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Short and long-term exposure to ocean acidification in limpets from the Castello Aragonese vent systems (Ischia Island, Italy)

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ABSTRACT

Ocean acidification (OA) is reported to entail a detrimental impact on calcifying organisms. Nevertheless, patellid limpets – *P. caerulea*, *P. rustica*, and *P. ulyssiponensis* – are able to persist in extremely low pH conditions inside the Castello Aragonese CO_2 vent systems (Ischia Island), suggesting that they may have developed tolerance to OA, through plasticity and/or adaptive mechanisms. The aim of this study is to evaluate the long-term strategies adopted by limpets that spent their entire life cycle in naturally acidified conditions and the short-term ones induced by a 30-day *in situ* transplant experiment.

Regarding native limpet populations, *P. caerulea* exhibited increasing size and higher energy resources in the extremely acidified site, potentially related to different food availability or to reduction in competition and/or predatory pressure; furthermore, no effects on oxidative stress, biomineralization and neurotoxicity occurred. Similarly, *P. ulyssiponensis* didn't exhibit any significant effects among different pH conditions regarding biochemical endpoints. Conversely, *P. rustica* displayed a significant modulation of almost all biochemical parameters, possibly due to its different position on the rocky shore. The short-term exposure of *P. caerulea* produced a decrease in protein content and an increase in glycogen content in the extreme acidified site, with an induction of superoxide dismutase and glutathione-S-transferases activities in the intermediate pH site.

Overall, our study revealed that different species of the same genus may have developed distinct responses to OA and suggested different mechanisms to cope with short and long-term exposure to low pH conditions.

1. Introduction

Anthropogenic activities entail a deleterious impact on ecosystems' health, especially in marine environments, negatively affecting their stability, biodiversity and functionality (Doney et al., 2012; Pecl et al., 2017). Among the various disturbances, ocean acidification (OA) stands out as one of the major threats to marine organisms.

Since the beginning of the Industrial Era, the concentration of carbon dioxide (CO_2) in the atmosphere has been rising from 280 ppm to the current level of 421.73 ppm (Lan et al., 2025), mainly due to anthropogenic activities. About 30 % of this atmospheric CO_2 is absorbed by oceans, where it determines an alteration of seawater chemistry and a significant reduction in the pH levels of the global surface seawater (Leung et al., 2022; Simonetti et al., 2022). According to the Intergovernmental Panel on Climate Change (IPCC), at the current rate and within the "business as usual scenario", the atmospheric CO_2 level may reach 800 ppm by the end of the century (2100), which would correspond to a decrease of the global ocean pH level of 0.4–0.5 units (from the current level of 8.1 to 7.7), entailing remarkable effects on the marine biota (Gattuso et al., 2015; Melzner et al., 2020; Vargas et al., 2022; IPCC, 2023; Shi and Li, 2024).

Several research highlighted that OA negatively impacts marine invertebrates at multiple level of biological organization, affecting primarily physiological mechanisms, such as acid-base homeostasis, respiration/gas exchange, and calcification, but also metabolism, behavior, life history traits, including fertilization, larval development

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and settlement, and reproduction, which subsequently have an impact on organisms' survival and fitness, ultimately affecting marine ecosystem health and functioning (Kroeker et al., 2010; Lannig et al., 2010; Wäge et al., 2016; Gao et al., 2019; Gray et al., 2022; Leung et al., 2022; Medeiros and Souza, 2023; Shi and Li, 2024).

So far, greater attention has been given to marine calcifying organisms (coccolithophores, corals, coralline algae, gastropods, bivalves, sea urchins, crustaceans, etc.), since OA entails an increase in hydrogen ions (H^+) and decrease in carbonate ions (CO_3^{2-}) concentrations. These reactions limit the availability of calcium carbonate ($CaCO_3$), which is a main component of shells, skeletons and other calcareous structures and it is formed starting from CO_3^{2-} ions (Buth, 2016; Figuerola et al., 2021). Moreover, the increase in H^+ ions and the consequent acidification of seawater lead to corrosion of calcareous structures, which generally appear thinner and more fragile, with a significant impact on their structural integrity and mechanical strength (Langer et al., 2014; Barclay et al., 2020).

The alteration of seawater chemistry induced by OA has also an effect on the regulation of pH levels in intra and extracellular fluids, which requires an active regulation of acid-base homeostasis, to guarantee adequate physiological functioning, especially at the interface tissue-shell. Additionally, OA is a well-established pro-oxidant agent for several marine organisms, both calcifying and non-calcifying, that can induce an alteration of the oxidative balance, mainly through the enhancement or the inhibition of antioxidant and detoxification enzymes, although with a remarkable species-specificity, even within the same taxon (Leung et al., 2022; Simonetti et al., 2022; Shi and Li, 2024).

Nonetheless, some organisms can develop regulatory and compensatory mechanisms that may be induced to buffer OA effects and to maintain physiological homeostasis, allowing them to live under acidified conditions; however, these strategies require energy (Thor and Dupont, 2015; Foo et al., 2020; Teixidó et al., 2020; Signorini et al., 2023, 2024; Asnicar et al., 2025). Therefore, energy balance represents a key element of stress tolerance and adaptation, notwithstanding that this might occur at the expense of other important processes, with potential consequences at population or ecosystem-level (Sokolova et al., 2012; Pan et al., 2015; Liao et al., 2019; Leung et al., 2022).

Albeit being fundamental in predicting the potential consequences in the future anthropogenic oceans, these mechanisms remain largely overlooked. Moreover, as evinced from the meta-analysis on 985 scientific products performed by Leung et al. (2022), most of the studies carried out on the effects of OA on marine calcifiers mainly focuses on short-term exposure, in laboratory conditions, often manipulating pH level improperly, and without taking into consideration the habitat complexity and the role of species interactions in shaping potential tolerance to OA. Within this context, CO_2 vent systems represent a valid alternative, that enable to avoid most of laboratory study drawbacks and to improve the ecological relevance. Indeed, CO_2 vent systems represent natural laboratories to investigate the effects of OA at ecosystem level, since they are environments naturally acidified by the volcanic carbon dioxide emitted from the seafloor (Foo et al., 2018; D'Alessandro et al., 2024). Studying organisms able to persist and even thrive under natural OA conditions of CO_2 vents allows to consider the development of potential acclimatization and/or adaptation strategies, contributing to formulate hypotheses of more realistic future environmental scenarios (Hall-Spencer et al., 2008; Cigliano et al., 2010; Mecca et al., 2020; Rodríguez-Romero et al., 2021).

Among shallow CO_2 vent systems, the Castello Aragonese, located in the North-East part of Ischia Island and within the Regno di Nettuno Marine Protected Area (Italy, central-western Tyrrhenian Sea), is characterized by a natural pH gradient on both north and south sides of the islet. Previous studies have extensively characterized the physico-chemical conditions of this vent, highlighting that gas emissions mainly consist of carbon dioxide (~90 %), nitrogen (3.2–6.6 %), oxygen (0.6–0.8 %), and methane (0.2–0.8 %), while toxic sulfur compounds are absent (Foo et al., 2018; Gambi et al., 2020).

At the Castello Aragonese vent systems, it is possible to find some calcifying species that are able to survive under ocean acidification conditions. Among these, limpets belonging to the genus *Patella* represent one of the few calcifiers that can persist even in the extreme acidified site (N3: pH = <7.4). Specifically, *Patella caerulea* (Linnaeus, 1758), *Patella rustica* (Linnaeus, 1758), and *Patella ulyssiponensis* (Gmelin, 1791) represent the three main and most abundant limpet species of the vent (Hall-Spencer et al., 2008; Rodolfo-Metalpa et al., 2011; Foo et al., 2018). These species occupy different vertical areas of the rocky shores: *P. rustica* is present in the upper midlittoral and in the supralittoral, while *P. caerulea* and *P. ulyssiponensis* occur from the mid to the lower midlittoral (Lima et al., 2006; Battelli, 2017; Reguera et al., 2018; Seabra et al., 2019). Moreover, they display different morphologies and colors of shells and feet, which, combined with their different distribution on the substrate, contribute to taxonomically identifying them (Fig. S1).

Patella species are grazing gastropods that occupy rocky intertidal areas of the East Atlantic and Mediterranean coasts in temperate latitudes. They are considered keystone species due to their primary role in structuring and regulating the ecological balance of intertidal communities (Vafidis et al., 2020). The fact that these three calcifying species, apparently vulnerable to OA, can persist in the extreme environment of the Castello Aragonese vent systems, demonstrates that they have developed tolerance to reduced pH conditions through plasticity and/or adaptive mechanisms (Vafidis et al., 2020; Foo et al., 2018). Up to now, most of the studies carried out in *Patella* species in relation to ocean acidification mainly focused on distribution, morphological traits, and calcification rates, but there is a lack of knowledge on the molecular and biochemical mechanisms that could underlie acclimatization and/or adaptation to OA. Furthermore, as explained above, several studies are carried out in laboratory conditions, without considering the complex and dynamic network of direct and indirect interactions with other species, besides natural fluctuations of abiotic parameters (Cornwall and Hurd, 2016; Leung et al., 2022).

