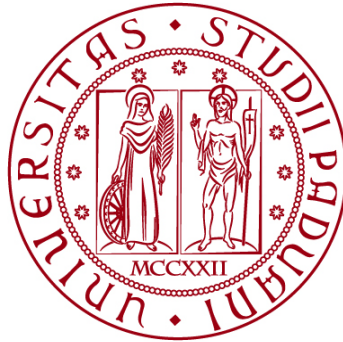


UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea in Biologia



ELABORATO DI LAUREA

**Effects of seawater acidification on the physiological
performance of the intertidal gastropod
*Phorcus articulatus***

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1.INTRODUCTION

1.1 Ocean acidification

Among the consequences of the increasing anthropogenic pressures on marine ecosystems, atmospheric carbon dioxide (CO₂) emissions represent one of the most critical environmental challenges. The current rate of atmospheric CO₂ increase, largely driven by fossil-fuel combustion and industrial and agricultural activities, is unprecedented in geological history, making the global scale of its effects highly concerning (Doney et al. 2012).

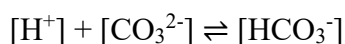
This atmospheric CO₂ accumulation is naturally buffered by oceanic uptake, which absorbs nearly one-third of the anthropogenic carbon emissions. While this process mitigates atmospheric warming and prevents the occurrence of an even more severe climate change scenario, the excessive uptake of CO₂ into the ocean is not a benign phenomenon. Instead, it alters fundamental chemical balances and leads to a decline in seawater pH, a process collectively defined as Ocean Acidification (OA).

In fact, as a result of oceanic CO₂ absorption, the global average surface ocean pH has decreased by approximately 0.1 units, from ~ 8.21 to 8.10, between the beginning of the Industrial Revolution and the dawn of the new millennium (Feely et al. 2004; Sabine et al. 2004; Doney et al. 2009). Recent monitoring confirms that this decline is accelerating, reaching a global annual mean surface seawater pH of 8.04 in 2024 (European Environment Agency 2025). Under the high-emission Representative Concentration Pathway (RCP) 8.5 scenario, over the course of the 21st century, ongoing carbon uptake is expected to worsen OA, causing open ocean surface pH to drop by an additional 0.3 units by the 2081-2100 period, relative to 2006-2015 (IPCC 2019)¹.

This decline in ocean pH is the result of changes in the seawater inorganic carbon system. As CO₂ dissolves in seawater, it first reacts with water molecules (H₂O) to form carbonic acid (H₂CO₃), a weak acid that rapidly dissociates into bicarbonate (HCO₃⁻) and hydrogen ions (H⁺):



The subsequent increase in H⁺ drives pH towards lower, more acidic values. The excess H⁺ ions react with available carbonate ions (CO₃²⁻) to form additional HCO₃⁻, depleting the pool of free carbonate:



Therefore, the overall effect of the dissolution of CO₂ in seawater results in an increase in the concentrations of H⁺, H₂CO₃ and HCO₃⁻ ions, reducing the carbonate ions (CO₃²⁻) availability (Raven et al. 2005). At the same time, the reaction between

¹ IPCC: Intergovernmental Panel on Climate Change

most of the hydrogen ions generated by CO₂ dissolution and carbonate ions reduces the concentration of free hydrogen ions, resulting in a much smaller change in pH than would otherwise be expected. This buffering effect comes at the cost of a substantial consumption of carbonate ions, which entails two relevant consequences in the context of OA. One is the progressive weakening of the carbonate buffer system itself, reducing its capacity to mitigate further pH changes as increased amounts of CO₂ are taken up by the oceans. Another consequence of the decrease in carbonate ion concentration concerns the chemistry of carbonate minerals, as the reduced carbonate availability hinders their formation while promoting their dissolution (Feely et al. 2004; Raven et al. 2005).

From a biological perspective, the impaired formation of carbonate minerals is particularly relevant, since calcium carbonate (CaCO₃) is the most abundant biogenic mineral, with calcite and aragonite serving as the dominant polymorphs secreted by marine calcifying organisms to build their shells and skeletons (Weiner and Dove 2003).

Organisms such as corals, molluscs and echinoderms are particularly vulnerable to OA because they rely on the biologically controlled precipitation of calcium carbonate to produce and maintain their protective structures.

The physiological impacts on these calcifiers depend strictly on the CaCO₃ saturation state (Ω_{CaCO_3}), which is expressed as:

$$\Omega = [\text{Ca}^{2+}] [\text{CO}_3^{2-}] / K_{\text{sp}}$$

where K_{sp} represents the apparent stoichiometric solubility product for either aragonite or calcite (Fabry et al. 2008). Although aragonite and calcite differ in their solubility, reflected in their different K_{sp} values, their saturation horizons (Ω_{arag} and Ω_{cal}) respond similarly to acidification and are collectively evaluated as Ω_{CaCO_3} (Bach 2015).

In this context, since the Ca²⁺ concentration in the ocean is relatively constant and varies primarily with salinity, fluctuations in Ω_{CaCO_3} are largely determined by changes in CO₃²⁻ concentration.

Saturation values above 1 ($\Omega_{\text{CaCO}_3} > 1$) thermodynamically favour CaCO₃ precipitation and the formation of calcified structures, whereas undersaturated conditions ($\Omega_{\text{CaCO}_3} < 1$) create highly corrosive environments that promote structural dissolution (Fabry et al. 2008).

Under natural conditions, CaCO₃ saturation states vary with temperature and pressure, showing natural vertical and latitudinal gradients. For instance, surface tropical waters are generally supersaturated ($\Omega > 1$) due to higher temperatures, lower pressure and greater carbonate ion availability. By contrast, deep and high-latitude waters exhibit lower saturation states due to higher hydrostatic pressure and lower temperatures, which increase CO₂ solubility (Fabry et al. 2008; Figuerola et al. 2021).

However, OA is currently disrupting these natural patterns, both by decreasing CaCO_3 saturation states and by causing the carbonate saturation horizon, the thermodynamic tipping depth below which seawater chemical conditions favour CaCO_3 dissolution, to rapidly shoal (Figuerola et al. 2021).

These progressive changes in CaCO_3 saturation conditions are already detectable across the global ocean and are expected to become more pronounced under future high-emission scenarios. In line with IPCC projections, the global area-averaged Ω_{arag} is predicted to drop by 47% by the end of the century (L. Jiang et al. 2023).

1.2 Effects of OA on calcifying organisms

Although the chemistry behind OA was deeply analysed several decades ago, extensive investigations on its impact on marine organisms began only about 20 years ago (Shi and Li 2024). It is now widely recognized that OA-driven changes in seawater chemistry result in increasingly unfavourable conditions for calcifying organisms, affecting multiple aspects of their biology.

Firstly, as already mentioned, declining carbonate ion availability in seawater directly compromises calcification and, consequently, the production of CaCO_3 structures. Moreover, especially in areas experiencing undersaturated conditions, even the preservation of already formed shells and skeletons may be threatened, as these structures become more susceptible to dissolution (Byrne and Fitzer 2019).

Evidence from studies conducted on different taxa of shelled molluscs confirms the occurrence of these effects (Fitzer et al. 2015; Gazeau et al. 2007; Harvey et al. 2013; Marshall et al. 2019; Ries et al. 2009). For instance, impaired calcification was reported by Fitzer et al. (2015) following a 9-month exposure of the mussel *Mytilus edulis* Linnaeus, 1758 to low-pH conditions (pH 7.31 and 6.70), resulting in a thinner shell aragonite layer as well as changes in shell shape. Similarly, a comparison between two benthic gastropod species, *Nerita chamaeleon* Linnaeus, 1758 and *Planaxis sulcatus* (Born, 1778), from acidified and reference sites showed altered shell shape and reduced shell size in individuals from acidified environments; importantly, *N. chamaeleon* specimens from these environments also exhibited greater shell erosion, with the average eroded surface exceeding 40% in CaCO_3 -undersaturated waters, compared to less than 10% in snails from reference sites (Marshall et al. 2019).

