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Behavioural and eco-physiological responses of the mussel *Mytilus galloprovincialis* to acidification and distinct feeding regimes

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ABSTRACT: The carbon dioxide taken up by the ocean is increasing as levels of atmospheric carbon dioxide increase, thus lowering the ocean pH and altering the carbonate system. In this laboratory study, we evaluated the physiological responses of juvenile mussels *Mytilus galloprovincialis* from Galician waters (NW Iberian Peninsula) exposed to control (500 µatm) and elevated (800 or 1200 µatm) seawater pCO_2 conditions under 2 different feeding regimes (optimal and suboptimal). Shell properties such as compressive strength and composition (organic matter and aragonite:calcite ratio) were negatively affected by high seawater pCO_2, regardless of food availability. This result suggests that water chemistry is a main driver for shell development. Under the optimal feeding regime, mussel feeding rates increased in response to elevated pCO_2, presumably as a strategy to maintain a high strength of attachment. In contrast, mussels on the suboptimal diet showed weak attachment and narrow valve opening at the highest pCO_2 condition. Thus, our results suggest that with optimal food availability, mussels were resilient to water acidification with respect to feeding activity, valve opening and attachment strength. Under a suboptimal diet, however, the ability of mussels to respond to acidification was compromised. These results highlight complex ecophysiological interactions for calcifying organisms subjected to climate change.

KEY WORDS: Marine mussels · Acidic conditions · Diet · Interaction · Behaviour

1. INTRODUCTION

Since the beginning of the industrial revolution, atmospheric levels of carbon dioxide (CO_2) have been increasing at rates faster than previously experienced in the history of the Earth (https://earthobservatory.nasa.gov/Features/GlobalWarming). The partial pressure of CO_2 in the atmosphere (pCO_2) has increased from 280 µatm at the start of the Industrial Revolution to a current level of about 408 µatm (https://www.noaa.gov/) and is expected to rise above 1100 µatm by the end of this century, which would lead to a decrease in seawater pH of approximately 0.3–0.4 units (IPCC 2014). The oceanic sink represents about 48% of the total fossil-fuel and cement-manufacturing emissions (Sabine et al. 2004), which in turn lower the pH of seawater and cause shifts in seawater carbonate chemistry in a process known as ocean acidification (OA) (Zeebe & Wolf-Gladrow 2001, Doney et al. 2009). OA can alter many biochemical and physiological processes in marine organisms such as growth, ingestion, calcification, reproduction, behaviour, survival, gene expression and biological interactions (Gazeau et al. 2007, Kroeker et al. 2010, Clements & Hunt 2015 among others). However, when organisms are frequently exposed to low pH in their natural

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environments, the impact of acidification does not appear to be very important (Melzner et al. 2009, Hiebenthal et al. 2013), suggesting local adaptation.

Calcifying organisms are particularly vulnerable to OA (Hendriks et al. 2010, Kroeker et al. 2013). Carbonate is vital for the bio-mineralization of calcium carbonate ($CaCO_3$) in their protective shells, and elevated seawater pCO_2 shifts the carbonate system speciation towards a decreased concentration of carbonate ions (CO_3^{2-}) as well as saturation states of the biologically important $CaCO_3$ minerals calcite (ΩCa) and aragonite (ΩAr). Recently, the role of another carbonate system species (i.e. HCO_3^-) has been highlighted (Thomsen et al. 2015).

Any strategy to cope with a lower pH in seawater would be energetically demanding (Thomsen et al. 2013, Pan et al. 2015), and therefore factors such as energetic status and food availability are of great importance. According to Ramajo et al. (2016a), in around half of laboratory experiments that monitored responses of larvae, juvenile and adult calcifiers to OA and that were included in a meta-analysis (Kroeker et al. 2013), the animals were not supplied with food. The few studies which have investigated the interactive effects between OA and food availability have shown that the negative effects of acidification may disappear when the organisms are supplied with optimal food resources, e.g. as in highly productive ecosystems (Melzner et al. 2011, Thomsen et al. 2013, Ramajo et al. 2016a,b).

Other factors apart from food availability, e.g. temperature, salinity and hypoxia-anoxia, may also act as global marine stressors and interact with OA. The link between temperature and food supply is one of the most studied in the literature and is reported to affect a number of responses, from filtration, absorption and utilization rates of available food to distinct physiological activities (Zippay & Helmuth 2012). Seawater warming is one of the most commonly studied drivers, and synergistic patterns of interactions with OA have been identified (Orr et al. 2005, Feely et al. 2008). However, in areas characterized by upwelling systems, like the Atlantic Galician coast in the NW Iberian Peninsula at the northernmost limit of the Eastern North Atlantic Upwelling System (Wooster et al. 1976), the waters are characteristically cold, nutrient-rich and CO_2-rich (Feely et al. 2008). In such systems, calcifiers may be less vulnerable to low saturation states for carbonate minerals and may benefit from abundant food supplies (Peterson et al. 1988, Figueiras et al. 2002), thus enabling them to develop energy-demanding biological mechanisms to resist OA (Hendriks et al. 2015).

As OA may primarily affect the formation of the $CaCO_3$ shell in calcifiers, other eco-physiological responses have received less attention—such as the strength of byssal attachment in mussels (which ensures settlement and survival of sessile organisms) and valve opening behaviour, which are linked to other vital functions such as foot extension, gas exchange, feeding and excretion. Studies concerning the impact of OA on feeding activity (clearance rates, CRs) show highly variable signals ranging from negative through neutral to positive (Fernández Reiriz et al. 2011, 2012, Gazeau et al. 2013, Navarro et al. 2013, 2016, Ramajo et al. 2016b). Regarding attachment strength, recent studies have revealed that the byssus filaments may also be negatively affected by OA in different ways (O'Donnell et al. 2013, Zhao et al. 2017, Babarro et al. 2018). Clements et al. (2018), however, noted that such negative effects on byssal attachment are not universal, and other factors such as condition index may play an important role.

