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

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Short title: acidification effect on mussels depends on food

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 μ atm) and elevated (800 or 1200 μ atm) seawater $p\text{CO}_2$ conditions under two 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 $p\text{CO}_2$, regardless of food availability. This result indicated water chemistry as a main driver for shell development. Under the optimal feeding regime, mussel feeding rates increased in response to elevated $p\text{CO}_2$, presumably as a strategy to maintain a high strength of attachment. By contrast, mussels under the suboptimal diet showed weak

attachment and narrow valve opening at the highest $p\text{CO}_2$ condition. In conclusion, under optimal food availability, mussels were resilient to water acidification when considering metrics such as 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.

Keywords: 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 faster rates than previously experienced in the history of the Earth (<https://earthobservatory.nasa.gov/Features/GlobalWarming>). The partial pressure of CO_2 in the atmosphere ($p\text{CO}_2$) has increased from 280 μatm since 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 environments, the impact of acidification does not appear to be very important (Melzner et al. 2009, Hiebenthal et al. 2013), suggesting local adaptation.

1 Calcifying organisms are particularly vulnerable to OA (Hendriks et al. 2010,
2 Kroeker et al. 2013). Carbonate is vital for the bio-mineralization of calcium
3 carbonate in the protective shells, and elevated seawater $p\text{CO}_2$ shifts the carbonate
4 system speciation towards a decreased concentration of carbonate ions (CO_3^{2-}) as
5 well as saturation states of biologically important CaCO_3 minerals like calcite (Ω_{Ca})
6 and aragonite (Ω_{Ar}). Recently, the role of another carbonate system species (i.e.,
7 HCO_3^-) has been highlighted (Thomsen et al. 2015).

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

18 Other factors apart from food availability, e.g., temperature, salinity and
19 hypoxia-anoxia, may also act as global marine stressors and interact with OA. The link
20 between temperature and food supply is one of the most evidenced in the literature
21 and is reported to affect a number of responses like filtration, absorption and
22 utilization rates of available food to distinct physiological activities (Zippay & Helmuth
23 2012). Seawater warming is one of the most commonly studied drivers, and
24 synergistic patterns of interactions with OA have been identified (Orr et al. 2005,
25 Feely et al. 2008). However, in areas characterised by upwelling systems like Atlantic
26 Galician coast (NW Iberian Peninsula) at the northernmost limit of the Eastern North
27 Atlantic Upwelling System (Wooster et al. 1976), the waters are characteristically
28 cold, nutrient-rich and CO_2 -rich (Feely et al. 2008). In such systems, calcifiers may be
29 less vulnerable to low saturation states for carbonate minerals and may benefit from

1 abundant food supplies (Peterson et al. 1988, Figueiras et al. 2002), thus enabling
2 them to develop energy-demanding biological mechanisms to resist OA (Hendriks et
3 al. 2015).

4
5 As OA may primarily affect the formation of calcium carbonate shell in calcifiers,
6 other eco-physiological responses have received less attention. For example, the
7 strength of byssal attachment in mussels (which ensures settlement and survival of
8 sessile organisms) and valve opening behaviour that are linked to other vital
9 functions such as foot extension, gas exchange, feeding and excretion. Studies
10 concerning the impact of OA on feeding activity (clearance rates) show highly variable
11 signals ranging from negative through neutral to positive (Fernández Reiriz et al.
12 2011, 2012, Gazeau et al. 2013, Navarro et al. 2013, 2016, Ramajo et al. 2016b).
13 Regarding the attachment strength, recent studies have revealed that the byssus
14 filaments may also be negatively affected by OA in different ways (O'Donnell et al.
15 2013, Zhao et al. 2017, Babarro et al. 2017). Clements et al. (2018), however, noted
16 that such negative effect on byssal attachment is not universal and other factors such
17 as condition index may play an important role.

