



Biological modulation of shell $\delta^{13}\text{C}$ in *Mytilus galloprovincialis* and *Phorcus* sp.: Divergent responses between CO_2 vents and laboratory mesocosms

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Abstract. Stable isotopes are fundamental proxies for deciphering past environmental conditions and carbon cycle perturbations. However, applying these geochemical tools with a forward-looking perspective can also help predict the resilience of marine biota in future acidified oceans. This study investigates carbon uptake and biomineralization pathways under reduced pH in two ecologically keystone calcifying species: the bivalve *Mytilus galloprovincialis* and the gastropod *Phorcus* sp. To unravel these mechanisms, we adopted a dual approach, comparing isotopic trajectories ($\delta^{13}\text{C}$) under strictly controlled laboratory mesocosms with observations from natural CO_2 vent systems in Ischia, Italy. Our results reveal a striking discrepancy between settings. Under laboratory conditions, both species exhibited a significant depletion in shell $\delta^{13}\text{C}$ as pH decreased, directly tracking seawater carbonate chemistry. Conversely, specimens from the volcanic vents displayed a paradoxical $\delta^{13}\text{C}$ enrichment at low-pH sites (pH ~ 7.4). These divergent trajectories indicate that in complex natural environments, the geochemical signal of ocean acidification can be overridden by metabolic overprints and trophically driven vital processes. Consequently, while laboratory experiments are essential to isolate kinetic fractionation, natural analogues remain crucial to capture the biological resilience and complex ecological feedback of future oceans. Future research combining these geochemical trends with direct, in vivo physiological assessments will be essential to precisely constrain the underlying metabolic kinetics and refine predictive metabolic models.

1 Introduction

Since the mid-20th century, the "Great Acceleration" has driven an unprecedented shift in the Earth System, characterized by the rapid intensification of anthropogenic pressures that have pushed key environmental indicators beyond Holocene variability. This trajectory has resulted in the transgression of several planetary boundaries, most notably within the climate



change, biosphere integrity, and nitrogen cycle domains (Rockström et al., 2009; Steffen et al., 2015; Steffen, 2005). Within this global context, marine ecosystems have undergone significant transformations as a consequence of climate change (Doney et al., 2012; Hoegh-Guldberg and Bruno, 2010; Wernberg et al., 2025). Among multiple, interacting stressors, ocean acidification (OA) has emerged as one of the most pervasive, silent and often overlooked threats. Although the large-scale ecological implications of OA are still not fully understood, evidence shows that calcifying marine invertebrates, such as corals, molluscs and echinoderms, are among the most vulnerable taxa to alterations in seawater carbonate chemistry (Clements and Darrow, 2018; Fitzer et al., 2016; Hill and Hoogenboom, 2022; Hurd et al., 2020; Thomsen et al., 2015).

For instance, the oceanic uptake of anthropogenic CO₂ has partially mitigated atmospheric carbon accumulation and the associated rate of climate warming (Gattuso et al., 2015; Nunes, 2023). However, this process has also resulted in a progressive decline in surface seawater pH and carbonate ion (CO₃²⁻) availability, thereby reducing aragonite and calcite saturation states (Ω). These chemical shifts can impair the capacity of marine organisms to precipitate calcium carbonate. While most calcifiers do not directly use seawater CO₃²⁻ for shell or skeleton formation, relying instead on the metabolic processing of CO₂ or bicarbonate, their mineralizing efficiency remains strictly linked to external chemistry. A mechanistic understanding of calcification pathways in key taxa is therefore essential to clarify how different components of the seawater inorganic carbon system influence physiological processes and species-specific sensitivity to ocean acidification (Hurd et al., 2020; Liu et al., 2018).

Among the carbonatic structures produced by marine taxa, mollusk shells have attracted considerable scientific interest across multiple disciplines, serving both as archives for reconstructing past environmental conditions in paleoclimate studies (Álvarez-Fernández et al., 2022; Yan et al., 2014) and as valuable tools for predicting biological responses under future climate change scenarios (Coleman et al., 2014; Nishida et al., 2018). Owing to their accretionary growth and sensitivity to environmental parameters, molluscan shells represent effective model systems for investigating the effects of changing seawater chemistry on biomineralization processes (Checa et al., 2007; Fitzer et al., 2014; Langer et al., 2018).

One of the traditional approaches used to decode past environmental conditions and organismal responses is the analysis of light stable isotopes, particularly oxygen and carbon (Carabel et al., 2009; West et al., 2006). Stable oxygen isotopic (δ¹⁸O) signatures recorded in mussel shells have been shown to reliably record environmental parameters such as temperature and salinity (Dämmer et al., 2020; H. et al., 2001; Milano et al., 2020). In contrast, the interpretation of stable carbon isotopic composition (δ¹³C) in shell carbonate remains more complex and debated, as it reflects the combined influence of ambient Dissolved Inorganic Carbon (DIC), metabolic carbon incorporation, physiological processes associated with shell formation, and broader biogeochemical carbon cycling among environmental compartments (Gillikin et al., 2006, 2007; McConnaughey and Gillikin, 2008).

In this study, we focused on calcifying taxa belonging to two different molluscan classes, Gastropoda and Bivalvia, with distinct ecological roles and life-history strategies. This comparative approach allows the assessment of species-specific and functional responses to environmental stressors, across divergent calcification modes and lifestyles.