In this view, the aim of the present study is to evaluate the long-term strategies adopted by limpets - *P. caerulea*, *P. rustica*, and *P. ulyssiponensis* - that spent their entire life cycle in naturally acidified conditions and the short-term ones induced by an exposure to OA through a *in situ* transplant experiment at the Castello Aragonese vent systems, that was carried out only with *P. caerulea*, due to its higher abundance. Therefore, the null hypotheses were:

1) There are no significant differences among the three limpet species (*P. caerulea*, *P. rustica*, and *P. ulyssiponensis*) across the different sites of the Castello Aragonese vent system and San Pietro promontory;
2) There are no significant differences among *P. caerulea* individuals transplanted along the acidification gradient at different sites.

To address these hypotheses, morphometric characteristics and a suite of biomarkers related to energy metabolism, oxidative stress and damage, biomineralization and neurotoxicity were carried out to provide valuable insights into the tolerance and potential adaptive mechanisms developed to survive in extremely harsh conditions.

2. Materials and methods

2.1. Long-term exposure to OA of P. caerulea, P. rustica and P. ulyssiponensis

2.1.1. Study area and sampling

The study area of the Castello Aragonese CO_2 vent systems is located on the north-east coast of Ischia Island, Italy (40°73′25.81''N, 13°96′51.03''E). It is a volcanic islet characterized by a natural pH gradient on both north (N) and south (S) side, where three different pH sites have been identified: N/S1 with ambient pH of 8.1, N/S2 with pH level of ~7.7, representative of the condition projected for the end of the

century (2100), and N/S3 marked by pH values lower than 7.4, indicative of an extreme acidified condition.

The pH level inside the Castello Aragonese vent area has been deeply characterized through continuous monitoring in previous studies (Hofmann et al., 2011; Kroeker et al., 2011; Foo et al., 2018; Gambi et al., 2020; Teixidó et al., 2018, 2024). Furthermore, a single measurement of pH, temperature, salinity, and pressure was carried through a multiprobe (Hanna instruments HI9828) contemporary to the limpet collection in February 2023 (Table S1).

The promontory of San Pietro (40°44′47.6″ N, 13°56′40.42″ E, approximately 4 km from the Castello Aragonese vent systems) was used as an additional ambient pH site (SP: pH = 8.1), potentially comparable to N1, since it presents the same environmental parameters (Ricevuto et al., 2015; Migliaccio et al., 2019) (Table S1).

Individuals of *P. caerulea*, *P. rustica*, and *P. ulyssiponensis* (Gastropoda, Mollusca) were collected by snorkeling from the lower intertidal to the subtidal rocky zone, along the northern pH gradient of the Castello Aragonese vent systems (N1 – N2 – N3), with the permission of the Marine Protected Area 'Regno di Nettuno' (Ischia Island, Italy), in February 2023. Between 3 and 22 individuals per site and per species were detached from the rocky shores through a knife, according to their availability and in order not to negatively affect the natural populations (Table S3). Species were taxonomically identified and morphometric characteristics, such as shell length, height and weight of all individuals were measured using a caliber (Performance Tool W80150 6-Inch Plastic Caliper). Considering the shape of limpet shells, volume was calculated by approximating the area occupied by each limpet to a cone area, following the formula:

$$\text{Volume} = \left[\pi \times (\text{L}/2)^2 \times \text{H}\right] \Big/ 3$$

where "L" and "H" are respectively the "length" and the "height" of the limpet shell.

Samples were subsequently kept at −80 °C in order to analyze the biochemical parameters.

2.1.2. Biomarkers

The analyses related to biochemical parameters were carried out separately on the foot and on the viscera, which included gills and digestive gland, since they represent two of the main tissues involved in the antioxidant response to environmental stressors, as well as in nutrient intake, energy metabolism, and immune defense (Khan et al., 2018–2021; Liang et al., 2022; Yu et al., 2023). All the biochemical endpoints were normalized on wet weight, due to significant variations in protein content among sites.

Approximately 80 mg of viscera/foot tissue were dissected and homogenized individually in a 100 mM potassium phosphate buffer (KCl 100 mM, EDTA 1 mM, dithiothreitol 1 mM and protease inhibitors 1:10, pH 7.4) in a ratio 1:5 w/v with TissueLyser II QIAGEN® set at a frequency of 30/s for 90 s. Samples were centrifuged at 15,000×*g* at 4 °C for 30 min and supernatants were stored at −80 °C for the measurements of protein and glycogen contents (PROT, GLY; both viscera and foot), reactive oxygen species content (ROS; viscera), antioxidant and detoxification enzyme activities (superoxide dismutase SOD, catalase CAT, glutathione-S-transferases GSTs; viscera), the level of lipid peroxidation (LPO; viscera), neurotoxicity endpoint (acetylcholinesterase AChE; both viscera and foot), and biomineralization enzyme activities (alkaline phosphatase ALKP, acid phosphatase ACP; foot) following the methods described by Signorini et al. (2024).

LPO measurements required a distinct extraction; about 50 mg of viscera were homogenized in a ratio 1:5 w/v in 20 % w/v trichloroacetic acid with TissueLyser II QIAGEN® set at a frequency of 30/s for 90 s and centrifuged at 10,000×*g* at 4 °C for 20 min. Supernatants were incubated for 25 min at 96 °C with trichloroacetic acid (TCA, 20 % w/v) and thiobarbituric acid (TBA, 0.5 % w/v). The reaction was stopped on ice and the absorbance was read at 532 nm. The colorimetric assay was carried out following the method described by Buege and Aust (1978), through the quantification of thiobarbituric acid reactive substances (TBARS), formed during the reaction between TBA and LPO by-products (ie malondialdehyde, MDA). LPO levels are expressed as nmol MDA g ww^{-1}.

Both ALKP and ACP activities were measured following the methods described by González et al. (1994) and Moreira et al. (2016). An aliquot of each sample (about 50 mg) was homogenized in Tris-HCl buffer (50 mM, pH 8.2 for ALKP and 4.5 for ACP) at a ratio 1:4 w/v using the TissueLyser II QIAGEN® set at a frequency of 25/s for 2 min and then centrifuged at 10,000×*g* for 20 min at 4 °C. After an incubation of 30 min at 20 °C with p-nitrophenylphosphate (pNPP, 6 mM), the reaction was stopped with sodium hydroxide (NaOH, 0.1 M) and the absorbance was read at λ = 410 nm. Enzyme activities are expressed as nmol min^{-1} g ww^{-1}, using 4-nitrophenol as a standard (0–1 mg ml^{-1}; $r^2 > 0.99$).

2.2. Short-term exposure to OA of P. caerulea

The *in situ* transplant experiment was carried out solely with *P. caerulea* due to its higher abundance and because of the complexity of the experimental set-up performed in field.

In January 2024 three natural volcanic rock tiles (30 × 30 cm) per site were attached along the pH gradient of the Castello Aragonese vent systems (N1 – N2 – N3). The tiles were attached to the rocky wall by scuba divers using an underwater drill (Nemo® Hammer Drill 50 m), a 20 cm steel threaded rod for each tile, and a two-component epoxy resin (Yachting Veneziani, Subcoat S Comp-A and Comp-B) to ensure the structure's stability throughout the entire experiment (Fig. 1.1).

The tiles were settled in the sites for seven months in order to let the growth of the biofilm and to guarantee adequate food provisioning to the transplanted limpets. At the end of August 2024, specimens of *P. caerulea* with heterogeneous dimensions were randomly collected from an ambient pH site (pH = 8.1) and transplanted in each one of the different sites involved (N1 – N2 – N3). The ambient pH site was located along the bridge that connects the Castello Aragonese to the mainland (40°43′55.4"N 13°57′38.7"E) and it reflects the same physico-chemical characteristics, such as exposure, temperature, salinity, etc. Specifically, each tile hosted up to four limpets, with a total of twelve individuals per site, in order to minimize competition and to guarantee a suitable number of replicates for robust experimental results. After the limpets were positioned, a metal cage was placed over the structure, to prevent their escape and to avoid predation and/or competition with other individuals native of the site (Fig. 1.2). During the exposure period, pH and temperature were monitored using loggers (HOBO® pH and Temperature Data Logger) deployed in N1, N2, and N3 (Table S2, Fig. S2).