18 Monitoring shell movements with high-frequency, non-invasive (HFNI)
19 electromagnetic-based biosensors is a novel technique recently used to document
20 acute and long-term effects of elevated CO₂ (Clements & Comeau 2018). Although OA
21 was not found to have clear effects on shell movements in several species (see review
22 in Clements & Comeau 2018), such a real-time behavioural response may provide a
23 bio-monitoring tool for coastal shellfisheries in relation to OA or other global
24 stressors (Andrade et al. 2016, Comeau et al. 2019) because it provides clues to
25 understanding several vital activities that may be altered in response to sub-lethal
26 exposure.

27 Short-term OA experiments are well documented and particularly valuable for
28 studying OA as a driver of eco-physiological changes in bivalve molluscs. Here, we
29 aimed to investigate the impact of OA on the tissues representative of i) the

attachment strategy (byssus) and ii) protection (shell). For this purpose, behavioural and eco-physiological responses in the 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. According to similar previous 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. MATERIAL AND METHODS

2.1. Collection and maintenance of mussels

Juvenile specimens of the mussel *Mytilus galloprovincialis* (30.69 ± 0.28 mm, shell length, mean $\pm$ standard deviation) 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 of natural filtered seawater system (1 μ m) at 15°C and with a 12:12 h (light: dark) photoperiod cycle, resembling natural conditions. The system supplied approximately 1 mg/L of seston as a mixture of two phytoplankton cultures of *Isochrysis galbana* clone T-Iso and *Rhodomonas lens* (50-50% in weight).

2.2. Experimental design for CO₂ and food availability

1 After an acclimation period of 10 days in the laboratory, the mussels were
2 exposed to a modified carbonate system for three weeks, in order to detect any
3 synergistic effects between type of feeding regime and acidified environments. For
4 each food supply test, twelve 9-L rectangular tanks (34×23×19 cm; length x width x
5 height) were used, i.e., four tanks per $p\text{CO}_2$ treatment (three tanks with mussels +
6 one control without mussels). Eighteen juvenile specimens of *M. galloprovincialis*
7 were placed in each tank and eventually formed clusters. Three header 200-L tanks
8 containing the desired concentrations of food were used to supply the experimental
9 tanks via peristaltic pumps (ISMATEC^R). The header tanks with food, peristaltic pumps
10 and experimental tanks were connected by tubing arranged in a random order.
11 Mussels were allowed to establish primary attachment to experimental units (glass
12 plates 24×19 cm; length x width) for one week, and any individuals that did not
13 produce byssus were removed and replaced.

14 Control $p\text{CO}_2$ levels were determined using measured $p\text{CO}_2$ levels in the Rías
15 recorded between June 2017 and June 2018 following Gago et al. (2003), who
16 mapped the spatial and seasonal variability in $p\text{CO}_2$ by dividing the Ría de Vigo into
17 three distinct regions. The mean $p\text{CO}_2$ value estimated from the superficial
18 measurements in five stations between June 2017-June 2018 in the Ría of Vigo was
19 $478\pm119 \mu\text{atm}$. Thus, we set the present $p\text{CO}_2$ value to $500 \mu\text{atm}$.

20 Apart from the current level of CO_2 in these coastal waters, the $p\text{CO}_2$ values
21 tested as cause of the probable future acidification were 800 and $1200 \mu\text{atm}$.
22 Atmospheric air and pure CO_2 gas were previously mixed in separate tanks before
23 being constantly bubbled through the experimental tanks, which also helped to
24 homogenize the water held at a constant temperature ($15\pm1 \text{ }^\circ\text{C}$). Gas concentrations
25 were logged continuously using LI-COR 6262 CO_2 gas analyzers and the
26 measurements were used to adjust the gas mixture through software-controlled
27 solenoid valves.

