



Physiological and metabolic plasticity in *Patella caerulea* enables survival in the CO₂ vent systems of the Castello Aragonese (Ischia Island)

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ABSTRACT

Ocean acidification (OA) represents a major threat to marine ecosystems, causing detrimental effects mainly on calcifying organisms. However, the limpet *Patella caerulea* is one of the few calcifiers that can inhabit the naturally acidified areas of the Castello Aragonese vent systems (Ischia Island, Italy). Its presence suggests that this species may have developed tolerance or adaptive strategies to cope with OA.

Nevertheless, the specific biological mechanisms remain largely unknown. To address this gap of knowledge, in our study we conducted physiological and metabolomics analyses on resident limpet populations collected along the acidification gradient of the Castello vent systems. Additionally, we investigated the same mechanisms in specimens transplanted for 30 days from ambient pH conditions to the different pH sites of the vent.

Only during summer, OA increased respiration rates in limpets from the most acidified site and, simultaneously, reduced excretion rates and likely protein catabolism, probably to preserve more energy resources while coping with this environmental stress. Furthermore, the individuals up-regulated carnitine metabolism, potentially enhancing energy production through β -oxidation, and several metabolites involved in osmoregulation, oxidative stress, and nucleic acid mechanisms. Similar results were obtained also in limpets transplanted to low pH sites.

Overall, our results suggest that limpets exposed to acidified conditions may have developed tolerance strategies to maintain energetic reserves and allocate them among metabolic processes, which are fundamental in maintaining biological and ecological traits and distribution when facing environmental disturbance such as OA.

1. Introduction

Ocean acidification (OA) emerges as a consequence of global industrialization and climate change in marine environments. OA is generated from the constant increase of carbon dioxide (CO₂) emissions in the atmosphere, primarily from anthropogenic activities. Oceans, acting as a sink, absorbed about 30 % of the CO₂ emitted, resulting in an alteration of the chemical balance and a significant reduction of seawater pH levels. Without the buffering action of oceans, the levels of CO₂ in the atmosphere would exceed by 80 ppm the current value of 426.40 ppm. If current trends continue under the “business as usual” scenario, pH levels are projected to decrease from the current level of 8.1

to 7.7 by the end of the XXI century (Terhaar et al., 2022; Sutton et al., 2022; Jiang et al., 2023; Lan et al., 2025).

The ongoing pH reduction will negatively impact several marine species, compromising different physiological and metabolic functions, as well as life-history traits, with serious consequences for their survival and reproduction. In particular, OA will profoundly affect organisms with calcareous structures (Kroecker et al., 2010; Leung et al., 2022). Indeed, the absorption of CO₂ by oceans alters seawater carbonate chemistry, thereby reducing the availability of calcium carbonate (CaCO₃) to calcifiers. Consequently, more CaCO₃ is needed to buffer the seawater pH reduction imposed by OA, which is therefore unavailable for building, growing, and maintaining their calcareous exoskeleton and

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shells (Figuerola et al., 2021; Ma et al., 2023). The adverse effects of OA on organisms have been widely described, showing thinner and more fragile structures, with weaker mechanical strength and resistance, entailing serious consequences on their growth, fitness, and survival. Although the intensity of response appears highly species-specific, OA can impact negatively on several other processes, including osmoregulation, oxidative status, metabolism, physiological performance, behavior, and up to population dynamics and community structure (Pan et al., 2015; Hall-Spencer and Harvey, 2019; Leung et al., 2022; Simonetti et al., 2022). However, when facing an environmental disturbance, organisms may be able to respond to these alterations through compensatory mechanisms, even though additional energy is required to recover and maintain homeostasis, and this might influence other key biological functions. Within this scenario, the capability to balance energetic demand and energy allocation, and therefore energetic trade-offs, represents a key-driving factor in the ability of organisms to cope with environmental disturbances and in determining their fitness (Sokolova, 2021; Leung et al., 2022).

However, most of the studies examine the effects of OA under laboratory-controlled conditions, without including the interactions with surrounding abiotic and biotic factors and without considering potential acclimation and adaptive strategies that these organisms may develop over time. In this framework, CO₂ vent systems represent a “window into the future”, which provide OA proxies and allow the formulation of more realistic hypotheses regarding the impact that pH decrease will exert on marine ecosystems in the coming decades. Indeed, vents are sites naturally-acidified by the CO₂ emitted from the seabed due to the terrestrial volcanic activities (Cornwall and Hurd, 2016; Aiuppa et al., 2020; Foo and Byrne, 2021; Vargas et al., 2022). Among the several shallow CO₂ vent systems identified in the Mediterranean Sea, the Castello Aragonese of Ischia Island (Italy) stands out as one of the most studied and well-characterized. Along the northern and southern sides of this volcanic islet, a pH gradient has been identified, with three sites characterized by three different pH values (Foo et al., 2018; Cocozza di Montanara et al., 2024; Teixidó et al., 2024).

The mollusk *Patella caerulea* (Linnaeus, 1758), a gastropod endemic of the Mediterranean Sea, is a keystone species able to modify the stability of the ecosystems and the organization and structure of marine benthic communities (Burgos-Rubio et al., 2015; Ferranti et al., 2018). It represents one of the few calcifying organisms living along the acidification gradient of the Castello vent, even in the sites with lower pH (Hall-Spencer et al., 2008; Rodolfo-Metalpa et al., 2011; Foo et al., 2018; Aliende et al., 2023). A previous study on this and other congeneric species (*P. rustica* and *P. ulysiponensis*) mainly focused on the impact of OA on morphological and biochemical parameters referring to energetic reserves, oxidative stress, biomineralization, and neurotoxicity (Signorini et al., 2025), revealing that organisms living under OA conditions didn't trigger any of the studied endpoints, while short-term exposure induced compensatory mechanisms to potentially counteract the adverse effects induced by low pH. However, the most relevant result regarded energy related parameters (glycogen and protein contents) and body dimensions, which were higher under acidified conditions, potentially due to a modification in biofilm coverage, which may have benefited from the natural CO₂ enrichment of the vent systems, providing more energy storage to allow survival and tolerance to OA conditions. In view of these results, it appears fundamental to deeper investigate the bioenergetic aspects underpinning the maintenance of homeostasis, which will provide insights about animal performance, plasticity, and evolutionary adaptation to global changes (Pan et al., 2015; Sokolova, 2021).