Beyond these structural impacts, increased CO_2 dissolution and the related shifts in seawater chemistry affect marine organisms at the physiological level as well. In fact, when CO_2 levels increase in seawater, CO_2 reacts with their internal body fluids, leading to a reduction in pH and causing hypercapnia (Fabry et al. 2008). This stress condition imposes energetic costs on organisms, since the newly prioritized activation of compensatory mechanisms aimed at preserving acid-base homeostasis reduces the resources available for energy-demanding functions such as reproduction, growth and biomineralization (Byrne and Fitzer 2019; Figuerola et al. 2021).

As a result, the energetically expensive production of calcified structures may be altered or reduced (Byrne and Fitzer 2019), with potential effects on calcification rates (Fabry et al. 2008), as well as on shell/skeleton structural properties (Byrne and Fitzer 2019).

Thus, considering the mechanisms discussed above, OA can affect calcification through both a direct chemical pathway, related to low carbonate availability, and an indirect physiological pathway, linked to energy reallocation (Byrne and Fitzer 2019).

For this reason, calcification-related traits can be considered informative indicators of an organism's capacity to tolerate acidification. In addition, given the impact of acidified conditions on physiological processes, several other physiological responses can be used as valid proxies to assess the extent of OA effects on organismal physiology (Fabry et al. 2008; Byrne and Fitzer 2019).

For instance, the physiological rates of respiration, excretion and filtration were assessed in the gastropod *Crepidula fornicata* (Linnaeus, 1758) exposed to increasingly acidified conditions. The stability of these parameters across all tested treatments suggested that this species can maintain its energy metabolism and rely on effective acidosis-buffering mechanisms, thus showing metabolic tolerance to acidification (Noisette et al. 2016).

A different physiological response to low pH was observed in juvenile *Ruditapes decussatus* (Linnaeus, 1758). They showed decreases in both ingestion and respiration rates, indicating lower energy intake and metabolic depression; conversely, increased ammonia excretion suggested enhanced protein catabolism, which, together with a low O:N ratio, is indicative of metabolic stress. Overall, although juveniles of this species were able to survive at reduced seawater pH exposures, alterations in their physiological parameters pointed to constrained energy availability for growth and other vital functions (Fernández-Reiriz et al. 2011).

1.3 Habitat influence on responses to OA

As illustrated by the examples above, not all marine calcifiers respond to OA in the same way; rather, there is considerable variation in their sensitivity. Such differences may be related to several factors, including the taxonomic group they belong to, their developmental stage, and their physiological ability to compensate for reductions in extracellular pH (Kroeker et al. 2010).

Another element that may play a role in organismal response to OA is the habitat they live in: species inhabiting areas which are naturally subjected to fluctuations in environmental conditions may be better able to tolerate and/or adapt to changes in CO₂ levels than those living in regions characterized by more stable carbonate chemistry (Figuerola et al. 2021).

For instance, coastal ecosystems show high long-term pH variability, in contrast to the more linear and uniform pH changes observed in open-ocean waters. This pH heterogeneity is not only driven by the acidification-promoting air-sea CO₂

exchange, but also by land-derived inputs, which can affect coastal pH in different ways. More specifically, freshwater inflows, which are often acidic and supersaturated with respect to CO₂, tend to dilute seawater alkalinity after entering the sea, thereby reducing its buffering capacity. Moreover, the riverine delivery of nutrients and organic carbon can shift ecosystem metabolism either towards enhanced primary production, leading to pH increase, or towards respiration, leading to pH decrease (Carstensen and Duarte 2019).

Among coastal ecosystems, rocky intertidal shores represent the most widespread littoral habitats on wave-battered coasts. They are particularly exposed to rapid shifts in several environmental conditions, since their location between land and sea exposes them to alternating periods of tidal submersion and aerial exposure (Baharuddin et al. 2018; Wolfe et al. 2020; Martínez et al. 2015). Consequently, organisms found on rocky shores experience periodic, tidally driven fluctuations in temperature, pH, oxygen availability and salinity. These parameters remain relatively stable during submersion but can become extremely variable during emersion, when desiccation stress, osmotic challenges and exposure to wind and breaking waves become additional threats to these species (Horn et al. 2021; Baharuddin et al. 2018).

Despite these stressors, these dynamic habitats commonly host diverse groups of resistant organisms, such as algae, lichens, barnacles and molluscs (Baharuddin et al. 2018). Their ability to withstand emersion-related stress plays a key role in shaping their physiology and, in turn, their distribution patterns along the intertidal gradient (Horn et al. 2021).

1.4 Gastropods of the genus *Phorcus*

In this context, molluscs represent one of the most abundant and ecologically relevant groups, as their communities substantially contribute to rocky-shore food-web dynamics and nutrient recirculation. Moreover, several molluscan species are well suited to serve as bioindicators of human-related environmental disturbances (Martínez et al. 2015).

This is exemplified by marine gastropods of the genus *Phorcus* Risso, 1826, a group of herbivorous snails belonging to the family Trochidae Rafinesque, 1815, which are an integral part of rocky-shore communities across the Mediterranean Sea and the Northeastern Atlantic Ocean.

Their ecological importance in rocky-shore ecosystems mainly lies in their role as grazers feeding on algal biofilms. By scraping the rocky substrate with their radula, they exert fundamental control not only over algal cover, but also over the overall structure of intertidal communities, since, in their absence, the excessive development of seaweed beds could alter the abundance and distribution of rocky-shore species.

Beyond their functional relevance, *Phorcus* gastropods exhibit several ecological and biological features that make them useful sentinel species, including wide distribution, long life span, reduced mobility, easy identification and sampling, and

suitable size for tissue analysis. Thus, they can be reliably used to assess the health status of the habitats they contribute to regulating, and to determine the consequences of anthropogenic impacts on coastal ecosystems, such as pollution and climate change (Sousa et al. 2018).

Notably, their calcifying nature gives them additional value as biomonitoring organisms for OA, since the formation of their spirally coiled shell in the embryonic stage, as well as its maintenance throughout life, depend on the secretion of calcium carbonate by the mantle (Marshall et al. 2019; Sousa et al. 2018).

In this regard, the assessment of OA effects on *Phorcus* species can be carried out not only by evaluating changes in shell integrity (Marshall et al. 2019), but also by monitoring physiological endpoints potentially reflecting energy balance and metabolic performance under acidified conditions (Noisette et al. 2016).

Conducting this type of investigation would help achieve a better understanding of the actual impact of climate-change-driven OA on intertidal calcifiers. Indeed, their physiological adaptations in response to the pH conditions, typical of rocky shores, may enhance their resilience to the increases in CO₂ concentrations expected in the coming decades. At the same time, the adaptation to extreme abiotic conditions could lead them to live close to their physiological tolerance limits, which could instead make them more vulnerable to pH changes exceeding their usual range of variability. If this hypothesis holds, intertidal ecosystems may be among the first to be affected by perturbations associated with climate change, and could therefore serve as valuable environments to assess its ecological effects (Sousa et al. 2018; Wolfe et al. 2020).

