



Ocean acidification will not affect the shell strength of juveniles of the commercial clam species *Chamelea gallina*: Implications of the local alkalinization of seawater

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ABSTRACT

Ocean acidification (OA) is expected to decrease the strength of bivalves' shells, especially during the early stages of development, with negative consequences to the resilience of natural populations and the economy. The objectives of the present study were to assess the long-term effect of increasing $p\text{CO}_2$ after 217 days of exposure under controlled conditions of pH of ~8.2, 8.0, and 7.7 on the strength and integrity of shells of juveniles of the commercial striped venus clam *Chamelea gallina*. Shell strength was estimated through compression tests and integrity through scanning electron microscopy (SEM) and dispersive X-ray analyses (EDX). The results showed that under increasing $p\text{CO}_2$ the shell strength of juveniles is unaffected, which could be related to the locally elevated total alkalinity of seawater with respect to other parts of the coastal lagoon. However, despite this, it was also observed that the juvenile clams exposed to elevated $p\text{CO}_2$ decreased their shell thickness and increased the porosity of their prismatic layer. Under future OA conditions, these changes could eventually compromise the integrity of the shells, becoming more vulnerable to the attack of predators and breakable during fishing operations. Future studies should address the plasticity of the organisms and the effect of the alkalinization of seawater on the resilience of shellfish juveniles under global change conditions.

1. Introduction

Ocean acidification, as a consequence of the absorption by the ocean of anthropogenic $p\text{CO}_2$, will decrease pH and the CaCO_3 saturation state of seawater (Ω) in the coming decades, making it more difficult for calcifiers to form their calcareous structures (Gazeau et al., 2011). Most studies have found that due to the lowering of pH and changes in the carbonate system of seawater under OA, the shells tend to lose integrity becoming thinner (Gaylord et al., 2011; Bressan et al., 2014), weaker (Welladsen et al., 2010; Beniash et al., 2010; Wright et al., 2018), and more porous (Meng et al., 2018; Knights et al., 2020), compromising their vital protective roles (Byrne and Fitzer, 2019).

In upwelling areas, such as the West Coast of the USA, due to the

lowering of pH shellfish growers are already experiencing difficulties raising shellfish from larvae and a high mortality of the seeds (Barton et al., 2015). At the same time, in the north of Chile, a decreasing trend in scallop landings has been observed over the last two decades (Lagos et al., 2021; Abarca-Ortega et al., 2022), and global change also threatens the northern bay scallop (*Argopecten irradians*) fisheries on the Atlantic coast of the USA (Scanes and Byrne, 2023). The early developmental stages of mollusks are expected to be particularly sensitive to global changes with important repercussions on natural populations and the shellfish fisheries and aquaculture industry (Barton et al., 2015; Tomasetti et al., 2023). Previous studies suggest a link between the natural variability of the CaCO_3 saturation state of seawater and the commercial production of oyster larvae in the hatcheries (Barton et al.,

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2015). However, calcification is not only driven by the carbonate ion concentration but also by the reduction of the seawater pH, which alters the proton gradient between internal cellular reservoirs and external bulk water, making it difficult to maintain pH homeostasis, decreasing the saturation state of seawater and the ability of marine organisms to secrete CaCO₃ skeletons, a process known as calcification (Cyronak et al., 2016).

The study of shell strength through compression experiments can provide relevant information on the effect of global change on the structural integrity of shellfish, as this method allows the evaluation of the maximum force a shell can withstand before breaking (Johnson, 2021). Also, the organic matrix or organic content of the mollusks, which includes organic components soluble in aqueous solutions and constituents not soluble in water and organic solvents, is closely involved in biomineralization processes and has a role in determining the mechanical properties of the shells (Krampitz et al., 1983). Even though shellfish calcification is biologically controlled, environmental factors can also affect this process modifying the amount and composition of shell organic components, the structural organization, and the morphology and mineralogy, such as the calcium (Ca) to carbon (C) ratio of the shells (Grenier et al., 2020). Changes in the Ca/C ratio can provide insights into potential changes in the mineral composition, density, and structural integrity of the shells. Monitoring this ratio helps us understand the impact of elevated pCO₂ exposure on the biomineralization process and the potential consequences on the strength and integrity of the clam shells under ocean acidification conditions.

Ocean Acidification (OA) is expected to increase the disorder of the CaCO₃ crystal deposition, resulting in a decrease in the shell strength and integrity of different life forms of commercial shellfish species (Byrne and Fitzer, 2019), such as oyster larvae, juveniles, and adults (Beniash et al., 2010; Meng et al., 2018; Wright et al., 2018; Chandra Rajan et al., 2023), mussel larvae, juveniles, and adults (Gaylord et al., 2011; Fitzer et al., 2014; Li et al., 2015; Knights et al., 2020; Zhao et al., 2020), and scallop juveniles and adults (Auzoux-Bordenave et al., 2020; Alma et al., 2020). However, there are divergences across OA studies, sometimes for the same species, which could also be related to a diverse range of confounding factors that influence the response of the organisms to OA, such as differences in temperature (Mackenzie et al., 2014), salinity (Lassoued et al., 2021), pCO₂ level, CaCO₃ saturation state, the total alkalinity of seawater (Sordo et al., 2021) or food availability (Leung et al., 2019), among others (see Supplementary Table 1). For instance, in a study of 183 days, Mackenzie et al. (2014) found that the shell strength of adults of the mussel species *Mytilus edulis* was unaffected by increasing pCO₂, while in a study of 60 days, Li et al. (2015) found that the shell strength of *M. edulis* adults decreased with pCO₂. This can be related to the fact that experiments were done under different temperatures (12–16° vs. 19–25°), pH ranges (pH 7.66–7.99 vs. 7.8–8.1), and diets (*I. galbana* vs. instant algae shellfish diet).

