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Living under natural conditions of ocean acidification entails energy expenditure and oxidative stress in a mussel species

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ABSTRACT

We investigated the health conditions of the Mediterranean mussel *Mytilus galloprovincialis* recruited in the CO_2 vents system of Castello Aragonese at Ischia Island (Mediterranean Sea). Individuals of *M. galloprovincialis* were sampled in three sites along the pH gradient (8.10, 7.7 and up to <7.4). Untargeted metabolomics and biochemical endpoints related to energetic metabolism, oxidative stress/damage, neurotoxicity and immune defense were analyzed. Corrosion of the valves occurred at low pH. A separation of the metabolome was observed along the pH gradient. Metabolites belonging to amino acids, nucleosides, lipids and organic osmolytes were significantly reduced in the organisms from the most acidified sites. The content of reactive oxygen species and the activity of glutathione peroxidase were reduced in organisms from the acidified sites compared to ambient pH, and no oxidative damage was induced. Overall results suggested the presence of an energy cost underpinning long-term survival in acidified conditions for this species.

1. Introduction

One of the most relevant factors that contribute to global climate change is the increasing atmospheric carbon dioxide concentration (CO_2), which has risen from 278 to 421 ppm after the industrial revolution, because of anthropogenic activities (NOAA-GML-ESRL, 2024). The global oceans, acting as a sinkhole and mitigator, have absorbed approximately 31 % of CO_2 emissions, notwithstanding that it entailed relevant modifications of seawater carbonate chemistry and pH level, leading to the phenomenon known as ocean acidification (OA) (Gattuso et al., 2015; Vargas et al., 2022). Estimates based on the Intergovernmental Panel on Climate Change (IPCC) suggest that atmospheric CO_2 levels may reach 800 ppm by the end of the century (2100), which could correspond to a reduction of the global ocean pH level of 0.30–0.32, that will decrease from the actual level of 8.1 to 7.8 (for the Representative Concentration Pathway RCP 8.5) (Liao et al., 2019; Findlay and Turley, 2021).

Ocean acidification alters biodiversity, trophic interactions and other ecosystem processes, even though the biological effects of OA are not uniform among different marine organisms (Vargas et al., 2022; Teixidó et al., 2024). Acid-base homeostasis, respiration/gas exchange, signaling mechanisms, digestion, energy metabolism, physiology, immune response, behavior and calcification processes, represent some of the mechanisms that can be negatively impacted by OA in different marine taxa, with consequences on growth rates, survival and reproductive success (Lannig et al., 2010; Range et al., 2014; Bressan et al., 2014; Wang et al., 2016; Gambi et al., 2016; Munari et al., 2016, 2018, 2019, 2020a, 2020b; Khan et al., 2020; Melzner et al., 2020; Zhao et al.,

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2020; Palombo et al., 2023; Shang et al., 2023).

In particular, marine organisms producing calcareous structures (shells, exoskeletons and calcareous endoskeletons), such as for instance mollusks, corals and echinoderms, are known to be sensitive to the detrimental effects of OA (Kroeker et al., 2013; Duarte et al., 2015; Thomsen et al., 2017; Rajan et al., 2021; Medeiros and Souza, 2023). Indeed biomineralization, which represents a key physiological process in defining sensitivity to OA and pH thresholds of organisms, is extremely expensive from an energetical point of view. Therefore in an acidified environment, organisms may have a shift in the energy budget in order to counteract the dissolution and erosion of calcifying structures (Lannig et al., 2010; Melzner et al., 2020).

However, a great variability in the biological responses to OA emerges from literature, even among closely related bivalve species, probably due to species-specific pH-threshold/sensitivity and to different experimental laboratory conditions (i.e. time of exposure, life stage, pH conditions) (Thomsen et al., 2015; Duarte et al., 2015; Zhao et al., 2017; Shang et al., 2023). Moreover, most experiments were performed under controlled laboratory conditions, exposing organisms from ambient pH sites to acidified conditions for a limited period of time. Although these studies are important for understanding the susceptibility of organisms to OA, they are not able to predict potential acclimation and/or adaptation processes, which allow some species to persist under low pH conditions.

On the other hand, investigations conducted in naturally acidified systems, such as hydrothermal vents, characterized mainly by CO_2 emissions, offer a unique opportunity to study *in situ* the effects of OA on calcifiers, and to investigate their different responses and the development of local adaptations. Earlier studies from these naturally acidified systems mainly focused on biomineralization (Rodolfo-Metalpa et al., 2011), size (Garilli et al., 2015; Aliende et al., 2023), and feeding (Connell et al., 2017). Nonetheless, the actual molecular and physiological mechanisms underpinning the response to OA and their role in the potential tolerance and acclimation/adaptation to this phenomenon, are still largely unknown. In particular, research carried out in the CO_2 vents system of the Castello Aragonese at Ischia (Mediterranean Sea, Italy) highlighted a reduction in the diversity, biomass and trophic complexity of benthic marine communities in the low pH conditions (form 7.8 to <7.4) (Foo et al., 2018; Teixidó et al., 2018), mainly due to the absence/reduction of calcifying organisms, such as calcareous algae, gastropods, decapods and echinoderms (Kroeker et al., 2011, 2013; Ricevuto et al., 2012; Vizzini et al., 2017). However, few calcifiers are still able to cope and survive in the most acidified areas of the vents, such as spirorbid polychaetes (Lucey et al., 2015), limpets (Hall-Spencer et al., 2008; Aliende et al., 2023), sea urchins (Kroeker et al., 2013), as well as juveniles of the Mediterranean mussel *Mytilus galloprovincialis* (Ricevuto et al., 2012). In particular, *M. galloprovincialis*, quite common in artificial collectors as a juvenile stage (max 3 mm length) even in the most acidified zones of the vents (Cigliano et al., 2010; Ricevuto et al., 2012), is missing as an adult (Hall-Spencer et al., 2008; Kroeker et al., 2011). However, in June 2021 specimens of *M. galloprovincialis* were detected for the first time in the Castello Aragonese vents system in adult stage (approx. 2–3 cm size).