For the transplant experiment it was not possible to use San Pietro site as an ambient pH collection site of limpets, as transporting them to the Castello area would have caused excessive stress, hindering their proper and stable attachment to the tiles. Besides, significant differences of biochemical endpoints were observed between the two ambient pH sites taken into consideration (San Pietro and N1) in the native limpet populations.

The transplant experiment lasted 30 days, and at the end of it, limpets were carefully removed from each tile and survival rates were calculated. Limpets were dissected and kept at −80 °C to evaluate energy metabolism (GLY, PROT), oxidative stress (ROS, SOD, CAT, GSTs), biomineralization (ALKP, ACP), and neurotoxicity (AChE) endpoints, following the methods described above (see Paragraph 2.1.2) (N = 8). Finally, the biofilm coverage was photographed, collected from each tile and stored at −80 °C to measure PROT and GLY contents (Fig. S3).

2.3. Statistical analysis

In order to test differences within the three species (*P. caerulea*, *P. rustica*, and *P. ulyssiponensis*) among the three different sites of the

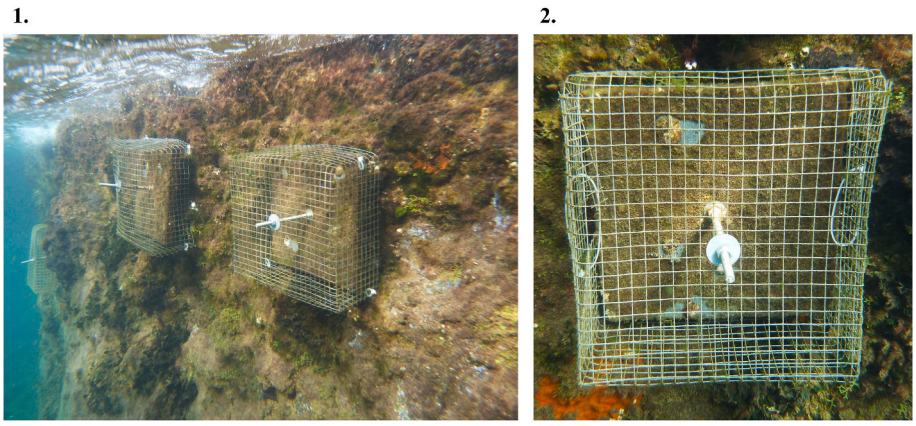

Fig. 1. Experimental set-up of the *in situ* transplant experiment carried out with *P. caerulea*. Three cages per site were attached to the rock (1). Four limpets per tile were placed on the biofilm developed for 7 months on the tile (2).

Castello Aragonese vent systems (N1 – N2 – N3) and San Pietro, concerning energy metabolism reserves (GLY, PROT), oxidative stress and damage (ROS, SOD, CAT, GSTs, LPO), biomineralization (ALKP, ACP), and neurotoxicity (AChE) endpoints, a generalized linear model (GLM) was applied, followed by the *post-hoc* Tukey test. This statistical test was chosen since the hypothesis of normality and homogeneity of variance were verified and rejected through the Shapiro-Wilk test and the Levene's test (p-Value $\leq$0.05) (Table S5).

A separate GLM was performed on the morphometric characteristics (shell length, soft-tissue weight and volume) of the three different species considered, due to the higher number of individuals that was available (Table S3; S4).

The analyses were carried out using "glm ()" and "emmeans ()" functions from the "DHARMa" and "emmeans" package of R software, version 2024.4.1.0 (https://www.R-project.org/), considering "Species" and "Site" as covariable. Moreover "leveneTest ()" and "shapiro.test ()" functions from the "car" package of R software were used to test homogeneity of variance and normal distribution.

Non-Metric Multidimensional Scaling (NMDS) was performed on biochemical endpoints since it allows to visualize complex multivariate datasets in space (Dexter et al., 2018). NMDS was carried out applying Bray-Curtis dissimilarity method (Bray and Curtis, 1957) and using "metaMDS ()" and "envfit ()" functions from the "vegan" package of R software.

Finally, all plots were created with the "ggplot2()" package in R software.

For the *in situ* transplant experiment, a GLM followed by the *post-hoc* Tukey test was performed on the biochemical data (GLY, PROT, ROS, SOD, CAT, GSTs, AChE, ALKP and ACP), considering only "Site" as a covariable (Table S6). Finally, the non-parametric statistical test Kruskal-Wallis followed by Dunnett's *post-hoc* test was chosen to test differences in PROT and GLY contents of the biofilm coverage among different pH sites (Table S7). These tests were carried out using "kruskal.test ()" and "dunn.test ()" packages of R software.

3. Results

3.1. Effects of long-term exposure to OA of P. caerulea, P. rustica, and P. ulyssiponensis

3.1.1. Morphometric characteristics

Among the three different limpet species considered in this study, only *P. caerulea* displayed a significant variation in its dimensions according to the different pH sites inside the Castello Aragonese vent systems and San Pietro. Specifically, limpets collected from the extreme acidified site N3 showed a significant increase in shell length, soft-tissue weight and volume in comparison with the limpets sampled from the external ambient pH site San Pietro and the internal ambient pH site N1 (Fig. 2.1). Moreover, significant differences between the ambient pH sites San Pietro and N1 were detected regarding shell length and soft-tissue weight. In the other two species the morphological parameters were increased in organisms collected from N3 with respect to the other sites involved, albeit not significantly (Figs. 2.2 and 2.3).

Furthermore, the GLM analysis revealed that the species *P. caerulea* was significantly different from both *P. rustica* (length: p = <0.0001; soft-tissue weight: p = <0.0001; volume: p = <0.0001) and *P. ulyssiponensis* (length: p = 0.000281; soft-tissue weight: p = 0.0148), according to all the morphometric parameters measured, highlighting an effect of the variable "Species" (Table S4).

3.1.2. Energy reserves

Protein content was homogeneous among the different sites in *P. caerulea* and *P. ulyssiponensis*; on the contrary, *P. rustica* displayed a significant modulation of this parameter in samples extracted from foot and viscera tissues, in most cases with higher values in the extreme acidified site N3 with respect to N1 and N2. Nevertheless, the GLM analysis didn't identify "Site" as a significant factor in modulating protein content among the different pH sites (Fig. 3.1, Table S5). On the contrary, this model revealed a significant effect of the factor "Species" between *P. caerulea* and *P. ulyssiponensis* (PROT viscera: p = <0.0001; PROT ALKP: p = 0.00166; PROT ACP: p = 0.000247). In addition, in *P. ulyssiponensis* a significant increase in the amount of proteins extracted from foot tissue for the alkaline and acid phosphatase analyses was assessed, with a significant decrease of this parameter in N2 with respect to San Pietro.

With respect to glycogen content, the GLM analysis revealed a significant effect of the factor "Site", which indicates that this energy-related endpoint varies similarly among the different pH sites, regardless of the species considered (GLY viscera: SP: p = 0.00847, N2: p = 0.01216; N3: p = <0.0001; GLY foot: p N3 = 0.020473) (Table S5). Regarding the factor "Species", *P. rustica* occurred to be significantly different from *P. caerulea* for both GLY quantification (GLY viscera: p = <0.0001; GLY foot: p = <0.0001), while *P. ulyssiponensis* only regarding GLY content measured in foot tissue (p = 0.0444). Indeed, there's an evident increasing trend in GLY content along the pH gradient of the Castello Aragonese vent systems (Fig. 3.2). In particular, in *P. caerulea* GLY content occurred to be significantly higher in the extreme acidified site N3 compared to the ambient pH sites, specifically with N1 in viscera tissue and with San Pietro in foot tissue, following the trend observed for the morphometric characteristics. In *P. rustica,* organisms collected from N3 showed higher level of GLY than those registered from N1 and N2 in both tissues, notwithstanding that in both cases, no significant differences were detected between San Pietro and N3. Conversely, no alteration of GLY content was observed in *P. ulyssiponensis*.