65 Species of the genus *Phorcus* Risso, 1826 are herbivorous marine gastropods (Gastropoda: Vetigastropoda) belonging to the
family Trochidae Rafinesque, 1815, and are widely distributed on rocky shores throughout the Mediterranean Sea. As benthic
grazers, these molluscs play a key role in structuring rocky shore communities through their control of algal biomass. Unlike
limpets and mussels, topshells are mobile and actively forage during low tide, exhibiting rapid behavioral responses to
70 changing environmental conditions. Their vertical distribution along the shore is primarily influenced by tolerance to
temperature fluctuations and desiccation stress (Sousa et al., 2018). In contrast, *Mytilus galloprovincialis* Lamarck, 1819
(Mollusca: Bivalvia: Mytilidae) is a sessile, gregarious marine bivalve widely distributed along temperate rocky shores, which
strongly influences benthic community structure. As a foundation species, *Mytilus* spp. play a key ecological role by modifying
habitat complexity, facilitating species interactions, and contributing to energy and matter fluxes in coastal ecosystems (Seed
and Suchanek 1992). Owing to its broad environmental tolerance and ecological importance, *M. galloprovincialis* has been
75 extensively used as a model organism in ecological, physiological, and environmental change studies (Anestis et al., 2010;
Coppola et al., 2017; Lassoued et al., 2021; Romero-Freire et al., 2020).

In this context, the present study aimed to investigate the effects of OA on *M. galloprovincialis* and *Phorcus* sp. under both
laboratory and field conditions, such as shallow-water CO₂ vent systems, by applying a stable isotope labelling approach to
trace carbon partitioning during exposure period.

80 Comparative studies that integrate laboratory experiments with observations from naturally acidified environments, such as
CO₂ vent systems, represent a powerful approach for advancing our understanding of ocean acidification effects. Laboratory
experiments allow the control of individual variables and facilitate the identification of mechanistic responses to reduced pH.
On the other hand, natural CO₂ vents allow a natural exposure of organisms to acidified conditions within ecologically complex
settings, where multiple biotic and abiotic factors interact. Combining these approaches improves ecological realism, enables
85 the validation of experimental results under natural conditions, and helps identify potential biases associated with
experimentally manipulated systems. As a result, integrated field–laboratory frameworks provide a more robust assessment of
organismal responses and adaptive capacities under future ocean acidification scenarios.

2 Material and Methods

2.1 Natural acidified condition: CO₂ vents system

90 2.1.1 Study Sites and Sample collection

The field study was conducted along the northeastern coast of Ischia Island (Tyrrhenian Sea, Italy), focusing on the shallow-
water CO₂ vent systems of Castello Aragonese (40°43'55.33"N 13°57'50.68"E; 40°43'55.92"N 13°57'51.99"E; 40°43'56.64"N
13°57'54.52"E) and Vullatura (40°44'00.68"N 13°57'36.67"E; 40°44'08.81"N 13°57'29.25"E; 40°44'09.88"N 13°57'28.20"E;



Fig. 1). These sites are characterized by persistent geogenic CO₂ emissions that create well-defined natural pH gradients along the rocky shores.

The present area has been deeply characterized, from a biogeochemical perspective (Cigliano et al., 2010; Gambi et al., 2026; Inguaggiato and Pecoraino, 2000; Kumar et al., 2020; Signorini et al., 2025; Teixidó et al., 2018) and following established classification for these systems (e.g., Foo et al. 2018), three distinct pH zones were identified:

- Ambient pH (~8.1): representing current physico-chemical state of the local coastal waters;
- Medium pH (~7.7): consistent with global projections for the end of the 21st century;
- Low pH (< 7.4): representing localized hyper-acidified conditions.

The gas emissions are primarily composed of CO₂ (90-95%), with minor contributions of N₂ (3-6%) and trace amounts of other gases, which makes these vents ideal for isolating the effects of ocean acidification (Foo and Byrne, 2021). In these systems, the pH reduction is driven by an increase in DIC, while Total Alkalinity (TA), temperature, and salinity remain relatively constant across the gradient. This results in a significant increase in the DIC/TA ratio, leading to a progressive decline in the saturation states of both aragonite (Ω_{ara}) and calcite (Ω_{cal}).

To ensure a robust control, the San Pietro promontory (40°44'47.6" N, 13°56'40.42" E), located approximately 4 km from the Castello Aragonese, was selected as an additional reference site (pH = 8.1). This site shares comparable biogeochemical parameters with the Castello's ambient zones but is unaffected by volcanic inputs.

To address the study's objectives within these natural laboratories, two distinct experimental strategies were employed, accounting for different exposure timescales and species-specific life histories.

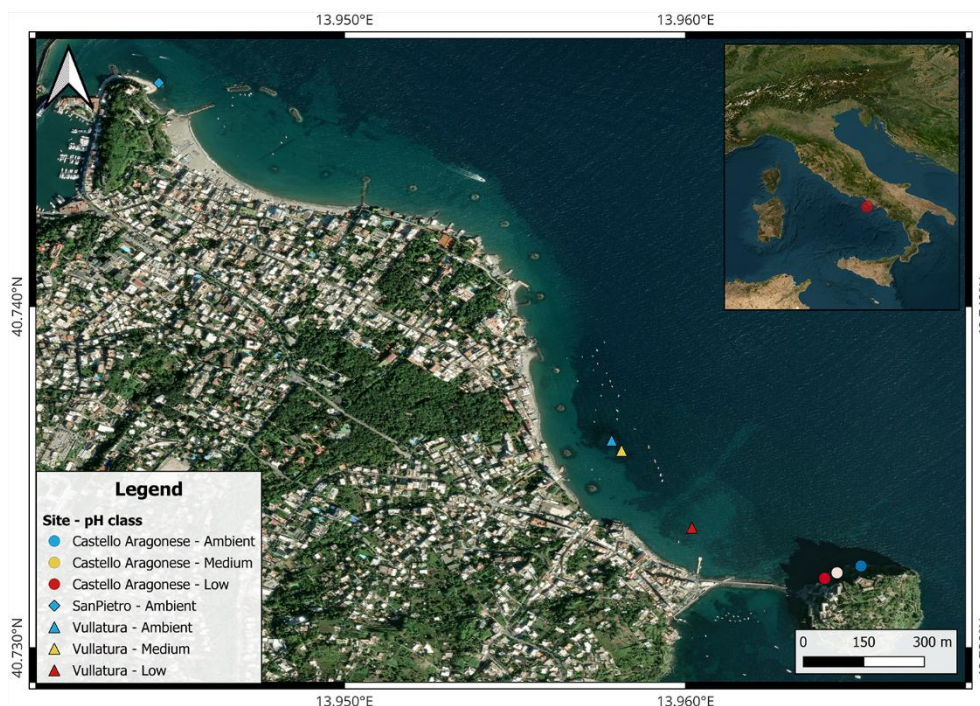


Figure 1. Map of the study area at Castello Aragonese and Vullatura (Ischia, Italy). Colored dots represent sampling stations, with colors indicating the mean pH gradient (red: low pH; yellow: medium pH; blue: control). The inset shows the location of the study site within the Gulf of Naples. Basemap source: Esri World Imagery | Powered by Esri.