1 The seawater salinity was checked weekly (8410 Portasal, Guildline). Seawater
2 samples were collected (also every week) for duplicate total alkalinity (A_T) analysis. A_T
3 was measured using a one endpoint method, with an automatic potentiometric
4 titrator (Metrohm 809 Titrando and Dosino 800 Metrohm) equipped with a combined
5 glass electrode (Pérez & Fraga 1987a). The samples were transferred with the aid of a
6 Knudsen pipette (~50 mL) to an open Erlenmeyer flask for potentiometric titration
7 with 0.1 M HCl. The final titration volume was determined by two pH readings after
8 the endpoint of 4.45 was reached (Mintrop et al. 2000). Certified reference material
9 (CRM, batch #163) for CO_2 in seawater (provided by A. Dickson, Scripps Institution of
10 Oceanography, University of California, San Diego, CA, USA) was used to quantify the
11 analytical error. The alkalinity of the test samples was $2214 \pm 10 \mu\text{mol}\cdot\text{kg}^{-1}$ and of the
12 CRM, $2212.6 \pm 0.4 \mu\text{mol}\cdot\text{kg}^{-1}$. Seawater samples were collected twice weekly from all
13 tanks directly into special optical glass spectrophotometric cells of 28 mL and 100 mm
14 of path length, for pH determination. These cells were held in a thermostatic bath at
15 25.0°C for approximately one hour before the analysis. The pH was measured using
16 the spectrophotometric method described by Clayton & Byrne (1993). Briefly, this
17 method consists of adding 75 μL of m-cresol purple to the seawater sample and
18 measuring its absorbance at three wavelengths. Absorbance measurements were
19 performed with a Shimadzu spectrometer (UV-2401PC). The pH values were
20 calculated following the equations described by Dickson et al. (2007), who included a
21 correction factor for the difference between seawater and the acidity indicator.
22 Seawater A_T , salinity, temperature and pH were used to calculate other seawater
23 parameters (Table 1) using CO2SYS (van Heuven et al. 2011) with dissociation
24 constants for carbonic acid (Lueker et al. 2000), and the constants for borate and
25 hydrofluoric acid (Dickson 1990, Pérez & Fraga 1987b). It was noted that TA was
26 relatively lower in optimal feeding conditions, which may suggest that the activity of
27 $CaCO_3$ fixation by mussels is active and leads to a significant reduction in TA relative
28 to suboptimal conditions. This would suggest that in conditions more unfavorable to

1 calcification, mussels are even able to fix more CaCO_3 . Short-term experiment like the
2 present one does not allow to confirm this fact with actual measures of shell growth.

3 The experimental diets were produced by manipulating the two microalgae
4 cultures (*Isochrysis galbana* clone T-Iso and *Rhodomonas lens*) together to yield
5 particle load values of 1.4 ± 0.14 and 0.3 ± 0.07 mg/L. These distinct food availability
6 conditions represent respectively optimal (approximately 6% DW individual per day)
7 and suboptimal (1.5% DW individual per day) diets for the experimental mussels.
8 These seston loads used in the experiment reflect the natural range encountered in
9 the Galician Rías (Babarro et al. 2000). Food was supplied to the mussels at a
10 continuous flow rate of 9 mL min^{-1} from the header tanks for each feeding regime.
11 The flow rate applied enabled removal of ammonium and other waste products due
12 to mussel metabolism or bacteria.

14 **2.3. Mussel Responses**

15 **2.3.1. Clearance rate, byssus strength and valve opening behaviour**

16 Clearance rates (CR) were measured as the volume of water cleared of known
17 suspended algal cells volume (T-ISO and *Rhodomonas lens*) following Cranford et al.,
18 (2016). An appropriately calibrated PAMAS laser particle counter (Model S4031GO
19 Seawater, 2017: 421-0289) was used for this purpose. As the phytoplankton cells
20 used in this study are within known size range distribution, the PAMAS was initially
21 set to determine the particle size distribution between 1 to 20 μm . Nevertheless, the
22 actual range monitored to determine clearance rates was set to 4.5-7.5 μm ,
23 according to highest retention efficiency values for the mussels (above 4 μm ;
24 Møhlenberg & Riisgård 1978, Cranford et al. 2016). The CR was measured twice a
25 week during the experiment. Flow (feeding) was stopped 1-hour before PAMAS
26 measurements. The mussels were left undisturbed on the bottom of the tanks, i.e.,
27 attached on glass substrates. A known volume of phytoplankton cells of the two
28 microalgae used in the experiment was added to each tank, and PAMAS
29 measurements began after 5 minutes, thus ensuring good mixing. Such volume of