Nevertheless, in a recent analysis of OA studies by Connell and Leung (2023), the authors reported more negative effects on the pioneering OA studies. Still, as the number of studies and scientific knowledge increased over the years, this tendency has changed. This is partially due to the short duration and insufficient acclimation of the organisms during the early studies and to the techniques used (acid-base additions vs. CO₂ bubbling) (Connell and Leung, 2023). Also, in the biggest meta-analysis on OA experiments ever done, Leung et al. (2022) found that over 70% of the 5153 observations reviewed recorded a non-negative effect of high CO₂ on the growth and calcification, implying that the impacts of OA on calcifiers are less harmful than initially thought as their adaptability has been underestimated.

Even though OA is expected to decrease shell strength and integrity, recent studies suggest that in naturally acidified areas, like natural CO₂ vents, some calcifiers can adapt to stressful conditions and compensate for the lowering of pH by maintaining or even enhancing their shell strength and growth (Leung et al., 2019). Also, other authors have observed that despite an increase in the intracellular precipitation of

amorphous calcium carbonate with a consequently diminished shell ultrastructure and a reduction in the crystallographic control of the formation of their shells, some species can maintain shell formation (Fitzer et al., 2015, 2016), repair shell damage (George et al., 2022), and compensate the lowering of pH by changing their shell thickness (Coleman et al., 2014; Fitzer et al., 2015; Leung et al., 2019), and shape (Fitzer et al., 2015).

The striped venus clam occurs in the infralittoral zone and is distributed mainly from the Gulf of Cádiz to the Mediterranean, Adriatic, and Black Seas (Pereira et al., 2007). Despite the commercial and gastronomical value of *Chamelea gallina* and the great importance of its fishery in several countries like Italy, Spain, Turkey, and Portugal, there is no aquaculture for this species. The recent over-exploitation of *Chamelea gallina*, the periodic recruitment failures, the irregular mortality events, and the unexpected annual fluctuations in stock abundance have raised growing concerns about the survival of these bivalve communities (Guarino et al., 2019). In southern Portugal, the striped venus clam is one of the target shellfish species supporting a commercial dredge fishery (Gaspar et al., 2004) which could be compromised due to the degradation of the natural environment. In addition, the shell of *C. gallina* is mainly composed of aragonite, one of the most soluble forms of CaCO₃. Therefore, in a future high pCO₂ scenario, this species is expected to grow weaker shells and become more vulnerable to the impairment of their shells during fishing.

Currently, there is almost no information on the effect that global change will have on *C. gallina* juveniles (Bressan et al., 2014; Sordo et al., 2021), so it is of great importance to study how changing environmental factors will affect the shell strength and integrity of this commercial clam species. This research is an extension of a previous work (Sordo et al., 2021) to investigate the resilience and growth of striped venus clam juveniles under future OA conditions. The objective of the present study was to assess the effect that future pCO₂ levels will have on the shell strength and integrity of *C. gallina*. We hypothesize that shell strength and integrity will decrease under high pCO₂ conditions. However, in highly alkaline seawater ocean acidification may not affect the shell strength of marine calcifiers.

2. Methods

2.1. Experimental design, seawater parameters, and biological material

Juveniles of *Chamelea gallina* were obtained from broodstock at the IPMA-Molluscan Aquaculture Experimental Station of Tavira (EEMT) in southern Portugal. The quantity of dry microalgae administered was 1% of the individual dry weight and was adjusted several times during the experiment. Juveniles were fed through a continuous flow system with a binary diet of *Isochrysis aff. galbana* or *T-iso* (now known as *Tisochrysis lutea*; see Bendif et al., 2013) and *Chaetoceros calcitrans* (6.5 x 10⁶ and 3 x 10⁶ cells mL⁻¹ of *T-iso* and *C. calcitrans* respectively).

The CO₂ enrichment system is installed at the EEMT, station located at Tavira, Southern Portugal (37°7'17.73"N, 7°37'12.19"W). At the EEMT, the seawater is pumped from the Ria Formosa coastal lagoon and goes into a preliminary 200,000 L concrete decantation reservoir. Seawater was previously aerated in two 2000L tanks to guarantee proper oxygenation before entering the three 250L header tanks at different pCO₂ concentrations (see experimental design in Supplementary Materials 2). During the 217 days of the experiment, the salinity and temperature were not manipulated and changed seasonally. The carbonate chemistry of the seawater was manipulated by injecting a CO₂-air mix with air stones into the header tanks. The CO₂ flux was controlled using a pH stat (PH450G-A-A/UM, Yokogawa, Japan) coupled to a solenoid valve that opens and closes when the pH level is below or above the set point. Each header tank presented a water pump to supply the seawater into the experimental aquariums per treatment. Three levels of pCO₂ were tested, a control level without enrichment (pH = 8.2), an intermediate level (pH = 8.0), and a high pCO₂ treatment

(pH = 7.7), based on the near-future projections for the worst-case scenario RCP 8.5 (IPCC, 2021). Each 250 L header tank was connected to four 20L aquariums where the organisms (200 individuals per replicate) were gradually acclimated to different $p\text{CO}_2$ conditions. The replicates at the different $p\text{CO}_2$ concentrations were interspersed to avoid confounding factors. Seawater was supplied at a constant flux of 1.2 L h^{-1} . This was an open water system to minimize metabolic waste products and maintain adequate oxygen levels (for further details see Sordo et al., 2021).

Temperature, pH, and salinity were monitored daily throughout the experiment in the aquariums, header tanks, and source tanks using a multi-probe (handheld multi-probe Pro-plus 605596, YSI, USA). Seawater was sampled for Total Alkalinity (TA) at the header and source tanks. The TA was determined using the potentiometric titration method, as in APHA (1976). Sub-samples of 100 ml were titrated with H_2SO_4 0.05 M using a manual titrator (multi dosimat 645; Metrohm, Herisau, Switzerland), and the pH was measured using a pH meter (HI 8418 printing pH meter, HANNA, USA) with a Cole-Parmer pH electrode calibrated in the National Bureau of Standards (NBS scale).