2.1.2 Mussel Watch field experiment

- During the autumn of 2024 (from the end of August to the end of October), an *in situ* transplant experiment was performed to assess the short-to-medium-term effects of OA on the carbon isotopic signatures of newly deposited shell carbonate. Individuals of *M. galloprovincialis* were translocated and deployed in small cages (26x18x12 cm) at ~5 m depth at both the Castello Aragonese and Vullatura sites. The 60-day duration was selected to allow for sufficient new shell growth under the local pH conditions. The pH levels were continuously recorded at the deployment stations using pH-data loggers.
- At the end of the two-month exposure, the molluscs were anesthetized by keeping them on ice for a few minutes and subsequently the shell was separated from the soft tissues. Shell samples were transported to the Department of Geosciences at the University of Padova for subsequent isotopic analyses.



2.1.3 *Phorcus* sp. populations

130 To assess the impact of OA on the isotopic carbon uptake across the entire life cycle, we analyzed native populations of the
gastropod *Phorcus* sp. Unlike the translocated mussels, these individuals were collected from populations naturally established
within the vent systems, representing a chronic exposure to acidified conditions from the larval stage through the juvenile,
sub-adult, and adult phases. Adult individuals were collected by snorkeling from the lower intertidal to the upper subtidal
rocky zones along a pH gradient located on the northern coast of Castello Aragonese and at the San Pietro reference site,
135 whereas the Vullatura site was not included due to the absence of shallow-water rocky substrates suitable for the collection of
the species.

To minimize ecological impact on natural populations while ensuring statistical relevance, 3 up to 15 individuals were sampled
per site, depending on local availability. Notably, at the most acidified station (Castello - Low pH), *Phorcus* sp. specimens
were found to be scarce and significantly reduced in size; consequently, they were excluded from the sampling.

140 The shell samples were frozen and transported to the Department of Geosciences at the University of Padova for subsequent
isotopic analyses.

2.2 Acidification laboratories conditions

2.2.1 Experimental set up

145 Two experiments were conducted in two different years, one for each species.

In the first experiment, which began in August 2024 and lasted until October 2024, only juvenile mussels were used (mean
length ~1.50 cm).

Specimens of *M. galloprovincialis* were collected along the west coast of the northern Adriatic Sea (near Chioggia, Italy) and
immediately transferred and maintained in large aquaria with aerated natural seawater at ambient salinity (33.7 PSU),
150 temperature (28.5°C) and pH (8.2 units). After a two-week acclimation period, twenty juvenile mussels were randomly
transferred into each replicate plastic tank within an experimental flow-through system. The system consisted of a main
reservoir filled with natural seawater (approximately 1000 l), allowing suspended matter to settle and the seawater to
equilibrate to the controlled laboratory temperature. Seawater was then pumped (flow rate of 30 ml min⁻¹) into six experimental
tanks (approximately 50 l each) containing the juvenile mussels. In three of the six tanks (Treatment 1; Treatment 2; Treatment
155 3), pH was reduced to experimental values by bubbling CO₂ using an automatic control system (Neptune System, Apex A3
System) connected to pH electrodes. The remaining three tanks were maintained under ambient conditions and served as
controls. The experimental pH in the treatment tanks was set to simulate the level of ocean acidification projected for the end
of the century under the IPCC RCP8.5 scenario (pH 7.7 ± 0.05). Mussels fed on phytoplankton and organic matter naturally
suspended in the continuously flowing seawater and were additionally supplied once every two days with 2ml of a mix of



160 microalgae suspension (*Isochrysis galbana* ~33.3%, *Nannochloropsis* ~31%, *Tetraselmis* ~18%, *Phaeodactylum* ~18%),
thereby increasing algal concentration in each experimental tank. Shell samples were collected, as described above, after 3
months of exposure and stored at 4 °C.

Similar to the first one, the second experiment was conducted during the autumn of 2025 and involved adult specimens of
Phorcus sp., collected at the same site. After a two-week acclimation period under ambient conditions, ten individuals were
165 transferred into each replicate plastic tank, filled with ~20 L of natural seawater. The experimental flow-through system and
pH manipulation were identical to those used in the mussels experiment. Gastropods were fed the epiphytes algae growing on
rocks collected from the sampling site, replenished weekly. This experimental setup was maintained for eight weeks, which
allowed the gastropods to have sufficient measurable shell growth.

Shell samples from both species were then delivered to the Department of Geosciences of University of Padova for further
170 analysis.