Monitoring shell movements with high-frequency, non-invasive (HFNI) electromagnetic-based biosensors is a novel technique recently used to document acute and long-term effects of elevated CO_2 (Clements & Comeau in press). Although OA was not found to have clear effects on shell movements in several species (see review in Clements & Comeau in press), such a real-time behavioural response may provide a bio-monitoring tool for coastal shellfisheries in relation to OA or other global stressors (Andrade et al. 2016, Comeau et al. 2019) because it provides clues to understanding several vital activities that may be altered in response to sub-lethal exposure.

Short-term OA experiments are well documented and particularly valuable for studying OA as a driver of eco-physiological changes in bivalve molluscs. Here, we aimed to investigate the impact of OA on the tissues representative of (1) the attachment strategy (byssus) and (2) protection (shell). For this purpose, behavioural and eco-physiological responses in juveniles of *Mytilus galloprovincialis* were integrated to obtain a better understanding of the resilience of mussels. Food availability represented a secondary stressor to investigate any synergies with OA. Based on previous similar studies, we can hypothesize that optimal food supply would make mussels at least partly more capable of balancing any impairment driven by OA (Melzner et al. 2011, Thomsen et al. 2013, Ramajo et al. 2016a). This research is of particular interest in relation to the upwelling-influenced coastal waters of Galicia (NW Iberian Peninsula), where mussels have been successfully cultivated for

decades. Given the socioeconomic and ecological importance of this bivalve in coastal aquaculture in the Atlantic region of Spain, understanding how future seawater conditions may affect cultured species is of vital importance.

2. MATERIALS AND METHODS

2.1. Collection and maintenance of mussels

Juvenile specimens of the mussel *Mytilus galloprovincialis* (shell length mean ± SD: 30.69 ± 0.28 mm) were collected from a raft in the subtidal zone of the mouth of the Ría de Arousa (NW Spain), the site closest to open Atlantic Ocean influence, in autumn 2017. The specimens were immediately transported to the IIM-CSIC laboratory, where epibionts were removed from the shells and byssal threads carefully cut from the ventral margin to prevent damaging the byssus gland or foot organ. The mussels were maintained in an open-through flow system of natural filtered seawater (1 µm) at 15°C with a 12 h light:12 h dark photoperiod cycle, resembling natural conditions. The system supplied approximately 1 mg l^{-1} of seston as a mixture of 2 phytoplankton cultures of *Isochrysis galbana* clone T-Iso and *Rhodomonas lens* (50–50 % in weight).

2.2. Experimental design for CO_2 and food availability

After an acclimation period of 10 d in the laboratory, the mussels were exposed to a modified carbonate system for 3 wk in order to detect any synergistic effects between type of feeding regime and acidified environment. For each food supply test, 12 rectangular tanks (9 l; length × width × height: 34 × 23 × 19 cm) were used, i.e. 4 tanks per pCO_2 treatment (3 tanks with mussels + 1 control without mussels). A total of 18 juvenile specimens of *M. galloprovincialis* were placed in each tank and eventually formed clusters. Three 200 l header tanks containing the desired concentrations of food were used to supply the experimental tanks via peristaltic pumps (ISMATEC^R). The header tanks with food, peristaltic pumps and experimental tanks were connected by tubing arranged in a random order. Mussels were allowed to establish primary attachment to experimental units (glass plates, length × width: 24 × 19 cm) for 1 wk, and any individuals that did not produce byssus were removed and replaced.

Control pCO_2 levels were determined using measured pCO_2 levels in the Rías recorded between June 2017 and June 2018 following Gago et al. (2003), who mapped the spatial and seasonal variability in pCO_2 by dividing the Ría de Vigo into 3 distinct regions. The mean pCO_2 value estimated from the superficial measurements in 5 stations between June 2017 and June 2018 in the Ría of Vigo was 478 ± 119 µatm. Thus, we set the present pCO_2 value to 500 µatm.

In addition to the current level of CO_2 in these coastal waters, the pCO_2 values used as probable future acidification were 800 and 1200 µatm. Atmospheric air and pure CO_2 gas were previously mixed in separate tanks before being constantly bubbled through the experimental tanks, which also helped to homogenize the water held at a constant temperature (15 ± 1°C). Gas concentrations were logged continuously using LI-COR 6262 CO_2 gas analyzers, and the measurements were used to adjust the gas mixture through software-controlled solenoid valves.

Seawater salinity was checked weekly (8410 Portasal; Guildline Instruments). Seawater samples were collected (also every week) for duplicate total alkalinity (A_T) analysis. A_T was measured using a one endpoint method, with an automatic potentiometric titrator (809 Titrando and 800 Dosino; Metrohm) equipped with a combined glass electrode (Perez & Fraga 1987a). The samples were transferred with the aid of a Knudsen pipette (~50 ml) to an open Erlenmeyer flask for potentiometric titration with 0.1 M HCl. The final titration volume was determined by 2 pH readings after the endpoint of 4.45 was reached (Mintrop et al. 2000). Certified reference material (CRM; batch #163) for CO_2 in seawater (provided by A. Dickson, Scripps Institution of Oceanography) was used to quantify the analytical error. The alkalinity was 2214 ± 10 µmol kg^{-1} for the test samples and 2212.6 ± 0.4 µmol kg^{-1} for the CRM. Seawater samples were collected twice weekly from all tanks and placed directly into special optical glass spectrophotometric cells (volume: 28 ml; path length: 100 mm), for pH determination. These cells were held in a thermostatic bath at 25.0°C for approximately 1 h before analysis. The pH was measured using the spectrophotometric method described by Clayton & Byrne (1993). Briefly, this method consists of adding 75 µl of *m*-cresol purple to the seawater sample and measuring its absorbance at 3 wavelengths. Absorbance measurements were performed with a Shimadzu spectrometer (UV-2401PC). The pH values were calculated following equations described by Dickson et al. (2007), who included a correction factor for the difference between seawater and the acidity indicator. Seawater