To fill this knowledge gap, we investigated the physiological performance (respiration and ammonia excretion rates, and O:N ratios), the metabolome (using the high-throughput techniques of untargeted metabolomics), and the ATP synthesis capacity (Electron Transport System assay) of resident limpet populations of *P. caerulea* that were sampled in the naturally acidified sites of the Castello Aragonese vent

systems. We selected these biological endpoints because they represent valuable proxies of the organisms' metabolic state and bioenergetic trends, allowing us to further explore the onset of stress tolerance and/or adaptation mechanisms to this environmental disturbance. The non-invasive physiological endpoints were measured over one year to assess their variation under different environmental conditions. Additionally, we evaluated the short-term responses induced by OA through an *in situ* transplant experiment.

2. Materials and methods

2.1. Sampling of organisms and *in situ* transplant experiment

Specimens of *P. caerulea* were collected from the infralittoral to the midlittoral areas of rocky shores (Sedano et al., 2020; Vafidis et al., 2020), along the northern side of the Castello Aragonese CO₂ vent systems of Ischia Island (40°73'25.81"N, 13°96'51.03"E) and from San Pietro bay (40°44'47.6"N, 13°56'40.42"E), already employed as an external ambient pH site (pH = 8.1) in several previous OA studies (Wäge et al., 2017; Aliende et al., 2023; Signorini et al., 2023). The northern pH gradient of the Castello vent mainly consists of three pH sampling sites, which have been thoroughly characterized in previous studies: N1 with an ambient pH value of 8.1, N2 characterized by an intermediate pH level of 7.7, representative of pH conditions that could be reached by the end of the century (2100), and N3 with an extremely low pH level lower than 7.4 (Kroecker et al., 2011; Teixidó et al., 2018, 2024; Signorini et al., 2025). All the sites considered in this study display similar temperature, salinity, and hydrodynamic conditions (Foo et al., 2018; Gaglioti et al., 2019; Migliaccio et al., 2019).

Four samplings were carried out: 08/30/2023 = summer (25.6 °C), 11/08/2023 = autumn (19.8 °C), 01/31/2024 = winter (13.8 °C), 04/09/2024 = spring (19.4 °C), with the superficial seawater temperature that varied from 14.9 to 27.7 °C across the year (Fig. S1). Physiological endpoints were measured immediately after collection. At the end of each analysis, resident limpets were brought back to their original site, in order not to negatively impact the natural populations. Only during the sampling carried out in winter, organisms were dissected and stored at −80 °C for subsequent analyses (Table S1).

Furthermore, an *in situ* transplant experiment was performed to evaluate the short-term responses of *P. caerulea* to OA. Seven months prior to the beginning of the experiment, 3 natural volcanic rock tiles per site were attached to the rocky shore to let a biofilm grow. In August 2024, specimens with heterogeneous dimensions were collected along the bridge that joins the Castello Aragonese to the mainland (40°43'55.4"N 13°57'38.7"E, pH = 8.1) and transplanted for 30 days along the pH gradient of the vent (N1 – N2 – N3). Each tile hosted up to 4 individuals (N = 12 × site). Additionally, a metal cage was tightly fixed above each tile to avoid external predation and/or competition. After 30 days, limpets were carefully removed from the tiles to measure physiological parameters. Afterwards, organisms were dissected and stored at −80 °C for subsequent analyses (Table S1). The detailed methods used in the experiment follow our previous work (Signorini et al., 2025).

During the experiment, pH, temperature, and redox potential were measured using loggers (HOBO® pH and Temperature Data Logger) positioned in N1, N2, and N3 (Table S2).

2.2. Physiological measurements

Respiration rate (RR), ammonia excretion rate (ER), and O:N ratio were analyzed as physiological endpoints in resident limpet populations of *P. caerulea*, performing multiple measurements across different seasons of the year and at the end of the 30-day transplant experiment (09/30/2024 = early autumn, 23 °C).

2.2.1. Respiration rate (RR)

Specimens of *P. caerulea* sampled from the three sites of the vent and

from San Pietro were individually put inside a glass respirometric chamber (0.05 L) containing seawater collected at each sampling site, with the related pH values. For 1 h, RR of each limpet was assessed by measuring oxygen concentration every 20 min through a fiber optic oxygen sensor (PICO-O₂, PyroScience, GmbH, Aachen, Germany), connected to a computer and combined with contactless sensor spots glued to the glass (OXSP5-SUB, PyroScience). Temperature was constantly checked and maintained stable for the whole duration of the experiment. A chamber filled with seawater only was used as a blank. RR was then calculated following the formula properly adapted from the methods described by Widdows and Johnson (1988):

$$RR = [(C_0 - C_f) \times (V_c - V_s)]/t$$

where “C” represents the concentration of oxygen dissolved in water at the beginning (0) and at the end (f) of the analysis, “V” represents the volume of the chamber (c) and of the sample (s), and “t” represents the duration of the analysis. V_s was calculated by rounding the space occupied by a limpet to the shape of a cone. Results are expressed as $\mu\text{mol O}_2 \text{ hour}^{-1}$.

2.2.2. Ammonia excretion rate (ER)

After completing each RR assay, the seawater present in each respirometric chamber was collected and stored at -20°C to measure ammonia excretion rate (ER). ER was calculated by determining the N-NH_4^+ concentration in 10 ml of seawater following the spectrophotometric assay of Solórzano (1969) and Munari et al. (2020). Ammonia was used as a standard ($0\text{--}18 \mu\text{g L}^{-1}$; $r^2 > 0.99$), and ER was calculated following the formula:

$$ER = [(C_s - C_b) \times (V_c - V_s)]/t$$

where “C” represents the N-NH_4^+ concentration measured in the sample (s) or in the blank (b), and “t” represents the duration of the analysis. Results are expressed as $\mu\text{mol [N-NH}_4^+] \text{ hour}^{-1}$.