Accordingly, we took the opportunity to collect *M. galloprovincialis* adults in the Castello vents, settled likely after a successful recruitment event, in order to investigate different metabolic and biochemical endpoints aiming at assessing the health conditions of mussels naturally exposed to OA. On these organisms we performed untargeted metabolomics, which is a high throughput technique that allows the quantification of metabolites synthesized by organisms and it provides information on their metabolic conditions, enabling a better understanding of the early effects of environmental disturbances and their physiological responses (Sinclair et al., 2019). Furthermore, OA is a well-known prooxidant agent, which may represent a source of oxidative stress that entails production of additional reactive oxygen species and a higher energetic consumption and metabolic rate (Munari et al., 2018; Valvassori et al., 2019). Therefore, different endpoints related to oxidative stress and damage, energy metabolism, neurotoxicity and immune defense were analyzed, to evaluate the initial molecular response of organisms to this environmental disturbance.

The main strength and novelty of the current project lies in the use of mussel populations inhabiting a natural system representative of future marine acidified scenario. Our approach allows one to gain insights into the different responses that organisms display to survive under natural low pH conditions, as well as their potential acclimation, by combining traditional biochemical techniques with metabolomics. This approach allows to acquire information that can be extrapolated to projected natural scenarios, enhancing our understanding of processes that enable biodiversity to persist under altered environmental conditions.

2. Materials and methods

2.1. Sites and sampling

Mytilus galloprovincialis (Lamarck, 1819) specimens (approx. 2–3 cm length) were collected from intertidal rocky shores along the southern pH gradient of the Castello Aragonese CO_2 vents systems (40°43′57.9″ N, 13°57′51.8″ E), with the permission of the Marine Protected Area 'Regno di Nettuno' (Ischia), in June 2021. In the Mediterranean Sea, this species releases gametes in late winter and early spring (Seed, 1976). Therefore, the sampling period was chosen to eliminate any bias related to spawning. Mussels were collected in three sampling sites located on the south side of the Castello vents, characterized by different pH conditions, in detail: the ambient pH site (pH ~ 8.0, hereafter 'S1'), the site with moderate CO_2 emissions (pH ~ 7.7, hereafter 'S2') and the site characterized by high venting activity (pH < 7.4, hereafter 'S3'). Several previous studies have intensively characterized the physico-chemical conditions of this vents system, avoiding the interaction of low pH/elevated pCO_2 with other environmental factors, such as for instance salinity, temperature (both similar to the ambient, not acidified seawaters), and absence of toxic sulfur compounds (Foo et al., 2018). In particular, both the two acidified sites, the S3 (intense venting and extreme low pH mean values) and S2 (moderate venting and low pH values) showed high pH fluctuations both in space (Kerrison et al., 2011) and time on a daily base (Hofmann et al., 2011, Kroeker et al., 2011; Teixidó et al., 2018, 2024). Therefore, one should consider that organisms living in these sites are exposed not only to mean low pH values, but also to high fluctuations of this parameter and OA conditions in general. Twelve individuals per site were collected by snorkeling on the rocky intertidal zone. Samples were stored at −80 °C to perform metabolomics and biochemical assays.

2.2. Shell surface erosion

Valves of *M. galloprovincialis* individuals were measured and photographed in order to determine the proportion of shell surface erosion and to assess the percentage of specimens with eroded valves on the total number in each site. Images were elaborated through ImageJ software, selecting and cutting out the borders of the eroded part and dividing this area by the total area of the shell. Data are expressed as the ratio between the eroded part and the total area of the valve (Fig. S1).

2.3. Untargeted metabolomics

The metabolomic analysis was carried out at the Unitech OMICs platform, the mass spectrometry facility of the University of Milano (Milan, Italy). The metabolites were extracted from the lyophilized tissue of the digestive gland of 3 individuals per each site of the Castello vents (S1 – S2 – S3). Digestive gland was analyzed because it represents one of the main site of intracellular digestion and energy storage in mussels (Shang et al., 2023). Each sample was first pulverized through a pestle and then added with 500 μL of ice-cold methanol:ethanol solution

(1:1, *v*/v), vortexed for 30 s, shaken for 90 min at 37 °C and finally centrifuged at 10,000 rpm for 10 min at 4 °C. Pellet was discarded and the supernatant was evaporated under a stream of nitrogen. The residue of each sample was finally resuspended in 50 μL of mobile phase (formic acid 0.1 %/acetonitrile), shaken, centrifuged, filtered and diluted again in ratio 1:2. In parallel, a pooled sample was prepared mixing 10 μL from each sample and a blank was also processed substituting water for the lyophilized sample.

All samples have been analyzed using an ExionLC™ AD system connected to TripleTOF™ 6600 System equipped with Turbo V™ Ion Source with ESI Probe (SCIEX, MA, USA). Chromatographic separation was achieved on CORTECS UPLC T3 C18–2.1 × 150 mm × 1.6 μm (Waters®) using a two mobile phases made of formic acid 0.1 % and acetonitrile with formic acid 0.1 %, respectively. MS spectra were collected over a *m/z* range of 50–1200 Da in positive and negative polarity, operating in IDA® mode. Data were analyzed using SCIEX OS 1.4 software (SCIEX™), implemented with two functions: FormulaFinder and LibraryView™ ver. 1.0. Each sample was injected in duplicate and the average value of the corresponding areas was reported and normalized for the mg of the extracted sample. The metabolite identifications (ID) were obtained based on the value of m/z achieved and determined in high resolution mode. Statistical analysis was performed using MetaboAnalyst ver. 5.0. applying one-way ANOVA, Partial Least Squares - Discriminant Analysis (PLS-DA) and hierarchical clustering. The PLS regression is performed using the plsr function provided by R pls package. Hierarchical clustering is performed with the hclust function in package stat.