A_T, salinity, temperature and pH were used to calculate other seawater parameters (Table 1), using CO2SYS (van Heuven et al. 2011) with dissociation constants for carbonic acid (Lueker et al. 2000) and the constants for borate and hydrofluoric acid (Dickson 1990, Perez & Fraga 1987b). The A_T values were relatively lower in the optimal feeding conditions, which may suggest that $CaCO_3$ fixation by mussels was active, leading to a significant reduction in A_T relative to the suboptimal conditions. This would suggest that in conditions less favorable for calcification, mussels are even able to fix more $CaCO_3$. However, our short-term experiment did not allow us to confirm this fact with actual measures of shell growth.

The experimental diets were produced by manipulating the 2 microalgae cultures (*I. galbana* clone T-Iso and *R. lens*) together to yield particle load values of 1.4 ± 0.14 and 0.3 ± 0.07 mg l^{-1}. These distinct food availability conditions represent, respectively, optimal (approximately 6% of the dry weight [DW] of an individual mussel per day) and suboptimal (1.5% DW ind. d^{-1}) diets for the experimental mussels. These seston loads used in the experiment reflect the natural range encountered in the Galician Rías (Babarro et al. 2000). Food was supplied to the mussels at a continuous flow rate of 9 ml min^{-1} from the header tanks for each feeding regime. This flow rate enabled removal of ammonium and other waste products due to mussel metabolism or bacteria.

2.3. Mussel responses

2.3.1. Clearance rates, byssus strength and valve opening behaviour

CRs were measured as the volume of water cleared of known suspended algal cells (T-ISO and *R. lens*) following Cranford et al. (2016). An appropriately calibrated PAMAS laser particle counter (Model S4031GO containing an HCB-LD-50/50 light-scattering sensor) was used for this purpose. As the phytoplankton cells used in this study were within a known size range, the PAMAS was initially set to determine the particle size distribution between 1 and 20 µm. Nevertheless, the actual range monitored to determine CRs was set between 4.5 and 7.5 µm, according to the highest retention efficiency values for the mussels (above 4 µm; Møhlenberg & Riisgård 1978, Cranford et al. 2016). The CR was measured twice a week during the experiment. Flow (feeding) was stopped 1 h before PAMAS measurements. The mussels were left undisturbed on the bottom of the tanks, i.e. attached on glass substrates. A known volume of phytoplankton cells of the 2 microalgae used in the experiment was added to each tank and PAMAS measurements began after 5 min, thus ensuring good mixing. This volume of phytoplankton served as a trigger for initial CR measurements under static (not continuously feeding) conditions just to restore the values before the flow-through was ceased in both optimal and suboptimal feeding regimes. The homogeneity of the phytoplankton concentration within experimental tanks was aided by the CO_2–air agitation system (see Section 2.2) and confirmed by noting the concentration of microalgae across different sections of the tank (right to left and bottom to top). The PAMAS was immediately set up to monitor the decrease in the number of algal cells as a function of time for 20 min. Control tanks with no mussels were also monitored to identify any reduction in phytoplankton due to deposition on the bottom and to correct the experimental slopes of those tanks with mussels; any decrease was subsequently corrected by the slope of the control tank. The CR was determined from the linear decrease in algal concentration (verified as a straight line in a semi-log plot) over time, with the formula proposed by Riisgård & Seerup (2003):

Table 1. Experimental seawater chemistry parameters: salinity, pH, pCO_2, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), total alkalinity (A_T), calcite and aragonite saturation states (ΩCa and ΩAr). Values are means ±SD

Diet treatment	Salinity (ppt)	pH	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	A_T (µmol kg^{-1})	ΩCa	ΩAr
Optimal								
500 µatm	34.90 ± 0.06	7.957 ± 0.004	506 ± 4	1970 ± 8	132.1 ± 1.2	2299 ± 10	2.34 ± 0.02	1.500 ± 0.014
800 µatm	34.92 ± 0.04	7.769 ± 0.010	814 ± 20	2056 ± 8	89.5 ± 1.8	2280 ± 7	1.58 ± 0.03	1.017 ± 0.021
1200 µatm	35.01 ± 0.06	7.609 ± 0.006	1202 ± 21	2099 ± 16	63.3 ± 0.8	2258 ± 16	1.13 ± 0.02	0.726 ± 0.009
Suboptimal								
500 µatm	34.78 ± 0.25	7.971 ± 0.005	488 ± 2	1973 ± 10	138 ± 0.6	2315 ± 12	2.43 ± 0.02	1.560 ± 0.015
800 µatm	34.75 ± 0.27	7.810 ± 0.005	743 ± 6	2075 ± 17	100 ± 0.6	2323 ± 19	1.76 ± 0.02	1.130 ± 0.015
1200 µatm	34.87 ± 0.28	7.621 ± 0.005	1195 ± 14	2156 ± 18	67 ± 0.6	2324 ± 19	1.20 ± 0.01	0.768 ± 0.008

$$CR = \lambda V/n \quad (1)$$

where V is the volume of water in the tank, λ is the slope of the regression line (semi-log plot) of the reduction in algal concentration over time in the aquarium with mussels and n is number of mussels per tank (here, n = 18). Only regression coefficients (r^2) greater than 0.9 were considered for the analyses.