2.2.3. O:N ratio

The O:N ratios were calculated by dividing the amount of oxygen consumed (RR) by the amount of ammonia excreted (ER) from the same organism during the same period, as indicated by Langenbuch and Pörtner (2022) and Guo et al. (2022).

2.3. Electron transport system (ETS)

ETS activity was assayed according to the procedures described by King and Packard (1975) and by De Coen and Janssen (1997) on specimens of *P. caerulea* from both long- (resident) and short- (transplanted) term exposure to OA. ETS from the long-term exposure was measured only in specimens collected during winter.

Foot tissue was used to measure the potential metabolic activity since it represents the main muscle tissue in patellid limpets, where ETS activity and energy metabolism are expected to be more modulated (Sun et al., 2020; Giménez-Escamilla et al., 2024).

About 80 mg of foot tissue were homogenized in a Tris-HCl extraction buffer (Tris 0.1 M, PVP15 % w/v, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 153 μM , Triton-X 0.2 % v/v; pH 8.5) in a 1:4 ratio (weight/volume) with the Tissue-Lyser II QIAGEN® set at a frequency of 30/s for 180 s. Homogenates were then centrifuged at $3,000 \times g$ at 4°C for 20 min and supernatants were stored at -80°C to measure ETS activities. ETS activity was measured following the reaction of samples with a mixture of nicotinamide adenine dinucleotide (NADH, 1.7 mM) and nicotinamide adenine dinucleotide phosphate (NADPH, 250 μM) and with iodonitrotriazolium (8 mM) for 10 min and reading the absorbance at 490 nm through a multiplate spectrophotometer. Activities are expressed as $\text{nmol min}^{-1} \text{ g ww}^{-1}$ (wet weight).

2.4. Untargeted metabolomics

Metabolomics analyses were carried out only on limpet populations resident in the vent systems during winter season and on transplanted limpets. The analyses were carried out at the Unitech OMICs platform of the University of Milano (Milan, Italy). Metabolites were extracted from the lyophilized tissue of the foot of 8 individuals per site (4 pools of 2 individuals *per site*). Foot tissue was chosen since it plays a key role in energy storage (Tikunov et al., 2010; Giménez-Escamilla et al., 2024). The analyses were carried out according to the methods thoroughly described in Signorini et al. (2023, 2024) and more details are provided in the SM.

2.5. Statistical analysis

The non-parametric Kruskal-Wallis test was chosen to assess differences in physiological endpoints (RR, ER, and O:N ratio) and ETS activities in *P. caerulea* from different sites (SP – N1 – N2 – N3) and seasons (autumn – winter – spring – summer) of both resident populations and transplanted organisms. This non-parametric statistical approach was selected because the hypotheses of normality and homogeneity of variance were rejected, as assessed through the Shapiro-Wilk and Levene's test ($p\text{-value} \leq 0.05$), respectively. The Kruskal-Wallis test was then followed by Dunn's *post-hoc* test for pairwise comparisons. The analyses were carried out using the “shapiro.test()”, “leveneTest()”, “kruskal.test()”, and “dunn.test()” packages of the R software, version 2024.4.1.0 (<https://www.R-project.org/>). All plots were created using the “ggplot2()” command in R software.

The statistical analysis for the untargeted metabolomics data was performed using MetaboAnalyst ver. 6.0 software (<https://www.metaboanalyst.ca/>). A One-way Analysis of Variance (ANOVA) was applied to compare different sites (SP – N1 – N2 – N3), followed by Fisher LSD *post-hoc* test for pairwise comparisons. Furthermore, MetaboAnalyst was used to perform Partial Least Squares – Discriminant Analysis (PLS-DA) and hierarchical clustering to better visualize the results.

3. Results

3.1. Physiological parameters

3.1.1. Respiration rate (RR)

The RR measurements taken across different seasons showed a significant effect of both “Site” ($p = 0.00369$) and “Season” ($p = 0.01$) factors, with significant modulations of this parameter in spring and summer seasons in vent-resident limpet populations of *P. caerulea* (Fig. 1; Table S3). Specifically, limpets analyzed during spring displayed a significant decrease in RR in N1 and N2 with respect to San Pietro, while no differences were detected in the extreme acidified site N3 ($p = 0.01815$). On the contrary, in summer, limpets collected in N3 significantly boosted RR in comparison with all the other sites taken into consideration ($p = 0.0001648$). No significant variations were assessed in autumn ($p = 0.5214$) and winter ($p = 0.6157$). In all seasons except summer, San Pietro displayed higher RR values than all vent sites, even though this difference was not always significant. Finally, both acidified sites displayed significant modulations of RR across the different seasons (N2: $p = 0.02658$; N3: $p = 0.002689$) (Table S4).

Finally, transplanted individuals didn't significantly modulate RR, notwithstanding a general increasing trend can be observed along the pH gradient ($p = 0.2369$) (Table S3).

3.1.2. Ammonia excretion rate (ER)

The ER measurements displayed a significant effect of the factor “Season” ($p < 0.0001$), while no modulation was detected for the factor “Site” ($p = 0.0836$) (Fig. 2; Table S3). Vent-resident limpets collected in autumn and summer significantly modulated their ER across the

