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Characterizing antioxidant defense and energy metabolism proxies in a neglected Mediterranean ecosystem engineer: *Sabellaria spinulosa* (Leuckart, 1849)

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ABSTRACT

Littoral environments are highly productive systems that commonly face intense anthropogenic pressures. Polychaete sabellariid (Annelida) reef ecosystems play crucial roles in coastal environments by providing essential habitat, sustaining biodiversity, mitigating erosion and, thus, conveying valuable ecosystem services. Despite their ecological importance, these sedentary organisms and their physiological state are poorly studied, representing a real gap for assessing ecosystem health and resilience. This study aims at providing valuable baseline data by investigating size-related variations in antioxidant defense mechanisms and energy metabolism in the reef-building polychaete *Sabellaria spinulosa* (Leuckart, 1849). Specimens were collected from veneer-type bioconstructions growing along the northern Adriatic coast of Italy (Mediterranean Sea) during spring seasons in a two-year field survey. We analyzed key enzymatic biomarkers in adult specimens of *S. spinulosa* characterized by different sizes. Small-size individuals displayed higher activities of antioxidant enzymes (CAT, GPx, SOD, GSR, and GST) and pyruvate kinase (PK). Conversely, larger specimens exhibited elevated phosphofructokinase (PFK) activity, indicating a metabolism probably more prone to energy storage and stress resilience. The consistency of the observed enzymatic patterns across two sampling years highlights robust, size-dependent physiological differences in *S. spinulosa*. By establishing this clear physiological baseline, our findings underscore the suitability of this species as a sentinel for environmental monitoring. Its widespread distribution allows for large-scale comparative assessments, while its ecological tolerance enables the detection of the sub-lethal stress responses that are crucial for early-warning biomonitoring programs.

1. Introduction

Biomonitoring programs are essential tools for assessing ecosystem health and to evaluate the ecological quality status of marine environment. A key component of these biomonitoring programs is the integration of multiple biomarkers to provide a comprehensive assessment of ecosystem health. Biomonitoring approaches have evolved to incorporate multiple biomarkers, as they provide insights into the biological effects of pollutants on marine communities (Cajaraville et al., 2000; Hochachka and Somero, 2002; Hylland et al., 2008; Livingstone, 1993; Narbonne et al., 2005). These biochemical proxies serve as a crucial connection, serving as early warning indicators of environmental stress and enable the evaluation of organismal and ecological quality status (Dahlhoff, 2004; Dahlhoff, 2002).

Marine organisms have evolved sophisticated cellular defense mechanisms to maintain homeostasis when facing both natural and anthropogenic stressors (Canesi and Viarengo, 1997; Viarengo et al., 1990). Among these, the antioxidant defense system play a crucial role in preventing oxidative damage by neutralizing reactive oxygen species (ROS) through a coordinated network of enzymatic and non-enzymatic components (Benedetti et al., 2021; Lesser, 2006; Regoli and Giuliani, 2014). These defense systems primarily rely on key enzymes, such as

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catalase (CAT) that mediates the decomposition of hydrogen peroxide into water and oxygen, and glutathione peroxidase (GPx) that reduces both hydrogen peroxide and organic peroxides using glutathione as a cofactor. Superoxide dismutase (SOD) represents the first line of organism defense against oxygen free radicals by catalyzing the dismutation of superoxide anion into hydrogen peroxide and oxygen. The defense system is complemented by glutathione S-transferases (GST), which conjugate glutathione with various electrophilic substrates, and glutathione reductase (GSR) regulating the reduced glutathione pool essential for cellular redox homeostasis (Benedetti et al., 2021; Vasseur and Leguille, 2004).