1.5 *Phorcus articulatus* in the Venice Lagoon

There are nine recognized living species belonging to the genus *Phorcus*, with a well-defined geographical separation between the five species occurring in the Atlantic Ocean and the remaining four in the Mediterranean Sea (Sousa et al. 2018). *Phorcus articulatus* (Lamarck, 1822) (Figure 1) is an endemic species to the Mediterranean Sea, where its distribution is known throughout the basin, except for the Bosphorus Strait and the Black Sea. It can be commonly observed along most Mediterranean rocky shores, mainly occupying the intertidal zone and the upper subtidal. It feeds on plant detritus and algae growing on rocky substrates, with macroalgae of the genus *Cladophora* Kützinger, 1843 and the species *Ulva lactuca* Linnaeus, 1753 also reported as potential food sources.

Individuals of this species are often found in aggregations of variable density, frequently clustered together and sometimes mixed with similar species sharing the same habitat, such as *Phorcus turbinatus* (Born, 1778).

The morphological similarity between *P. articulatus* and its Mediterranean congeners may complicate species identification, making it necessary to focus on its main shell-related diagnostic features. More specifically, *P. articulatus* is characterized by a thick, spirally-coiled shell with a swollen base and six whorls separated by a shallow suture. Shell coloration is variable, but it presents a typical

pattern consisting of one or more articulated red and white bands of irregular width, separated by intervals showing a fine hatching of wavy lines or violet to dark-grey zigzags (Matejcekova et al. 2025).

One of the most distinctive environments inhabited by this gastropod species is the Venice Lagoon (Russo 2017).

The Venice Lagoon is a shallow coastal water body (average depth 1.5 m) which extends in an arch approximately 55 km long and 13 km wide, representing the largest lagoonal system in Italy, and ranking among the widest transitional areas in the Mediterranean Sea (Solidoro et al. 2010; Camatti et al. 2023; Russo 2017).

It is connected to the Adriatic Sea through three different inlets -named Lido, Malamocco and Chioggia- through which seawater and sediments enter and exit the lagoon, resulting in a water renewal time of about a few days for the areas located near the inlets, and up to one month for the most internal zones (Camatti et al. 2023).

The Venice Lagoon is a highly heterogeneous system that comprises distinct landforms -including sand-mud banks, marshes, fishing basins and a network of channels originating from the three inlets- whose dynamics are primarily shaped by a semidiurnal tidal regime, which consists of two high and two low tides of unequal amplitudes (Camatti et al. 2023; Russo 2017; Solidoro et al. 2010).

However, tidal exchange with seawater is not the only natural driver affecting the lagoon's water properties, since freshwater discharges from 11 main inflows and other minor rivers also contribute to determining the spatial and temporal variability of salinity, temperature, carbonate chemistry, oxygen concentration and nutrient availability. These environmental variables are also strongly influenced by the pervasive and multifaceted human pressures on the lagoon: since the late 19th and early 20th centuries, several anthropogenic stressors have increasingly acted alongside natural drivers in governing the structure and functioning of the entire lagoon ecosystem (Solidoro et al. 2010), making the Venice lagoon one of the Italian marine and coastal areas most impacted by human-related activities (Camatti et al. 2023).

Eutrophication and pollution caused by industrial, agricultural and urban wastewater inputs, together with fishing activities (especially clam harvesting), mariculture, channel dredging and maritime transport, are some of the factors threatening this entire system. These disturbances combine with the pronounced dynamism of transitional environments, resulting in a complex and highly diversified lagoonal ecosystem that evolves in response both to natural and man-induced stressors (Solidoro et al. 2010; Camatti et al. 2023).

For this reason, the occurrence of marine species in the challenging and highly variable lagoon environment, including *P. articulatus*, may indicate a marked capacity to adapt to fluctuating conditions (Russo 2017).

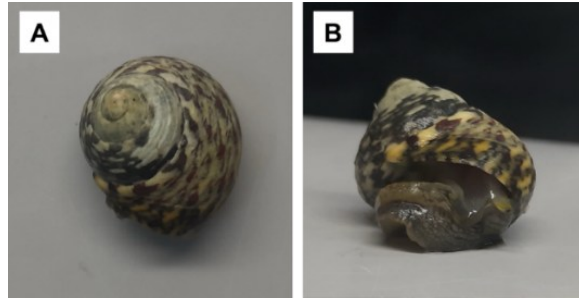


Figure 1. Representative specimen of *P. articulatus* investigated in this study. (A) Top view of the shell.

(B) Lateral view of the specimen, with the animal partially protruding from the shell.

1.6 Aim of the study

The present thesis aimed to investigate the physiological response of the gastropod species *P. articulatus* following a two-month exposure to acidified conditions.

The effects of low pH values on this shelled mollusc were assessed by measuring three response variables indicative of the overall physiological status of the organism: respiration rate, excretion rate, and feeding rate, which respectively reflect metabolic maintenance costs, nitrogen elimination, and energy intake from the environment (Zhang et al. 2016).

Despite the ecological relevance of *P. articulatus*, experimental data regarding its performance under acidification are currently lacking. By evaluating the three parameters mentioned above, this study seeks to address this knowledge gap, providing a comprehensive overview of the energy allocation strategy and physiological condition displayed by this species under low-pH-induced stress.

Since organismal responses to marine acidified environments are species-specific (Zhang et al. 2016), characterizing the metabolic profile of *P. articulatus* could represent a useful addition to the mosaic of biological patterns documented in recent decades, and may ultimately help broaden the current understanding of the extent to which OA might affect the fitness of intertidal calcifiers.

2. MATERIALS AND METHODS

2.1 Sample collection

Mature individuals of the gastropod *P. articulatus* were collected in July 2025 from a rocky shore site in the Venice Lagoon at Chioggia (45°13'08.08"N, 12°16'42.74"E), northern Italy (Figure 2).

Specimens were collected manually from the rocky substrate during low tide, as this species mainly occurs in the intertidal and upper subtidal zones.

Abiotic conditions during the sampling period were obtained from the databases made available by the Department of Biology of the University of Padua, whose Hydrobiological Station of Chioggia facility daily monitors selected physico-chemical parameters of the waters of the southern sector of the Venice Lagoon. In July 2025, average measured water temperature, salinity, pH and dissolved oxygen were respectively 26.37 °C, 35.23 psu, 8.22 units and 5.55 mg/L (Department of Biology, University of Padua, n.d.).

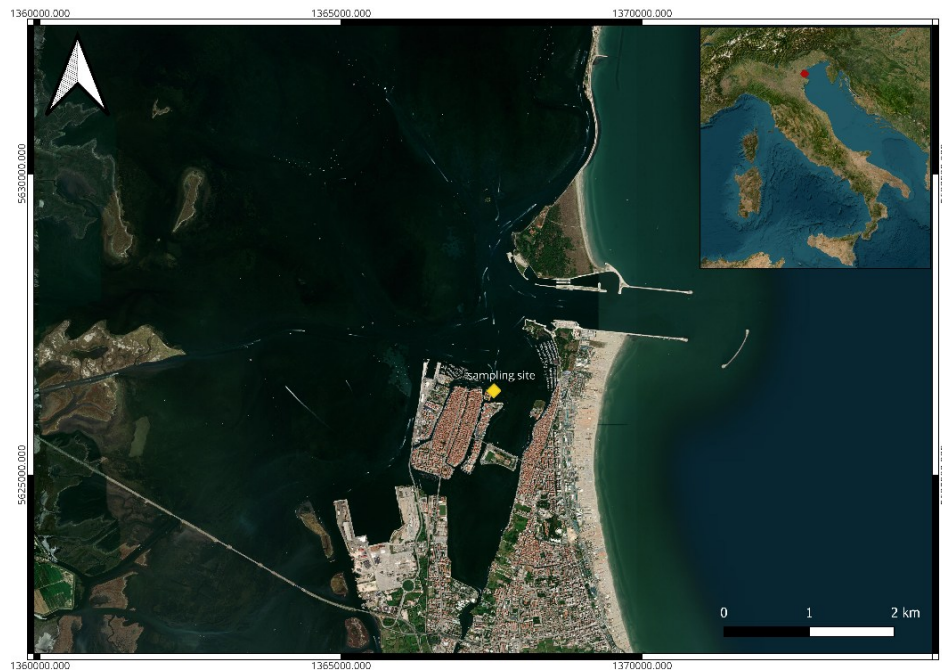


Figure 2. Map of the study area in Chioggia (southern part of the Venice Lagoon, Northern Italy). The yellow square indicates the sampling site. The inset shows the location of the study area within Italy. Basemap source: Esri World Imagery | Powered by Esri.