2.4. Biomarker analysis

Proteins and glycogen content (GLY), antioxidant and detoxification enzymes activities (superoxide dismutase SOD, catalase CAT, glutathione-S-transeferases GSTs, glutathione peroxidase GPx) and tissue levels of reactive oxygen species (ROS), were measured in the gills from six individuals of *M. galloprovincialis*, following the methods described by Della Torre et al. (2017) and Morosetti et al. (2020). Gills were chosen since they represent the first interface between the organism and the external environment, being therefore more susceptible to environmental changes and more prone to oxidative stress (Khan et al., 2021). Further biochemical analysis related to energy metabolism (electron transport system activity, ETS), neurotoxicity (measurement of acetylcholinesterase activity, AChE), immune defense (measurement of acid phosphatase activity, ACID P) and oxidative damage (measurement of lipid peroxidation, LPO) were carried out on the soft tissues of six individuals. Detailed description of all procedures is reported in supplementary materials.

2.5. Statistical analysis

To test differences among mussels collected along the pH gradient concerning i) shell surface erosion rates, ii) activities of antioxidant and detoxification enzymes (ROS, GPx, CAT, SOD, GSTs), iii) level of oxidative damage (LPO), iv) energy-related endpoints (GLY, ETS, Protein), v) neurotoxicity (AChE), and vi) immune defense (ACID P), a Linear Model was used, considering the site of origin of the mussels as single fixed factor (three levels corresponding to S1, S2 and S3 sites). The analysis was carried out using "lm()" and "anova()" functions form the base package of the R software, version 4.2.3 (https://www.R-project.org/) (Logan, 2010). This method was chosen since the assumption of data normality was tested by the Shapiro-Wilk test for each experimental condition and most of the resulting *p*-values led to the rejection of the normality hypothesis ($p \leq 0.05$). Furthermore, it is well-suited for analyzing complex, non-normally distributed data, and allows us to determine the significance of the relationships between predictor variables and response variables. The anova() function returns an ANOVA table, allowing us to visualize the *p*-Value associated with the significance of the categorical predictor variable (i.e., the site of origin of mussels) on the response variables of interest. Only results with *p*-Value ≤0.05 were considered statistically significant. Additionally, we calculated effect size as Eta squared (η^2). The Principal Component Analysis (PCA) was carried out to integrate and visualize the results of the whole biomarker dataset, including energy metabolism, oxidative stress, neurotoxicity and immune defense endpoints. This analysis was carried out using the function "prcomp" from the "FactoMineR" package of the R software.

3. Results

3.1. Effects on growth and on shell surface erosion

The size of individuals of *M. galloprovincialis* was similar in all the three sites sampled. Specifically, the mean shell length was 2.529 ± 0.160 cm in S1, 2.783 ± 0.194 cm in S2 and 2.700 ± 0.341 cm in S3.

The graph in Fig. 1A shows a significant increase in both the portion of eroded part of the valves of mussels and in the incidence of organisms that displayed shell surface erosion along the pH gradient ($p = 0.0005$; $F = 9.257$). In particular, 54 % of the individuals sampled in S1 showed surface erosion in their valves; on the contrary, S2 entailed shell erosion in the 93 % of organisms and S3 in the 100 % of sampled organisms (Fig. 1B Fig. S1).

3.2. Effects on the metabolome

A distinct separation in the profile of metabolites in *M. galloprovincialis* sampled along the pH gradient of the Castello vents has been detected, as shown in Fig. 2A. In Fig. 2B it is represented the result of the hierarchical cluster analysis, where it is possible to detect the metabolites that were up or down-regulated among the three different groups/stations. The majority of metabolites appeared to be down-regulated from S1 to S2 and S3.

The One-way ANOVA analysis showed a significant modification in the level of 26 known and 2 unidentified metabolites (Table 1; Fig. S2). Regarding the known metabolites, multiple amino acids, nucleosides, lipids and organic osmolytes were significantly modulated in the three different groups. Specifically, all metabolites appeared down-regulated following the pH decrease, except for the nucleoside adenosine, which exhibited a significant increase in its content in S2 and S3 in comparison with S1.

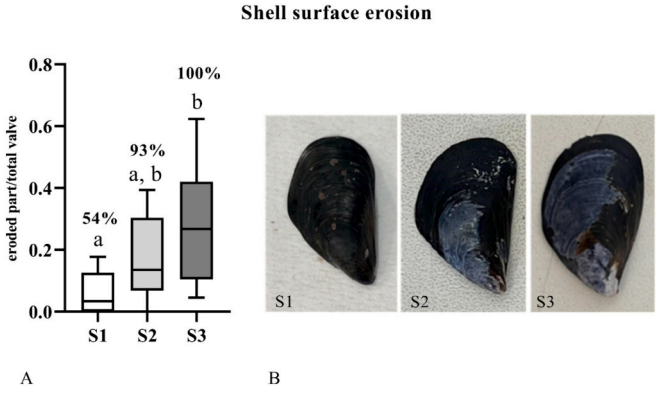

Fig. 1. Mean ± standard deviations (S.D.) ($N = 12$) of the ratio between the eroded area and the total area of the valve in individuals sampled from the three sites, S1 – S2 – S3. Percentages reported above bars represent the occurrence of erosion features in mussels for each site of the vent. Different letters mean statistically significant differences among the three different groups (*p*-Value ≤0.05) (A). Increasing of shell erosion features in the valves of three individuals of *M. galloprovincialis* collected from the sites of the vent, S1 - S2 - S3 (B).