However, a primary challenge in applying these biomarkers is accounting for their natural variability. The enzymatic activities can vary significantly among different taxa and also within species depending on life stage, reproductive status, and environmental conditions (Abele and Puntarulo, 2004). The modulation of the enzymatic activities represents a critical adaptive response, particularly in sessile organisms that cannot escape unfavorable conditions (Viarengo et al., 2007), making them good indicators of the ecological quality of marine ecosystems (Laitano and Fernández-Gimenez, 2016). Furthermore, age- and size-related variations in antioxidant defenses, characterized by decreased antioxidant capacity, suggest complex relationships between life history and oxidative stress resistance (Costantini, 2014). On the other hand, energy metabolism markers, such as phosphofructokinase (PFK) and pyruvate kinase (PK), serve as indicators of metabolic status and energy allocation strategies (Salomon et al., 2000).These markers vary in response to multiple environmental variables and physiological organism states: for example, changes in enzyme activities could reflect adaptation to stress conditions, growth demands and maintenance costs (Dowd et al., 2015; Hochachka and Somero, 2002). Therefore, knowledge of basal physiological conditions in foundation species is mandatory for recognizing the possible effect induced by stressors during biomonitoring (Barrick et al., 2016). This knowledge is commonly lacking for many ecologically important, non-commercial invertebrate species, representing a significant research gap.

At temperate latitudes, some sessile sedentary organisms – despite being understudied in terms of ecotoxicological responses - play a crucial role in structuring and maintaining littoral ecosystem functionality also temporary trapping some pollutants (e.g. Microplastics - da Costa et al., 2021; Lo Bue et al., 2023; Mancin et al., 2022). This is the case of the polychaetes from the genus *Sabellaria* (Lamarck, 1818). *Sabellaria spinulosa* (Leuckart 1849) and *Sabellaria alveolata* (Linnaeus, 1767) are among the most important reef-building species in European temperate waters (Curd et al., 2020; Gravina et al., 2018; Gruet, 1986; Pearce et al., 2014), with their habitats being recognized as conservation priorities under international frameworks such asthe OSPAR Convention and the EU Habitats Directive. As ecosystem engineers, they create complex biogenic structures that enhance biodiversity and provide essential ecosystem services (Bonifazi et al., 2019; Dubois et al., 2005; Dubois et al., 2002; Ventura et al., 2024). During the bioconstruction process, they agglutinate sandy grains taken from the substrate stabilizing littoral sediments along the shoreline, thus protecting the coast from erosion by waves, tides and drift currents (Lisco et al., 2017). In particular, the species *S. spinulosa*, abundantly distributed along both the Adriatic coasts of the Mediterranean and the English coasts of the Atlantic and the North Sea, is characterized by a remarkable ecological adaptability, being able to rapidly colonize both natural and artificial substrates (Firth et al., 2015; Gravina et al., 2018; Lo Bue et al., 2023). It also shows a wider tolerance to varying environmental conditions, such as temperature and depth (Pearce, 2017). Its rapid colonization ability is facilitated by a peculiar reproductive strategy, where the larvae preferentially settle on existing bioconstructions, being attracted by organic compounds secreted during tube-building by already established individuals (Jensen and Morse, 1990; Pawlik, 1986; Wilson, 1970). Moreover, its annual life cycle, with a long spring-summer reproductive period (Lezzi et al., 2015; Wilson, 1970) combined with a lifespan thought to be several years (Pearce, 2017), creates a complex multigenerational reef structure.

The first study recording biochemical markers in the reef-building polychaete *S. alveolata* revealed that the worms from retrograding bioconstructions showed less stress than those from prograding areas, likely due to both reduced competition and better food access (Curd et al., 2019). This could be considered as evidence on how the reef structure could control the organism health status, emphasizing the need to understand their natural physiological condition before interpreting possible responses to environmental stressors. Nonetheless, in situ monitoring and specific analytical techniques are time consuming and very expensive. Many ecotoxicity studies have traditionally focused on commercially important invertebrate species (e.g. mussels and oysters - (Beyer et al., 2017; Buck et al., 2023) as model organisms. This research bias has been further reinforced by the practical demands of biomarker analysis; the spectrophotometric enzymatic assays, while standard laboratory methods, are inherently time-consuming and can be costly in terms of reagents when applied at scale. This commonly leads researchers to focus on established model organisms, creating a fundamental knowledge gap regarding the baseline physiology of key habitat-forming species, which this study aims to close. Without a clear understanding of how natural factors like size influence biomarker activity, it is impossible to distinguish natural variation from a response to environmental stressors.