1 phytoplankton served as a trigger for CR measurements at the starting time under
2 static (not continuously feeding) conditions just to restore the values before the flow-
3 through was ceased in both optimal and sub-optimal feeding regimes tested. The
4 homogeneity of phytoplankton concentration within experimental tanks was aided by
5 the CO₂-air agitation system (see before) and confirmed by noting the concentration
6 of microalgae across different sections of the tank (right to left and bottom to top).
7 The PAMAS was immediately set up to monitor the decrease in the number of algal
8 cells as a function of time for 20 min. Control tanks with no mussels were also
9 monitored to identify any reduction in phytoplankton due to deposition on the
10 bottom and correcting the experimental slopes of those tanks with mussels. Any
11 decrease was subsequently corrected by the slope of the control tank. The CR was
12 determined from the linear decrease in algal concentration (verified as a straight line
13 in a semi-log plot) over time, with the formula proposed by Riisgård & Seerup (2003):

$$14 \quad CR = \lambda V / n,$$

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

19
20 The byssus strength of the mussels was measured by connecting one individual
21 from the cluster to a dynamometer (Digital Force Gauge DN431 with peak hold
22 indication, resolution of 0.01 N). Groups of 5-6 mussels were analysed per tank at the
23 end of the experimental time. When sampling individual mussels, care was taken to
24 avoid disturbing the neighbouring mussels. Therefore, the mussels immediately
25 adjacent to those selected for dislodgement were not considered when the byssal
26 threads were interconnected (Babarro & Comeau 2014).

27
28 For each $p\text{CO}_2$ treatment, in two of the three experimental tanks containing
29 mussels, four individuals were connected to a non-invasive valvometry system ($n=8$
30 mussels per experimental condition). A coated Hall element sensor (HW-300a, Asahi

Kasei, Japan) fitted to a small electrical cable (1.5 mm diameter, 4.9 g per m) was attached to a valve with cyanoacrylate glue. A magnet (4.8 mm diameter by 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 the byssus strength within the mussel cluster were used to determine the 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 and Abad 2013 for other specifications). Shell strength was calculated from the maximum force measured in the curves and was then normalized by the shell thickness, measured with a micro-calliper (Mitutoyo $0\text{--}25 \pm 0.01 \text{ mm}$) 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 can be simply 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 (SGR)

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

$SGR = \ln (F_{\text{length}} / I_{\text{length}}) \times t^{-1}$, where F_{length} and I_{length} 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 $p\text{CO}_2$ (three levels: 500, 800 and 1200 μatm) and feeding regime (two levels: optimal and suboptimal) on mussel clearance rates as a response variable were tested at the cluster level by repeated measures ANOVA. For this purpose, differences between groups in CR over time (3 weeks) (measured twice per week; see before) were analysed.

Byssus attachment strength, valve opening, shell compressive strength, organic matter of the shell, Ar:Ca ratio and specific growth rate variability were analyzed by two-way ANOVA with the $p\text{CO}_2$ level and food regime as independent factors.

Prior to statistical analysis, the tank was considered a random experimental unit and was tested, in both repeated measures ANOVA and two-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 variances were examined by respectively the Shapiro-Wilk W -test and Levene's test. Whenever the assumptions of analysis of variance were violated, the 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 tests in multiple comparisons. All values

1 shown in the figures are mean $\pm$ SD. All analyses were performed with STATISTICA 7.0
2 software (Tulsa, OK 74104 USA) and IBM SPSS Statistics 23.