The carbonate system of seawater was calculated from the measured pH, temperature, salinity, and TA using the software CO_2SYS (Lewis and Wallace, 1998) with the constants of Mehrbach et al. (1973) (refitted by Dickson and Millero, 1987). After 217 days of exposure to three $p\text{CO}_2$ different levels, to preserve the integrity of the shells until the analyses, the juvenile clams were frozen at -18° . For the shell integrity analyses, a total of 96 clams were used for the analyses ($n = 32$ per $p\text{CO}_2$ treatment). Two days before the analyses, the clams were defrosted, the flesh was separated from the shells, and the shells were rinsed with distilled water and left to dry at room temperature. The valves were carefully inspected to verify their full integrity. The right valves were used to assess the biometry and the organic content of shells, while the left valves were used to determine the maximum load sustained by the shells or shell-failure load.

2.2. Biometric measurements

Biometric measurements were taken at the beginning and end of the experiment. At the start of the experiments, the juveniles were five months old and presented lengths from 4.96 to 8.77 mm and widths from 5.15 to 9.71 mm. The shell length, width and thickness of 96 right valves ($n = 32$ per $p\text{CO}_2$ treatment) were measured using a digital Vernier caliper ($0\text{--}100 \text{ mm} \pm 0.02 \text{ mm}$). The thickness of the right valves was measured at the thickest part of the distal edge of the shells, avoiding the curvature of the valve. The individual weight of the shells after air-drying for two days was estimated using a high-resolution scale (MX5 Microbalance, Mettler Toledo, USA).

2.3. Organic content of shells

The right valves of 32 individuals per $p\text{CO}_2$ treatment were used to analyze the organic content of shells. The shells were oven-dried to a constant weight at 80°C for 24 h. Then, the dried shells were reduced to ashes in a muffle furnace for 24 h at 450°C (Furnace 6000, Thermolyne, USA). The dry and ash weights of shells were measured with a high-resolution scale (MX5 Microbalance, Mettler Toledo, USA). The organic content of shells was estimated as ash-free dry weight (AFDW) by subtracting the ash weights from the dry weights of the shells.

2.4. Shell strength measurements

To test the effect of long-term exposure to high $p\text{CO}_2$ on juveniles of *Chamelea gallina*, the shell strength of 32 left valves per $p\text{CO}_2$ level was estimated using an Electro-Mechanical Tension and Compression Testing Machine (Instron 1011). The left shell valve of each individual was placed on the crushing plate, and compression was applied at the highest point of each shell until breakage. The experiments were carried

out at a constant loading rate of 5 mm/min with a maximum force cell of 5000 N. The applied force required to break each shell was measured in Newtons ($\text{N} = \text{kg m s}^{-1}$) and continuously recorded on graph paper.

2.5. SEM and energy dispersive X-ray analyses (EDX)

The effect of high $p\text{CO}_2$ on the shell morphology was analyzed by scanning electron microscopy (SEM) using a Vega3 Tescan equipment (Tescan, Brno, Czechia), operating at an accelerating voltage of 15 kV and a magnification of 40x, 200x, and 5000x. For these analyses, three shells per $p\text{CO}_2$ treatment ($n = 3$) were cleaned and fixed on a brass stub using double-sided tape and then coated with a gold/palladium (Au/Pd) thin film by sputtering, using the sputter coater equipment (Quorum Technologies). In order to assess the degree of dissolution of the shells, a categorization scheme developed by Bednaršek et al. (2022) based on the literature on crystalline structure was followed.

The elemental analyses of calcium and carbon atoms were performed at three shells per treatment ($n = 3$), and the Ca/C ratio was assessed (wt %). The measurements were carried out at the central area and ventral margin of the internal side of the shells using the energy dispersive X-ray analysis (EDX) (Xflash 6|30 from Bruker).

2.6. Statistics

The software R (R Core Team, 2023) via RStudio (Posit team, 2024) was used for the analysis of covariance (ANCOVA), and SigmaPlot (version 12.5 Systat Software, Germany) was used to perform the rest of the statistical analyses. The morphometric parameters, the required force to break the shells, the organic content of shells, and concentrations of the calcium to carbon (Ca/C ratio) at the central and ventral areas of the prismatic layer were compared among $p\text{CO}_2$ treatments using a one-way analysis of variance (ANOVA). When significant differences were found ($P < 0.05$), a post hoc test was applied (Student-Newman-Keuls Method, SNK) to explore the differences among $p\text{CO}_2$ treatments. Whenever the normality and equal variance assumptions were not met (Shapiro-Wilk test), a square root or log transformation was performed. A Kruskal–Wallis analysis of variance (ANOVA on ranks) was applied when data presented equal variance but did not meet the assumptions of normal distribution and the Dunn's test was applied for the multiple comparison procedure. Analysis of covariance (ANCOVA) was used to further assess the linear relationship between the shell strength, i.e. force (in N) required to break the shells, and the different morphometric features, namely, length, weight, width, and thickness, and the organic content of the shells, for the different levels of the qualitative variable being analyzed, i.e. the $p\text{CO}_2$ level. Two distinctive outliers and influential observations were removed.

3. Results

3.1. Seawater parameters

The mean values of the carbonate system for the three different levels of $p\text{CO}_2$ are presented in Table 1. The total alkalinity values were stable at the source and header tanks and ranged from 3492.4 to $3631.1 \mu\text{mol kg}^{-1}$. Temperature, salinity, and the total alkalinity of seawater did not differ among $p\text{CO}_2$ treatments. The mean pH values at the different $p\text{CO}_2$ treatments were 8.28 ± 0.001 in the control system, 8.09 ± 0.002 in the intermediate, and 7.75 ± 0.008 in the high $p\text{CO}_2$. The temperatures recorded at all experimental aquaria and header tanks followed the natural variations observed at Ria Formosa Coastal Lagoon, ranging from $16.76 \pm 0.56^\circ\text{C}$ in winter to $22.07 \pm 0.56^\circ\text{C}$ at the end of the experiment in summer of 2020. During the experiment, the salinity values changed seasonally and ranged from 33.82 ± 0.09 to 35.62 ± 0.05 (see Sordo et al., 2021 dataset available at 10.1594/PANGAEA.937477).