2.2 Experimental setup

The animals were maintained to acclimate for two weeks in a plastic tank (~1000 L) filled with natural seawater collected from the Chioggia lagoon and maintained under near-ambient conditions, approximately corresponding to pH 8.1, 26 °C and 33 psu.

Rocks from the collection site with epiphytic algae were provided as food.

After acclimation, twelve individuals were transferred into each replicate plastic aquarium (20 L) filled with natural seawater.

Two treatments differing in pH level were considered: three control tanks ($\sim$ pH 8.1) and three CO₂-enriched tanks (pH 7.7 ± 0.05).

Thus, a total of 72 individuals were used in this experiment.

Each set of replicate aquaria was supplied from an 80-L upper tank containing natural seawater under the respective treatment regime.

In the control header tank, pH reflected current conditions at the sampling site, varying naturally over time in accordance with lagoon conditions ($\sim$ pH 8.1), whereas in the CO₂-enriched header tank, pH was maintained at 7.7 to simulate the RCP8.5 scenario for the year 2100 (IPCC 2019).

The elevated-CO₂ treatment was achieved via an APEX-based control system (Neptune Systems, Morgan Hill, California, USA) with dedicated probes and CO₂ diffusers.

Temperature and salinity were identical in both upper tanks and followed natural seasonal variations.

From each upper tank, seawater flowed continuously into the three corresponding replicate aquaria through dedicated tubing, ensuring a constant supply of treatment-aligned seawater to the animals.

Environmental parameters (temperature, salinity, and pH) were periodically measured in each replicate aquarium using a multiparametric probe to verify that temperature and salinity matched ambient conditions across all aquaria.

Each replicate aquarium was also equipped with an oxygen aerator, which provided a constant supply of oxygen to the water.

The room housing the tanks was equipped with a lighting system programmed to simulate the seasonal circadian light cycle, as well as an air-conditioning system set to 25 °C to maintain stable indoor conditions throughout the experiment. Gastropods were fed the epiphytes on rocks from the collection site, which were replenished weekly. Seawater was renewed weekly ($\sim$ 70% change) to prevent accumulation of faeces and metabolic waste.

This experimental setup was run for eight weeks (Figure 3).

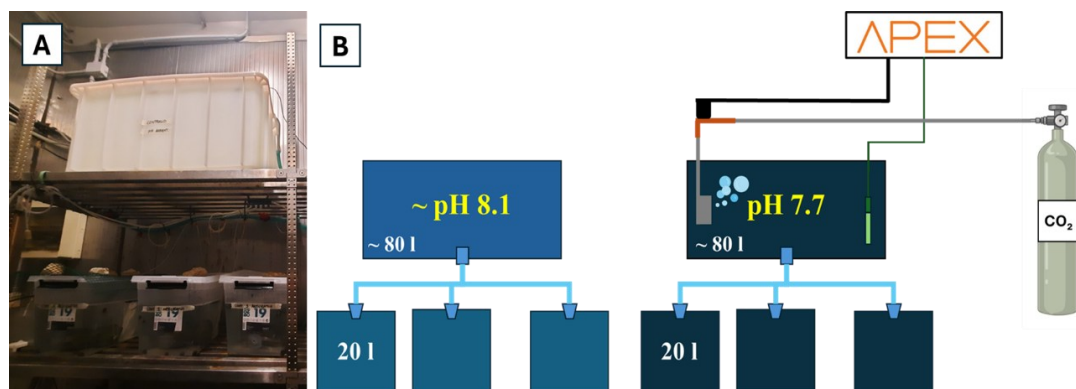


Figure 3. Experimental setup:

(A) Control treatment setup, showing the upper tank supplying seawater to replicate aquaria through a flow-through system. An identical structural arrangement was used for the CO₂-enriched treatment.

(B) Schematic representation of the entire seawater delivery system. In the CO₂-enriched treatment upper tank, pH regulation was achieved using an APEX-based control system comprising a probe, a solenoid valve, and a CO₂ diffuser.

2.3 Experimental sampling and measurements

Gastropods were sampled at three different time points during the experiment: at the beginning (T0), after four weeks (T1), and at the end (T2).

The individuals collected at all three time points were starved for the same duration prior to dissection. Additionally, at T2, physiological measurements were performed before the starvation and subsequent dissection.

At the beginning of the eight-week experiment (T0), two sets of three gastropods each were randomly taken from the initial pool of 72 acclimated animals. After a 24-h starvation period, the volume of each individual was measured by water displacement, after which the shell was removed, and the soft tissue was collected into two portions: an anterior part (consisting of head, mouth and foot), and a posterior part (consisting of the visceral mass) (Figure 4).

The anterior tissues from the three animals within each set were pooled together and then divided into three aliquots, which were then stored at -80 °C for later laboratory analyses (which will not be addressed in the present work). The same procedure was applied to the posterior tissues. The shells of the gastropods were also kept for subsequent investigation of their carbon isotopic composition.

After four weeks (T1), three individuals per replicate aquarium were removed from the experimental setup, for a total of 18 gastropods.

After a 24-h starvation period, the volume and mass of each individual were measured, and the animals underwent the same procedure as at T0.

At the end of the eight-week experiment (T2), a total of 24 individuals were taken (four animals per replicate aquarium). Respiration, excretion and feeding rate assessments were conducted on the selected organisms.

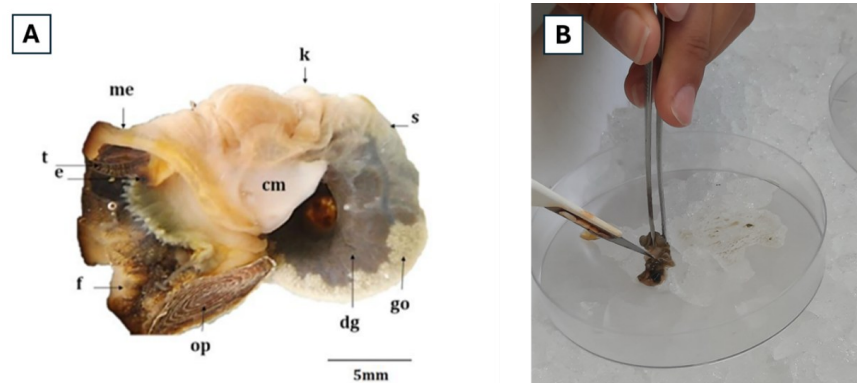


Figure 4. (A) Main anatomical components of a gastropod of the genus *Phorcus* after shell removal. me: mantle edge; t: tentacle; e: eye; f: foot; op: operculum; dg: digestive gland; go: gonad; cm: columellar muscle; s: stomach; k: kidney (Fahmi et al. 2023).