The byssus strength of the mussels was measured by connecting one individual from the cluster to a dynamometer (Digital Force Gauge DN431 with peak hold indication, resolution of 0.01 N). Groups of 5–6 mussels per tank were analyzed at the end of the experiment. When sampling individual mussels, care was taken to avoid disturbing the neighbouring mussels. Therefore, the individuals immediately adjacent to those selected for dislodgement were not considered when the byssal threads were interconnected (Babarro & Comeau 2014).

For each pCO_2 treatment, 4 individuals in 2 of the 3 experimental tanks containing mussels were connected to a non-invasive valvometry system (n = 8 mussels per experimental condition). A coated Hall element sensor (HW-300a; Asahi Kasei) fitted to a small electrical cable (1.5 mm diameter, 4.9 g m^{-1}) was attached to a valve with cyanoacrylate glue. A magnet (4.8 mm diameter × 0.8 mm high) was then glued to the other valve so that it was located on the opposite side of the Hall sensor. For other specifications of this valvometry system, see Comeau et al. (2018). Valve opening was monitored throughout the experimental period.

2.3.2. Shell compressive strength, organic matter of the shell and aragonite:calcite ratio

The same individuals used to measure byssus strength within the mussel cluster were used to determine shell compressive strength. The left valve was chosen for all analyses. The compressive force required to crack the shell was measured using a universal testing machine (Instron 5566), with 1 kN load cell and at a rate of 2 mm s^{-1}; the shells were arranged horizontally (see Babarro & Abad 2013 for other specifications). Shell strength was calculated from the maximum force measured in the curves and was then normalized by shell thickness, measured with a micro-calliper (Mitutoyo 0–25 ± 0.01 mm) at the highest point of the shell where the force was applied.

Three mussels were collected from the other mussel clusters in the tanks after measurement of byssus strength. The organic matter (OM) (periostracum) and calcite and aragonite contents of the shell were determined following Addadi et al. (2006). OM was determined using the gravimetric method, by calculating the loss of organic weight after calcination at 500°C for 48 h; calcium, magnesium and strontium content were measured after hydrochloric acid extraction in an inductively coupled plasma optical emission spectrometer (ICP-OES). Semi-quantitative phase proportions were determined for mixtures of carbonate minerals by powder X-ray diffraction, as outlined by Davies & Hooper (1963) for calcite and aragonite. All elemental and compound analysis was performed at CACTI, analytical services from University of Vigo.

2.3.3. Specific growth rate

The length, width and height of all mussel shells were measured at the beginning and end of the experiment. The mean (±SD) values for each mussel cluster in each tank were then obtained. Specific growth rate (SGR) of the mussels was calculated as follows:

$$SGR = \ln(L_f/L_i) \times t^{-1} \quad (2)$$

where L_f and L_i represent the shell lengths at the end and beginning of the experiment, respectively, and t is the duration of the experimental period (22 d) (Christensen et al. 2015).

2.4. Statistical analysis

The effects of pCO_2 (3 levels: 500, 800 and 1200 µatm) and feeding regime (2 levels: optimal and suboptimal) on mussel CRs as a response variable were tested at the cluster level by repeated measures ANOVA. For this purpose, we analyzed differences between groups in CRs over time (3 wk) (measured twice wk^{-1}; see Section 2.3.1). Byssus attachment strength, valve opening, shell compressive strength, OM of the shell, aragonite:calcite (ΩAr:ΩCa) ratio and SGR variability were analyzed by 2-way ANOVA with pCO_2 level and food regime as independent factors.

Prior to statistical analysis, tank was considered a random experimental unit and was tested, in both repeated measures ANOVA and 2-way ANOVAs, for any significant effect on mussel responses. After confirmation of the lack of any effect of the experimental unit (tank), the analyses were repeated for the factors under study. Normality and homogeneity of vari-

ances were examined by Shapiro-Wilk's *W*-test and Levene's test, respectively. Whenever the assumptions of analysis of variance were violated, data were log or rank transformed (Conover 2012). Homogeneous groups were established *a posteriori* with the Bonferroni adjusted level for distinct sample sizes and Tukey's tests for multiple comparisons. All values shown in the figures are means ± SD. All analyses were performed with STATISTICA v.7.0 software (StatSoft) and SPSS Statistics 23 (IBM). Results were considered significant at $p \leq 0.05$.

3. RESULTS

3.1. Clearance rates, byssus strength and valve opening behaviour

The CRs of the mussels subjected to distinct pCO_2 levels and the corresponding food levels are shown in Fig. 1A. Both pCO_2 and food availability factors had interactive effects on the CRs of individuals (pCO_2 × diet; $p < 0.001$; Table 2) so that the effect of pCO_2 depended on the amount of food supplied in the experiment. In mussels supplied the optimal diet, CRs increased significantly with increasing pCO_2 (i.e. by 60 and 80% at pCO_2 800 and 1200 µatm, respectively) (Fig. 1A). In contrast, no increase in CR was observed in mussels supplied the suboptimal diet, and the values (between 0.67 and 0.79 l h^{-1} for all pCO_2 levels tested) were significantly lower than for optimally fed mussels (Fig. 1A).