The primary aim of this work is to provide environmental data for enzymatic biomarkers in *S. spinulosa* across two distinct size classes. By combining antioxidant enzyme profiling and energy metabolism markers in small (< 1 cm) and large (> 1 cm) individuals, we tested the hypothesis that investigated biomarker status differs significantly with organism size. By characterizing the natural variability of these physiological parameters, we aim at establishing reference values and specific patterns in enzymatic responses in our target species.

2. Materials and methods

2.1. Study site, sampling and laboratory preparation

The sampled site (Misano Adriatico: 43°59′05.8"N; 12°41′35.7″E) is located along the northern Adriatic coast of Italy (Fig. 1A). Here, the coast mainly consists of flat, fine-grained sandy beaches, characterized by a semidiurnal and microtidal regime (neap tidal range ± 0.15 m; spring tidal range ± 0.4 m - Harley et al., 2016). Man-made breakwaters placed parallel to the coast protect the shoreline from erosion by waves and littoral drift. In the subtidal environment at about 0.5–1 m depth, veneer-type bioconstructions of *S. spinulosa* grow on the breakwaters, forming several small patches, 30–50 cm wide and 10–100 cm thick.

The adopted analytical protocol followed the plan synthesized in Fig. 1B and D three discrete bioconstruction portions (about 200 g each) were collected from different patches during two distinct sampling events (i.e., 2 April 2023 and 26 March 2024).

In laboratory, the living specimens were carefully extracted from their arenaceous tubes by means of a steel nipper, then they were placed on a glass petri dish for morphometric measurements (total body length) at the stereomicroscope, considering 50 individuals for each sampling event. Only the worms that exhibited a full development of the metameric bodies and of the prostomial palae were isolated during sorting, as these morphological features indicate adult specimens (See Fig. S1 available as Supplementary Materials, for SEM observations on adult specimens). Total body length was selected as the primary size metric for this physiological study. While other parameters such as opercular crown diameter are standard for Sabellariids, our paramount concern was preserving the integrity of our biochemical samples, as enzyme activity is highly susceptible to handling stress and tissue degradation. Body length is a rapid, non-invasive measurement, that minimizes specimen manipulation and the potential for stress-induced artifacts. In contrast, accurately measuring the opercular crown is a more time-