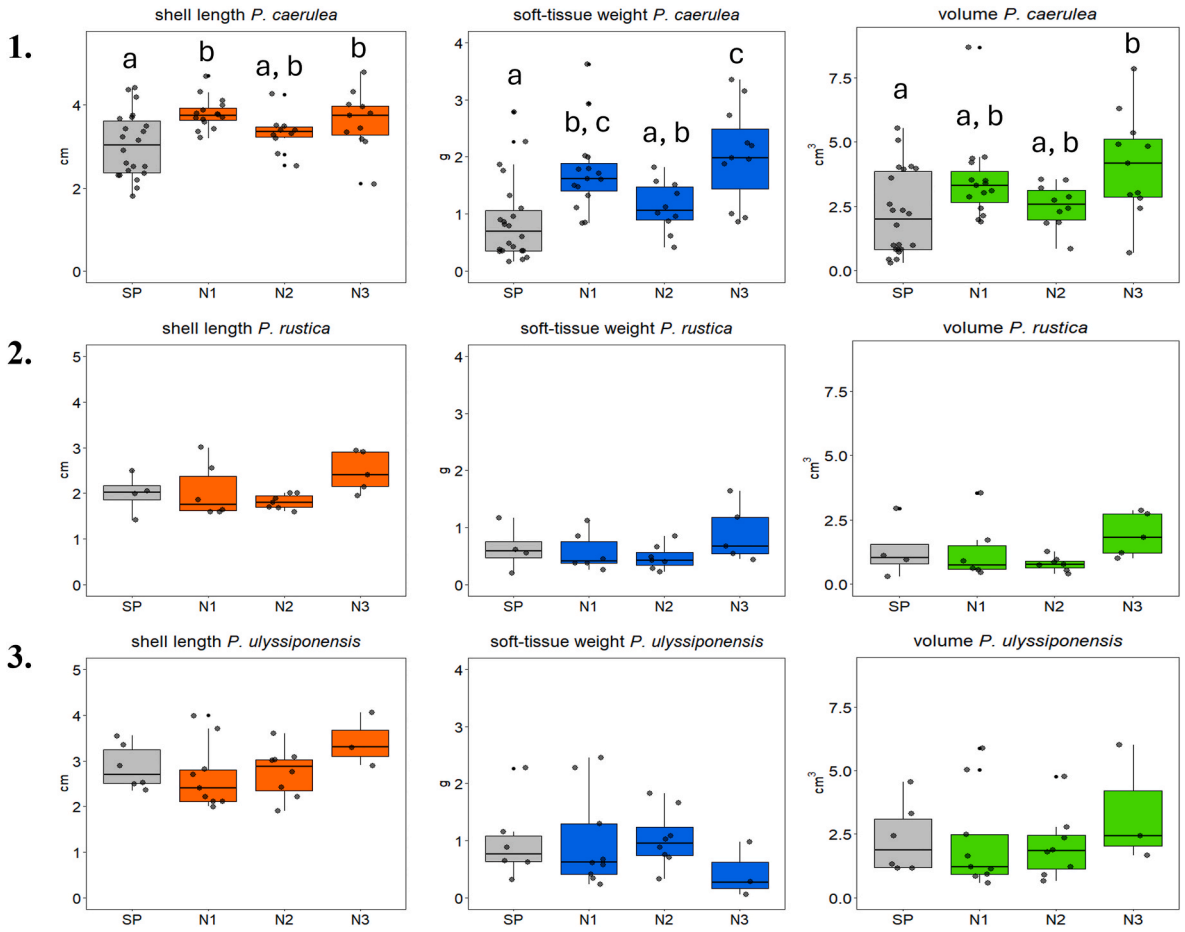

Fig. 2. Boxplots of shell length, soft-tissue weight and volume of *P. caerulea* (1), *P. rustica* (2) and *P. ulyssiponensis* (3) collected from the three different sites of the Castello Aragonese vent systems (N1 – N2 – N3) and the ambient pH site San Pietro (SP). The number of individuals used for each species and site is reported in Table S3. Dots indicate independent biological replicates inside each site. Different letters indicate statistically significant differences among the different sites (*p*-Value ≤0.05).

3.1.3. Oxidative stress and damage

The GLM analysis revealed that *P. rustica* occurred to be significantly different from *P. caerulea* for all oxidative stress parameters according to the factor "Species" (ROS: $p = <0.0001$; SOD: $p = <0.0001$; CAT: $p = <0.0001$; GSTs: $p = 0.000204$; LPO: $p = 0.000204$), while the factor "Site" appeared to be significant only for ROS content, with regards to N1 and N3 ($p = 0.0497$) (Table S5).

In particular, a general increasing trend in the total content of ROS was observed in the very low pH site N3 of the vent in all three species, even though it was not significant in *P. caerulea* and in *P. rustica*. On the contrary, *P. ulyssiponensis* displayed a significant increase in ROS content in all the sites inside the vent area compared to the external ambient pH site San Pietro (Fig. 4.1).

The SOD activity was significantly decreased in N3 with respect to San Pietro, whereas *P. caerulea* and *P. ulyssiponensis* didn't significantly modulate their activities (Fig. 4.2).

An opposite trend was obtained for CAT activity, which was modulated only in *P. rustica*, with a significant decrease in the extreme acidified site N3 in comparison with all the other sites analyzed, while for the other species no differences were detected (Fig. 4.3).

GSTs activity didn't differ among the different sites in any of the three species taken into consideration (Fig. S4).

Similar considerations can be taken regarding oxidative damage, the levels of LPO did not differ among the three limpet species across the various pH sites examined (Fig. S5).

3.1.4. Biomineralization

Neither *P. caerulea* nor *P. ulyssiponensis* displayed significant variations in biomineralization endpoints, while *P. rustica* showed a significant increase only in ALKP activity in the extreme acidified site N3 with respect to the other two sites inside the Castello Aragonese vent systems (Figs. 5.1 and 2). Overall, the GLM analysis highlighted no significant effects of factor "Site", whereas regarding factor "Species", in both cases *P. ulyssiponensis* occurred to be significantly different from *P. caerulea* (ALKP: $p = 0.00821$; ACP: $p = 0.000422$), while *P. rustica* only for ALKP activity ($p = <0.0001$) (Table S5).

3.1.5. Neurotoxicity

Solely the AChE activity measured in the viscera of *P. rustica* resulted to be significantly modulated among the different sites considered (Fig. 6.1). Specifically, AChE activity significantly decreased in limpets collected from N3 with respect to N1 and San Pietro. A similar trend can be detected also in the foot tissue, even though it was not significant (Fig. 6.2). GLM analysis revealed a significant effect of factor "Species", indicating that AChE activities significantly vary between *P. caerulea* and *P. rustica* regardless of the "Site" (AChE viscera: $p = <0.0001$; AChE foot: $p = <0.0001$) (Table S5). Similarly, also AChE activity measured in the foot in *P. ulyssiponensis* is significantly different from *P. caerulea* for the factor "Species" ($p = 0.00924$).

3.1.6. Non-Metric Multidimensional Scaling of biochemical endpoints

The NMDS analysis carried out on the biochemical endpoints highlighted a clear separation in the response to OA in the three different