Table 1

Carbonate system parameters for each $p\text{CO}_2$ level tested. Salinity (Sal.), temperature (T), pH (NBS scale), and total alkalinity (TA) were measured, while the rest of the parameters were calculated using the software CO_2SYS . The values are expressed as means \pm SE ($n = 4$).

Rearing system	Sal.	T ($^{\circ}\text{C}$)	pH	TA ($\mu\text{mol. kg}^{-1}$)	HCO_3^- ($\mu\text{mol. kg}^{-1}$)	CO_3^{2-} ($\mu\text{mol. kg}^{-1}$)	$p\text{CO}_2$ (μatm)	Ω aragonite
2000L source tank	33.93 \pm 0.0	22.50 \pm 0.0	8.15 \pm 0.005	3492.43 \pm 3.1	2838.93 \pm 3.1	285.00 \pm 2.8	670.30 \pm 7.9	4.50 \pm 0.04
Control CO_2	33.93 \pm 0.0	23.00 \pm 0.0	8.28 \pm 0.001	3631.11 \pm 16.4	2756.83 \pm 14.5	383.21 \pm 1.1	480.13 \pm 3.8	6.07 \pm 0.02
Intermediate CO_2	33.92 \pm 0.0	23.20 \pm 0.0	8.09 \pm 0.002	3495.18 \pm 4.5	2904.96 \pm 7.0	257.55 \pm 1.0	799.91 \pm 5.8	4.08 \pm 0.02
High CO_2	33.93 \pm 0.0	23.10 \pm 0.0	7.75 \pm 0.008	3494.00 \pm 2.7	3195.93 \pm 7.5	130.09 \pm 2.4	1872.73 \pm 12.7	2.06 \pm 0.04

3.2. Biometry

After 217 days at different $p\text{CO}_2$ concentrations, the shell length was not significantly affected by CO_2 (one-way ANOVA; $F = 1.567$, $P = 0.214$), and neither was the width (one-way ANOVA; $F = 1.17$, $P = 0.315$). The only morphometric variable that significantly decreased with $p\text{CO}_2$ concentration was the thickness of the shells (one-way ANOVA; $F = 8.201$; $P < 0.001$) (Table 2). Significant differences were observed between the control and both the high ($P < 0.001$) and intermediate $p\text{CO}_2$ treatments ($P = 0.021$). However, there were no significant differences between intermediate and high $p\text{CO}_2$ conditions ($P = 0.096$). Also, the differences among treatments for the shell's weight were non-significant (one-way ANOVA; $F = 0.186$, $P = 0.831$).

3.3. Organic content

There were no significant differences between the organic content of shells under control conditions and shells exposed to intermediate and high $p\text{CO}_2$ conditions (one-way ANOVA, $F = 0.267$; $P = 0.766$). Shells under control conditions had a mean organic content (\pm SE) of 2.01 \pm 0.09 mg, and those under intermediate and high $p\text{CO}_2$ conditions of 2.00 \pm 0.10 mg and 1.93 \pm 0.09 mg, respectively.

3.4. Shell strength

The mean force required to break the shell was similar across treatments, with the control shells breaking at a mean load (\pm SE) of 1624.52 \pm 97.80 N, while those of the intermediate and high $p\text{CO}_2$ treatments broke at a force of 1641.94 \pm 90.58 and 1742.58 \pm 73.53 N respectively. Despite this, the results showed that increasing $p\text{CO}_2$ did not have a significant effect on the shell strength of the juveniles of *Chamelea gallina* (one-way ANOVA; $F = 0.884$, $P = 0.417$). The control and high $p\text{CO}_2$ conditions were the treatments that differed the most ($P = 0.209$), while the control and intermediate treatments differed the least ($P = 0.406$). To verify if shell resistance changed with length, the shells were separated into two length categories, from 7 to 8 mm and from 9 to 11 mm. Nevertheless, for both length categories, non-significant differences were observed (7–8 mm: $P = 0.991$; 9–11 mm: $P = 0.121$).

Table 2

Shell morphometric parameters of *Chamelea gallina* juveniles cultivated at different CO_2 concentrations and pH levels. Mean values \pm SE ($n = 32$) for shell weight (air-dried), length, width, and thickness following a 217 days of exposure to three different levels: control $p\text{CO}_2$ (pH \sim 8.2), intermediate $p\text{CO}_2$ (pH \sim 8.0), and high $p\text{CO}_2$ (pH \sim 7.7).

Parameters	(units)	Treatments		
		Control CO_2	Intermediate CO_2	High CO_2
Weight	(mg)	71.90 \pm 3.19	72.61 \pm 3.26	75.38 \pm 4.28
Length	(mm)	9.05 \pm 0.15	8.79 \pm 0.79	8.71 \pm 0.14
Width	(mm)	8.15 \pm 0.12	7.99 \pm 0.12	7.88 \pm 0.13
Thickness	(mm)	0.67 \pm 0.02	0.61 \pm 0.01	0.58 \pm 0.01

3.5. Relationship between shell strength and shell morphometric features and organic content

Shell strength (force, N) was significantly and linearly related to morphometric features and organic content (Table 3). The positive relationships were more pronounced/steeper in the case of the shell thickness followed by shell weight, organic content, and width, and increased with $p\text{CO}_2$ (Fig. 1). Even though there were apparent differences among levels of $p\text{CO}_2$ (e.g. Fig. 1A–D, E), these were non-significant (ANCOVA, $P > 0.38$) (Table 3).