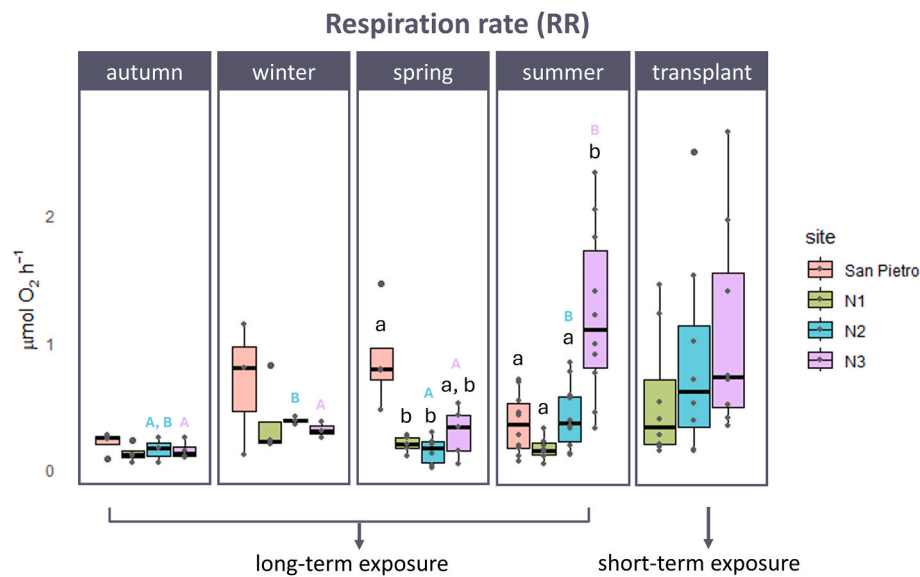


Fig. 1. Boxplots of RR measured in *P. caerulea* collected from the Castello Aragonese vent systems and from San Pietro (long-term exposure) and of the transplant experiment (short-term exposure). Dots represent biological replicates inside each site. Different lower case letters indicate statistically significant differences among the sites and across season; different capital letters indicate statistically significant differences of sites across different seasons (p -value ≤ 0.05).

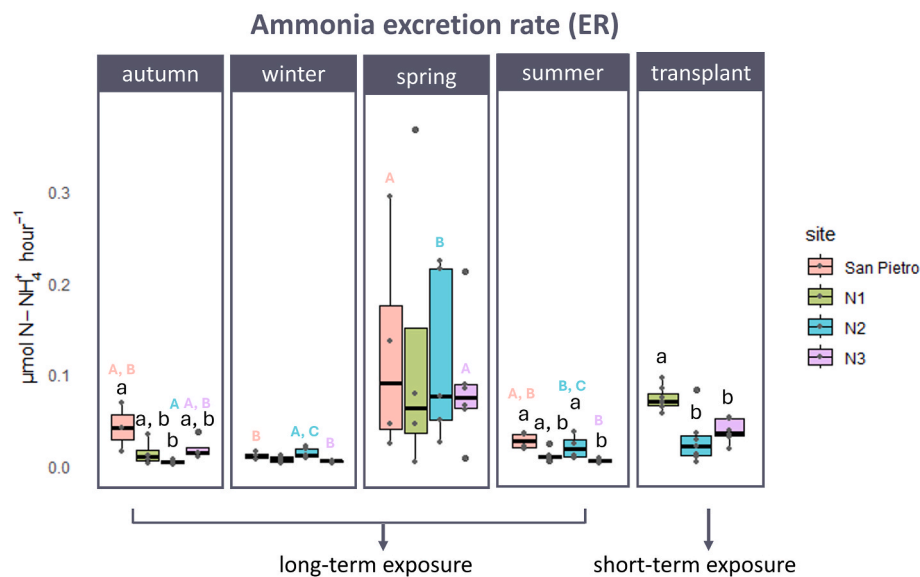


Fig. 2. Boxplots of ER measured in *P. caerulea* collected from the Castello Aragonese vent systems and from San Pietro (long-term exposure) and of the transplant experiment (short-term exposure). Dots represent biological replicates inside each site. Different lower case letters indicate statistically significant differences among the sites and across seasons; different capital letters indicate statistically significant differences of sites across different seasons (p -value ≤ 0.05).

different pH sites. In particular, specimens of *P. caerulea* collected in N2 significantly reduced their ER with respect to San Pietro in autumn ($p = 0.02393$), while, during summer, the site N3 displayed a significant decrease in ER in comparison with both San Pietro and N2 ($p = 0.01437$). On the contrary, no significant variations were detected in winter ($p = 0.05519$) and spring ($p = 0.9708$). Furthermore, both San Pietro and the low pH sites of the vent exhibited significant modulations of ER across the different seasons (SP: $p = 0.04671$; N2: $p = 0.002788$; N3: $p = 0.004593$) (Table S4).

Finally, the transplant induced a significant reduction of ER in both N2 and N3 in comparison to N1 ($p = 0.002437$) (Table S3).

3.1.3. O:N ratio

The O:N ratio highlighted a significant effect of factor “Season” ($p < 0.0001$), while factor “Site” did not show any significant effect ($p =$

0.6489) (Fig. S2; Table S3). Specifically, San Pietro exhibited higher O:N ratio than the intermediate pH site N2 during spring ($p = 0.03984$), while in summer the extreme acidified site N3 significantly increased its O:N ratio with respect to San Pietro and N1 ($p = 0.02584$). No significant differences were detected in autumn ($p = 0.1462$) and winter ($p = 0.4222$).

Furthermore, the transplant experiment showed a significant increase in O:N ratio in both acidified sites with respect to N1 ($p = 0.01155$) (Table S3).

3.2. Electron transport systems (ETS)

The long-term exposure to OA did not induce any significant variations of ETS activities in limpets collected at the Castello Aragonese vent systems ($p = 0.1151$) (Fig. 3A; Table S3). Conversely, the short-term

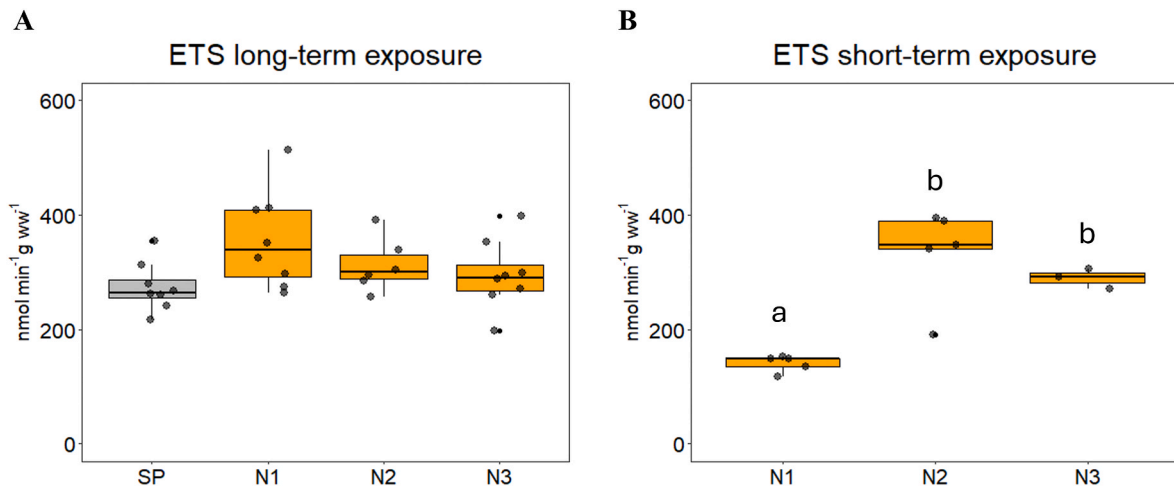


Fig. 3. Boxplot of ETS activities measured in *P. caerulea* collected from the Castello Aragonese vent systems and from San Pietro (A) and of the transplant experiment (B). Dots represent biological replicates inside each site. Different letters indicate statistically significant differences among the sites (p -value ≤ 0.05).