The variability in byssus strength for mussels exposed to different pCO_2 and food regimes is shown in Fig. 1B. The 2-way ANOVA revealed a highly significant impact of food regime but also a small interaction effect (pCO_2 × diet) (Table 2). Byssus attachment was stronger (and similar among pCO_2 levels) in mussels supplied the optimal diet, with values ranging more narrowly between 7.1 and 7.9 N for all

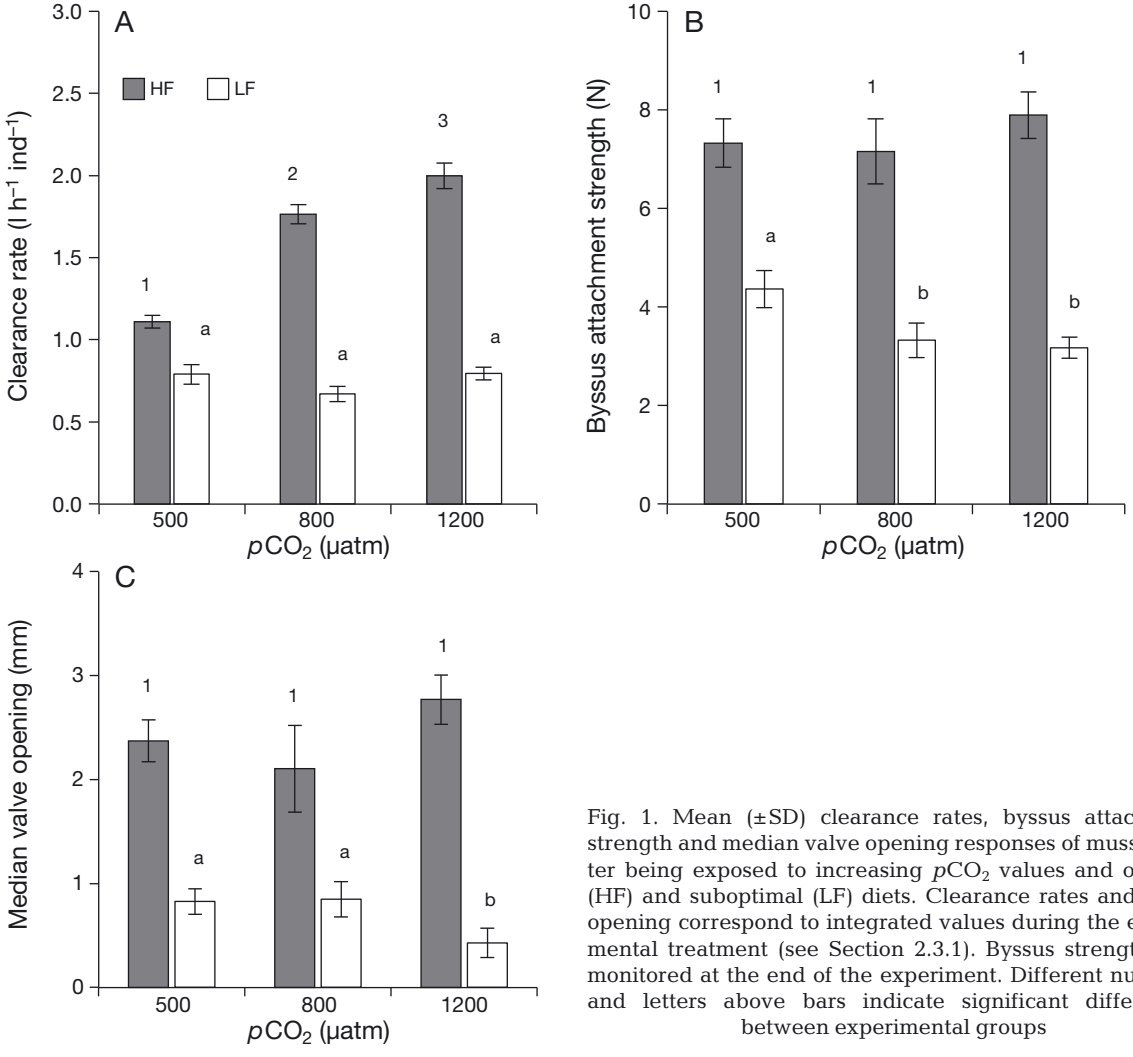

Fig. 1. Mean (±SD) clearance rates, byssus attachment strength and median valve opening responses of mussels after being exposed to increasing pCO_2 values and optimal (HF) and suboptimal (LF) diets. Clearance rates and valve opening correspond to integrated values during the experimental treatment (see Section 2.3.1). Byssus strength was monitored at the end of the experiment. Different numbers and letters above bars indicate significant differences between experimental groups

Table 2. Results of repeated measurements ANOVA (clearance rates) and 2-way ANOVAs to determine the effect of acidification values (pCO_2) and feeding regime (diet) on distinct mussel responses as indicated. When indicated, dependent variables were rank-transformed prior to analysis. Tank was previously included in the analyses as a random experimental unit (see Section 2.4) but subsequently removed when confirming the non-significant effect. Values in **bold** are statistically significant ($p < 0.05$); ns: not significant

Factor	df	*F*	p
Clearance rates			
pCO_2	2	24.689	**<0.001**
diet	1	498.854	**<0.001**
pCO_2 × diet	2	32.898	**<0.001**
Error	12		
Byssus strength (rank-transformed)			
pCO_2	2	2.014	ns
diet	1	143.451	**<0.001**
pCO_2 × diet	2	3.856	**<0.05**
Error	97		
Median valve opening (rank-transformed)			
pCO_2	2	1.459	ns
diet	1	55.288	**<0.001**
pCO_2 × diet	2	4.684	**<0.05**
Error	39		
Shell compressive strength (rank-transformed)			
pCO_2	2	10.315	**<0.001**
diet	1	2.524	ns
pCO_2 × diet	2	0.641	ns
Error	102		
Organic matter of the shell			
pCO_2	2	5.992	**<0.01**
diet	1	2.011	ns
pCO_2 × diet	2	1.290	ns
Error	47		
ΩAr:ΩCa ratio			
pCO_2	2	14.023	**<0.001**
diet	1	0.013	ns
pCO_2 × diet	2	0.219	ns
Error	47		
Specific growth rate, SGR (rank-transformed)			
pCO_2	2	1.386	ns
diet	1	28.553	**<0.001**
pCO_2 × diet	2	0.472	ns
Error	12		

pCO_2 values than for suboptimally fed mussels (3.1–4.4 for all pCO_2 values; Fig. 1B). In contrast, in mussels supplied the suboptimal diet the byssus attachment strength was slightly lower (23–27%) at the highest pCO_2 values than in the water tanks with 500 µatm pCO_2, as reflected in the dependency of both factors in the statistical analysis (Table 2; pCO_2 × diet; $p < 0.05$).