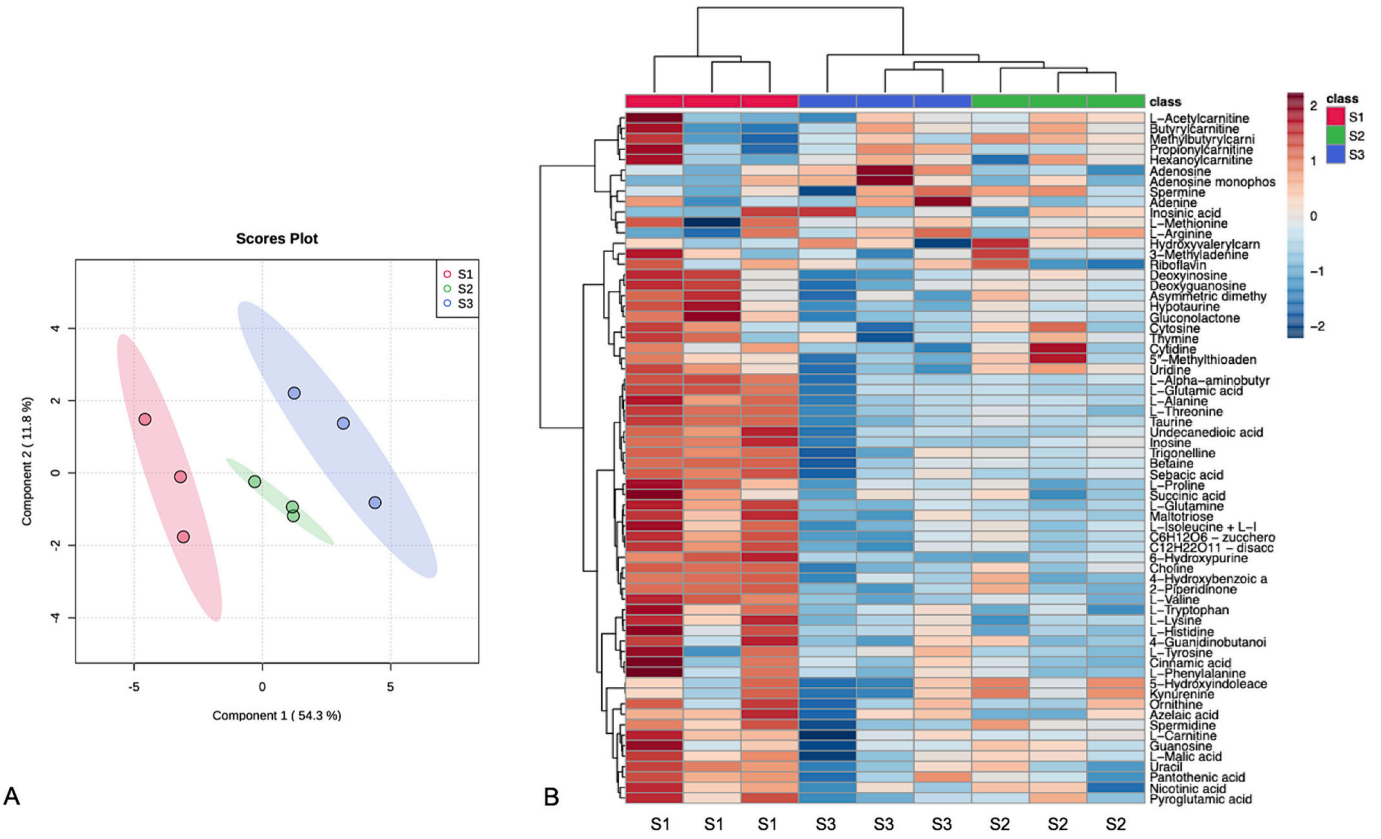

Fig. 2. Scores plot between selected PCs, the explained variances of the components are shown in brackets (A). Clustering results shown as heatmap (distance measure using Euclidean and clustering algorithm using ward.D) (B).

3.3. Effects on oxidative stress and damage, neurotoxicity and immune defense

The total content of ROS exhibited a significant decrease in S3 ($p = 0.05$; $F = 4.145$; η^2 0.479) (Fig. 3A). A similar trend has been observed for the activity of GPx, which displayed a significant reduction between S1 and S2 ($p = 0.0276$; $F = 5.494$, η^2 0.558) (Fig. 3B). Moreover, CAT showed a decreasing trend in activity as well, even though not significant, due to the high inter-individual variability in S1 and S2 (Fig. 3C, Table S3). Finally, no differences were detected for SOD and GSTs activities related to the OA gradient within the vents system (Fig. 3D, E, Table S3).

Concerning oxidative damage, the level of lipid peroxidation did not differ among organisms from the three groups/stations (Fig. 3F, Table S3).

AChE activity, an index of neurotoxicity, was not significantly affected by OA (Fig. 3G, Table S3). Similarly, the activity of ACID P, associated with immune defense, did not show any significant effect (Fig. 3H, Table S3).

3.4. Effects on energy metabolism

Regarding energy metabolism endpoints, GLY content was significantly reduced in mussels sampled in S3 in comparisons with S1 ($p = 0.0339$; $F = 5.049$, η^2 0.492) (Fig. 4A). Conversely, ETS activity and the protein content did not show significant differences among sites (Fig. 4B,C, Table S3).

3.5. Principal component analysis of biochemical endpoints

The PCA plot revealed a clustering of three different sites, with the ambient pH site S1 more separated from the acidified sites S2 and S3 (Fig. 5), notwithstanding that high dispersion of data is observed, due to high biological inter-individual variability.

4. Discussion

In this study, we provided for the first time the metabolic and biochemical modifications occurring in individuals of *M. galloprovincialis* naturally exposed to high pCO_2/low pH conditions at the Castello Aragonese vents system. The fact that this species, that would be normally considered vulnerable to ocean acidification, is able to inhabit this environment, gives the opportunity to better investigate the mechanisms underpinning tolerance and acclimation/adaptation to OA and to evaluate potential trade-offs between resistance and growth/development. However, as stated before, this species was never observed as an adult (2–3 cm size) in the most acidified areas of the Castello vents before June 2021 (Foo et al., 2018), but only as a recruit in artificial structures (Cigliano et al., 2010; Ricevuto et al., 2012). Therefore, we hypothesize that a strong and successful recruitment event occurred and allowed some of the most resistant individuals of *M. galloprovincialis* to survive and grow under low pH conditions. In May 2023, in fact, the mussels were no more observed in S3 and were reduced to few individuals in S2 (Gambi M.C., personal observations), suggesting a threshold to long-term exposure to OA, which remain critical for this species. Such threshold represents a time physiological limit of an organism to be able to cope and resist to extreme low and low pH conditions, and in the Castello vents have been observed also for other organisms, such as the polychaete *Sabella spallanzani* (Turner et al., 2015; Ricevuto et al., 2016).