2.2.2 Stable isotope analyses

Stable carbon and oxygen isotope analyses were performed on the shell aperture of *Phorcus* sp. and on the growing shell
margin of *M. galloprovincialis*. For *Phorcus* sp., additional analyses were carried out at the apex, on the mid-spire, and at the
175 shell aperture to investigate ontogenetic variations in the isotopic signal. All samples consisted of multiple shell specimens.
Stable isotope analyses were performed on a variable number of individuals from each sample (typically 10-15 specimens,
depending on specimen availability). This approach accounts for the inter-individual isotopic variability commonly observed
among shells and enables robust statistical evaluation of the isotopic data. Stable isotope measurements were carried out at the
Department of Geosciences, University of Padova, using a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo
180 Fisher Scientific) coupled to an automated GasBench II continuous-flow preparation device. Approximately 200–300 µg of
powdered shell carbonate was weighed into a headspace vial and flushed with helium. Subsequently, 100% phosphoric acid
was added to each vial and allowed to react, releasing CO₂ for isotopic analysis. Instrument calibration and quality control
were performed using three in-house carbonate standards: Carrara marble MAQ1 ($\delta^{13}\text{C} = +2.58\text{‰}$; $\delta^{18}\text{O} = -1.15\text{‰}$ VPDB),
SUPP ($\delta^{13}\text{C} = -49.69\text{‰}$; $\delta^{18}\text{O} = -16.38\text{‰}$ VPDB), and Monzoni marble GR1 ($\delta^{13}\text{C} = +0.68\text{‰}$; $\delta^{18}\text{O} = -10.44\text{‰}$ VPDB).
185 Repeated analyses of the standards throughout the analytical sessions yielded analytical precisions better than $\pm 0.06\text{‰}$ for $\delta^{13}\text{C}$
and $\pm 0.09\text{‰}$ for $\delta^{18}\text{O}$. All isotope values are reported in standard delta (δ) notation relative to the Vienna Pee Dee Belemnite
(VPDB) international reference standard.



2.3 Statistical Analysis

190 All statistical analyses were performed using R (R Core Team, 2025). Prior to hypothesis testing, all data were screened for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test, using the functions from the “car” package. For all tests, the significance level was set at $\alpha = 0.05$.

In order to test any differences among two difference acidified condition (controlled laboratory setting and natural acidified conditions at the CO₂ vents) on two calcifying organisms, the bivalve *M. galloprovincialis* and the gastropod *Phorcus* sp., two
195 distinct analytical approaches were followed:

- Field Experiment: for the bivalve *M. galloprovincialis*, to compare differences in shell $\delta^{13}\text{C}$ a Generalized Linear Models (GLMs) was used, via the “glm()” function, with Site (Vullatura and Castello Aragonese) and pH Treatment (Ambient, Medium and Low pH conditions) as fixed factors.

For the gastropods *Phorcus* sp. collected at Castello Aragonese and at the reference site San Pietro, a Two-way Analysis of
200 Variance (ANOVA) was conducted under a crossed design to evaluate the effects of Treatment (Ambient vs. Medium pH) and Growth Stage (ontogenetic stages: Juvenile/Protoconch, Sub-adult, and Adult/Aperture) on shell $\delta^{13}\text{C}$. The interaction term (Treatment x Growth Stage) was included to determine if the ontogenetic isotopic trajectory differed between pH regimes.

- Laboratory Experiment: for the bivalve, differences in $\delta^{13}\text{C}$ between control and acidified treatments were assessed using a One-way ANOVA. While significant deviations from homoscedasticity were observed in the *Phorcus* sp.
205 laboratory dataset; consequently, a Welch's t-test was employed to compare $\delta^{13}\text{C}$ signatures between control and acidified treatments, as this method is robust against unequal variances.

Post-hoc comparisons were performed using the “emmeans()” function from the emmeans package, applying the Tukey's Honest Significant Difference (HSD) test to identify differences between growth stages and treatment-stage combinations. Results are reported as mean values $\pm$ standard error (SE) unless otherwise specified.

210

3 Results

3.1 Mussel Watch field experiment

The data regarding the mussels exposed at the CO₂ vents system were verified using a Shapiro-Wilk test ($W = 0.978$, $p = 0.061$), satisfying the normality assumption. The carbon stable isotope ($\delta^{13}\text{C}$) of *M. galloprovincialis* shells exhibited site-specific responses across the pH gradient. Under “Ambient” pH conditions, the $\delta^{13}\text{C}$ of Vullatura shells were significantly
215 lower ($p = 0.036$) than the Castello ones.



Focusing on the two sites independently, the GLM for the Castello showed how $\delta^{13}\text{C}$ remained stable across all pH treatments ($p > 0.05$). While, at Vullatura site, the model and then a post-hoc comparisons revealed a significant enrichment in shell $\delta^{13}\text{C}$ under “Low” pH conditions, which differed significantly from the “Ambient” level (post-hoc, $p < 0.05$, Table 1, Fig. 2).

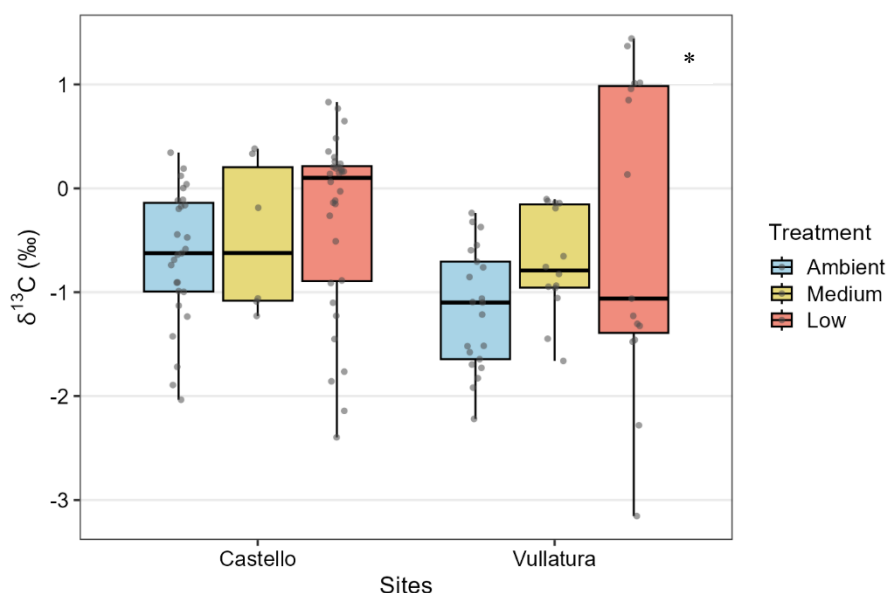


Figure 2. Boxplots of $\delta^{13}\text{C}$ signatures (expressed as $\delta^{13}\text{C}$ ‰) in *M. galloprovincialis* shells exposed to different pH levels: Ambient pH (~ 8.1 ; light blue), Medium pH (~ 7.7 ; yellow), and Low pH (< 7.4 ; red). The asterisk indicates statistically significant differences among the different sites ($p\text{-Value} \leq 0.05$).