3.6. SEM and EDX

The shell of *C. gallina* contains two main layers, a homogeneous inner layer formed by irregular granules and a more compacted outer layer (Fig. 2). Also, on the SEM image of the transversal cut of the shell, it was possible to distinguish a transition zone between the inner and outer layers.

Changes in the shell's structure were observed by SEM analysis and were accentuated with increasing $p\text{CO}_2$ concentration. Images from the external ventral margin of the shells showed uniform growth lines under control conditions, and more defined growth lines under intermediate conditions, while under high $p\text{CO}_2$ conditions the growth lines were uneven and less defined (Fig. 3). Based on the SEM images taken from the center of both sides of the shells, the external layer of the clams exposed to control conditions appeared to present a more ordered alignment of the crystals, whereas the acidified shells presented a more disordered alignment of the crystals with increasing $p\text{CO}_2$ and a higher porosity of the grains (Fig. 4).

Following the dissolution scale proposed by Bednaršek et al. (2022), the prismatic surfaces of the control shells were practically intact and presented a smoother appearance than the shells exposed to intermediate and high $p\text{CO}_2$ conditions, which presented a higher porosity and a moderate dissolution more accentuated at the external side of the prismatic layer (between Type I and Type II in the scale proposed by Bednaršek et al., 2022). These changes were more noticeable in the juveniles exposed to high $p\text{CO}_2$ conditions, which presented a rougher surface of the shell (Fig. 4).

The results from the dispersive X-ray analyses (EDX) measured at the inner side of the shells showed that the relative concentration of the Ca/C ratio did not change with $p\text{CO}_2$ concentration at the central area of the shells (one-way ANOVA, $P = 0.761$). At the ventral margin the Ca/C ratio did not change between the control with the intermediate and high $p\text{CO}_2$ treatments (Fig. 5). The differences observed were between the intermediate and high $p\text{CO}_2$ treatments (ANOVA on ranks; $H = 9.67$, $P = 0.008$; Dunn's test). The shells exposed to an intermediate level presented the highest Ca/C concentration, while the shells exposed to high $p\text{CO}_2$ conditions presented the lowest Ca/C concentrations at the ventral margin of the inner side of the shells.

4. Discussion

The long-term exposure to high $p\text{CO}_2$ did not affect the shell strength of juveniles of *Chamelea gallina*. The results suggest that under intermediate and high $p\text{CO}_2$ levels, juveniles coped with OA and compensated for the loss of integrity under OA conditions. However, after a

Table 3Results for the ANCOVA models relating Force (N) vs. $p\text{CO}_2$ level [CO_2] and shell morphometric features (covariates).

Model	Term	df	SS	MS	F	P-value
Force (N) vs. Length (mm)	[CO_2]	2	391657.8	195828.9	0.815	0.446
	Length	1	762450.6	762450.6	3.173	0.078
	Residuals	88	21147539.4	240312.9		
Force (N) vs. Organic content (mg)	[CO_2]	2	341555.6	170777.8	0.824	0.442
	Organic	1	3554444.7	3554444.7	17.145	<0.001
	Residuals	90	18658382.8	207315.4		
Force (N) vs. Weight (g)	[CO_2]	2	391657.8	195828.9	0.959	0.387
	weight	1	3939705.8	3939705.8	19.293	<0.001
	Residuals	88	17970284.2	204207.8		
Force (N) vs. Width (mm)	[CO_2]	2	391657.8	195828.9	0.829	0.440
	Width	1	1126708.1	1126708.1	4.771	0.032
	Residuals	88	20783281.9	236173.7		
Force (N) vs. Thickness (mm)	[CO_2]	2	391657.8	195828.9	0.978	0.380
	Thickness	1	4288363.5	4288363.5	21.416	<0.001
	Residuals	88	17621626.5	200245.8		

long-term exposure of 217 days to elevated $p\text{CO}_2$ conditions, it was observed an increase in the porosity of the surface of the shells and a decrease in the shell thickness which was intensified under high $p\text{CO}_2$ conditions. All of the other parameters were statistically homogeneous. The slight losses of integrity to compensate for the negative effect of OA could have unknown consequences in the near future that could compromise the resilience of this species, with important repercussions for bivalve fisheries and natural populations.

Other authors have also found that OA will increase or not affect the shell strength of juveniles and adults of mussels (Mackenzie et al., 2014; George et al., 2022; Mele et al., 2023), adult oysters (Mele et al., 2023), juvenile scallops (Lagos et al., 2021; Abarca-Ortega et al., 2022; Córdova-Rodríguez et al., 2022), juvenile clams (the present study) or sea snails (Duquette et al., 2017; Leung et al., 2019, 2020). However, as in the present study, previous works also found that even though some species were able to compensate for the negative effect of OA on their shell strength, the extra energy expended to keep up high calcification rates caused some minor structural changes, such as decreases in growth, on the integrity and organic content of the shells (Duquette et al., 2017; Lagos et al., 2021; Abarca-Ortega et al., 2022; Córdova-Rodríguez et al., 2022; the present study). Some of these experiments took place in areas exposed to upwelling (Lagos et al., 2021; Abarca-Ortega et al., 2022; Córdova-Rodríguez et al., 2022) and in CO_2 vents (Duquette et al., 2017; Leung et al., 2019, 2020). In these areas, the organisms are already adapted to future conditions and are more resilient to future changes, especially under moderate CO_2 levels (Leung et al., 2020) when enough food is available (Ramajo et al., 2016; Leung et al., 2019).

The present study found that the best predictor of shell strength in compression experiments with juveniles of *C. gallina* is shell thickness. In a study about the shell mechanical properties of adults of *C. gallina* collected at different latitudes through compression tests, Guarino et al. (2019) also found that shell strength depends on changes in shell thickness. The low correlation of shell strength with the other variables may also be related to the technique used. In a previous study with the bivalve species *Callista chione*, Vasconcelos et al. (2011) found that in compression experiments, the best predictor for shell strength is thickness, while in compaction experiments, the best predictor is shell weight. When comparing both methods, the authors suggested that the correlation coefficients were higher in compression experiments than in compaction experiments. Because of this, future studies should compare both methods to verify if the best predictors and correlation coefficients change in the same way independently of the bivalve species used during the experiments.