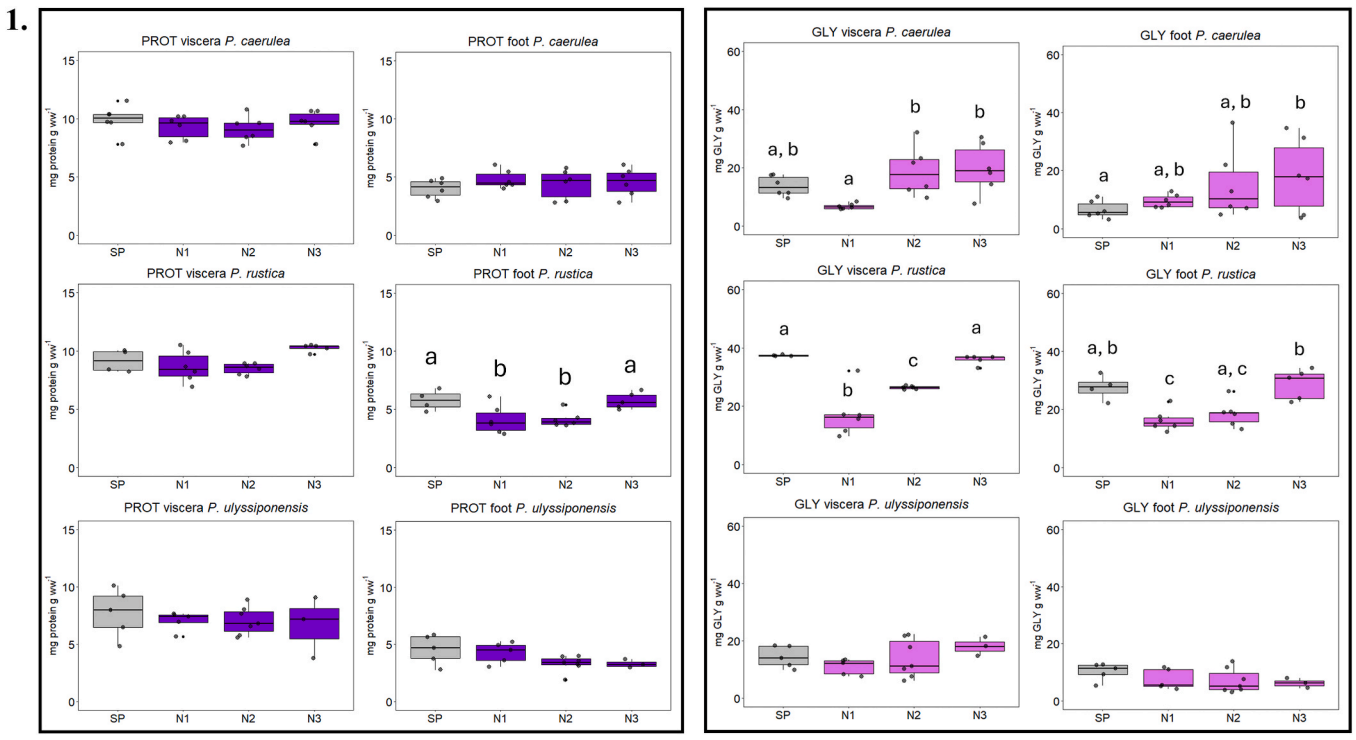

Fig. 3. Boxplots of protein (PROT) (1) and glycogen (GLY) (2) content in viscera and foot tissues of *P. caerulea*, *P. rustica* and *P. ulyssiponensis* collected from the three different sites of the Castello Aragonese vent systems (N1 – N2 – N3) and the ambient pH site San Pietro (SP). Dots indicate independent biological replicates inside each site. The number of individuals used for each species and site is reported in Table S3. Different letters indicate statistically significant differences among the different sites (p-Value $\leq$0.05).

limpet species ("Species"). Nevertheless, no clear clusterization was observed in relation to the different pH sites ("Site"), potentially due to high biological inter-individual variability (Fig. 7.1). The direction of the arrows indicates that most of the biochemical endpoints are modulated in the species *P. rustica* and only partially in *P. caerulea* (Fig. 7.2).

3.2. Effects of short-term exposure to OA of P. caerulea and energy-related endpoints of biofilm coverage

At the end of the transplant experiment, all sites displayed the same mortality (about 24.9 %), with no significant differences among the different pH conditions.

Concerning energy metabolism, a significant decrease in protein content obtained from viscera tissue was assessed in the limpets transplanted from ambient pH to very low pH (N3) conditions ($p = 0.00251$), while GLY content extracted from the same tissue, significantly increased in N3 ($p = 0.0042$) (Fig. 8.1, Table S6).

The short-term exposure to ocean acidification induced an imbalance of the oxidative status without entailing an increase in reactive oxygen species production. All antioxidant and detoxification enzymes displayed the same trend with an increase in their activities in the intermediate pH site N2 with respect to N1 and N3, notwithstanding it was significant only for SOD ($p = 0.00624$) and GSTs ($p = 0.00304$), but not for CAT ($p = 0.220$) (Fig. 8.2, Table S6).

With respect to biomineralization endpoints, only ACP displayed a significant increase in specimens of *P. caerulea* transplanted from ambient pH condition to the very low pH site N3 ($p = 0.00178$), while no significant differences were assessed for ALKP activities ($p = 0.973$) (Fig. 8.3, Table S6).

Finally, the analysis of AChE in viscera tissue revealed a significant reduction in the activity between the ambient pH site N1 and the extremely acidified site N3 ($p = 0.00282$) (Fig. 8.4).

With respect to biochemical analysis carried out on biofilm, a significant increase in PROT content was observed only in the extreme acidified site N3 with respect to the other sites involved ($p = 0.00432$), while GLY content remained stable across different pH conditions ($p = 0.9898$) (Fig. S6, Table S7).

4. Discussion

When exposed to altered environmental conditions that deviate from their equilibrium, organisms are able to modulate their responses through acclimatization or adaptation and depending on the changing environment. Acclimatization represents a short-term strategy that mainly occurs through phenotypic plasticity, and it provides resilience to environmental changes. In the long-term and under specific conditions, genetic adaptation or phenotypic accommodation might occur (Calosi et al., 2013; Lombardi et al., 2015; Foo and Byrne, 2016; Rodríguez-Romero et al., 2021). In view of this, we investigated the biochemical performances either of native limpet populations inhabiting the naturally acidified environment of the Castello Aragonese vent systems and of organisms subjected to a 30-day *in situ* transplant, to better investigate the potential mechanisms that may underlie the tolerance to OA and the ability to colonize naturally acidified sites.

4.1. Growth and energy reserves

Among the studied species, only *P. caerulea* exhibited more marked variations in size across the different pH sites, with a significant increase in shell length, soft-tissue weight and volume in the extreme acidified site N3 compared to the ambient pH sites. Samplings were conducted randomly, collecting specimens of heterogeneous dimensions, representative of the entire range of limpet populations observed in the field; consequently, the results are unlikely to be attributed to a bias. This result is in accordance with previous observations on *P. caerulea* and *P. rustica* from the CO_2 vent systems of Vulcano Island (Duquette et al., 2017). The fact that these differences were not significant in our study may be attributed to the reduced number of specimens collected at each

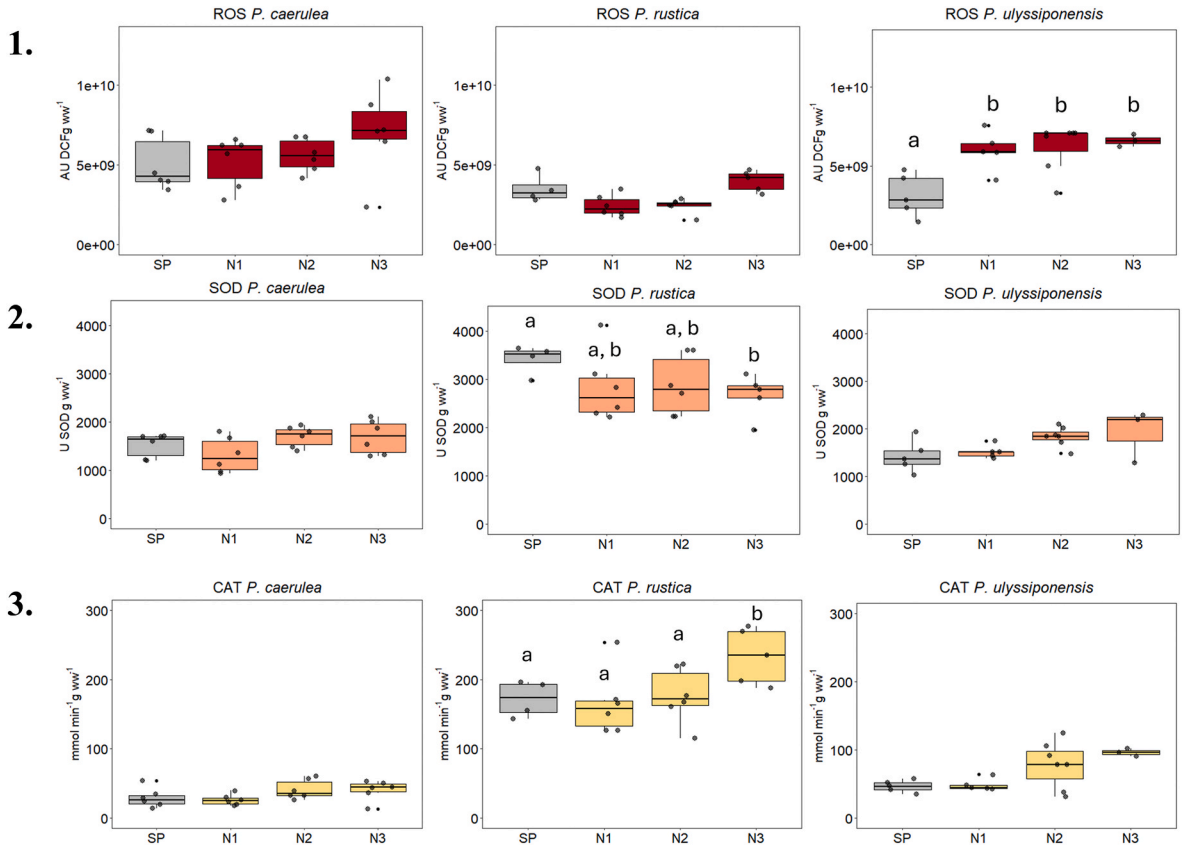

Fig. 4. Boxplots of reactive oxygen species content (ROS) (1), superoxide dismutase (SOD) (2) and catalase (CAT) (3) activities measured in *P. caerulea*, *P. rustica* and *P. ulyssiponensis* collected from the three different sites of the Castello Aragonese vent systems (N1 – N2 – N3) and the ambient pH site San Pietro (SP). Dots indicate independent biological replicates inside each site. The number of individuals used for each species and site is reported in Table S3. Different letters indicate statistically significant differences among the different sites (*p*-Value ≤0.05).