Following the 90 days laboratory experiment, *M. galloprovincialis* shells exposed to reduced pH (7.7) exhibited significantly different $\delta^{13}\text{C}$ values compared to the control group ($F_{1,69} = 34.79$, $p < 0.001$; Table 1). Specifically, the mean isotopic signature shifted from $-2.51 \pm 0.15\text{‰}$ in the control tanks to $-4.11 \pm 0.14\text{‰}$ in the acidified treatments (Table 1, Fig. 3).

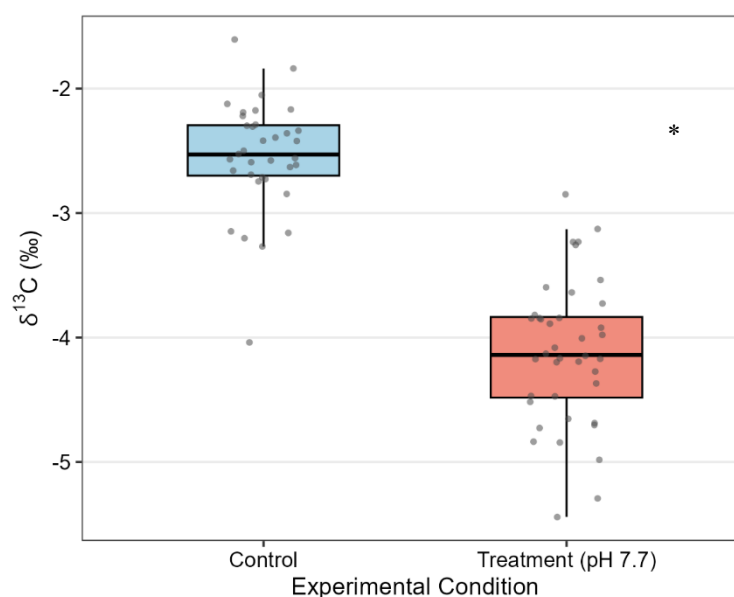


Figure 3. Boxplots of $\delta^{13}\text{C}$ signatures (expressed as $\delta^{13}\text{C}$ ‰) in *M. galloprovincialis* shells exposed to control and acidified laboratory conditions. The asterisk indicates statistically significant differences among the two conditions (p -Value ≤ 0.05).

Table 1. Statistical summary for *M. galloprovincialis* shell $\delta^{13}\text{C}$ analysis. The table reports the comparison between pH conditions from both the natural vent systems (spatial gradient: Castello and Vullatura sites across Ambient, Medium, and Low pH) and the controlled laboratory acidification experiments. Significance levels are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistically significant effects and contrasts ($p < 0.05$) are highlighted in bold.

Analysis	Factor	Estimate	Std. Error	t-value / W	p-value
<i>Mussel Watch – Field Experiment</i>					
Shapiro–Wilk test	Residuals normality	–	–	0.978	0.061
Global GLM model					
Dependent variable:					
$\delta^{13}\text{C}$	Site (Vullatura)	–0.520	0.245	–2.121	0.036*
Family: Gaussian					
	Castello				
Post-hoc pairwise	Ambient vs. medium	–0.173	0.380	–0.455	0.892
comparisons within site	Ambient vs. low	–0.347	0.220	–1.577	0.260
(Tukey-adjusted)	Medium vs. low	–0.174	0.375	–0.464	0.888
	Vullatura				



Analysis	Factor	Estimate	Std. Error	t-value / W	p-value
Between-sites comparisons (Tukey-adjusted)	Ambient vs. medium	−0.457	0.291	−1.573	0.262
	Ambient vs. low	−0.733	0.285	−2.576	0.030*
	Medium vs. low	−0.276	0.313	−0.883	0.652
	Castello vs. Vullatura				
	Ambient pH	0.520	0.245	2.121	0.036*
	Medium pH	0.236	0.411	0.573	0.568
	Low pH	0.133	0.264	0.506	0.614
	Laboratory experiment				
	Shapiro–Wilk test	Residuals normality	–	–	0.97624
Levene's test	Homogeneity of variance	df = 1, 73	–	F = 2.7943	0.09888
One-way ANOVA	Group	df = 1, 73	–	F = 169.7	<2e-16**

240 3.2 *Phorcus* sp. populations

Regarding the gastropods *Phorcus* sp. collected at the vent sites, prior to parametric statistical testing, the assumption of normality was successfully validated for all experimental combinations. Shapiro-Wilk normality tests conducted on each subgroup defined by site condition and ontogenetic stage yielded non-significant results ($p > 0.05$), with p -values ranging from 0.138 to 0.928 (Table 2), confirming that the dataset was suitable for analysis of variance.

245 The two-way ANOVA revealed no statistically significant differences in $\delta^{13}\text{C}$ signatures between the external reference control site (San Pietro) against the local control station (Castello Ambient) ($F_{1,12} = 0.45$, $p = 0.514$). Conversely, the pH treatment had a highly significant effect on the isotopic composition ($F_{1,12} = 12.81$, $p = 0.0038$), indicating a clear response of the shell carbonate to the acidified conditions at the vent sites (Fig. 4, Table 2).