(B) Dissection of an individual of *P. articulatus* used in this study, resulting in the separation of the body into an anterior and a posterior portion.

2.3.1 Respiration and excretion rate

To measure respiration rate, each individual was transferred into a 100 mL airtight chamber filled with seawater adjusted to their respective treatment conditions. Seawater used to fill the chambers was previously filtered to remove organic components that could have interfered with oxygen consumption measurements, then saturated with oxygen using an aerator.

Chambers were placed in a water bath (maintained at 21.0 ± 0.5 °C) for an 8-hour period, consisting of 4 hours of darkness followed by 4 hours of light exposure. After an initial 1-hour acclimation period, dissolved oxygen concentration was measured hourly using an optical dissolved oxygen probe (PyroScience, Aachen, Germany), resulting in four measurements under dark conditions and four under light exposure (Figure 5).

Before each measurement, the temperature of the water bath was entered into PyroScience, and the water in each chamber was gently stirred to ensure uniform conditions and to allow a stable and accurate oxygen record. For each set of four individuals (i.e., per replicate aquarium), a blank chamber without animals was run simultaneously with each set of animal chambers to account for background oxygen consumption.

Respiration rate (RR) for each individual was calculated according to the following equation:

$$RR = (\Delta[O_2]_{\text{corrected}} \times V) / (M \times \Delta t)$$

where $\Delta[O_2]_{\text{corrected}}$ is the change in oxygen concentration in the chamber, corrected for background changes measured in the blank run alongside the considered set of chambers;

V is the chamber volume (100 mL) corrected for the volume of the individual;

M is the wet flesh weight of the individual determined after dissection;

Δt is the incubation time (1 h).

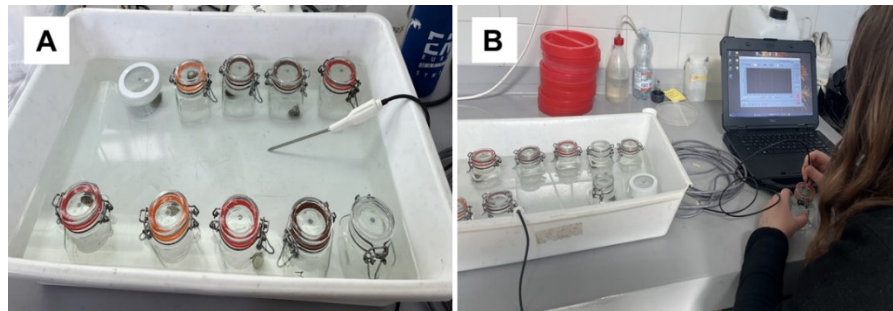


Figure 5. Experimental setup used to measure the respiration rate.

(A) Airtight chambers containing individual gastropods placed in a temperature-controlled water bath during the 8-h incubation period.

(B) Measurement of dissolved oxygen concentration in each chamber using an optical oxygen probe (PyroScience).

At the end of the 8-hour respiration incubation, the seawater from each chamber was collected to quantify ammonia (NH₃) excretion (measured as ammonium, NH₄⁺). For every animal, the chamber water was transferred into two 50-mL Falcon tubes and immediately frozen for later laboratory analysis of ammonium concentration. Seawater contained in blank chambers was also collected to account for background ammonium levels.

Ammonia excretion rate (ER) for each individual was calculated with the following equation:

$$ER = (\Delta[NH_4^+]_{\text{corrected}} \times V) / (M \times \Delta_t)$$

where $\Delta[NH_4^+]_{\text{corrected}}$ is the change in ammonium concentration in the chamber, corrected for the background concentration measured in the blank run alongside the considered set of chambers;

V is the chamber volume (100 mL) corrected for the volume of the individual;

M is the wet flesh weight of the individual determined after dissection;

Δ_t is the incubation time (8 h).

2.3.2 Feeding rate

To determine the feeding rate (FR), all individuals were starved for 24 hours. Prior to the feeding assay, their volume and fresh weight were measured.

Gastropods were then placed in 100 mL individual containers filled with seawater adjusted to their respective treatment conditions, each containing 1 g (fresh weight) of green algae (*Chaetomorpha* sp. and *Ulva* sp.) collected from the sampling site and gently blotted to remove excess water.

For each set of four individuals, a blank sample (i.e., containing only seawater and algae) was included to account for background algal mass loss (Figure 6).

After the 24-hour feeding period, the algae from each container were transferred onto aluminium foil and placed in a drying oven for approximately 6 hours to remove all moisture; the dried algal samples were then weighed.

To express algal consumption in terms of fresh weight, final fresh-weight equivalents were estimated from dry weights using the fresh-to-dry weight ratio calculated from the blank samples.

All animals were starved for an additional 24 hours to ensure consistency with the procedures at T0 and T1. Afterwards, their shells were removed, their flesh was weighed, and the animals were dissected following the same protocol described for T0.

Feeding rate (FR) for each individual was obtained according to the following equation:

$$FR = \Delta FW_{\text{corrected}} / (M \times \Delta_t)$$

where $\Delta FW_{\text{corrected}}$ is the algal fresh-weight consumption corrected for the corresponding blank;

M is the wet flesh weight of the individual determined after dissection;

Δt is the incubation time (24 h).



Figure 6. Setup of the feeding assay showing the five containers used for one set of individuals: four containers with gastropods and one blank container without animals. Each container was filled with seawater adjusted to the respective treatment conditions and supplied with 1 g (fresh weight) of green algae (*Chaetomorpha* sp. and *Ulva* sp.).

2.4 Statistical analysis

All statistical analyses and data visualizations were performed using R statistical software (v4.4.1; R Core Team, 2025). Prior to hypothesis testing, the data distributions were evaluated for normality using the Shapiro-Wilk test, and homoscedasticity was assessed using the F-test for equality of variances.

Data are expressed as mean $\pm$ standard deviation (SD).

To assess the effect of low pH on physiological parameters, differences between control and treated (pH 7.7) groups were analysed using an independent-samples Student's t-test. In cases of unequal variances, Welch's t-test was applied.

Non-parametric Mann-Whitney U tests were systematically performed alongside parametric tests as a robustness check whenever normality assumptions were not met.

Regarding respiration rates, differences between light and dark exposure within the same treatment condition were evaluated using a paired Student's t-test, performed alongside the non-parametric Wilcoxon signed-rank test for non-normal data distributions. Finally, a two-way ANOVA was conducted on respiration rate data to evaluate the main effects of treatment (pH level), light condition (light vs. dark), and their potential interaction.

For all statistical tests, the significance threshold was set at $\alpha = 0.05$.

3. RESULTS

3.1 Respiration rate

After the 8-week experimental period, the mean respiration rate (RR, expressed in mmol O₂ g⁻¹ h⁻¹) was 3.67 ± 3.40 (mean $\pm$ SD) in the control group under dark conditions and 2.91 ± 1.94 under light conditions, whereas in the treated group (pH 7.7) values were 5.60 ± 4.15 in the dark and 6.39 ± 3.46 in the light (Figure 7).

The Shapiro-Wilk test indicated a non-normal distribution of the data collected under dark conditions in both groups (Control: $W = 0.751$, $p = 0.003$; Treated: $W = 0.815$, $p = 0.014$), whereas data collected under light conditions satisfied the normality assumption (Control: $p = 0.567$; Treated: $p = 0.785$). For this reason, the paired t-test (which assumes normality of the differences) may be less reliable, so the Wilcoxon signed-rank test was used as a robust check that does not depend on this assumption.