Shell thickness was the only biometric variable that decreased with increasing $p\text{CO}_2$. This agrees with the results of Gazeau et al. (2011) who found that shell thickness is an early and more accurate indicator of the effect of ocean acidification on shellfish rather than other biometric

parameters. According to the review made by Johnson (2021), the resistance to crushing can depend more on shell shape than thickness. This agrees with the results from Fitzer et al. (2015) who presented the first assessment on the implications of mussel shell shape for functional protection under OA. The authors found that mussels grown under OA conditions reduced their shell thickness and also presented changes in shape. In future studies on the effect of OA on shell resistance, the relationship between shell thickness, shape, and strength, has to be further studied to verify whether the decrease in shell thickness observed is related to adaptive changes in the shape of the clams to become more resistant to crushing. However, in order to detect a possible change in the convexity of the shells, it would be necessary to calculate the shape index and perform statistical shape analyses comparing the surface representation in 3D of the shells (see Fitzer et al., 2015).

The increase in the mechanical resilience of the shell has been associated with a higher organic content (Leung et al., 2019), while degradation of the organic matrix is an indicator of a loss of the structural integrity and strength of the shells (Welladsen et al., 2010; Johnson, 2021). In the present study, there were no significant decreases in the organic content and shell size when exposed to elevated $p\text{CO}_2$, and therefore, the shell strength was unaffected. However, the increase in the porosity of the shells with $p\text{CO}_2$, especially on the SEM images taken from the external layer of the shells suggests that despite the high resilience of the juveniles there was a loss of integrity of their shells under OA conditions. The increase in the porosity and loss of shell thickness could be a compensatory mechanism to confront environmental distress and maintain the shell's biomechanical functionality. This agrees with previous studies which found that elevated $p\text{CO}_2$ increased the disorder of the shell crystals and had a negative effect on shell density, while other biomechanical properties remained unaffected (Lagos et al., 2021; Fitzer et al., 2015, 2016). The authors also suggested that the loss of integrity was a tradeoff to be able to maintain shell biomechanical properties through compensatory mechanisms, such as biopolymer plasticity (Lagos et al., 2021), adjustment of the building blocks to reduce nanotwin thickness and increase the incorporation of organic matter (Leung et al., 2020) or a change of the shape of their shells (Fitzer et al., 2015). As stated previously by Leung et al. (2022) in this new era of ocean acidification research is imperative to study not only the negative effects of OA but also the adaptive capacity of calcifiers to persist under global change conditions.

The results obtained in previous experiments done at the EEMT station also found that in the short term, the growth and survival of different shellfish species increased or were unaffected by OA (Range et al., 2011, 2012; Fernández-Reiriz et al., 2012; Sordo et al., 2021). The positive or neutral response to OA has been associated with the local alkalization of seawater ($3434.43 \pm 11.46 \mu\text{mol kg}^{-1}$) which is higher than in other parts of the Ria Formosa Coastal lagoon (2348 ± 7.10