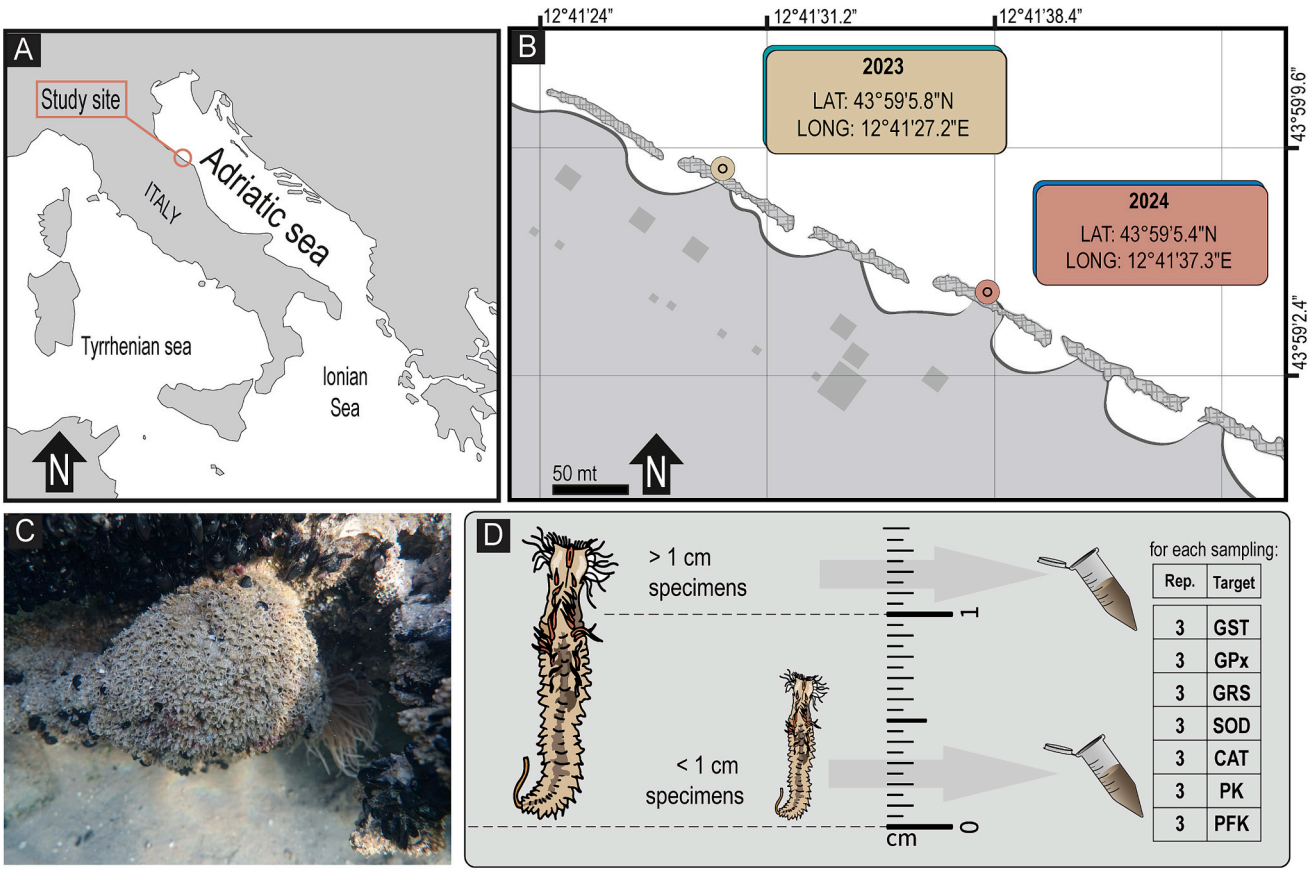

Fig. 1. Study area and experimental design. (A) Location of the study site in the northern Adriatic Sea, Italy. (B) Geographic coordinates of the sampling points for the years 2023 and 2024. (C) Misano Adriatico S. spinulosa bioconstruction during sampling activity. (D) Scheme of the experimental protocol, showing the two size classes (< 1 cm and > 1 cm) and the seven target enzymes analyzed, with three replicates for each sampling.

consuming process that would have prolonged handling, potentially compromising the quality of the primary enzymatic data. Based on morphometric analysis, worm individuals were subdivided into two groups: small (< 1 cm in body length) and large (> 1 cm). These classes were established to serve as a proxy for investigating physiological differences that may correspond to different life-history stages or other sources of intraspecific heterogeneity. Despite in the literature a sexual dimorphism is reported, with male adult specimens characterized by opaque white abdominal segments and adult females by light pink coloration (Culloty et al., 2010; Curd et al., 2019; Lezzi et al., 2015), in our samples, the adult specimens collected during both sampling events lacked clearly distinguishable sexual features. Therefore, we analyzed individuals without any sex-based separation.