The comparison between dark and light conditions, performed using a paired t-test, did not reveal statistically significant differences in either the control group ($t = 0.760$, $df = 11$, $p = 0.463$; Wilcoxon: $p = 0.666$) or the treated group ($t = -1.218$, $df = 11$, $p = 0.249$; Wilcoxon: $p = 0.290$).

The comparison between control and treated animals, performed using an independent-samples t-test, showed a statistically significant difference under light conditions ($t = -3.038$, $df = 22$, $p = 0.006$; Mann-Whitney: $p = 0.007$), with higher RR values in the treated individuals. Under dark conditions, the difference did not reach statistical significance, although a similar trend was observed ($t = -1.248$, $df = 22$, $p = 0.225$; Mann-Whitney: $p = 0.069$).

These results were confirmed by the two-way ANOVA (Treatment $\times$ Light condition), which showed a significant main effect of treatment ($F_{(1,44)} = 7.89$, $p = 0.007$), but no significant effect of light condition ($F_{(1,44)} < 0.001$, $p = 0.987$) and no significant interaction between the two factors ($F_{(1,44)} = 0.643$, $p = 0.427$). Overall, these findings indicate that at the end of the exposure to pH 7.7, an increase in respiration rate compared to the control is observed, regardless of light condition, as shown in Figure 7.

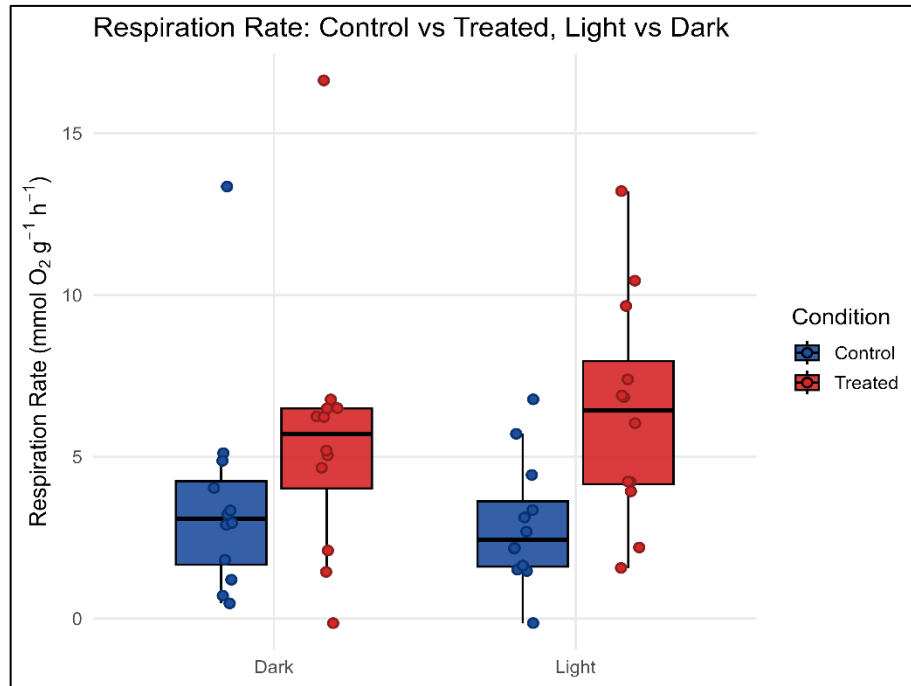


Figure 7. Boxplots of respiration rate (RR, expressed in $\text{mmol O}_2 \text{ g}^{-1} \text{ h}^{-1}$) of *P. articulatus* exposed to 8 weeks of control (pH 8.1, blue) and acidified (pH 7.7, red) laboratory conditions, evaluated under dark and light phases. Horizontal lines within boxes represent median values. Individual measurements are represented as dots ($n = 12$ per treatment).

3.2 Ammonia excretion rate

The mean ammonia excretion rate (ER, expressed in $\text{mmol NH}_4^+ \text{ g}^{-1} \text{ h}^{-1}$) was 0.0044 ± 0.0018 (mean $\pm$ SD) in the control group and 0.0061 ± 0.0037 in the treated group (pH 7.7), suggesting a tendency toward higher values in treated individuals, although with greater variability in this latter group (Figure 8).

The Shapiro-Wilk test confirmed a normal distribution in both groups (Control: $p = 0.963$; Treated: $p = 0.095$), whereas the F-test revealed a statistically significant difference in variance between the two groups ($F = 0.247$, $p = 0.029$), indicating greater data dispersion in the treated group. For this reason, Welch's t-test, which does not assume homogeneity of variance, was applied.

The comparison between control and treated animals did not reveal a statistically significant difference in ammonia excretion rate (Welch's t-test: $t = -1.460$, $df = 16.11$, $p = 0.163$), a result also confirmed by Student's t-test ($t = -1.460$, $df = 22$, $p = 0.158$). Overall, these findings indicate that, unlike what was observed for the respiration rate, exposure to pH 7.7 does not lead to a statistically significant alteration in ammonia excretion rate, although a trend toward higher and more variable values was observed in the treated group (Figure 8).



Figure 8. Boxplots of ammonia excretion rate (ER, expressed in mmol NH₄⁺ g⁻¹ h⁻¹) of *P. articulatus* exposed to 8 weeks of control (pH 8.1, blue) and acidified (pH 7.7, red) laboratory conditions. Horizontal lines within boxes represent median values. Individual measurements are represented as dots (n = 12 per treatment).

3.3 Feeding rate

The mean feeding rate (FR, expressed in g algae g⁻¹ animal h⁻¹) was 0.0005 ± 0.0028 (mean $\pm$ SD) in the control group and 0.0023 ± 0.0032 in the treated group (pH 7.7), suggesting a tendency toward higher feeding rate in treated individuals (Figure 9). The Shapiro-Wilk test confirmed a normal distribution of the data in both groups (Control: $p = 0.211$; Treated: $p = 0.873$), and the F-test showed no significant difference in variance between the two groups ($F = 0.778$, $p = 0.685$), indicating homogeneity of variance.

The comparison between control and treated animals did not reveal a statistically significant difference in feeding rate (Student's t-test: $t = -1.438$, $df = 22$, $p = 0.165$). Overall, these findings indicate that, consistent with the results obtained for ammonia excretion, exposure to pH 7.7 does not lead to a statistically significant alteration in feeding rate, although a trend toward higher consumption was observed in the treated group.

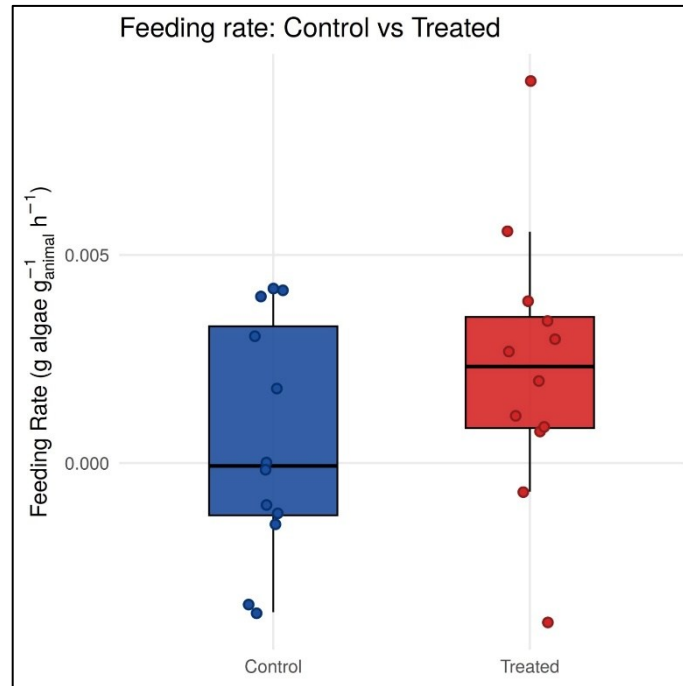


Figure 9. Boxplots of feeding rate (FR, expressed in $\text{g algae g}^{-1} \text{ animal h}^{-1}$) of *P. articulatus* exposed to 8 weeks of control (pH 8.1, blue) and acidified (pH 7.7, red) laboratory conditions. Horizontal lines within boxes represent median values. Individual measurements are represented as dots ($n = 12$ per treatment).