exposure to OA entailed a significant induction of ETS in both the acidified sites N2 and N3 with respect to the ambient pH site N1 ($p = 0.009641$) (Fig. 3B).

3.3. Untargeted metabolomics

3.3.1. Long-term exposure to OA

The PLS-DA plot shows a clear clusterization in the profile of analytes measured in the resident populations of *P. caerulea* collected from the Castello vent systems and San Pietro, with the sites of the vent arranged as a gradient (Fig. 4A). The One-way ANOVA analysis revealed a significant modulation in the level of 27 known and 9 unknown metabolites out of the 119 identified, including carnitine and 11 of its metabolites, various osmolytes, and nucleosides. Furthermore, all metabolites occurred to be significantly up-regulated along the acidification gradient of the vent systems as shown in the heatmap, except for isoxanthopterin and citric acid, which resulted significantly reduced in N2 and N3 in comparison with San Pietro and N1, respectively (Fig. 4B; Fig. S3; Table S5).

3.3.2. Short-term exposure to OA

The short-term exposure to OA induced a marked clusterization of metabolites measured in the sites of the Castello Aragonese vent systems (Fig. 5A). The statistical analysis revealed a significant modulation of 19

metabolites out of the 143 identified. Among these, only 12 were known and they were mainly related to carnitine and fatty acids metabolism, similarly to the long-term exposure. All metabolites were up-regulated in the acidified conditions of N3, except for dodecanedioic acid, tiglyl-carnitine, and 2 unknown metabolites, which occurred to be down-regulated in both N2 and N3 with respect to N1 (Fig. 5B; Fig. S4; Table S6).

4. Discussion

Energy plays a pivotal role in managing biological functions and fitness of marine calcifying organisms. Various environmental stressors, such as OA, may influence energetic processes, which lead to cascading effects on biomineralization, internal acid-base homeostasis, antioxidant capacity, metabolic pathways, physiology, life-history traits, and even population and community dynamics. A great amount of energy is generally necessary to support compensatory mechanisms that organisms may induce to cope with environmental disturbances. Therefore, the balance of energy distribution is fundamental in promoting tolerance and adaptation to the future ocean conditions (Sokolova et al., 2012; Leung et al., 2020, 2022; Simonetti et al., 2022; Sukhotin et al., 2024; Yoon et al., 2024). Nevertheless, the metabolic pathways involved in the response to OA have been poorly considered.

Accordingly, we investigated the physiological and metabolic

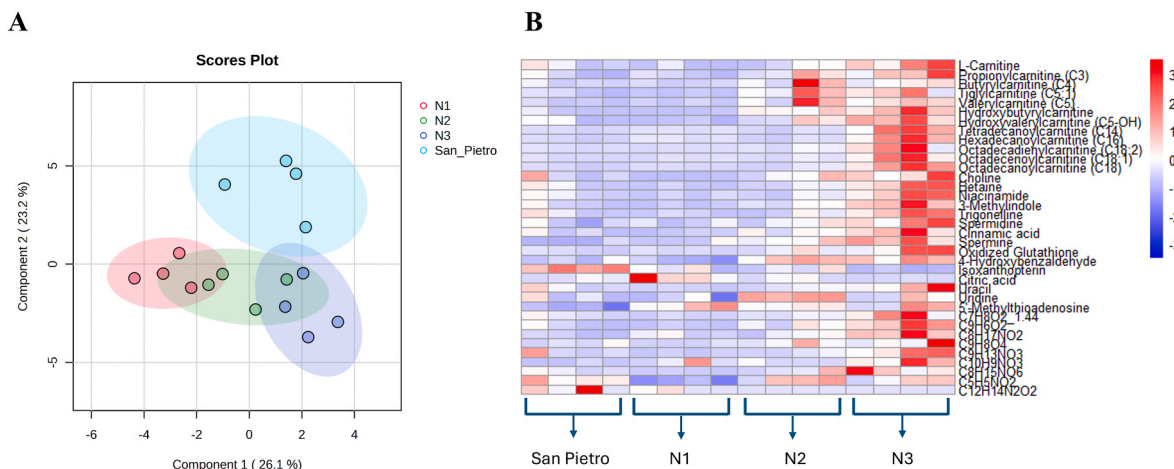


Fig. 4. The PLS-DA of resident limpet populations of *P. caerulea* collected from the Castello Aragonese vent systems and from San Pietro (A). Heatmap analysis of significantly modulated metabolites across sampling sites (B).