2.2. Enzyme assays

2.2.1. Preparation of homogenates

For each size class, three replicates were sorted from the individually measured worms. The worms, divided into dimensional categories, were weighed and ice-cold milliQ water was added at ¼ mass volume ratio. Then, samples were homogenized by the Medimachine II twice for 10 s at a constant speed of 100 rpm (Montanari et al., 2022). Obtained tissue homogenates were subdivided in aliquots then put within different tubes containing 1 mL for protein and enzymes assay (Curd et al., 2019).

2.2.2. Biomarkers of antioxidant enzyme activities

Part of the homogenates were diluted by 1/3 in ice-cold lysis buffer solution (specific buffer for each type of enzyme) and homogenized using a teflon/glass Potter homogenizer. Homogenates were incubated for 1 h before being centrifuged twice at 4000 rpm for 1 h at 4 °C and at 11700 rpm for 45 min at 4 °C. The resulting supernatants were subdivided into aliquots and stored at −80 °C until protein quantification and enzyme assays. Protein content was determined according to the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951).

2.2.2.1. Superoxide dismutase (SOD) and catalase (CAT) activity. Another set of homogenates were diluted 1/3 in 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.5 % Tween 20, 1 tablet of complete EDTA free protease inhibitor cocktail (Roche Diagnostics, Risch-Rotkreuz, Switzerland) in 25 ml of buffer, 10 mM NaF, 2 mM Na_3VO_4 as phosphatase inhibitor pH 7.4 (Curd et al., 2019; with some modifications: Tween 20 instead of Igepal; NaF and Na_3VO_4 as phosphatase inhibitors).

The SOD activity was measured following the method described by Prazeres et al. (2011) with some modifications. This method is based on the xanthine/xanthine oxidase assay and the reduction of cytochrome *c* (McCord and Fridovich, 1969; Okado-Matsumoto and Fridovich, 2001). The assay was carried with *Sabellaria* homogenate (50 μL in 950 μL of reaction buffer) containing potassium phosphate (KH_2PO_4, 50 mM), cytochrome *c* (10 μM), xanthine oxidase (50 μM), and EDTA (100 μM) with a solution pH of 7.8. The reaction began with the addition of 0.5 U of xanthine oxidase in a final volume of 1000 μL of the reaction mixture. Cytochrome c reduction was observed spectrophotometrically (550 nm) for 5 min. Results were expressed as units of SOD activity per milligram of protein (U mg^{-1} of protein), where 1 unit of SOD is defined as the activity causing 50 % inhibition of cytochrome *c* reduction at 25 °C (Ciacci et al., 2022). The CAT activity was evaluated by spectrophotometric evaluation following the decomposition of H_2O_2 at pH 7, 25 °C, at 240 nM as previously described (Ciacci et al., 2022). CAT is expressed in

U mg^{-1} protein where 1 U is the amount of enzyme necessary for catalyzing 1 μmol of H_2O_2 per min (using εH2O2, 39.4 mM^{-1} cm^{-1}).

2.2.2.2. Glutathione-related enzymes. GST, selenium-dependent glutathione peroxidase (Se-GPx), total glutathione peroxidase (GPx tot), and oxidized glutathione reductase (GSR) activities were evaluated as previously described (Canesi and Viarengo, 1997). Tissue sample homogenates obtained as described above were diluted 1/3 in 20 mM Tris–HCl buffer, pH 7.6, containing 0.5 M sucrose, 0.15 M NaCl, and centrifuged as before described. Aliquots of the supernatants were utilized for the spectrophotometric determination of enzyme activities. GST activity was evaluated with CDNB (1-chloro-2,4-dinitrobenzene) as a substrate. The reaction mixture (1 mL) contained 125 mM K-phosphate buffer, pH 6.5, 1 mM CDNB, and 1 mM GSH. The formation of S-2,4-dinitrophenyl glutathione conjugate was evaluated by monitoring the increase in absorbance at 340 nm. GSR activity was estimated in 1 mL of a reaction mixture containing 125 mM K-phosphate buffer, pH 7.5, 0.05 mM NADPH, and 1 mM GSH. The NADPH oxidation was evaluated by monitoring the increase in absorbance at 340 nm. GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of GSR and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm was measured. The Se-GPx activity was measured using H_2O_2 as the substrate for the oxidation of GSH. The molar extinction coefficients (ε) were set at 9.6 μmol/cm for GST and 6.2 μmol/cm for GSR, GPx, and Se-GPx.