The two-way ANOVA on *Phorcus* sp. shells revealed that both growth stage and pH treatment significantly influenced $\delta^{13}\text{C}$ signatures. The growth stage was the primary driver of isotopic variability ($F_{2,39} = 29.67$, $p < 0.001$), showing a consistent ontogenetic trend (Fig. 5). The treatment effect was also highly significant ($F_{1,39} = 13.16$, $p < 0.001$), indicating that environmental pH shifts the isotopic baseline. Notably, the interaction between treatment and growth stage was not significant ($p = 0.279$), suggesting that the ontogenetic carbon incorporation pattern remains stable across different pH environments.

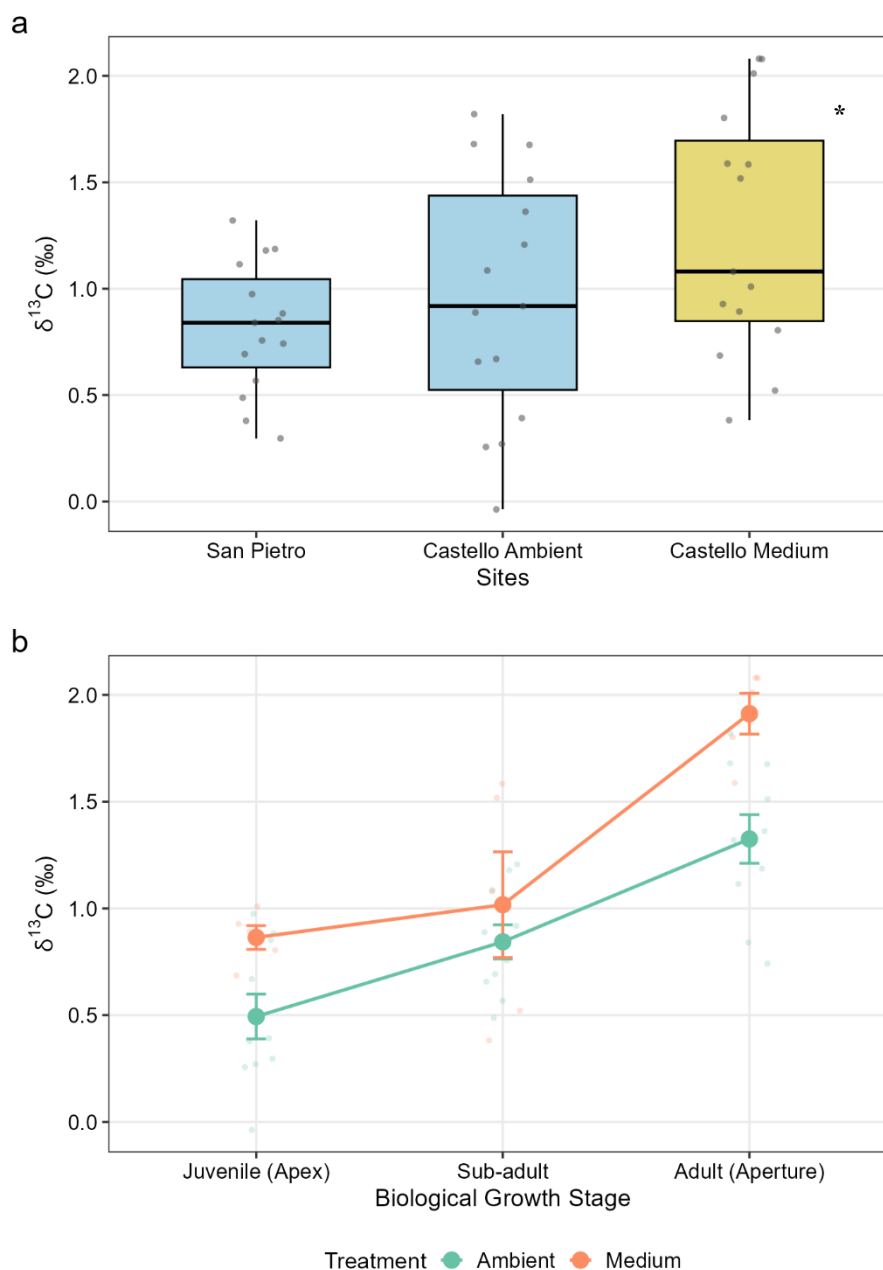
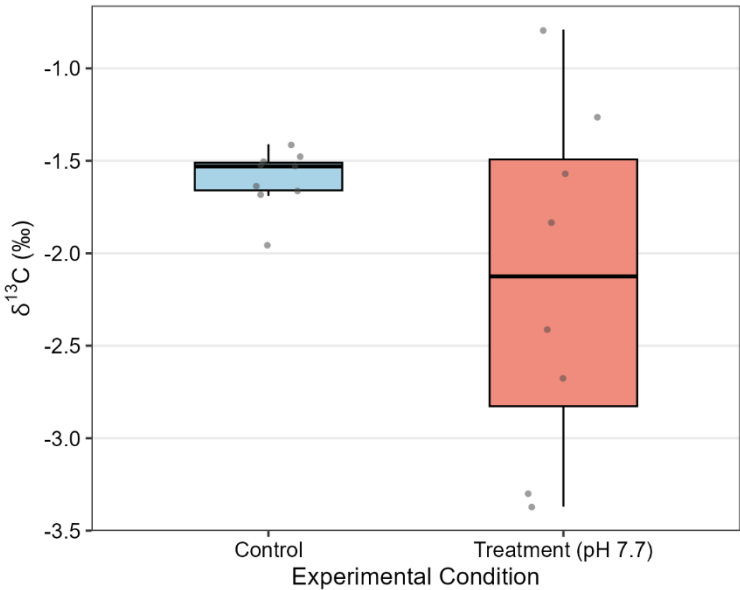


Figure 4. Boxplot of stable carbon isotope composition $\delta^{13}\text{C}$ in the shell carbonate of the gastropod *Phorcus* sp. across the natural vent gradient. The boxplot compares the reference control site (San Pietro, Reference Ambient pH; light blue) with the local control (Castello, Local Ambient pH; light blue) and the acidified station (Castello, Medium pH; yellow). The asterisk indicates statistically significant differences among the different sites (p -Value ≤ 0.05) a). Ontogenetic $\delta^{13}\text{C}$ trajectories in



Phorcus sp. The plot illustrates the variation in $\delta^{13}\text{C}$ values across three biological growth stages: Juvenile (Apex), Sub-adult, and Adult (Aperture). Central points represent the mean values for 'Ambient' (green) and 'Medium' (orange) treatments b).