4.1. Effects on metabolic pathways

OA causes a primary direct effect on organisms with calcareous

Table 1
List of metabolites significantly modulated in individuals of *M. galloprovincialis* sampled from the pH gradient of the Castello vents (S1 – S2 – S3).

Metabolite	f-Value	*p*-Value	Fisher's LSD
Amino acids			
L-Valine	34.99	0.0005	S1 – S2; S1 – S3
L-Threonine	28.03	0.0009	S1 – S2; S1 – S3
L-Glutamine	25.31	0.0012	S1 – S2; S1 – S3
L-Alpha-aminobutyric acid	24.99	0.0012	S1 – S2; S1 – S3
L-Glutamic acid	22.31	0.0017	S1 – S2; S1 – S3
L-Alanine	20.20	0.0021	S1 – S2; S1 – S3
L-Proline	9.32	0.0145	S1 – S2; S1 – S3
L-Lysine	9.19	0.0148	S1 – S2; S1 – S3
L-Isoleucine + L-Isoleucine	8.77	0.0166	S1 – S2; S1 – S3
L-Tryptophan	8.25	0.0191	S1 – S2; S1 – S3
Nucleosides			
6-Hydroxypurine	27.28	0.0010	S1 – S2; S1 – S3
Inosine	23.75	0.0014	S1 – S2; S1 – S3
Uridine	13.61	0.0059	S1 – S2; S1 – S3
Adenosine	8.16	0.0195	S1 – S2; S1 – S3
Deoxyinosine	7.95	0.0204	S1 – S2; S1 – S3
Sugars			
C12H22O11 - disaccharide	18.43	0.0028	S1 – S2; S1 – S3
C6H12O6 – monosaccharide	15.58	0.0042	S1 – S2; S1 – S3
Maltotriose	10.11	0.0120	S1 – S2; S1 – S3
Gluconolactone	11.30	0.0093	S1 – S2; S1 – S3
Lipids			
Undecanedioic acid	15.28	0.0045	S1 – S2; S1 – S3
Sebacic acid	10.18	0.0117	S1 – S2; S1 – S3
Osmolytes			
Choline	22.90	0.0015	S1 – S2; S1 – S3
Betaine	13.29	0.0063	S1 – S2; S1 – S3
Hypotaurine	10.81	0.0102	S1 – S2; S1 – S3
Taurine	43.07	0.0003	S1 – S2; S1 – S3
Trigonelline	8.88	0.0162	S1 – S2; S1 – S3
Miscellanea			
2-Piperidinone	10.90	0.0100	S1 – S2; S1 – S3
Spermidine	8.20	0.0191	S1 – S2; S1 – S3

structures, such as calcareous algae, mollusks, crustaceans and echinoderms. This is due to the fact that high pCO_2/low pH conditions lead to an imbalance of physiological functions as homeostasis and acid/base regulation and reduction in calcification rates, ultimately impacting organisms' growth and fitness (Simonetti et al., 2022). Furthermore, calcification is an extremely expensive mechanism, that requires additional energy expenditure in the maintenance and in order to counteract the dissolution of calcifying structures under OA conditions (Lannig et al., 2010). Although mussels display a periostracum in the outer shell layer, which better protects them from corrosion (Rodolfo-Metalpa et al., 2011), this study revealed a significant increase in both the eroded portion of the valve and in the occurrence of individuals of *M. galloprovincialis* displaying erosion features, in the acidified sites of the Castello Aragonese vents system (S2 - S3). In line with our results, significant shell dissolution and loss of periostracum was observed in *M. galloprovincialis* subjected to reduced pH (−0.3 units) for 10 months (Gazeau et al., 2014). Similar effects were also highlighted by Rodolfo-Metalpa et al. (2011) in mussels transplanted for 5 months at the Castello Aragonese vents and in other mollusk species, thriving in the acidified areas of the same vents system, such as *Phorcus turbinatus* (Hall-Spencer et al., 2008), *Patella* spp. (Rodolfo-Metalpa et al., 2011; Aliende et al., 2023), and *Columbella rustica* (Garrard et al., 2014).

These results could reveal the potential presence of an important energy expenditure to maintain elevated calcification to oppose shell dissolution, which has the potential to make their valves more fragile and therefore more vulnerable to mechanical damage and predation and also to pathogens infection (Lannig et al., 2010; Henry et al., 2020).

Overall, OA induced alterations in the profile of several metabolites in *M. galloprovincialis* along the pH gradient, suggesting that hypercapnia might interfere with different metabolic pathways, as already stated in other calcifying species, such as *Mytilus coruscus*, *Patinopecten yessoensis* and *Pocillopora damicornis* (Shang et al., 2023; Liao et al., 2019; Sogin et al., 2016). Among modified metabolites, organic osmolytes such as taurine, betaine and its precursor choline, were significantly reduced in the acidified sites, confirming that OA may induce disturbances in the osmotic balance of *M. galloprovincialis*. These metabolites in fact display important physiological roles in the osmotic regulation of different marine invertebrates (Liu et al., 2010), since their accumulation inside cells enables a major water retention, which could help to better counteract the detrimental effects of OA (Ramaglia et al., 2018).