3. RESULTS

3.1. Clearance rate, byssus strength and valve opening behaviour

3 The clearance rates (CRs) in the mussels subjected to distinct $p\text{CO}_2$ levels and
4 the corresponding food levels are shown in Figure 1A. Both $p\text{CO}_2$ and food
5 availability factors had interactive effects on CR of individuals ($p\text{CO}_2 \times \text{diet}$; $p < 0.001$;
6 Table 2) so that the effect of $p\text{CO}_2$ depended on the amount of food supplied in the
7 experiment. In mussels supplied the optimal diet the CR increased significantly
8 with increasing $p\text{CO}_2$ (i.e., by 60 and 80% at $p\text{CO}_2$ of 800 and 1200 μatm ,
9 respectively) (Figure 1A). By contrast, no increase in CR was observed in mussels
10 supplied the suboptimal diet, and the values (between 0.67 and 0.79 L h^{-1} for all
11 $p\text{CO}_2$ levels tested) were significantly lower than for optimally-fed mussels (Figure
12 1A).

13 The variability in the byssus strength of attachment for mussels exposed to
14 different $p\text{CO}_2$ and food regimes is shown in Figure 1B. The two-way ANOVA revealed
15 the highly significant impact of food regime tested but also the small effect of the
16 interaction term ($p\text{CO}_2 \times \text{diet}$) (Table 2). Byssus attachment was stronger (and similar
17 among $p\text{CO}_2$ levels) in mussels supplied the optimal diet, with values ranging more
18 narrowly between 7.1 and 7.9 N for all $p\text{CO}_2$ values than for suboptimally-fed mussels
19 (3.1-4.4 also for all $p\text{CO}_2$ values; Figure 1B). By contrast, in mussels supplied the
20 suboptimal diet the byssus strength of attachment was slightly lower (23-27%) at the
21 highest $p\text{CO}_2$ values than in the water tanks with $p\text{CO}_2$ of 500 μatm , as reflected in
22 the dependency of both factors in the statistical analysis (Table 2; $p\text{CO}_2 \times \text{diet}$;
23 $p < 0.05$).

Mean valve opening in the mussels, as indicated by monitoring during the whole experimental period and considering the impact of both $p\text{CO}_2$ and food availability factors, is presented in Figure 1C. Valve opening was significantly greater in the optimally-fed mussels (ranging between 2.10 and 2.77 mm for all $p\text{CO}_2$ values considered) than in the mussels supplied the suboptimal diet (0.43-0.85 for all $p\text{CO}_2$ values; Figure 1C; Table 2). As an independent factor, the $p\text{CO}_2$ level did not have any effect on valve opening, but the slightly significant interaction factor $p\text{CO}_2 \times \text{diet}$ ($p < 0.05$; Table 2) highlighted that differences in the feeding regime were greater at the highest $p\text{CO}_2$ levels (Figure 1C). This established that the most stressful scenario tested (highest $p\text{CO}_2$ conditions 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 $p\text{CO}_2$ of 500 μatm (Figure 1C).

3.2. Compressive strength, organic matter and Ar:Ca ratio in mussel shells

The variability in shell compressive strength of the mussels as a function of $p\text{CO}_2$ level and food regime tested is presented in Figure 2A. Two-way ANOVA revealed the highly significant impact of $p\text{CO}_2$ as an independent factor (Table 2). Neither the different feeding regimes used in the experiments nor the interaction term $p\text{CO}_2 \times \text{diet}$ affected the variability (Table 2). Shell strength decreased significantly (20-30%) from mean values of 82-80 N mm^{-1} (500 μatm of $p\text{CO}_2$) to around 55-69 N mm^{-1} in the more acidified waters in equilibrium with atmospheres of 800 and 1200 μatm (Figure 2A).

The two-way ANOVA considering shell organic matter showed that, similarly to shell strength, $p\text{CO}_2$ only had a significant effect as independent factor (Figure 2B). Neither food availability nor the interaction term $p\text{CO}_2 \times \text{diet}$ appeared to be significant factors (Table 2). The shell organic matter, which represented 5.2% of the total shell weight under $p\text{CO}_2$ conditions of 500 μatm , decreased by, on average, 6% for $p\text{CO}_2$ levels of 800 and 1200 μatm (Figure 2B).