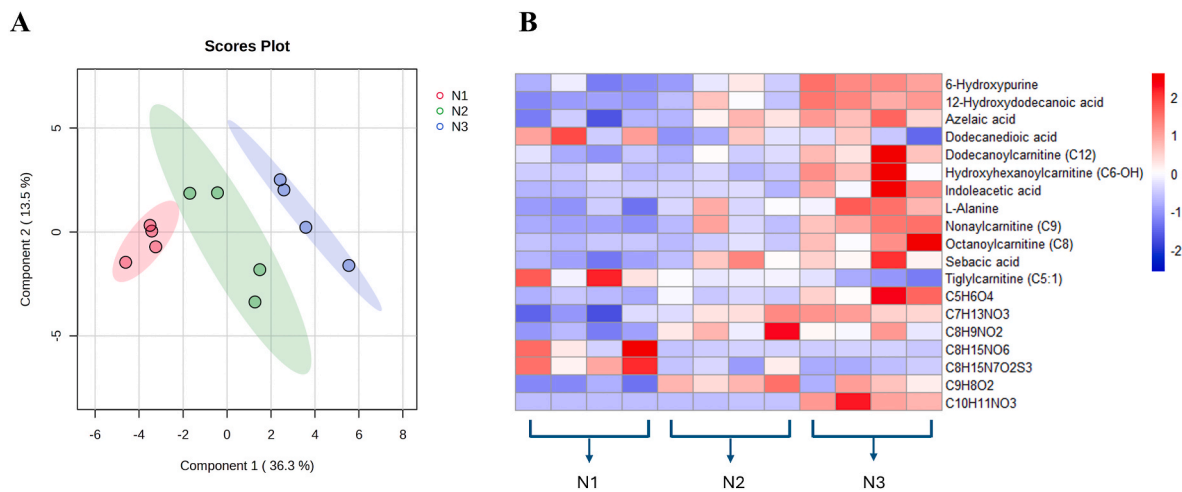


Fig. 5. The PLS-DA of limpet populations of *P. caerulea* collected from an ambient pH area and transplanted to the Castello Aragonese vent systems (A). Heatmap analysis of significantly modulated metabolites across sampling sites (B).

parameters of the calcifying species *P. caerulea* collected from the naturally acidified environment of the Castello Aragonese CO₂ vent systems (Ischia Island, Italy). Moreover, an *in situ* transplant experiment allowed to further investigate potential tolerance and/or adaptive mechanisms in response to acidified conditions.

4.1. Effects of OA on physiology

As reviewed by Medeiros and Souza (2023) and Alter et al. (2024), the physiological outcomes of OA among taxa are wide, leading to either induction or inhibition of metabolic parameters. Induction suggests an increase in energy demand to buffer the effects of OA and to maintain the homeostasis; on the contrary, inhibition may reflect a state of metabolic depression, that allows these organisms to preserve more energy resources and raise survival chance, notwithstanding that it may be effective only over short-term periods and that it might occur at the expense of important functions. In both scenarios, the main goal is achieving a balance between the development of tolerance mechanisms and the maintenance of energy trade-offs (Ferreira et al., 2025).

In our study, physiological performance was assessed by measuring respiration and ammonia excretion rates across different seasons, providing valuable insights into metabolic state as well as into the energy preserved and consumed by organisms exposed for both long- and short-term periods to naturally acidified conditions. In particular, the most relevant result is the significant increase in RR in the extremely low pH site N3 in summer with respect to all the other sites considered in this study. Summer is notably characterized by increasing temperatures and heat waves that are intensifying under the climate change scenario, which may represent an additional threat to marine organisms besides OA. This significant induction highlighted that the combination of hypercapnia and temperature can entail an increase in energy demand. Indeed, no significant difference in RR was observed among sites with different pH during the rest of the year. It is well documented that combined disturbances exert greater effects compared to individual stressors alone in crustaceans, bivalves, and gastropods (Dissanayake and Ishimatsu, 2011; Matoo et al., 2013; Melatunan et al., 2013; Gazeau et al., 2014; Valles-Regino et al., 2015; Stevens and Gobler, 2018; Clements and Hunt, 2023; Benítez et al., 2024). Similar findings were observed in the sea hare *Stylocheilus striatus*. This shelled heterobranch mollusk didn't exhibit alterations in metabolic rate under the exposure to elevated CO₂ and temperature individually, while the combination of the two stressors significantly increased oxygen consumption rates (Horwitz et al., 2020). Similarly, no modulation of respiratory performance was observed in the tropical gastropod *Gibberulus gibbosus* at

ambient temperature (Lefevre et al., 2015); the respiration rate measured in the amphipod *Cymadusa filosa* was affected by temperature but not by pH, while a significant interaction between the two stressors was highlighted (Ferreira et al., 2025). Overall, these findings suggest that OA does not affect the respiration rates of *P. caerulea* that inhabits the Castello Aragonese vent systems over the long-term period, while the combination of OA and temperature fluctuations may impact more intensively its survival and well-being. This is further supported by the significant variation in the profile of RR across different seasons only in the acidified sites. Further investigations are warranted to verify this hypothesis.

Short-term exposure to OA induced a non-significant increasing trend following the pH decrease. To date, few studies have investigated physiological performance in marine invertebrates exposed to natural acidified conditions through *in situ* transplant experiments, with findings showing either similar or opposite trends. For example, Calosi et al. (2013) found that some polychaete species displayed either a reduction or an increase of metabolism when acutely exposed to elevated pCO₂ within the Castello Aragonese vent systems. Similarly to our results, Harvey et al. (2016) reported a non-significant increase in oxygen consumption rate in *Hexaplex trunculus* from the Vulcano vent system (Sicily, Italy), when transplanted from ambient pH to low pH conditions (pH = 7.65).

The elevated RR measured here could underlie an increasing oxygen demand at cellular/tissue level in organisms exposed to stressful conditions, which may enhance aerobic ATP production through the mitochondrial electron transport chain. Indeed, mitochondria represent the main organelle responsible for oxygen consumption, ATP production, and biosynthesis of several cellular metabolites (Stumpp et al., 2019; Sukhotin et al., 2024). Accordingly, we evaluated metabolic capacity by measuring electron transport system activities, which is a biomarker commonly used in mollusks to assess the cellular respiratory potential of organisms (Freitas et al., 2017). No significant differences in ETS were detected in resident limpet populations. This result aligns with those of RR, since ETS analyses related to long-term exposure to OA were conducted on specimens collected in winter, when no significant variation in physiological parameters was observed. Conversely, a significant induction in ETS activity was assessed in specimens transplanted to N2 and N3 of the vent systems. Similarly, this result seems consistent with those of RR, considering the increasing trend along the OA gradient, which is however non-significant due to high inter-individual variability.