A significant reduction in some lipids and sugars content, which represent primary energy sources, was detected in the intermediate and in the extreme low pH sites of the Castello Aragonese vents system. Due to their role in energy storage and lipid metabolism, their decrease may be indicative of active consumption of these resources to cope with OA, suggesting the hypothesis that energy supply might not have compensated energy demands of mussels (Shang et al., 2023). These findings may point out a deleterious impact of OA on *M. galloprovincialis* individuals living in acidified conditions, since the ability to preserve sufficient energy resources when exposed to environmental stressors represents the species' capability to maintain its distribution and abundance (Lannig et al., 2010). In line with our observations, a significant decrease of protein and carbohydrate content was observed also in *M. galloprovincialis* kept at pH 7.63 for 17 days, supporting the findings that OA impacts on these important metabolic parameters, with potential adverse consequences on growth and survival of local population (Belivermiş et al., 2023). Indeed, Swezey et al. (2020) found that low lipid concentrations were correlated with a significant increase in mortality and vulnerability in the red abalone *Haliotis rufescens* exposed to high CO_2 concentrations, suggesting that lipid metabolism could play a critical role in influencing vulnerability to OA.

According to these findings, a significant reduction of taurine, its precursor hypotaurine, and of trigonelline was detected in the acidified sites of the vent. These metabolites are abundant in several seaweeds and in phytoplankton, with important anti-inflammatory and antioxidant functions (Terriente-Palacios and Castellari, 2022; McNabney et al., 2023). A decrease in their content might be correlated with a different food availability inside the vent area and/or with differences in assimilation processes, similarly to what has already been observed in the polychaete species *Platynereis* spp. (Signorini et al., 2023), since these organisms are filter-feeders and herbivorous, respectively. An analogous reduction in taurine was observed also by Shang et al. (2023) in *Mytilus coruscus* exposed to reduced pH conditions (7.7). Accordingly, Navarro et al. (2013) pointed out a significant reduction of clearance rate in *Mytilus chilensis* exposed to different high pCO_2 conditions, resulting in lower food absorption rate and efficiency. These findings suggest that OA can inhibit the clearance and absorption efficiency of mussels supporting the hypothesis that energy demand is not completely compensated by energy supply in these organisms, limiting the resources available for physiological and metabolic processes and potentially constraining them to deplete their lipid and carbohydrate reserves, as observed in the present work (Gu et al., 2019; Tang et al., 2022). This finding is further supported by the results of a recent study, in which a significant decrease of filtration rate was observed in *M. galloprovincialis* subjected to low pH (7.80, 7.50) for 80 days (Sezer et al., 2020).

Furthermore, a significant reduction in alanine content and a significant increase in adenosine level in low pH sites were observed. These findings better support the hypothesis of the presence of an energy cost underpinning resistance in acidified conditions. In particular, decrease in alanine levels may reflect an induction of gluconeogenesis, as already stated by Lannig et al. (2010) in *Crassostrea gigas*, and by Liao et al. (2019) in *Patinopecten yessoensis*, both reared under hypercapnic conditions. Indeed, gluconeogenic pathways entail the conversion of non-carbohydrate substrates, like amino acids, lactate and glycerol, into glucose, one of the main energy resources available for different metabolic pathways (Stark and Kibbey, 2014). This result is consistent with the significant decrease of GLY content observed in *M. galloprovincialis*, suggesting that gluconeogenesis may compensate and replenish