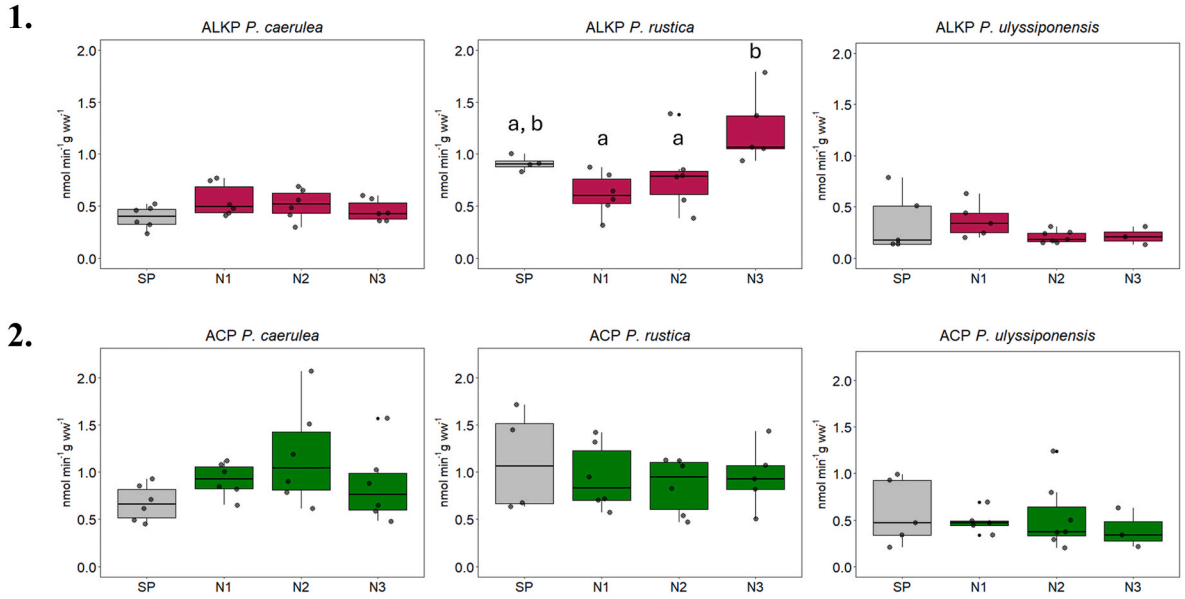

Fig. 5. Boxplots of alkaline (ALKP) (1) and acid (ACP) (2) phosphatase activities measured in *P. caerulea*, *P. rustica* and *P. ulyssiponensis* collected from the three different sites of the Castello Aragonese vent systems (N1 – N2 – N3) and the ambient pH site San Pietro (SP). Dots indicate independent biological replicates inside each site. The number of individuals used for each species and site is reported in Table S3. Different letters indicate statistically significant differences among the different sites (*p*-Value ≤0.05).

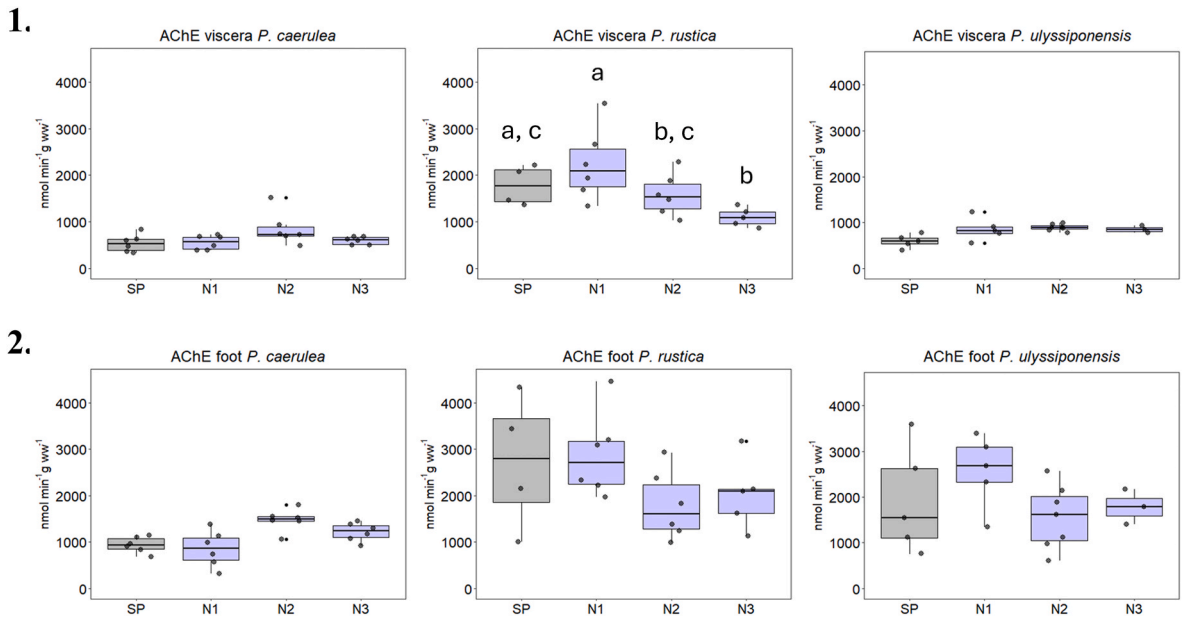

Fig. 6. Boxplots of acetylcholinesterase activity (AChE) measured in viscera (1) and foot (2) tissues in *P. caerulea*, *P. rustica* and *P. ulyssiponensis* collected from the three different sites of the Castello Aragonese vent systems (N1 – N2 – N3) and the ambient pH site San Pietro (SP). Dots indicate independent biological replicates inside each site. The number of individuals used for each species and site is reported in Table S3. Different letters indicate statistically significant differences among the different sites (p-Value $\leq$0.05).

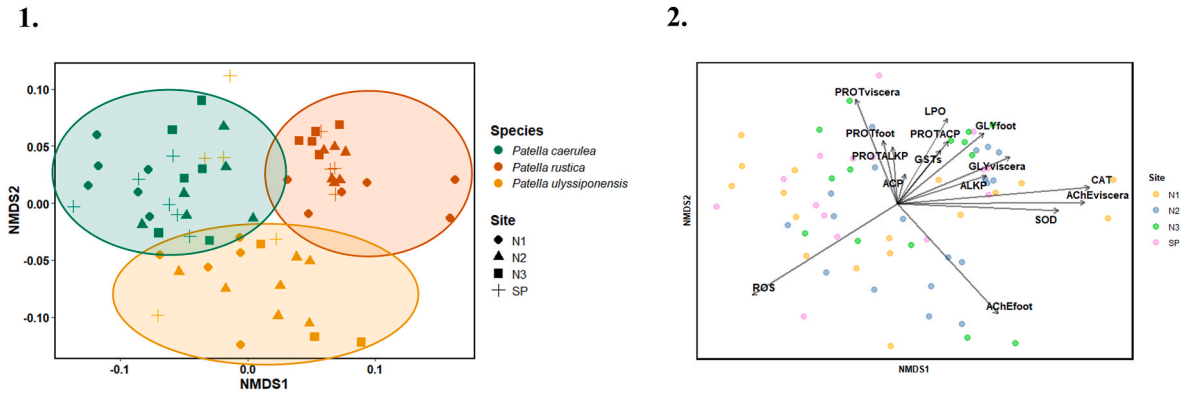

Fig. 7. Non-Metric Multidimensional Scaling analysis (NMDS) integrating all biochemical endpoints (PROT viscera, PROT foot, PROT ALKP, PROT ACP, GLY viscera, GLY foot, ROS, CAT, SOD, GSTs, LPO, AChE viscera, GLY foot, ALKP, ACP) of the three different species (*P. caerulea*, *P. rustica*, *P. ulyssiponensis*) sampled in the four different sites (SP – N1 – N2 – N3). Each point in the plot represents an individual limpet.

site, which was performed in order not to overly affect the natural populations.

The increasing sizes observed in limpets collected in extreme acidified conditions can be related either to an alteration of biotic relationship (predation and interspecific competition) or to a different nutrient availability and quality inside the acidified areas of the Castello vent.