265 In the laboratory experiment, *Phorcus* sp. specimens exposed to acidified conditions showed a more depleted mean $\delta^{13}\text{C}$ signature (-2.15‰) compared to controls (-1.60‰). However, this shift was not statistically significant (Welch's t-test: $t = -1.63$, $df = 7.35$, $p = 0.144$), primarily due to the high inter-individual variability and heteroscedasticity observed in the treated group (Levene's test: $p < 0.001$).



270 **Figure 5.** Boxplots of $\delta^{13}\text{C}$ signatures (expressed as $\delta^{13}\text{C} \text{‰}$) in gastropod *Phorcus* sp. shells exposed to control and acidified laboratory conditions. The asterisk indicates statistically significant differences among the two conditions ($p\text{-Value} \leq 0.05$).

275 **Table 2.** Statistical results for $\delta^{13}\text{C}$ isotopic signatures in *Phorcus* sp. shells. The table reports the comparison between pH treatments in controlled laboratory conditions and the multi-factor analysis of natural vent populations, including ontogenetic growth stages. Significance levels are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistically significant effects and contrasts ($p < 0.05$) are highlighted in bold.

Analysis	Factor	df	F / t	p-value	Significance
<i>Laboratory experiment</i>					



Analysis	Factor	df	F / t	p-value	Significance
Welch's t-test	Treatment (Control vs. Acidified)	7.35	t = -1.63	0.144	ns
Levene's test	Variance homogeneity	1, 15	F = 20.86	<0.001	***
Field (vents) – ontogenetic					
Two-way ANOVA	Treatment (pH level)	1, 39	F = 13.16	<0.001	***
	Growth stage (age)	2, 39	F = 29.67	<0.001	***
	Interaction (treatment × stage)	2, 39	F = 1.32	0.279	ns
Post-hoc (Tukey HSD)	Comparison		diff	adj. p-value	
	Intermediate vs. Juvenile (Old)		-0.62	<0.001	***
	Adult (Young) vs. Juvenile (Old)		-0.90	<0.001	***
	Medium:Old vs. Ambient:Old		0.59	0.026	*

280 4 Discussion

4.1 Natural acidified condition: CO₂ vents system responses

Characterizing isotopic fractionation and the preferential incorporation of light (¹²C) versus heavy (¹³C) carbon isotopes into the biogenic carbonate (CaCO₃) structures is crucial for establishing marine shells as high-resolution geochemical proxies. To achieve this issue, our dual experimental approach reveals a complex interplay between carbon system dynamics and biological modulation under reduced pH conditions and a gap of knowledge of carbon uptake mechanisms under reduced pH.

285 In the natural CO₂ vents systems, the δ¹³C signatures of *M. galloprovincialis* shells exhibited a significant and unexpected enrichment trend at “Low” pH site. Specifically, this increasing trend in δ¹³C was statistically significant at the Vullatura site, whereas at the Castello station, the enrichment was marginally non-significant but maintained a clear upward trajectory similar



to that observed at Vullatura. This result stands in stark contrast to the expected depletion associated with the influx of volcanic-derived Dissolved Inorganic Carbon (DIC), which is typically lightened in ^{13}C compared to standard marine pools, as previously documented across Eolian Islands and Ischia Island shallow vent systems (Vizzini et al. 2010, Ricevuto et al. 2015). A similar outline was observed in the gastropod *Phorcus* sp., supporting the hypothesis that in ecologically complex and extreme environments, biological and metabolic processes can modulate, or even override, the geochemical signal and the local environmental $\delta^{13}\text{C}$ (Manno et al., 2017; Signorini et al., 2024).

This overall isotopic enrichment is likely driven by both seasonal fluctuations and physiological responses to lower pH conditions. While seasonal $\delta^{13}\text{C}_{\text{DIC}}$ instability, ranging from 0.9‰ in winter to 1.4‰ during summer phytoplankton blooms in vent-affected areas (Hahn et al., 2011), modulates the baseline, it cannot fully explain the observed shell enrichment. Instead, we hypothesize that under acidification stress, *M. galloprovincialis* employs homeostatic strategies such as prolonged valve closure. This behaviour isolates the extrapallial fluid (EPF), altering calcification rates and triggering kinetic fractionation (Heinemann et al., 2012; Melzner et al., 2011). Such isolation promotes metabolic carbon incorporation into the shell, where respiratory CO_2 (derived from ^{13}C -enriched local biomass) becomes the dominant carbon source. Another plausible driver for the observed $\delta^{13}\text{C}$ enrichment is the influence of local primary productivity. Volcanic vents could act often as localized fertilizers; the elevated CO_2 availability stimulates intense photosynthesis in both phytoplankton and benthic biofilms (Agostini et al., 2018; Hall-Spencer et al., 2008). Since photoautotrophs preferentially sequester ^{12}C , the remaining DIC pool and the resulting organic biomass become markedly enriched in ^{13}C . As *M. galloprovincialis* (a filter-feeder) and *Phorcus* sp. (a grazer) assimilate this isotopically heavy organic matter, the respiratory CO_2 contributes an enriched signature to the internal carbon pool. This biological mechanism appears to counteract the negative isotopic baseline of volcanic CO_2 , illustrating a "reactive" rather than "passive" calcification strategy in these species. However, it is important to underline that no direct physiological measurements were conducted in this study to independently corroborate this interpretation. Likewise, no along-gradient measurements of $\delta^{13}\text{C}$ for DIC or of particulate organic matter (POM) were collected, precluding a quantitative partitioning of ambient versus metabolic carbon sources to shell carbonate.