4. DISCUSSION AND CONCLUSION

Respiration, excretion, and feeding rates were analysed to obtain a clear snapshot of the physiological response of *P. articulatus* to decreased pH. However, these three physiological processes were affected differently by the acidification conditions.

4.1 Respiration rate

Respiration rate (RR), reflecting O₂ consumption, was evaluated in *P. articulatus* in relation to both seawater acidification treatments and light-dark cycles. Overall, a significant increase in O₂ consumption was detected under low pH conditions, regardless of light exposure.

4.1.1 Effect of seawater acidification on respiration rate

Respiration rate differed significantly between the two treatments, with individuals exposed to pH 7.7 exhibiting markedly higher values. This increase in O₂ consumption may represent the macroscopic manifestation of a physiological mechanism documented to occur at the cellular level in certain mollusc groups to counteract hypercapnia-induced acidosis (W. Jiang et al. 2026; Parker et al. 2013). Indeed, in these organisms, low environmental pH leads to the acidification of both intra- and extracellular fluids; if not compensated, this disruption in acid-base balance can lead to damaging effects on the individual, including protein degradation and alteration of vital metabolic pathways, ultimately resulting in organismal mortality (Fernández-Reiriz et al. 2011; Melatunan et al. 2011; Parker et al. 2013). Maintaining internal pH homeostasis is therefore crucial, and it is achieved through the active transport of acid-base relevant ions across cell membranes in order to extrude H⁺ ions and simultaneously accumulate HCO₃⁻ in bodily fluids. This process relies on the action of transmembrane ion transport proteins (e.g. Na⁺/K⁺ -ATPase, H⁺-ATPase, and Na⁺/H⁺-exchanger), whose concerted action requires the utilization of ATP molecules. Consequently, the increase in respiration rate displayed by *P. articulatus* gastropods subjected to seawater acidification might reflect an upregulation of aerobic metabolism, as a higher O₂ consumption is required to synthesize the ATP necessary to sustain this physiological compensation mechanism (W. Jiang et al. 2026; Parker et al. 2013; Signorini et al. 2025; Leung et al. 2020).

Under these conditions, the overall energetic demand of basal metabolism drastically increases, forcing the enhanced ATP production to be entirely reallocated to the fulfilment of these maintenance requirements (W. Jiang et al. 2026; Lardies et al. 2014; Pan et al. 2015). As a result, the residual energy remaining after sustenance costs are met, also defined as Scope for growth (SFG), is severely reduced, at the expense of processes such as somatic growth and reproduction, which are both critical for long-term individual fitness and population persistence (Leung et al. 2020; Parker et al. 2013). For instance, in the bivalve *Ruditapes philippinarum* (A. Adams & Reeve, 1850), the substantial costs associated with the

cellular pH regulation mechanism have been estimated to cause a 158% increase in maintenance energy, resulting in a projected 34% reduction in somatic growth (dry flesh weight) (W. Jiang et al. 2026).

Another major strategy employed by many molluscs to survive internal acidosis is metabolic depression, characterized by a decrease in respiration rate and, consequently, in aerobic metabolism.

This response, directly opposing active ion regulation, enables the organism to lower its overall energy requirement, thereby matching ATP supply and demand (Parker et al. 2013; Melatunan et al. 2011; Carey et al. 2016).

The spectrum of molluscan physiological responses to OA thus spans from active acid-base compensation to metabolic depression, with a continuum of intermediate strategies across taxa (Signorini et al. 2025; Parker et al. 2013).

A clear example of slight metabolic depression under acidified conditions is provided by the intertidal gastropod *Littorina littorea* (Linnaeus, 1758), which exhibited a 31% reduction in oxygen consumption after a 30-day exposure to pH 7.6, prioritizing instead the activation of anaerobic metabolic pathways (Melatunan et al. 2011).

The pronounced variability in molluscan physiological responses to OA is well exemplified by comparing *L. littorea* with *P. articulatus*. In fact, despite sharing a similar intertidal lifestyle, being exposed to comparable low pH conditions (pH 7.6 and 7.7), and having food available, these two gastropod species displayed opposing physiological strategies. Whereas *L. littorea* undergoes a reduction in respiration rate and aerobic metabolism to lower both ATP demand and production (Melatunan et al. 2011), our results reveal an opposite trend in *P. articulatus*, which upregulates its O₂ consumption, likely to fuel energy-demanding active acid-base compensation.

This divergence in physiological responses under similar ecological and experimental conditions provides strong evidence supporting the high degree of species-specificity characterizing organismal strategies to cope with OA, rejecting the assumption that a comparable ecological niche implies the adoption of the same tolerance mechanism (Zhang et al. 2016; Kroeker et al. 2010; Noisette et al. 2016; Parker et al. 2013).

Because both physiological strategies entail long-term limitations, survival under future OA scenarios will not depend on the exclusive reliance on a single mechanism. Rather, the adaptive success of marine organisms will include key features such as metabolic flexibility and plasticity in ATP allocation (W. Jiang et al. 2026; Melatunan et al. 2011; Pan et al. 2015; Parker et al. 2013).

Metabolic flexibility refers to the ability to undergo continuous and reversible shifts in energetic processes in response to environmental variability (Lardies et al. 2014; Signorini et al. 2025). Meanwhile, ATP allocation plasticity enables the organism to channel cellular energy toward essential maintenance processes, without completely draining its overall energy budget (W. Jiang et al. 2026; Pan et al. 2015).

Both metabolic flexibility and ATP allocation plasticity are most prominently observed in marine species whose evolutionary history and life history are closely linked to unstable environments, such as intertidal zones, estuaries, and hydrothermal vents (Signorini et al. 2025; W. Jiang et al. 2026). Inhabiting these dynamic habitats and experiencing broad daily fluctuations in pH, salinity, and temperature, these organisms exhibit highly responsive and flexible gene regulatory systems that allow them to rapidly adjust their metabolic processes to changing external conditions (Carey et al. 2016; W. Jiang et al. 2026). Conversely, organisms adapted to environmentally stable habitats, such as the deep sea, having evolved under stable physicochemical conditions, are often incapable of dynamically adjusting their metabolism to rapidly shifting scenarios (W. Jiang et al. 2026; Zhang et al. 2016).