The mineral content of the shells expressed as aragonite:calcite ratio (Ar:Ca) only varied significantly with the variable $p\text{CO}_2$ in the experiment (Figure 2C; Table 2). Similarly to shell strength and organic matter, neither food availability nor its interaction term ($p\text{CO}_2 \times \text{diet}$) caused any important variation in the Ar:Ca ratio (Table 2). As the $p\text{CO}_2$ increased, the Ar:Ca ratio decreased by 7-8% and 18% for mussel shells exposed to 800 and 1200 $\mu\text{atm } p\text{CO}_2$, respectively (Figure 2C).

3.3. Specific growth rate (SGR)

The increase in shell length of the experimental mussels as SGR based on initial and final values of the cluster is illustrated in Figure 3. As expected, growth was almost nil in the suboptimally-fed mussels (Figure 3). By contrast, despite the relatively short-term exposure (i.e., 3 weeks), the shell length of the optimally-fed mussels increased ($0.031\text{--}0.070 \text{ day}^{-1}$; Figure 3). The two-way ANOVA revealed a significant impact of food availability on SGR, whereas neither $p\text{CO}_2$ nor the interaction term ($p\text{CO}_2 \times \text{diet}$) had any effect, despite the high variability noted (Table 2; Figure 3).

4. DISCUSSION

The aim of the study was to investigate the role of increased $p\text{CO}_2$ as a driver of behavioural and ecophysiological changes in juveniles of *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 as on the Galician coast (NW Iberian Peninsula). In general, the interactive effects between $p\text{CO}_2$ and food availability for short-term maintenance were significant for feeding activity of juveniles (i.e., clearance rates), as well as for valve opening behaviour and byssus strength of attachment, although to a lesser extent. Most acidic conditions

1 and suboptimal diet caused negative impact on these mussel's responses but not in
2 the case of optimal diet. By contrast, shell performance (compressive strength) and
3 other compositional values such as organic matter and mineral Ar:Ca ratio were only
4 affected by the acidification regardless of food supply.

5 Although investigating the real effects of OA may be difficult because multi-
6 generational exposure is required (McElhany 2017), the study findings highlight the
7 importance of food availability in counterbalancing the negative impact of OA. A
8 negative impact of acidification on byssus attachment strength of mussels has been
9 also reported in recent studies, occasionally linked to changes in filaments or
10 adhesive plaque properties (O'Donnell et al. 2013, Zhao et al. 2017, Babarro et al.
11 2017). However, a recent study showed that a negative effect of elevated $p\text{CO}_2$ on
12 byssus strength would not be universal when other factors such as condition index
13 are considered (Clements et al. 2018). As byssus and shell secretion are biologically
14 controlled by the mussels, the energetic status is very important. Accordingly, in
15 acidified waters mussels would need to derive more energy for intracellular pH
16 regulation, while other activities (e.g., protein synthesis) may be reduced. Such
17 energetic requirements to cope with acidification stress seem to be satisfied only by
18 the high food resources supplied in the present study. Optimally-fed mussels may be
19 capable of maintaining the attachment strength under acidification thanks to the
20 increased feeding rates (CR), which would enable greater energy uptake for
21 metabolic adaptations. Furthermore, together with this increased CR activity, valve
22 opening was also greater which in turn would have enabled correct foot
23 extension/activity for optimal byssus secretion. Indeed, valve opening variability was
24 mainly driven by differences in food availability. Similarly, Ballesta-Artero et al. (2017)
25 reported same result for valve opening in *Arctica islandica* in comparison to other
26 factors such as temperature and photoperiod.