Limpets are preyed upon a wide range of predators, including birds, fish, starfish, octopuses, decapods, and other gastropods (Lowell, 1986; Silva et al., 2008; Tyler et al., 2014). Notably, many of these predators are almost absent or display lower abundance in low pH areas, as evidenced by several studies conducted at the Castello Aragonese and in other similar vent systems (Kroeker et al., 2011; Garrard et al., 2014; Vizzini et al., 2017). With a top-down effect, limpets may be exposed to reduced predatory pressure, which could allow them to potentially survive and increase their body size. Furthermore, limpets are herbivorous grazers and most of their competitors are calcifying organisms as well, like trochids and sea urchins, which present lower abundance in the extremely acidified site of the Castello vent systems, similarly to their predators. Indeed, several manipulative experiments carried out in the field highlighted that increasing competitive interactions among herbivorous induced higher mortality and a reduction in shell growth and tissue mass (Branch, 1986; Silva et al., 2008). Overall, these findings support the idea that OA may act as a driving force in shaping the composition and dynamics of invertebrate communities (Hall-Spencer et al., 2008; Fabricius et al., 2011; Foo and Byrne, 2017).

Additionally, the increasing observed body size could be potentially related to a different food availability and quality and therefore to the increasing glycogen and protein contents observed in acidified conditions in both *P. caerulea* and *P. rustica* with respect to both ambient pH sites (San Pietro and N1). Moreover, these results are supported by the significant increasing protein content observed in the biofilm collected from N3. Multiple studies support the hypothesis that an increase in food supply represents a key driving factor for greater abundance of various invertebrate taxa. Indeed, patellid limpets are herbivorous grazers that feed on epilithic microbial biofilm associated with macroalgal species and algal propagules (Garrard et al., 2014; Burgos-Rubio et al., 2015). Primary producers, especially fleshy and non-calcareous algae, may thrive under OA, resulting in increased biomass and higher nutritional

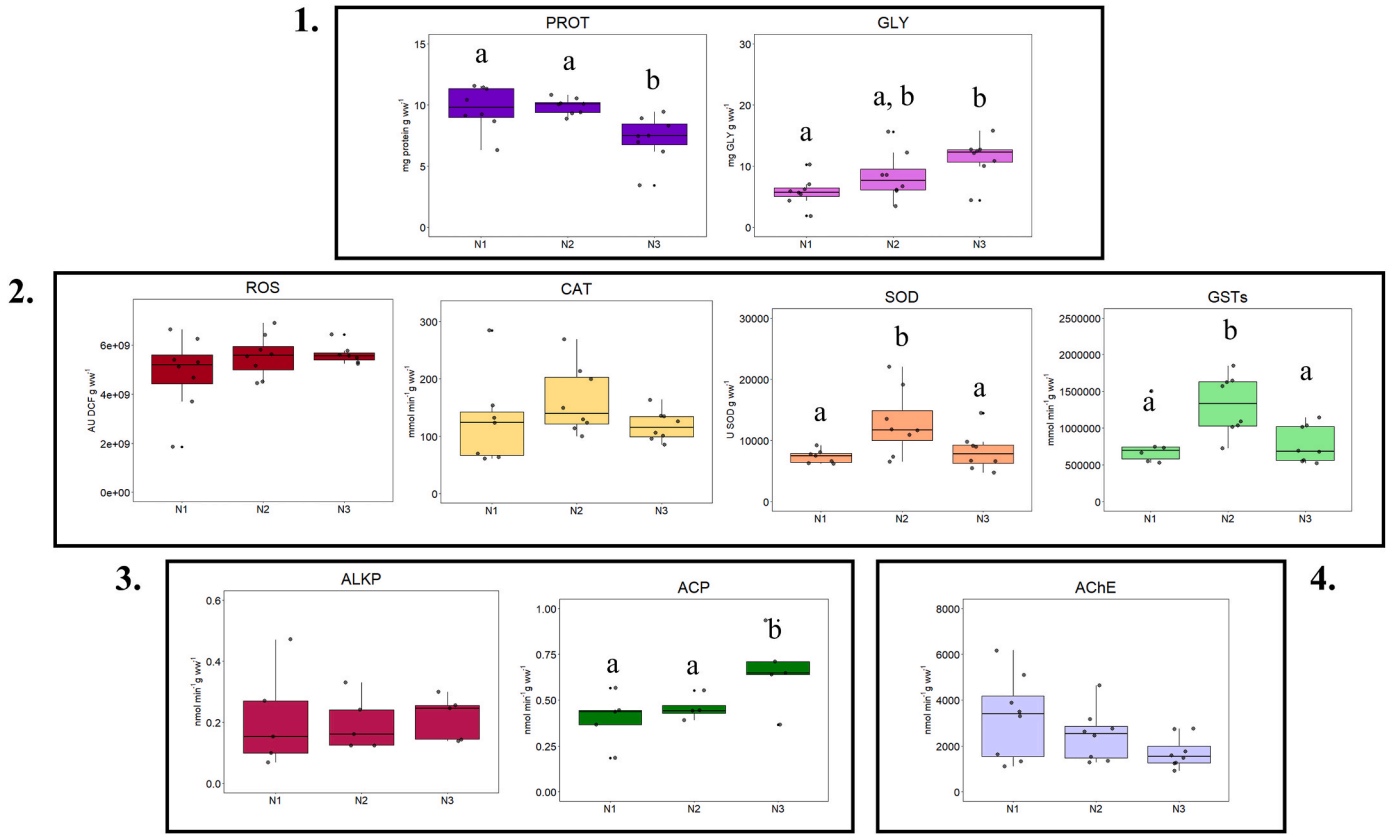

Fig. 8. Boxplots of energy metabolism (1), oxidative stress (2), biomineralization (3) and neurotoxicity (4) measured in *P. caerulea* transplanted from ambient pH condition to the three different pH sites of the Castello Aragonese vent systems (N1 – N2 – N3). Dots indicate independent biological replicates inside each site (N = 8). Different letters indicate statistically significant differences among the different sites (*p*-Value ≤0.05).

quality, with direct and indirect effects on the entire ecosystem (Melzner et al., 2011; Lidbury et al., 2012; Asnaghi et al., 2013; Thomsen et al., 2013; Johnson et al., 2015; Bergstrom et al., 2019; Leung et al., 2022). Thus, OA may enhance biomass and potentially nutritional quality of biofilm coverage in N3 enabling grazers, like limpets, to increase their body size and energy reserves.

Besides, significant increases in glycogen and protein contents were detected in *P. caerulea* and *P. rustica* collected from acidified conditions, which might represent a strategy to preserve more energy resources to counteract the negative effects of OA. A similar trend was observed in individuals of *P. caerulea* transplanted to the acidified site N3 of the Castello Aragonese vent systems, even though the variation was not significant. Energy is assimilated from nutrients or food intake and it is stored as reserves in form of lipids, proteins and carbohydrates (Sokolova et al., 2012; Koyama et al., 2020). The ability to preserve adequate levels of energy resources and to flexibly allocate them between metabolic processes facing environmental stressors, such as OA, represents a key element in maintaining life-history traits, distribution and abundance both locally and globally (Sokolova et al., 2012; Calosi et al., 2013). Indeed, the effects of OA are extremely extensive and diverse, involving physiological functions, metabolic pathways, behavior, and reproduction. These processes require greater amount of energy to maintain homeostasis or to recover under stressful conditions (Lannig et al., 2010; Sokolova et al., 2012; Leung et al., 2022; Simonetti et al., 2022). Based on this evidence, we would have expected a significant reduction in energy resources endpoints. Thus, the observed increase in GLY content under acidified conditions, may represent the organisms' ability to maintain a balance between energy demand and supply, representing a potential tolerance and/or adaptive strategy to cope with the bioenergetic shifts imposed by OA (Calosi et al., 2013).

This result is partially in contrast with various research conducted on other calcifying species, such as *Crassostrea virginica*, *Mizuhopecten yessoensis*, *Rochia nilotica*, and *Mytilus galloprovincialis*, where GLY displayed a decreasing trend in hypercapnic conditions (Dickinson et al., 2012; Liao et al., 2019; Zhang et al., 2021; Signorini et al., 2024). However, most of these studies were carried out in laboratory conditions, without considering the whole complexity of the environment and the interactions with biotic and abiotic factors. Moreover, energy homeostasis and tolerance limit may present a significant species specificity, highly dependent on the morphological, physiological, molecular, and genetic aspects of the species. For instance, specimens of *M. galloprovincialis* collected from the same CO_2 vent systems revealed opposite results to those obtained in this study for *Patella* species. Indeed, the first one is a filter-feeder species, while the second ones are herbivorous grazers. Therefore, as mentioned above, a different food strategy could be accountable for the observed differences and strategies developed by these species to oppose OA.