With regards to the ammonia excretion rate, no clear trend was revealed in vent-resident limpet populations. However, it is worth

noting that limpets from San Pietro always displayed higher RR and reduced ER values with respect to those from the Castello area, which were significant only during spring (RR) and autumn (ER). We observed similar discrepancies in our previous work, which focused on the effects of OA on several biochemical endpoints measured in *Patella* species collected from the same study sites (Signorini et al., 2025). San Pietro has been considered in multiple studies as an external ambient pH site, comparable to N1 of the Castello vent, displaying similar light, temperature, salinity, depth, hydrodynamic conditions, and rocky shore habitat (Kroeker et al., 2011; Calosi et al., 2013; Migliaccio et al., 2019). Nevertheless, other environmental factors might have played a role in influencing the physiological and biochemical performance of *P. caerulea* inhabiting San Pietro during the different seasons. For example, its proximity to the harbor of Ischia Island or the presence of one of the underwater sewage lines of the island (Della Torre et al., 2024). These conditions may influence chemical pollution levels and nutrient availability, with cascading effects on marine food webs (Fowles et al., 2018; Pieretti et al., 2020). Deeper investigations would be necessary to disentangle the net of abiotic and biotic factors that withstand the differences observed between San Pietro and the Castello area.

On the other hand, a significant reduction of ER occurred in individuals from the acidified sites with respect to those of N1 after the transplant experiment. The ER is a parameter that reflects energy decline by releasing nitrogen compounds starting from protein and nucleic acid catabolism (Munari et al., 2020; Ferreira et al., 2025). The observed reduction in ER may suggest an inhibition of protein and/or nucleic acid catabolism to potentially maintain energetic resources to cope with the higher energetic cost due to OA. Alternatively, the reduction in ER may be considered a strategy to preserve the hydric and ionic balance of the internal fluids. Indeed, free amino acids and their catabolites are used as intracellular osmolytes to protect organisms from the fluctuations of the extracellular osmolarity due to the acidified surrounding environment (Tikunov et al., 2010; Sokolova, 2021).

4.2. Effects of OA on the metabolome

Untargeted metabolomics analyses conducted on both long- and short-term exposures to OA highlighted the presence of a metabolic cost underpinning tolerance to naturally acidified conditions. *Patella caerulea* tried to compensate for this cost by up-regulating several metabolites involved in energetic processes.

The most relevant result is the significant up-regulation of carnitine and carnitine-related metabolites (11 for the long-term exposure and 5 for the transplant) in *P. caerulea* collected from the acidified sites N2 and N3 of the Castello Aragonese vent systems, which may be directly related to fatty acids metabolism and therefore energy production as an anti-stress response to OA. Indeed, carnitine displays a pivotal role as a carrier in transporting long, medium, and short-chain fatty acids in the form of acylcarnitine inside the mitochondrial matrix (“carnitine shuttle pathway”), where they enter the β -oxidation cycle. Biochemical reactions of β -oxidation reduce the number of carbon atoms to form acetyl-CoA, which subsequently enters the Krebs cycle through which ATP is produced. Fatty acids are energetically more efficient than polysaccharides or proteins, and they are fundamental in maintaining energetic homeostasis and in determining the organisms’ viability (Tikunov et al., 2010; Li et al., 2019; Huang et al., 2020; Fu et al., 2021; Späth et al., 2022; De Paula et al., 2023; Imbert-Auvray et al., 2023). Specifically, we observed a significant increase in carnitine and in carnitine-related metabolites, which are named acylcarnitines, such as hexadecanoylcarnitine, butyrylcarnitine, propionylcarnitine, etc. These molecules are composed of a long, medium, or short fatty acid bound to the carrier carnitine, which allows their transport to mitochondria, where they are converted into energy. Longer molecular chains correspond to a greater amount of energy produced to sustain survival and tolerance strategies under OA conditions.

In our previous work carried out on *P. caerulea* inhabiting the same vent systems, a significantly higher glycogen content was observed in limpets collected in low pH conditions. This finding may suggest that for these organisms it might be more effective to induce lipid metabolism rather than rely on polysaccharide reserves, which notably were not depleted, but instead increased, potentially also due to differences in food quality and availability among the pH sites of the vent (Signorini et al., 2025).

In support of this hypothesis, in our work it was also possible to detect a significant decrease of citric acid in resident limpet populations collected from acidified conditions. This metabolite is an intermediate of the Krebs cycle and it is produced from acetyl-CoA and oxaloacetate. A decrease in citric acid levels may result from a reduced availability of acetyl-CoA, which might be involved in the “carnitine shuttle pathway” for energy production. Indeed, carnitine regulates many cellular pathways by modifying the mitochondrial acetyl-CoA/CoA ratio (Wang and Lou, 2024). Moreover, in N2 and N3 we assessed a significant up-regulation of niacinamide, a carnitine precursor essential for cellular bioenergetics and metabolism. Therefore, its production might have been induced to increase carnitine synthesis and to fuel the energy processes. Furthermore, niacinamide has been detected in some micro and macroalgae, and since patellid limpets are herbivorous grazers, an up-regulation of this metabolite may be directly related to our hypothesis of an improved quality and/or quantity of food under OA scenario (Edelmann et al., 2019; Li et al., 2019; Orlandi et al., 2020).

Despite the great species-specificity, living in intermediate and extreme acidified conditions entails intensive energetic costs (Figueroa et al., 2021; Sokolova, 2021; Leung et al., 2022; Simonetti et al., 2022; Shi and Li, 2024). Biomineralization, osmoregulation, internal acid-base homeostasis, behavior, antioxidant activity, and signaling pathways represent some of the main processes affected by OA. Therefore, energetic trade-offs represent a fundamental functional trait closely related to organisms’ fitness and ecological and ecosystem processes, whose understanding can contribute to acknowledging their plasticity and adaptation to future ocean conditions.

The induction of energetic metabolism is not in line with results of RR, since no modulation of RR was observed in winter among the different sites. This discrepancy could be due to the fact that, even though they increase energy metabolism, at the same time they do not need to induce respiration. Indeed, the higher energetic costs could be partially compensated by the more favorable environmental conditions, in particular a modified quantity and/or quality of food, as hypothesized in our previous work (Signorini et al., 2025). In the vent systems we observed an increase of biofilm coverage in N2 and N3 with respect to N1. In line with our findings, some studies revealed that the biofilm benefits from seawater CO₂ enrichment in terms of biomass, photosynthetic activity, and nutritional value (Russell et al., 2013; Burgos-Rubio et al., 2015; Johnson et al., 2015). Since limpets graze on the epilithic biofilm grown on the rocky shore, this specific condition may be effective in balancing the negative effects of OA, allowing them to inhabit the vent systems and even display increased biomass and energy reserves.