27 *Mytilus galloprovincialis* may be rather resistant to acidification if it has
28 sufficient food available. Thus, optimal feeding enabled the mussels to maintain
29 wider valve opening and gain energy to sustain attachment, with a slight but

1 significant shell growth. Together these findings represent an example of energy-
2 demanding biological mechanisms to cope with OA stress (Hendriks et al. 2015). The
3 mussels used in this study were obtained from Galician Rías where the impact of the
4 Eastern North Atlantic Upwelling System has significant effects in terms of
5 fertilization with abundant food resources (Feely et al. 2008). The local habitat is
6 considered important for establishing complex interactions with OA treatments and
7 may modulate feeding responses of organisms living in estuarine waters (Saavedra et
8 al. 2018), as in the present study.

9
10 The acidification regime used in this survey did not have any significant effect
11 on mussel shell growth rate (SGR), probably because of the short period considered.
12 By contrast, food availability was the only factor affecting SGR, and the mussels grew
13 even under highest $p\text{CO}_2$ values, which corresponded to under-saturated values
14 relative to aragonite ($\Omega_{\text{Ar}} < 1.0$). Theoretically, low pH values would reduce the
15 calcification rate, shell deposition and/or increase shell dissolution (Michaelidis et al.
16 2005, Fernández Reiriz et al. 2012, Liu et al. 2017). However, calcification in mussels
17 is also influenced by food availability, demonstrating the real calcification capacity
18 even under saturated conditions ($\Omega_{\text{Ar}} < 0.5$) and high food availability (Thomsen et al.
19 2010, Thomsen & Melzner 2010). Similarly to byssus strength values in the present
20 study (see above), the effects of OA scenario on calcification and growth rates of
21 calcifiers were reported to be negative (Miller et al. 2009, Nienhuis et al. 2010,
22 Duarte et al. 2015), positive (Findlay et al. 2009, Iglesias-Rodriguez et al. 2008,
23 Gutowska et al. 2010) or neutral (Fabry et al. 2008, Ries et al. 2009, Range et al.
24 2011).

25 Studies reporting responses of other mytilids under OA scenarios have shown
26 how individuals secrete more brittle calcite outer shells and softer aragonite inner
27 shells (*M. edulis*; Fitzner et al. 2015, 2017 but see Gazeau et al. 2007, 2013, Hiebenthal
28 et al. 2013). Short-term OA exposures similar to our study noted significant effects on
29 net calcification or growth rates of juvenile *M. chilensis* after 20 days (Duarte et al.

2015) and shells of *M. californianus* larvae were weaker (12-20%) after only 5 and 8 days of acidification (540 and 970 μatm ; Gaylord et al. 2011). In the present experiment with juveniles of *M. galloprovincialis*, shell compressive strength decreased significantly under acidification (25%) and regardless of food availability. This result was accompanied, although to a much lesser extent, by decrease in shell organic matter (6%) and the Ar:Ca ratio (7-18%). This demonstrates the fragility of the shells of *M. galloprovincialis* juveniles under acidified environments and the potential negative consequences for the ecological interactions within communities, e.g., facing predatory actions. 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 microstructural 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 helping in the construction of the inorganic part 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 organic matter of the *M. galloprovincialis* shells maintained under highly acidified conditions and low food supply was also noted in comparison to high food although not statistically significant.

In summary, the study findings revealed that biological non-calcifying tissues of the mussel *M. galloprovincialis* may also be vulnerable to acidification under specific circumstances, e.g., scarcity of food. Byssus strength and behavioural (shell opening) and physiological (clearance rates) activities were affected very differently when