Moreover, the ontogenetic trajectory of *Phorcus* sp. further elucidates the shift in carbon uptake mechanisms during life history and offers additional insights into the interplay between environmental signals and biological factors. The transition from the protoconch (juvenile) to the aperture (adult) is characterized by a consistent $\delta^{13}\text{C}$ enrichment. In early life stages (apex), the lower $\delta^{13}\text{C}$ values likely reflect a stronger kinetic (vital) effect associated with the rapid calcification rates typical of juvenile growth, which tends to preferentially incorporate isotopically lighter, metabolically respired carbon. However, we can suppose that, as the individual reaches maturity and shell deposition slows, these vital effects attenuate, allowing the shell carbonate to more closely approach isotopic equilibrium with the ambient seawater DIC pool, a comparatively ^{13}C -enriched carbon source relative to the depleted signal associated with rapid juvenile calcification. This trend suggests a progressive reduction in the vital effects that characterize fast juvenile growth, allowing the equilibrium environmental signal to become more prominent as ontogeny advances.



Consequently, we can infer that biological modulation could effectively override geochemical processes in these high-CO₂ environments. Our findings suggest that the shell isotopic composition is not a passive reflection of the ambient seawater DIC pool.

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4.2 Laboratory acidified condition

Under controlled laboratory conditions, the $\delta^{13}\text{C}$ signatures of *M. galloprovincialis* showed a distinct depletion trend, from control (pH $\sim 8.0 \pm 0.05$) to acidified treatments (pH $\sim 7.5 \pm 0.05$). This observation aligns with previous studies (Lu et al., 2018; Mele et al., 2023), and describes a more direct and clear geochemical response of biogenic CaCO₃ to altered seawater carbonate chemistry. In the mesocosms experiment, the depletion in $\delta^{13}\text{C}$ suggests that the reduction in CO₃²⁻ and the resulting dynamics of the DIC pool act as the primary determinants of the shell's isotopic signature. This reinforces the hypothesis that in the absence of complex ecological variables the shell of *M. galloprovincialis* may function as a relatively passive recorder of the surrounding environmental chemistry, with a minimized metabolic overprint.

Conversely, while *Phorcus* sp. exhibited a comparable depletion trend, even if not statistically significant, its response was characterized by pronounced inter-individual variability, particularly under the low-pH treatments. This heterogeneity points to a high degree of physiological plasticity and distinct calcification strategies among individuals. Unlike the relatively uniform response observed in *M. galloprovincialis*, the isotopic fluctuations in *Phorcus* sp. reflect a biologically-regulated calcification mechanism heavily coupled with the metabolic energy budget (Leung et al., 2020). Under OA-induced stress, individuals may differentially allocate energy between basal maintenance and shell growth. Consequently, stochastic calcification events and variable rates of metabolic carbon incorporation into the extrapallial fluid likely led to a more heterogeneous isotopic record. Such inter-individual divergence highlights that while environmental $p\text{CO}_2$ provides the baseline, the final $\delta^{13}\text{C}$ value in *Phorcus* sp. is heavily modulated by the organism's specific metabolic status and energetic trade-offs.

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5 Conclusion

The striking discrepancy between our laboratory findings and the data obtained from the volcanic vent systems highlights a fundamental ecological divergence in biogenic fractionation. While laboratory specimens faithfully tracked the environmental $\delta^{13}\text{C}_{\text{DIC}}$ depletion, organisms inhabiting the vent systems exhibited a paradoxical ^{13}C enrichment. This contrast underscores the necessity of natural analogues for a holistic understanding of ocean acidification, as they provide a unique window into future coastal scenarios where complex biotic interactions are at play. Our results demonstrate that the negative geochemical signal of volcanic CO₂ may be frequently overridden by trophic-driven vital effects and enhanced local primary productivity (e.g., biofilm blooms). These findings suggest that while laboratory mesocosms remain essential for isolating the direct impact

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of pH on calcification kinetics, they may underestimate the capacity of biological and ecological feedback to decouple biogenic archives from the ambient seawater carbonate system. Ultimately, this study cautions against the oversimplification of geochemical proxies in reconstructed environments, highlighting that in complex natural systems, physiological plasticity and trophic dynamics can fundamentally modulate the isotopic signature of marine calcifiers.

Within this framework, while the current dataset provides a robust baseline for these isotopic shifts, coupling these geochemical trends with direct, *in vivo* physiological assessments offers a powerful framework for future research to precisely constrain the underlying metabolic kinetics. Similarly, high-resolution, along-gradient monitoring of DIC $\delta^{13}\text{C}$ and POM pools will offer an ideal opportunity to quantitatively partition the relative contributions of ambient versus metabolic carbon sources to the shell carbonate matrix.

Code and data availability

All underlying research data (stable isotope datasets) and R scripts will be made publicly available in PANGAEA and Zenodo repositories upon final acceptance of the manuscript. During the peer-review process, the complete dataset and code are available to reviewers through the Copernicus online editorial system via the "access limited to reviewers" option to ensure reproducibility.

Author contributions

AC handled Conceptualization, Investigation, Data Curation, and Writing the original draft. CA contributed to Resources, Formal Analysis, and Writing - review & editing. SR contributed to Writing - review & editing. IdA was involved in Conceptualization, Formal Analysis, and Writing - review & editing. MM participated during the Investigation. MGM, GdA, and VM secured Funding acquisition; VM also managed Project administration and contributed to Writing - review & editing, along with DdA. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

Disclaimer

The authors declare that no disclaimers are applicable to this study.



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