On the other hand, the metabolism of amino acids seems not affected. This result is in line with the absence of modulation of ER, which is a physiological parameter related to nitrogenous compounds such as proteins.

In the case of short-term exposure, it is more complex to formulate comparable hypotheses, despite carnitine remains a key factor also in specimens transplanted to the Castello Aragonese vent systems, making it a potential common mechanism under both long- and short-term exposure to OA. Notably, fewer metabolites were significantly modulated, potentially due to the fact that 30 days might not have been sufficient to induce a measurable metabolic shift in response to OA. For instance, several fatty acids, such as azelaic acid, indoleacetic acid, and sebacic acid, displayed higher concentrations in low pH conditions. This result was not expected, since the increase of β -oxidation should lead to a decrease in total lipid content as observed in resident populations. The

general higher level of fatty acids detected in *P. caerulea* after the transplant may be due to an increase of absorption, synthesis, metabolism, and/or storage of lipids, and, consequently, it may represent a strategy developed to face pulsed acidified conditions. Further investigations are needed to support this hypothesis.

The increase of energetic metabolites is in line with the significant increase of ETS and RR (even though not significant). This result suggests that short-term exposure to OA induces stress conditions in organisms, that should enhance respiration and ATP production. Conversely, ER decreased as a compensatory strategy to reduce protein catabolism, as already seen under life-long exposure. Ultimately, the O:N ratios revealed an increase in acidified conditions, which occurred to be significant in spring and summer, as well as at the end of the transplant experiment, with values above 50. The O:N ratio is a physiological parameter that is indicative of substrate catabolism, with higher ratios that generally underline a preference on lipid catabolism (Anestis et al., 2010; Wang et al., 2015; Ramaglia et al., 2018). Therefore, this result represents a further confirmation of the evidence that limpets mainly rely, as energetic resources, on lipids rather than proteins. A final consideration needs to be made for the up-regulation of metabolites related to osmoregulation, oxidative stress, and nucleic acid mechanisms, detected in the low pH sites of the vent. These processes represent some of the primary targets of OA, already modulated in calcifying and non-calcifying species collected from the same vent systems (Signorini et al., 2023, 2024). Trigonelline, betaine, and its precursor choline are osmolytes that are involved in osmoregulation in several marine species, with significantly higher concentrations in *P. caerulea* under acidified conditions. Since these metabolites can modify the ionic strength of cytosol and maintain the osmotic pressure within the cell, their up-regulation may represent a strategy to counteract the imbalance caused by hypercapnic conditions (Tikunov et al., 2010; Ramaglia et al., 2018; Imbert-Auvray et al., 2023) and support the results of ER. Additionally, OA can induce changes in the antioxidant system, as already assessed in several marine species (reviewed by Melzner et al., 2020; Simonetti et al., 2022). Within this study, we detected a significant increase in several metabolites associated with oxidative stress, including oxidized glutathione, cinnamic acid, and 4-hydroxybenzaldehyde. These compounds are known for their antioxidant properties and capability to inhibit free radical species, and therefore their induction might be a mechanism to cope with OA (Nobsathian et al., 2017; Jiang et al., 2024). Finally, metabolites involved in DNA/RNA mechanisms, such as uracil and uridine, were up-regulated in acidified conditions as well. This up-regulation may reflect an increased demand for nucleic acid synthesis or the activation of repair mechanisms in response to OA, which was already assessed in the polychaetes of the genus *Platynereis* from the same vent systems or in the crab *Metacarcinus magister* and the sea urchin *Strongylocentrotus intermedius* exposed to low pH conditions (Wanamaker et al., 2019; Li et al., 2020; Signorini et al., 2023). As stated above, the tight regulation and balance of these processes require a great amount of energy, further confirming the presence of an energetic cost, that allows survival and tolerance of *P. caerulea* to OA conditions.

Overall, our findings suggest that naturally acidified conditions entailed a metabolic expenditure in *P. caerulea*; nevertheless, these organisms seemed to compensate for this stressful condition by adjusting their energy budget through both physiological and metabolic modulation to potentially increase energy availability and better mitigate the adverse outcomes of hypercapnic conditions.

5. Conclusion

Based on the data concerning the population inhabiting the vent systems, we showed that surviving under low pH conditions implies phenotypic plasticity in physiological and cellular processes. In particular, the carnitine pathway emerged as a key cellular process to sustain the energetic demand requested to face the stress induced by OA. The high energetic demand needed to tolerate acidic environment might

compromise the survival of the populations in the vent systems upon further anthropogenic/environmental disturbance, as suggested in previous studies (Nagelkerken and Connell, 2015; Rodríguez-Romero et al., 2021; Munari et al., 2022). The translocation experiment revealed that adult limpets might be able to colonize even the low pH site, at least for a short period of time. Nonetheless, based on our experimental data, it is not possible to predict whether adult immigrants might be able to survive, grow, and reproduce under OA in the long term. Further studies investigating the populations of the vent at the genetic level are warranted to verify the role of genetic adaptation besides plasticity in allowing the survival of organisms inside the vent.

This study improves the current knowledge concerning the fine mechanisms that promote survival under OA conditions, which is fundamental in understanding how environmental disturbances might shape the marine biodiversity in the future.

CRedit authorship contribution statement

Silvia Giorgia Signorini: Writing – original draft, Investigation, Formal analysis, Data curation. **Marco Munari:** Writing – review & editing, Resources, Conceptualization. **Fabio Crocetta:** Writing – review & editing, Validation. **Valerio Matozzo:** Writing – review & editing, Resources, Investigation. **Ilaria D’Aniello:** Formal analysis. **Lara Nigro:** Formal analysis. **Manuela Fontana:** Writing – review & editing, Formal analysis. **Fiorenza Faré:** Writing – review & editing, Resources, Data curation. **Camilla Della Torre:** Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2025.123155>.

Data availability

Data will be made available on request.

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