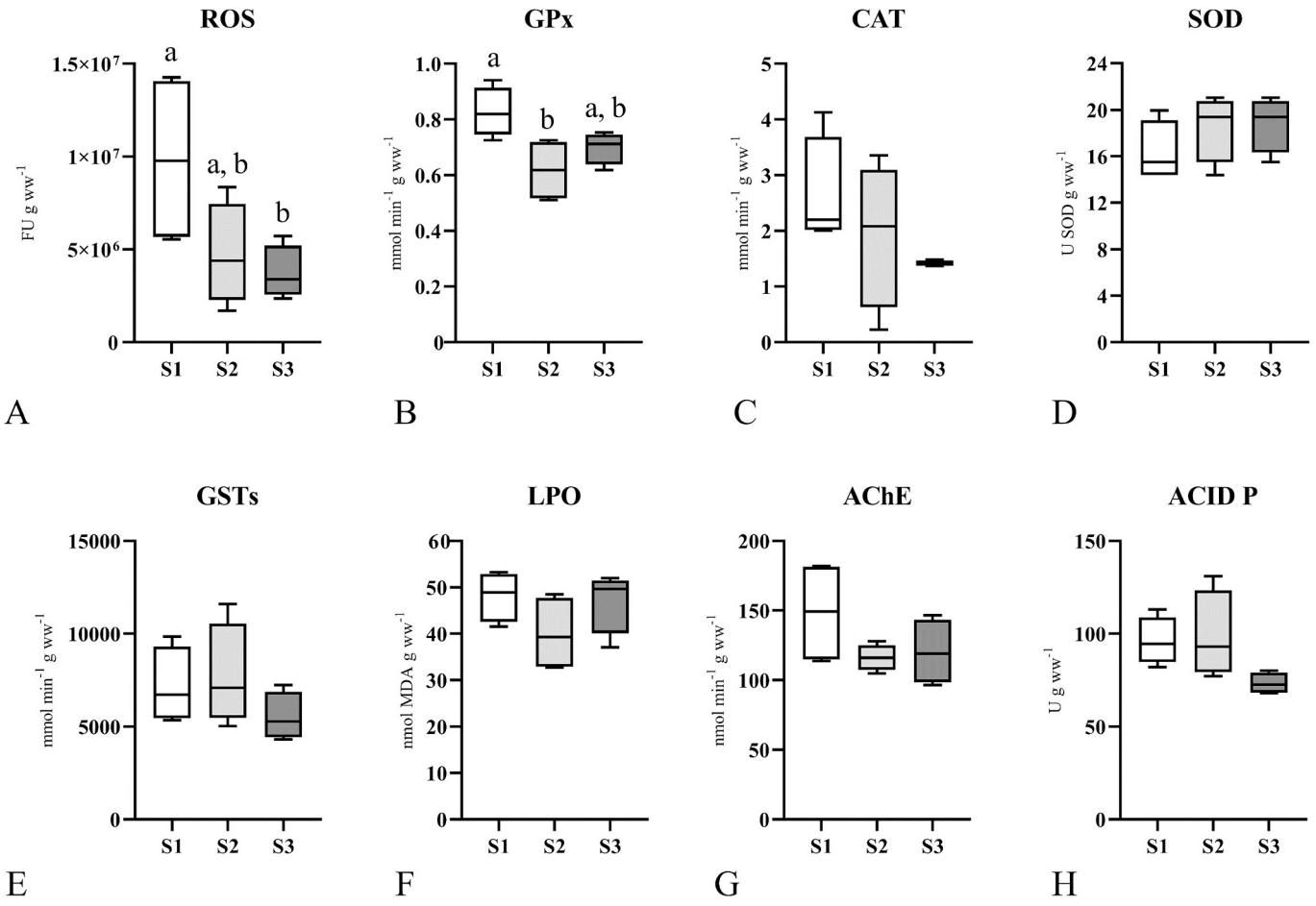

Fig. 3. Basal activities of antioxidant and detoxification enzymes and basal level of oxidative damage. Mean ± standard deviation (S.D.) ($N = 6$) of ROS content (A), of the activity of GPx (B), CAT (C), SOD (D), GSTs (E), level of LPO (F), activity of AChE (G) and ACIPD P (H) measured in *M. galloprovincialis* sampled from three sites S1 – S2 – S3. Different letters mean statistically significant differences among different groups (p-Value ≤0.05).

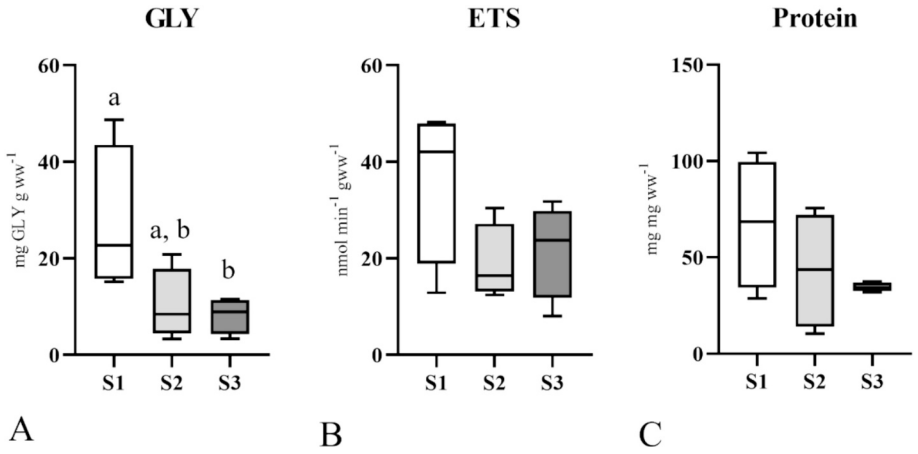

Fig. 4. Basal activity of energy-related endpoints. Mean ± standard deviations (S.D.) (N = 6) of GLY content (A), ETS activity (B) and total protein content (C) measured in *M. galloprovincialis* sampled from three sites S1 – S2 – S3. Different letters mean statistically significant differences among different groups (p-Value ≤0.05).

glycogen consumption (Lannig et al., 2010). In support of the energy expenditure observed, adenosine occurred to be up-regulated in acidified sites. Adenosine is a byproduct of the breakdown of high-energy purine like ATP, and, as a consequence, its increase may be indicative of an active consumption of ATP by these organisms to cope with reduced pH conditions (Borea et al., 2018; Willis et al., 2022). This hypothesis requires further confirmation, since the analysis of Electron Transport Systems, which is correlated with ATP production, did not display any significant variation in different sites. Few studies are present in the literature investigating this mechanism in invertebrates: for example, Willis et al. (2022) observed that adenosine has the potential to induce metabolic depression during periods of energetic stress in the

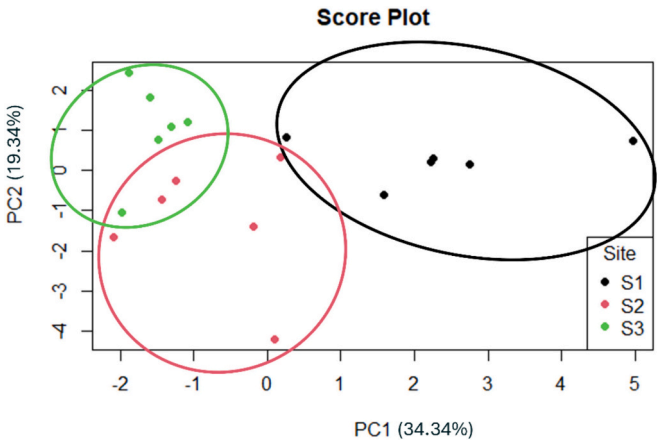

Fig. 5. Plot of principal component analysis integrating all different biochemical endpoints (GLY, ETS, Protein, ROS, GPx, CAT, SOD, GSTs, LPO, AChE, ACID P) measured in *M. galloprovincialis* sampled from three sites S1 – S2 – S3 ($n = 6$). The explained variances of the components are shown in brackets.

giant freshwater prawn *Macrobrachium rosenbergii*. Similar results were assessed in studies with other mollusks species, the oyster *Crassostrea gigas* and the scallop *Patinopecten yessoensis*, in which it was pointed out that reduced pH levels are likely to interfere with their energy metabolism, by significantly decreasing ATP and glycogen levels (Lannig et al., 2010; Liao et al., 2019).

In addition, a significant decrease of several amino acids was detected, according to the pH decrease. Similar to our findings, previous research detected that elevated pCO_2 can strongly affect amphipods, sponges, Antarctic diatoms, Arctic copepods, reef-building corals and mussels, like *Mytilus edulis*, by entailing a reduction in amino acids and protein contents and in the expression of genes involved in protein and energy metabolism (Duarte et al., 2016; Botté et al., 2019; Duncan et al., 2022; Thor et al., 2022; Li et al., 2023). This result might be indicative of inhibition of protein synthesis mechanisms, possibly aiming at preserving energy resources under environmental stressful conditions, since protein synthesis is an ATP-consumption process. Another explanation for amino acids decrease might be due to an induction of cellular protein catabolism, caused by cytosolic and lysosomal proteolysis, aimed at recovering energy to cope with elevated pCO_2, even though protein depletion generally occurs later, acting as a "last resort fuel" as stated by Shang et al. (2023).