Nevertheless, inhabiting dynamic habitats like the intertidal zone does not ensure complete protection against present and future OA, mainly because of the fundamental nature of this phenomenon, which manifests as a chronic, continuous stressor rather than a transient fluctuation. In this context, possessing intrinsic metabolic flexibility is unquestionably advantageous, yet it does not automatically guarantee survival when pH levels are permanently altered beyond a species' evolutionary norm (W. Jiang et al. 2026; Parker et al. 2013; Melatunan et al. 2011). The observed upregulation of respiration in *P. articulatus* could suggest that this species, and specifically the population inhabiting the Venice Lagoon, possesses a rapid capacity to respond to environmental shifts. However, this observation represents merely a snapshot of an immediate physiological response that accounts for active compensatory mechanisms. The current data do not clarify whether, over extended periods, *P. articulatus* exhibits sufficient metabolic adaptability to dynamically adjust its energy budget, or if the prolonged reliance on this energetically demanding strategy will lead to chronic energy deficits (W. Jiang et al. 2026; Leung et al. 2020). Evaluating the species' true persistence will therefore require multi-generational, multi-stressor studies to verify if short-term active compensation can effectively translate into successful long-term acclimatization (Parker et al. 2013).

4.1.2 Effect of light and dark conditions on respiration rate

Regarding photoperiodic effects, respiration rates in *P. articulatus* showed no statistically significant differences between light and dark conditions across treatments. In natural habitats, this species has been documented to exhibit heightened nocturnal activity associated with grazing on hard substrates (Cheour et al. 2014). Based on this behavioural pattern, an increase in O₂ consumption during dark exposure might have been expected under experimental conditions as well, as a reflection of its natural activity cycle.

The empirical data revealed that O₂ demand remained remarkably consistent regardless of the lighting regime. This trend was observed across both control and acidified treatments, which suggests that this outcome may be tied to specific

constraints of ex situ testing, particularly concerning the respirometry chambers. During respiration measurements, individuals were in fact maintained in small containers where wide-range movement was physically restricted. Since in natural habitats the nocturnal activity peak of *P. articulatus* is primarily driven by substantial spatial movements associated with active foraging, the absence of such spatial and feeding opportunities under laboratory conditions may have suppressed this nocturnal dynamism.

Furthermore, the absence of additional complex environmental cues in the experimental regime, such as tidal cycles and wave action, which work in tandem with photoperiod to regulate temporal orientation, likely contributed to flattening their natural behavioural rhythmicity (Sousa et al. 2018).

In light of these considerations, the measured respiration rate, which remained constant between dark and light conditions, can be hypothesized to reflect the organism's baseline maintenance costs (Routine Metabolic Rate, RMR), rather than accounting for O₂ consumption linked to locomotory activity peaks (Active Metabolic Rate, AMR) (Lardies et al. 2014; Leung et al. 2020).

In line with the mechanisms discussed in the previous section, this Routine Metabolic Rate was consistently higher in gastropods from the acidified treatment due to the elevated energetic demands of cellular acid-base homeostasis under hypercapnic stress (W. Jiang et al. 2026; Lardies et al. 2014; Parker et al. 2013).

4.2 Ammonia excretion rate and feeding rate

Parallel to O₂ consumption, nitrogenous waste excretion via ammonia excretion rate (ER) and energy acquisition via feeding rate (FR) were evaluated to obtain a comprehensive picture of the energy budget of *P. articulatus* under acidification stress.

4.2.1 Ammonia excretion rate

Unlike respiration rate, ammonia excretion rate showed no statistically significant differences between gastropods exposed to control and acidified conditions. This lack of significant variation indicates that *P. articulatus* does not rely on endogenous protein catabolism to supply the extra ATP required for active acid-base regulation under hypercapnia (Noisette et al. 2016; Leung et al. 2020; Signorini et al. 2025; Zhang et al. 2016).

Instead, a plausible explanation -as observed by Signorini et al. (2025) in *Patella caerulea* Linnaeus, 1758- could be that *P. articulatus* successfully preserves its functional protein structures by preferentially utilizing other, energetically more efficient substrates to fuel its heightened maintenance costs, such as carbohydrates and lipids.

Nevertheless, a trend toward higher and more variable values was observed in the group exposed to acidification. Given that this pattern reflects a subtle, non-statistically significant tendency rather than a massive surge in ammonia excretion, it may not signify active, energy-yielding protein catabolism. Instead, this evidence

could point toward the deployment of a physiological acid-base buffering strategy through “proton trapping”. In this pathway, lipophilic NH_3 diffuses out of the cell and rapidly binds with external H^+ ions to form charged NH_4^+ which is impermeable to the cell membrane. By sustaining an outward concentration gradient for NH_3 , this mechanism promotes its continuous passive excretion while trapping protons in the external fluid, thereby alleviating internal acidity (W. Jiang et al. 2026; Noisette et al. 2016).

However, while proton trapping is advantageous in contributing to internal pH maintenance, this process inherently requires a continuous supply of nitrogen for ammonia production. In the short term, the organism can draw from its intracellular pool of free amino acids (FAAs). Over extended periods, however, if energy and nitrogen replenishment through feeding are not scaled up, this pool is progressively depleted, forcing the organism to harvest nitrogen from structural proteins (Signorini et al. 2025; Parker et al. 2013; Fernández-Reiriz et al. 2011).

Thus, while effective as an acute compensatory response, this strategy could become potentially deleterious under chronic hypercapnic stress (Fernández-Reiriz et al. 2011; W. Jiang et al. 2026; Parker et al. 2013).

4.2.2 Feeding rate

Similarly to ammonia excretion rate, feeding rate did not differ significantly between pH treatments. This experimental evidence aligns with the hypothesis advanced in the previous section, namely that the immediate physiological response of *P. articulatus* fits within a framework of active cellular ion compensation rather than metabolic depression. Indeed, the latter is typically characterized by a sharp decline in food intake rather than sustained feeding activity (Parker et al. 2013; Fernández-Reiriz et al. 2011; Noisette et al. 2016).

However, in a scenario of heightened metabolic costs associated with active ionic regulation, the absence of increased energy ingestion could lead to a chronic energy deficit and eventual metabolic collapse over extended timeframes.

Indeed, if food intake remains constant, even optimal ATP allocation plasticity cannot prevent resource depletion. Once the energetic costs of cellular acid-base regulation are met, insufficient energy remains for somatic growth and reproduction (Leung et al. 2020; Parker et al. 2013; W. Jiang et al. 2026). Consequently, to complement the previous discussion, long-term survival under future OA requires more than metabolic flexibility and allocation plasticity: it also depends on high food availability and the capacity to increase feeding rates (Signorini et al. 2025; Parker et al. 2013; W. Jiang et al. 2026; Noisette et al. 2016).

4.3 Conclusion

This experimental study provided a characterization of the physiological response of the intertidal gastropod *P. articulatus* following a two-month exposure to pH 7.7. This was achieved through the simultaneous evaluation of three key physiological endpoints: respiration rate, ammonia excretion rate, and feeding rate. The empirical

results indicate that hypercapnic stress significantly altered the physiological status of the species, driving a marked increase in respiration rate while feeding and ammonia excretion rates remained virtually constant between control and acidified treatments. These findings suggest the adoption of an energetically demanding, active cellular acid-base compensation mechanism. Despite this, the lack of significant variation in ammonia excretion suggests that the species does not substantially rely on protein catabolism, consistent with the hypothesis that internal pH regulation can be sustained without increased food intake.

However, while these empirical findings provide a clear picture of the physiological strategy deployed by *P. articulatus* in the short term, they reveal little about how the organism will respond over extended temporal scales under chronic OA.

To definitively resolve its long-term survival strategies and evaluate the species' true persistence, future studies should be conducted over broader temporal scales and account for the combined effects of OA with other environmental variables, such as rising temperatures. Additionally, incorporating environmental cues, like tidal cycles, into the experimental design could help bridge the gap between laboratory conditions and natural habitats, thereby providing increasingly precise and reliable predictions regarding the organism's true response in a future OA scenario.

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