1 mussels were exposed to the combined effects of OA and food supply. *Mytilus*
2 *galloprovincialis* may be negatively impacted as consequence of weakened byssal
3 attachment, lower capacity to process food particles or partially closing the valves (all
4 aspects potentially interrelated) when low pH and low food availability acted
5 together. Such negative impacts of OA were, however, counterbalanced when
6 mussels had optimal food resources available. This study was also able to identify a
7 negative impact on shell strength to a greater extent than any compositional value
8 monitored. This finding may indicate the greater vulnerability of the mussels to
9 predatory actions or any other cascade of events that are important to ecosystem
10 functioning. Future changes in both carbonate chemistry and ecosystem productivity,
11 especially those related to successful mussel cultivation areas, as in Galicia (NW
12 Iberian Peninsula), are of great interest. Mussels represent a significant component
13 of the food chain, cleaning the water and helping to recycle nutrients, functioning as
14 keystone engineers and habitat-forming species. Studies like the present one may
15 help to clarify and plan the future of aquaculture industries with simple approach to
16 gain understanding and ability to forecast the species' responses to environmental
17 heterogeneity covering natural and predicted variability. Future predictions for
18 climate change impact will be based on organism's responses that will help to protect
19 the health of natural areas under risk.

20
21
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FIGURE LEGEND

Figure 1. Clearance rates ($\text{L h}^{-1} \text{ ind}^{-1}$), byssus attachment strength (N) and median valve opening (mm) responses of mussels after being exposed to increasing $p\text{CO}_2$ values and optimal (HF) and sub-optimal (LF) diets. Clearance rates and valve opening correspond to integrated values during the experimental treatment (see Material and Methods). Byssus strength was monitored at the end of the experiment. Values are means $\pm$ SD. Distinct numbers (optimal food, HF) and letters (sub-optimal food, LF) mean significant differences between experimental groups.

Figure 2. Shell compressive strength (N mm^{-1}), organic matter of the shell (%) and aragonite:calcite (Ar:Ca) ratio values obtained for the shells of the experimental mussels after being exposed to increasing $p\text{CO}_2$ values and optimal (HF) and sub-optimal (LF) diets. All responses were monitored at the end of the experimental period (3 weeks). Values are means $\pm$ SD. For distinct numbers and letters, we refer to legend of Figure 1.

Figure 3. Specific growth rate (SGR, %) of the mussels maintained in laboratory under increasing $p\text{CO}_2$ values and optimal (HF) and sub-optimal (LF) diets. Values are means $\pm$ SD. For distinct numbers and letters, we refer to legend of Figure 1.

Figure 1

Figure 2

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Table 1. Experimental seawater chemistry parameters: salinity, pH, pCO₂ (μatm), bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻), total alkalinity (TA), calcite and aragonite saturation states (ΩCa and ΩAr. Values are means ±standard deviation.

Diet	Treatment	Salinity (ppt)	pH	pCO ₂ (μatm)	HCO ₃ ⁻ (μmol kg ⁻¹)	CO ₃ ²⁻ (μmol kg ⁻¹)	TA (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
Sub-optimal	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

Table 2. Results of Repeated measurements ANOVA (clearance rates, CR) and 2-way ANOVAs to determine the effect of acidification values (pCO₂) and feeding regime (diet) on distinct mussel responses as indicated. When indicated, dependent variables were rank-transformed prior to the analysis. Tank was previously included in the analyses as random experimental unit (see Material and Methods) but subsequently removed when confirming the non significant effect. Values in **bold** are statistically significant (**p<0.05**). ns, not significant.

Clearance rates (CR)				Byssus strength (rank-transformed)		
factor	df	F	p	df	F	p
pCO ₂	2	24.689	<0.001	2	2.014	ns
diet	1	498.854	<0.001	1	143.451	<0.001
pCO ₂ ×diet	2	32.898	<0.001	2	3.856	<0.05
Error	12			97		
Median valve opening (rank-transformed)				Shell compressive strength (rank-transformed)		
pCO ₂	2	1.459	ns	2	10.315	<0.001
diet	1	55.288	<0.001	1	2.524	ns
pCO ₂ ×diet	2	4.684	<0.05	2	0.641	ns
Error	39			102		
Organic matter of the shell				Ar:Ca ratio		
pCO ₂	2	5.992	<0.01	2	14.023	<0.001
diet	1	2.011	ns	1	0.013	ns
pCO ₂ ×diet	2	1.290	ns	2	0.219	ns
Error	47			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					

8
9
10