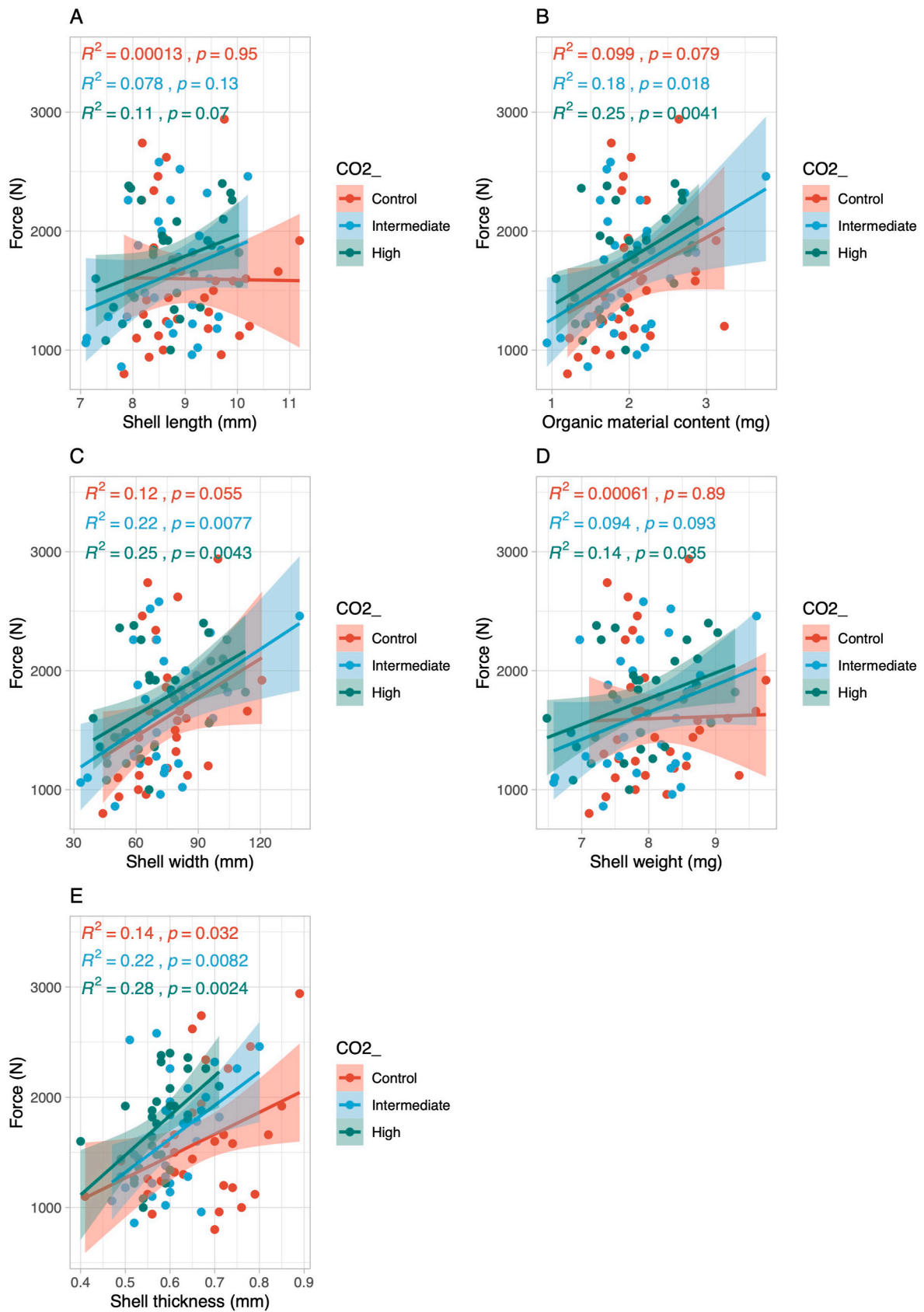


Fig. 1. Correlation of shell strength and length (A), organic material content (B), width (C), weight (D), and thickness (E) of the *Chamelea gallina* shells exposed to three different pCO₂ concentrations; control (pH~8.2), intermediate (pH~8.0) and high (pH~7.7) for 217 days (n = 32).

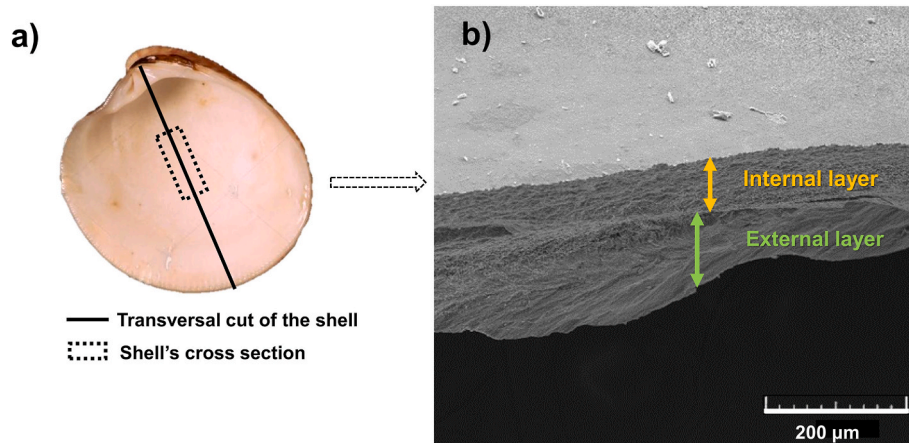


Fig. 2. Schematic representation of the transversal cut of a *C. gallina* shell (a) and SEM image where two main layers are depicted (outer and inner sections; 200x) (b).

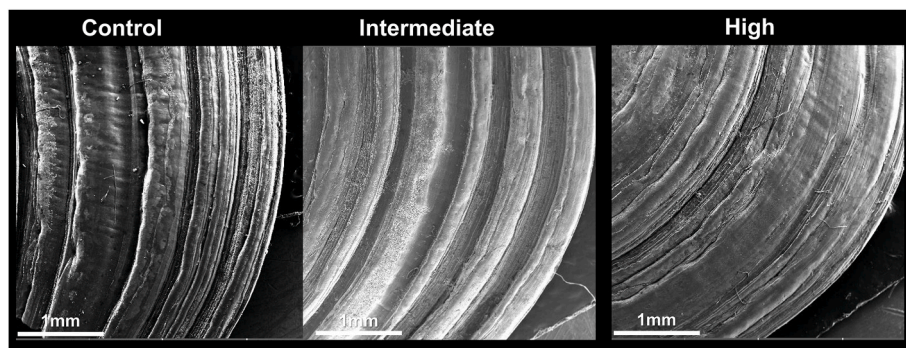


Fig. 3. Scanning electron microscopy (SEM) image of the ventral edge of the external side of the shells (40x) of *C. gallina* juveniles exposed to control (pH~8.2), intermediate (pH~8.0), and high $p\text{CO}_2$ (pH~7.7) concentrations.

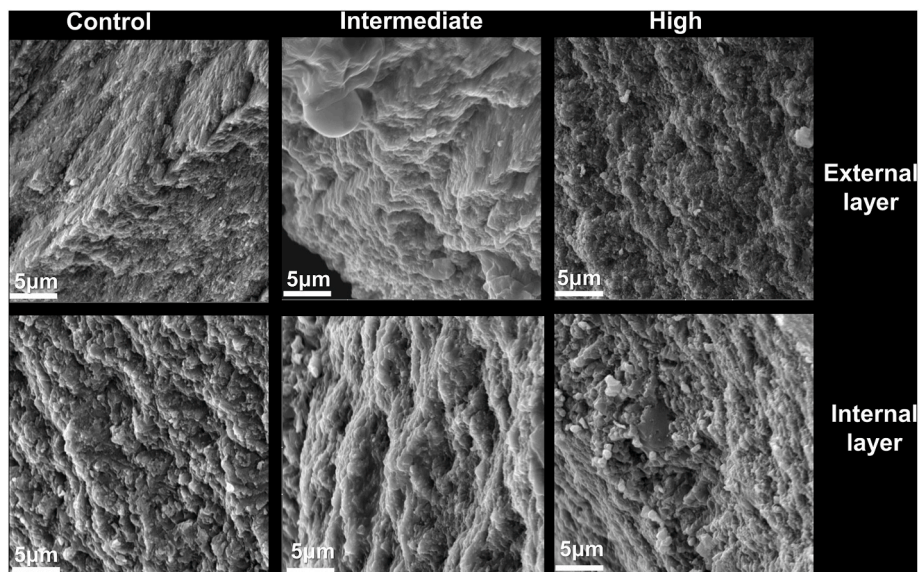


Fig. 4. Scanning electron microscopy (SEM) images taken from the central area of the external and internal layer of the *C. gallina* shells (5000x) exposed to different $p\text{CO}_2$ concentrations; control (pH~8.2), intermediate (pH~8.0) and high (pH~7.7) for 217 days (n = 3).

