19.500	12	1.627	

be similar to the response of intertidal individuals to anaerobic conditions.

According to De Zwaan et al. (1976), a pH decrease in the internal fluids causes a shift of mussel metabolism to partial anaerobiosis, with a consequent degradation of proteins. Due to the experimental design of this study, metabolic conditions of partial anaerobiosis were probably achieved, inducing the mussels to move from a state of anabolism to catabolism. Our results show significant differences in the excretion of ammonia (V_{NH4-N}) by *Mytilus galloprovincialis,* caused by the pH reductions, with larger values measured in mussels exposed to the most extreme pH reduction (ΔpH = −0.6). This increase in NH_4 excretion under reduced pH conditions can be interpreted as an intracellular pH regulatory mechanism. In fact, according to Boron (2004), greater excretion and protein degradation may support the production of HCO_3^- and, consequently, promote pH regulation. Also, Michaelidis et al. (2005), suggested that short-term (20 to 24 h) incubation of *M. galloprovincialis* under acidified conditions resulted in an increased excretion of ammonia, indicating net degradation of proteins. Thomsen & Melzner (2010) observed that NH_4^+ excretion in *M. edulis* rose with increasing pCO_2, and concluded that the decreased O:N ratios observed at the highest seawater pCO_2 indicated enhanced protein metabolism, which contributed to intracellular pH regulation.

Overall, these results suggest that *Mytilus galloprovincialis* could be a tolerant ecophysiotype to CO_2 acidification, at least in highly alkaline coastal waters. Nevertheless, mytilids are also able to dominate habitats with low alkalinity and high pCO_2 (Thomsen et al. 2010). The mechanisms by which these mussels are able to compensate their physiological responses (i.e. increased ammonium excretion and absorption efficiency) to long-term exposure to acidified seawater are probably the same as those which explain their versatility in aerobic and anaerobic environments. According to Kroeker et al. (2010), the biological effects of ocean acidification are generally large and negative, but the variation in sensitivity amongst organisms has important implications for ecosystem responses. Accordingly, given the widespread presence of *M. galloprovincialis* in many coastal systems worldwide, its physiological behaviour might be pre-adapted to cope with future ocean acidification scenarios.

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