Mean valve opening in the mussels during the whole experimental period, considering the impact of both pCO_2 and food availability factors, is presented in Fig. 1C. Valve opening was significantly greater in the optimally fed mussels (ranging from 2.10–2.77 mm for all pCO_2 values considered) than in the mussels supplied the suboptimal diet (0.43–0.85 mm for all pCO_2 values; Fig. 1C, Table 2). As an independent factor, pCO_2 level did not have any effect on valve opening, but the slightly significant interaction between pCO_2 and diet ($p < 0.05$; Table 2) highlighted that differences in the feeding regime were greater at the highest pCO_2 levels (Fig. 1C). This established that the most stressful scenario tested (highest pCO_2 and low food availability) was responsible for the lowest valve opening (0.43 mm), relative to valve opening of 0.83 mm in waters with pCO_2 at 500 µatm (Fig. 1C).

3.2. Compressive strength, organic matter and ΩAr:ΩCa ratio in mussel shells

The variability in shell compressive strength of the mussels as a function of pCO_2 level and food regime is presented in Fig. 2A. The 2-way ANOVA revealed a highly significant impact of pCO_2 as an independent factor (Table 2). Neither the different feeding regimes used in the experiments nor the interaction term (pCO_2 × diet) affected the variability (Table 2). Shell strength decreased significantly (20–30%) from mean values of 82–80 N mm^{-1} (500 µatm pCO_2) to around 55–69 N mm^{-1} in the more acidified waters in equilibrium with atmospheres of 800 and 1200 µatm (Fig. 2A).

The 2-way ANOVA considering shell OM showed that, similar to shell strength, pCO_2 only had a significant effect as an independent factor (Fig. 2B). Neither food availability nor the interaction term (pCO_2 × diet) appeared to be significant factors (Table 2). The shell OM, which represented 5.2% of the total shell weight under pCO_2 conditions of 500 µatm, decreased by 6% on average for pCO_2 levels of 800 and 1200 µatm (Fig. 2B).

The mineral content of the shells expressed as an ΩAr:ΩCa ratio only varied significantly with the variable pCO_2 in the experiment (Fig. 2C, Table 2). Similar to shell strength and OM, neither food availability nor its interaction term (pCO_2 × diet) caused any important variation in the ΩAr:ΩCa ratio (Table 2). As pCO_2 increased, the ΩAr:ΩCa ratio decreased by 7–8% and 18% for mussel shells exposed to 800 and 1200 µatm pCO_2, respectively (Fig. 2C).