$\mu\text{mol kg}^{-1}$), probably related to the sediments rich on shell debris that are below the station (see Sordo et al., 2021). However, Sordo et al. (2021) also found that after 200 days at moderate levels of acidification, the juvenile clams decreased their calcification and growth rates but not

their survival, while in the present study, even though the shell strength was unaffected, the clam juveniles exposed to the highest $p\text{CO}_2$ level decreased their shell thickness and present a moderate dissolution at the external layer of the shells, increasing their porosity. This agrees with

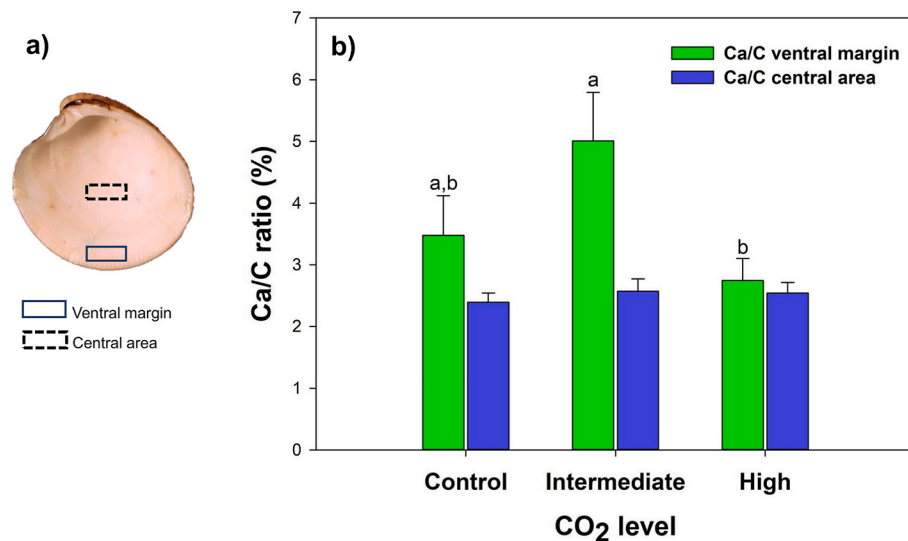


Fig. 5. EDX analysis (calcium and carbon ratio; Ca/C) at the central (a) and ventral margin (b) of the inner side of the *C. gallina* shells exposed to control (pH~8.2), intermediate (pH~8.0) and high $p\text{CO}_2$ concentrations (pH~7.7) for 217 days ($n = 3$). The letters indicate significant differences between CO_2 treatments (mean \pm SE).

the results of León et al. (2020) who found a moderate shell dissolution in all analyzed taxa despite a supersaturation of aragonite in the seawater suggesting that the loss of integrity of the shells was related to seasonal changes in the carbonate chemistry. Therefore, despite the high alkalinity conditions of SW, the long-term exposure to increasing $p\text{CO}_2$ conditions came with an “extra cost” that could eventually decrease the productivity of shellfish populations, with individuals growing thinner shells. Because of this, it is fundamental to consider the local conditions under which the organisms were bred and raised when interpreting the results in OA experiments.

The neutral and positive results to OA by different shellfish species cultivated at the station (Range et al., 2011, 2012; Fernández-Reiriz et al., 2012; Sordo et al., 2021; the present study) suggest that the alkalization of seawater could be an effective carbon dioxide removal approach (CDR) to mitigate the effect of OA on shellfish aquaculture and fisheries. For instance, the use of shell residues to alkalize the sediment in aquaculture areas, and the establishment and restoration of oyster reefs (Waldbusser et al., 2013), are potential solutions that increase biodiversity and support the reuse of residues from mollusk production, promoting the circular bioeconomy. Also, more efficient and selective dredges decrease the number of individuals damaged in catches and allow juveniles to escape rapidly decreasing their susceptibility to injuries due to the abrasion among animals and/or debris (Gaspar and Chicharro, 2007). Therefore, the implementation and use of more sophisticated fishing gear, such as the mechanical vibrating sieve, could minimize shell damage during fishing operations under future CO_2 conditions (Bargione et al., 2023).

Even though there were no differences in shell strength across treatments and there was a homogeneous response of most of the parameters measured, the minor structural changes observed in juveniles exposed to high $p\text{CO}_2$ conditions could eventually compromise the shell resistance of the clams. Under an intermediate $p\text{CO}_2$ level, juveniles seemed to counterbalance the negative effects of OA. Despite this, acidified juveniles suffered an increase in porosity and shell thickness losses. These changes and the increase in the positive relation between shell thickness and strength with $p\text{CO}_2$ concentration could be related to a change in the shape of the organisms conferring them a higher resilience to being broken. Nevertheless, further long-term experiments where the shell strength, shape, and other variables are measured simultaneously are necessary to confirm the plasticity of shellfish juveniles under global change conditions.

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CRedit authorship contribution statement

Laura Sordo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eduardo Esteves:** Writing – review & editing, Funding acquisition, Formal analysis. **Joana F.A. Valente:** Writing – review & editing, Investigation, Funding acquisition. **Jaime Anibal:** Writing – review & editing, Funding acquisition. **Catarina Duarte:** Investigation. **Nuno Alves:** Funding acquisition. **Teresa Baptista:** Resources. **Miguel B. Gaspar:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data is available on supplementary materials 2

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2024.106746>.

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