Besides, several nucleosides underwent a significant reduction in acidified conditions. This effect was observed also in the polychaete *Platynereis* spp. sampled in the same CO_2 vents system of Ischia (Signorini et al., 2023). Analogous results were assessed by Li et al. (2020) in sea urchin larvae (*Strongylocentrotus intermedius*) reared in acidified seawater, and by Wei et al. (2015) in oysters (*Crassostrea gigas*), where in fact nucleoside metabolism was one of the most affected pathways, making it a potential common mechanism underlying response to OA.

Overall, results support the hypothesis that OA may entail a mobilization and redistribution of energy storage and can cause the metabolic energy demand to exceed the energy accumulated by the organism, probably to maintain elevated biomineralization and acid-base regulations, two mechanisms that represent key physiological processes in defining sensitivity to OA and pH thresholds, and that are extremely expensive from an energetical point of view (Lannig et al., 2010; Melzner et al., 2020). As a consequence, the consumption of other important resources to maintain normal physiological metabolism could occur (Shang et al., 2020).

4.2. Effects on biochemical markers

As seen for the metabolome, a distinct separation of the response of biochemical parameters was observed, suggesting that the oxidative status and metabolic condition of the organisms from the acidified site is different with respect to organisms living at normal pH. Our results are in contrast with the general induction of the antioxidant system that usually occurs in marine invertebrates, especially in calcifying organisms, exposed to acidified conditions. For instance, the polychaete species *Hediste diversicolor* and *Diopatra neapolitana*, exposed to high pCO_2/low pH conditions, enhanced the activity of antioxidant and detoxification enzymes, mainly SOD and GSTs (Freitas et al., 2015, 2016). An induction of GPx was also observed in *M. galloprovincialis* exposed to OA (Hu et al., 2015). Nevertheless, all these studies were carried out under laboratory conditions, performing short-term exposures to OA in organisms that spent their entire life cycle in ambient pH environments. On the contrary, our study was conducted in mussels that have grown inside the CO_2 vent area, in different experimental conditions that could explain the discrepancy observed with respect to the other mentioned studies. This significant decrease observed in oxidative stress parameters could be due to the fact that *M. galloprovincialis* specimens sampled in the CO_2 vent system, might have been so compromised from a metabolic and physiological point of view, that they were not able to trigger the antioxidant machinery to cope with reduced pH conditions, as evidenced by the general disruption of energy endpoints. The lower metabolic rates can entail a decrease in ROS production and consequently in the activities of antioxidant and detoxification enzymes, notwithstanding that further investigations, potentially related to physiological endpoints, would be necessary to validate this hypothesis. In line with this hypothesis, Lesser (2016) observed a significant decrease in HSP70 expression, CAT activity, and protein and glycogen contents in *Mytilus edulis* exposed to thermal stress and elevated CO_2 concentration, suggesting the induction of metabolic depression.

Finally, based on the two enzymatic markers chosen for this study (AChE and ACID P activities), no significant evidence of neurotoxicity and immune impairment seems to occur in *M. galloprovincialis* from the acidified sites of the Castello vents system. Nevertheless, the decreasing trend along the pH gradient observed for AChE, albeit non-significant, parallels the significant reduction of choline content observed in mussels collected in the acidified sites. Since choline is a precursor of acetylcholine, we could hypothesize that a reduction of this osmolite could reflect in a decrease in AChE activity, finally impacting on the cholinergic functions of the organisms. Since AChE is not the only type of cholinesterase present in mussels (Brown et al., 2004), other cholinesterases (butyrylcholinesterase and propionylcholinesterase) could potentially be targeted by OA. Therefore, further investigations are warranted to confirm and support this observation.

5. Conclusion

This study represents the first investigation on the health conditions of individuals of *M. galloprovincialis* early recruited in the CO_2 vents system of Ischia Island, observed for the first time as adults in this vent area, a naturally acidified site representative of the real conditions that may occur in future marine environments, contributing to formulating hypothesis of more realistic future OA scenarios. Heavy erosion of the valves occurred, as expected. Untargeted metabolomics analysis highlighted the detrimental impact of OA on energy metabolism at the cellular level, through the alteration of biosynthesis and metabolism of several metabolites, notwithstanding that no effects were detected on mussel size. A slight imbalance of the antioxidant system was highlighted and the energy-related endpoints supported the results obtained by metabolomic analysis, confirming the presence of an energy cost underpinning survival under acidified conditions, which might compromise growth and fitness of the species in the long-term. This is supported by the observation that, while in artificial structures juveniles

of *Mytilus* were observed often (Cigliano et al., 2010; Ricevuto et al., 2012), adults were only observed in this occasion and in May 2023, they have disappeared from S3 and were reduced to a few individuals in S2, thus suggesting a threshold in the ability of this species to survive under pCO_2/low pH conditions on a long-time.

CRediT authorship contribution statement

Silvia Giorgia Signorini: Writing – original draft, Investigation, Formal analysis, Data curation. **Marco Munari:** Writing – review & editing, Conceptualization. **Lorenzo Federico:** Formal analysis. **Fiorenza Farè:** Writing – review & editing, Data curation. **Manuela Fontana:** Writing – review & editing, Investigation, Formal analysis. **Donatella Caruso:** Writing – review & editing, Data curation. **Rosa Freitas:** Writing – review & editing, Supervision, Resources. **Sofia Paciello:** Formal analysis. **Ilaria D'Aniello:** Formal analysis. **Maria Cristina Gambi:** Writing – review & editing. **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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.116470.

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