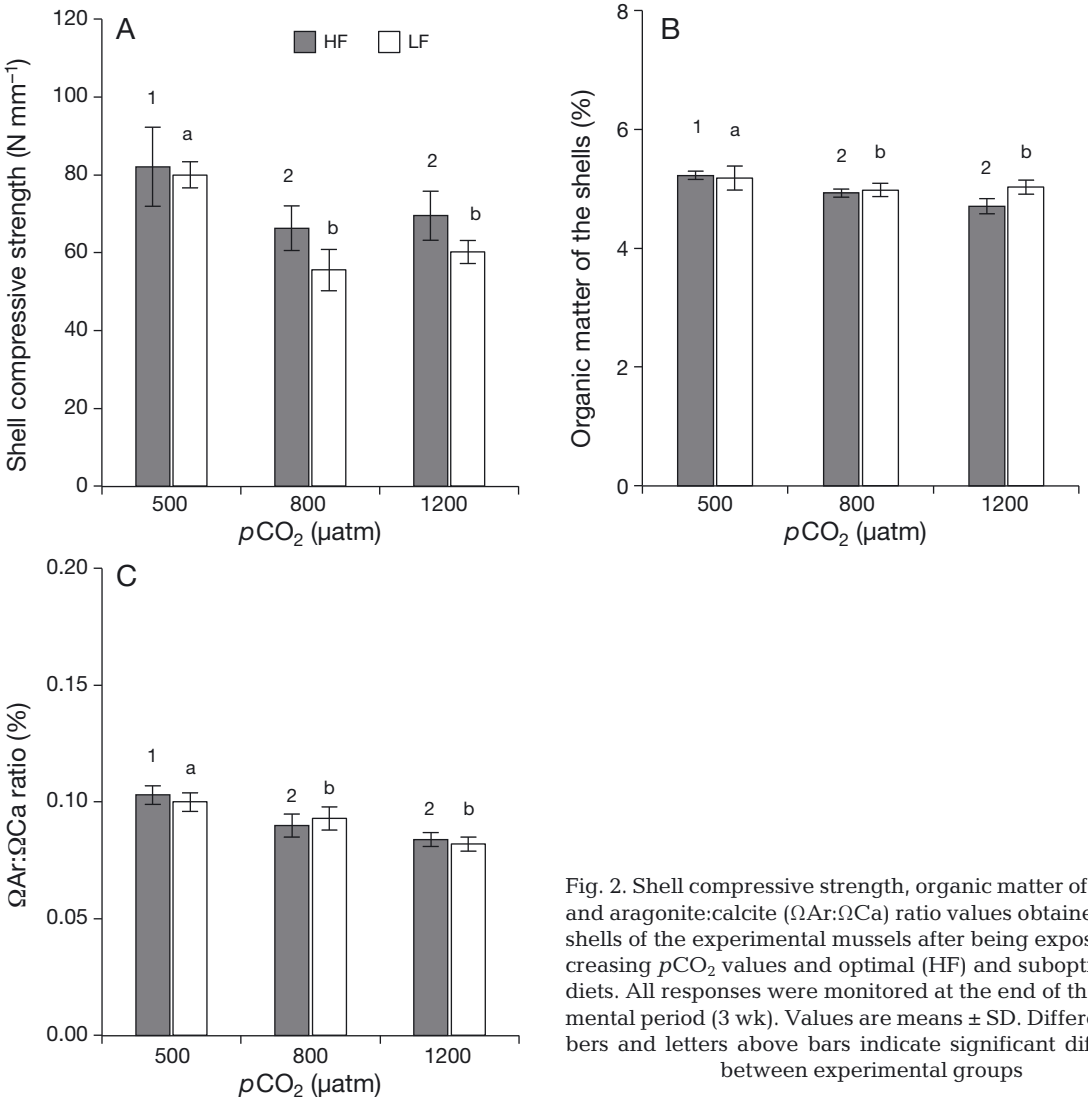

Fig. 2. Shell compressive strength, organic matter of the shell and aragonite:calcite (ΩAr:ΩCa) ratio values obtained for the shells of the experimental mussels after being exposed to increasing pCO$_2$ values and optimal (HF) and suboptimal (LF) diets. All responses were monitored at the end of the experimental period (3 wk). Values are means ± SD. Different numbers and letters above bars indicate significant differences between experimental groups

3.3. SGR

The increase in shell length of the experimental mussels as SGR based on initial and final values of the cluster is illustrated in Fig. 3. As expected, growth was almost nil in the suboptimally fed mussels (Fig. 3). In contrast, despite relatively short-term exposure (i.e. 3 wk), the shell length of the optimally fed mussels increased by 0.031–0.070 d^{-1} (Fig. 3). The 2-way ANOVA revealed a significant impact of food availability on SGR, whereas neither pCO$_2$ nor the interaction term (pCO$_2$ × diet) had any effect, despite the high variability noted (Table 2, Fig. 3).

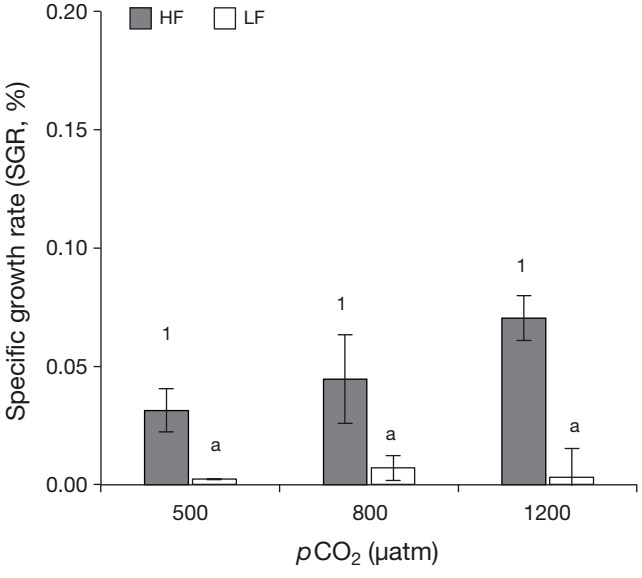

Fig. 3. Specific growth rate of the mussels maintained in laboratory under increasing pCO$_2$ values and optimal (HF) and suboptimal (LF) diets. Values are means ± SD. Different numbers and letters above bars indicate significant differences between experimental groups

4. DISCUSSION

The aim of this study was to investigate the role of increased pCO_2 as a driver of behavioural and ecophysiological changes in juvenile *Mytilus galloprovincialis* by considering interactive effects (if any) with the energetic resources available. Such potential synergy between acidification and food availability would be crucial in areas governed by natural upwelling systems, such as on the Galician coast of the NW Iberian Peninsula. In general, the interactive effects between pCO_2 and food availability for short-term maintenance were significant for feeding activity of juveniles (i.e. CRs), as well as for valve opening behaviour and byssus attachment strength, albeit to a lesser extent. Most of the acidic and suboptimal diet experimental conditions caused a negative impact on the mussels' responses, but not in the case of optimal diet. In contrast, shell performance (i.e. compressive strength) and other compositional values such as OM and mineral (ΩAr:ΩCa) ratio were only affected by acidification regardless of food supply.

Although investigating the real effects of OA may be difficult because multi-generational exposure is required (McElhany 2017), our study highlights the importance of food availability in counterbalancing the negative impacts of OA. A negative impact of acidification on mussel byssus attachment strength has also been reported in other studies, occasionally linked to changes in filaments or adhesive plaque properties (O'Donnell et al. 2013, Zhao et al. 2017, Babarro et al. 2018). However, a recent study showed that a negative effect of elevated pCO_2 on byssus strength would not be universal when other factors such as condition index are considered (Clements et al. 2018). As byssus and shell secretion are biologically controlled by the mussels, their energetic status is very important. Accordingly, in acidified waters mussels would need to derive more energy for intracellular pH regulation, while other activities (e.g. protein synthesis) may be reduced. Such energetic requirements to cope with acidification stress seemed to be satisfied only by the high food resource treatment in the present study. Optimally fed mussels may be capable of maintaining attachment strength under acidification thanks to increased feeding rates (i.e. CRs), which would enable greater energy uptake for metabolic adaptations. Furthermore, together with this increased CR activity, we found that valve opening was also greater — which in turn would have enabled correct foot extension/activity for optimal byssus secretion. Indeed, valve opening variability was mainly driven by differences in food availability. Similarly, Ballesta-Artero et al. (2017) reported the same result for valve opening in *Arctica islandica* in comparison to other factors such as temperature and photoperiod.