Similar considerations can be drawn for protein content, which significantly increased in *P. rustica* specimens and in the biofilm collected at the very low pH site N3, with respect to the ambient pH site of the vent. Proteins are an additional source of energy reserves available for organisms to face environmental challenging conditions, representing a tolerance strategy, comparably to the glycogen content trend observed in *P. caerulea* and *P. rustica* (Cruz et al., 2016; Garrard et al., 2022; Zhang et al., 2022). On the other hand, during the short-term exposure to OA of *P. caerulea*, protein content occurred to be significantly reduced in very low pH conditions, suggesting the hypothesis of an induction of protein catabolism to preserve more energy resources. Similarly, hypercapnic conditions led to an alteration of protein synthesis and degradation in different mollusks, polychaetes, and echinoderms (reviewed by Simonetti et al., 2022). This strategy may be effective in the short-term, enabling organisms to buffer acidified

conditions. However, in the long-term this strategy might entail significant cost on metabolism, growth and reproduction, and therefore it may be more advantageous to preserve and enhance their content, as observed in resident limpets inhabiting the Castello Aragonese vent systems for multiple generations (Calosi et al., 2013).

It should be noted that some of the significant differences concerning size and energy related endpoints in N3 are related to the external ambient pH San Pietro besides the internal ambient pH site N1. Additionally, several parameters significantly differed from the two ambient pH sites considered (San Pietro and N1). These findings could be potentially related to a different hydrodynamic exposure and nutrient availability of San Pietro as well as to its closer proximity to the harbor, which may have influenced the biological responses (Della Torre et al., 2024). Further analyses would be necessary to deeper investigate this aspect.

4.2. Cellular stress

Among the native limpet species exposed for long-term to OA, only *P. rustica* significantly induced CAT activity at the very low pH site (N3), with respect to ambient pH ones (San Pietro and N1). This antioxidant enzyme converts H_2O_2 into water and oxygen, thus it might have been activated to limit oxidative damage and prevent cascading effects on metabolism, physiology, and growth. Indeed, the lack of increased ROS production and the absence of lipid peroxidation in *P. rustica* could be attributed to the activation of the antioxidant system. Nevertheless, GSTs, another key component of detoxification pathway, did not exhibit any significant variation in its activity. Similar results related to oxidative status were obtained in many other marine invertebrates, both calcifying and non, when exposed to low pH conditions. For example the polychaetes *Diopatra neapolitana* and *Hediste diversicolor* significantly increased SOD, CAT and GSTs activities and induced LPO when exposed to OA (pH = 7.8, 7.3) (Freitas et al., 2015, 2016); the oyster *Magallana gigas* up-regulated the expression of proteins involved in oxidative response (pH = 7.6) (Cao et al., 2018); the copepod *Tigriopus japonicus* significantly increased ROS content and GSTs and glutathione reductase (GR) activities after an exposition to low pH conditions (pH = 7.5) (Lee et al., 2019) and similarly the mussel *Mytilus coruscus* exhibited a significant induction of SOD, CAT and GPx activities (pH = 7.3) (Huang et al., 2018).

However, antioxidant endpoints were modulated only in *P. rustica* and not in *P. caerulea* or *P. ulyssiponensis*. This pattern, also evident in the NMDS plot, where most biochemical endpoints clustered around *P. rustica* (Fig. 7.2), may be related to the different position that these limpet species occupy on the rocky intertidal shore. While *P. caerulea* and *P. ulyssiponensis* predominantly inhabit the mid-lower midlittoral zones, *P. rustica* settles higher on the shore, from the upper midlittoral to the supralittoral zone. This positional difference may have exposed *P. rustica* to more extreme conditions related to tidal fluctuations and air exposure, in addition to OA (Garrity, 1984; Mortnesen and Dunphy, 2016; Stickle et al., 2017; Sun et al., 2023). These multiple stressors may explain the intensified antioxidant responses observed in this limpet species.

In contrast to the long-term exposure to OA, the 30-day transplant experiment conducted on *P. caerulea* revealed an activation of the antioxidant and detoxification systems, particularly through increased SOD and GSTs activities at the intermediate pH site (N2). These specimens transplanted from ambient to low pH conditions activated oxidative stress enzymes to potentially buffer the effects of OA.

A similar 30-day *in situ* transplant experiment carried out at the Castello Aragonese vent systems on the fanworm *Sabella spallanzanii*, revealed an impairment of the overall capacity to neutralize hydroxyl radicals, with significant modulations of CAT and GPx activities, suggesting potential vulnerability to near-future OA conditions (Ricevuto et al., 2016). The same research group conducted a comparable 30-day field experiment on different polychaete species (*Platynereis dumerilii*, *Polyophthalmus pictus*, and *Syllis prolifera*) from the same vent systems. Results revealed a species-specific modulation of antioxidant capacity, with a significant reduction in the ability to neutralize oxyradicals mainly in *P. pictus* (Ricevuto et al., 2015).

Furthermore, the species *P. rustica* displayed an induction of alkaline phosphatase (ALKP) activity in the extremely acidified site N3 of the vent with respect to N1 and N2. ALKP is a multifunction protein, but, due to its primary role in biomineralization processes, this result could be indicative of a potential compensation mechanism to buffer very low pH conditions, notwithstanding that no significant differences were detected either in the other two species, or in *P. caerulea* transplanted to the vent area (Moreira et al., 2016; Zhang et al., 2018). Similar findings were already detected in other calcifying species exposed to hypercapnic conditions (Moreira et al., 2016; Huang et al., 2018). However, additional analyses would be necessary to further investigate the potential effects of OA on calcification and biomineralization processes at the molecular level.

Finally, similar conclusions can be drawn for acetylcholinesterase (AChE) activity. Notably, among the native limpets, only *P. rustica* exhibited a significant reduction in AChE activity along the pH gradient. Given AChE's pivotal role in neurotransmission, its inhibition can disrupt the normal breakdown of acetylcholine, leading to its excessive accumulation at cholinergic synapses and neuromuscular junctions. This imbalance may cause an impairment of signal transmission, potentially causing muscular dysfunction, reduced mobility, and behavioral alterations. Over time, this disruption could reduce the organisms' ability to cope with environmental stressors, forage effectively, or escape predators, ultimately impacting survival and fitness under ocean acidification conditions (Deidda et al., 2021; Xing et al., 2021; Daniel et al., 2022; Zaidi et al., 2022). However, further analyses would be necessary to deeper characterize this aspect and its potential consequences at organism and population levels.

Alternatively, the inhibition of AChE may reflect the reduction of acetylcholine and of its precursor choline, not considered in this study, but already assessed in *M. galloprovincialis* collected at the same CO_2 vent systems. Indeed, choline displays an important physiological role, being involved in osmotic balance, which represents one of the main targets of OA (Ramaglia et al., 2018). We might hypothesize that this metabolite may have been down-regulated under OA in limpets, subsequently impairing the activity of AChE; however, no data are available at the moment to either confirm or reject this hypothesis. Similar findings were assessed also in the gill tissue of the oyster *Magallana gigas* exposed to pH 7.6 (Cao et al., 2019), in the flatfish *Solea senegalensis* (see Sampaio et al., 2018) and in the bivalve *Latona cuneata* exposed to hypercapnic conditions (Priya et al., 2016).

5. Conclusion

Our results indicate that, individuals of *P. caerulea* and *P. ulyssiponensis* exposed for multiple generations to the acidified conditions of the Castello Aragonese vent systems have developed tolerance strategies that enable them to withstand these harsh conditions without significantly altering their biochemical parameters, whereas a modulation of biochemical endpoints was observed in *P. rustica*. Furthermore, *P. caerulea* and, to a lesser extent *P. rustica*, exhibited increased size and energy-related endpoints in extremely acidified conditions. These results may derive from higher quality and/or quantity of algal availability or reduced competition and predatory pressure. Based on this hypothesis, these findings highlight the need to further investigate energy metabolism aspects, which could help to better understand the compensatory mechanisms underlying tolerance to OA. In the short-term, OA triggered compensatory mechanisms primarily linked to oxidative stress and neurotoxicity.

Overall, these results highlight the importance of conducting research in naturally acidified environments, as it enables a more comprehensive understanding of the whole habitat complexity,

including species interactions and abiotic factors, as well as the development of potential tolerance and/or adaptive strategies. This research contributes to formulate more realistic hypotheses about the ability of species to persist in the future conditions of anthropic oceans.

CRediT authorship contribution statement

Silvia Giorgia Signorini: Writing – original draft, Investigation, Formal analysis, Data curation. **Marco Munari:** Writing – review & editing, Resources, Methodology, Conceptualization. **Fabio Crocetta:** Writing – review & editing, Validation. **Isabella Moro:** Writing – review & editing, Investigation. **Ilaria D'Aniello:** Formal analysis. **Lara Nigro:** Formal analysis. **Fiorenza Micheli:** Formal analysis, Resources, Writing – review & editing. **Camilla Della Torre:** Writing – original draft, Supervision, Funding acquisition, 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.2025.121874.

Data availability

Data will be made available on request.

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