M. galloprovincialis may be rather resistant to acidification if it has sufficient food available — as seen in our experiment, where optimal feeding conditions enabled the mussels to maintain wider valve opening and gain energy to sustain attachment, with a slight but significant shell growth. These findings represent an example of energy-demanding biological mechanisms to cope with OA stress (Hendriks et al. 2015). The mussels used in this study were obtained from Galician Rías, where the impact of the Eastern North Atlantic Upwelling System has had significant effects in terms of fertilization with abundant food resources (Feely et al. 2008). The local habitat is considered important for establishing complex interactions with OA treatments and may modulate feeding responses of organisms living in estuarine waters (Saavedra et al. 2018), as in the present study.

The acidification regime used in this study did not have any significant effect on mussel shell growth (i.e. SGR), probably because of the short period considered. In contrast, food availability was the only factor affecting SGR, and the mussels grew even under highest pCO_2 values, which corresponded to under-saturated values relative to aragonite ($\Omega Ar < 1.0$). Theoretically, low pH values would reduce the calcification rate, shell deposition and/or increase shell dissolution (Michaelidis et al. 2005, Fernández Reiriz et al. 2012, Liu et al. 2017). However, calcification in mussels is also influenced by food availability, demonstrating real calcification capacity even under saturated conditions ($\Omega Ar < 0.5$) and high food availability (Thomsen et al. 2010, Thomsen & Melzner 2010). Similarly to byssus strength values in the present study (see Section 3.1), the effects of OA on calcification and growth rates of calcifiers were reported to be negative (Miller et al. 2009, Nienhuis et al. 2010, Duarte et al. 2015), positive (Iglesias-Rodríguez et al. 2008, Findlay et al. 2010, Gutowska et al. 2010) or neutral (Fabry et al. 2008, Ries et al. 2009, Range et al. 2011).

Studies reporting responses of other mytilids under OA scenarios have shown how individuals secrete more brittle calcite outer shells and softer aragonite inner shells (*M. edulis*; Fitzer et al. 2015a,b but see Gazeau et al. 2007, 2013, Hiebenthal et al. 2013). Studies examining short-term OA exposures similar to our experiment noted significant effects on net calcification or growth rates of juvenile *M. chilensis* after 20 d (Duarte et al. 2015), and shells of *M. californianus* larvae were weaker (12–20%) after only

5 and 8 d of acidification (at 540 and 970 µatm; Gaylord et al. 2011). In the present experiment with juvenile *M. galloprovincialis*, shell compressive strength decreased significantly under acidification (25%), regardless of food availability. This result was accompanied, although to a much lesser extent, by decrease in shell OM (6%) and the ΩAr:ΩCa ratio (7–18%). This demonstrates the fragility of the shells of *M. galloprovincialis* juveniles under acidified environments and suggests potential negative consequences for ecological interactions within communities (e.g. predation). Comparatively, the lower magnitude of changes in organic or mineral layers may have profound impacts on shell performance and may be more closely related to shell dissolution than shell deposition (Nienhuis et al. 2010). Considering the gap between functionality and properties of the shell obtained here, it is plausible that other factors may have played a role. Changes in the crystallographic orientation of the mineral units that form the shell or other micro-structural changes such as size and elongation of prismatic structure are potential candidates (Milano et al. 2017).

Regarding the organic layer of the shell, protection against OA stress was demonstrated, thus aiding in the construction of the inorganic component by allowing calcification (Ries et al. 2009, Parker et al. 2013). As an example, an over-expression of a specific enzyme (chitin synthase) and changes in the biochemical composition of this organic layer (higher concentration of polysaccharides) were demonstrated for calcifiers maintained under low pH and low food supply (Ramajo et al. 2016b). In our study, a slight increase in OM of the *M. galloprovincialis* shells of individuals maintained under highly acidified conditions and low food supply was also noted compared to those provided with high food supply, although this difference was not statistically significant.

In summary, our findings revealed that the biological non-calcifying tissues of the mussel *M. galloprovincialis* may be vulnerable to acidification under specific circumstances, e.g. food scarcity. Byssus strength and behavioural (shell opening) and physiological (CRs) activities were affected very differently when mussels were exposed to the combined effects of OA and different levels of food. *M. galloprovincialis* was negatively impacted as a consequence of weakened byssal attachment, a lower capacity to process food particles and partial closing of the valves (all aspects potentially interrelated) when low pH and low food availability acted together. Such negative impacts of OA were, however, counterbalanced when mussels had optimal food resources available. This study also identified a negative impact on shell strength to a greater extent than any compositional value monitored. This finding may indicate a greater vulnerability of mussels to predatory actions or any other cascade of events that are important to ecosystem functioning. Future changes in both carbonate chemistry and ecosystem productivity, especially those related to successful mussel cultivation areas, as in Galicia (NW Iberian Peninsula), are of great interest. Mussels represent a significant component of the food chain, in addition to cleaning the water and helping to recycle nutrients, functioning as keystone engineers and habitat-forming species. Studies like the present one may help to clarify and plan the future of aquaculture industries with a simple approach to further our understanding and ability to forecast the species' responses to environmental heterogeneity, covering natural and predicted variability.

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