2.2.3. Metabolic enzyme activities (glycolytic enzymes)

The last part of homogenates were diluted in 3 volumes of 20 mM Tris–imidazole buffer, pH 7.2, containing 10 mM EDTA, 10 mM EGTA, 0.1 mM PMSF, 15 mM β-mercaptoethanol. The homogenates were then centrifuged at 20,000 ×*g* for 20 min. The resulting supernatants were utilized for the spectrophotometric determination of PFK (phosphofructokinase, E.C. 2.7.1.11) and PK (pyruvate kinase, E.C. 2.7.1.40) activities, expressed as n-moles NADH consumed per mg sample protein, as previously described (Ciacci et al., 2012). For PFK activity, optimal assay conditions involved a 20 mM imidazole–HCl buffer (pH 7.2) supplemented with 1 mM fructose 6-phosphate (F 6-P), 0.5 mM Mg–ATP, 5 mM MgCl2, 20 mM KCl, 0.15 mM NADH, 0.2 U/ml triosephosphate isomerase, 2.4 U/ml glycerol-3-phosphate dehydrogenase, and 0.4 U/ml aldolase (Canesi et al., 2001). For PK activity, the optimal assay conditions utilized a 20 mM imidazole–HCl buffer (pH 7.2) containing 2 mM ADP, 5 mM MgCl2, 20 mM KCl, 0.15 mM NADH, 7.5 mM phosphoenolpyruvate, and 1 U/ml lactate dehydrogenase (Canesi et al., 2001).

2.3. Data analyses

Statistical analyses were performed using the software R version 4.3.0 (R Core Team, 2024). Morphometric data and enzymatic activity measurements were firstly tested for normality using Shapiro-Wilk tests. Morphometric data were also treated using descriptive statistics including mean, standard deviation, median, quartiles, and coefficient of variation (showing the standard deviation as a percentage of the mean) by applying the 'tidyverse' and 'dplyr' packages (Wickham et al., 2019). Homogeneity of variance between the two sampling years was assessed using Levene's test from the 'car' package (Fox et al., 2001). Population size distributions were compared between the sampling years using the Kolmogorov-Smirnov test, which evaluates differences in both location and shape of the distributions. A preliminary analysis was conducted to assess the comparability of the two sampling years (i.e., 2023 and 2024) through Wilcoxon-Mann-Whitney testing. As no significant inter-annual variation was detected for the most of endpoints, the data were pooled to increase the statistical power for testing our primary hypothesis regarding the effect of organism size. Subsequently, differences in individual enzyme activities between the two size classes (< 1 cm and > 1 cm) were assessed on this pooled dataset using the non-parametric Wilcoxon-Mann-Whitney test, which was selected due to the non-normal distribution of the data.

Multivariate statistical analyses were conducted using the packages vegan v2.6–4 (Oksanen et al., 2001), ggplot2 v3.4.2 (Wickham, 2016). Eight metabolic enzyme activities were analyzed to assess differences between size classes and years: GST, PK, PFK, Se-GPx, GPx tot, SOD, CAT, GSR. Data were standardized using z-score transformation prior to analysis. To visualize multivariate patterns in enzyme activities, Principal Component Analysis (PCA) was performed.

To test for significant differences in enzymatic profiles, Permutational Multivariate Analysis of Variance (PERMANOVA - Anderson, 2001) was performed with 9999 permutations on Euclidean distance matrix. The analysis first tested the full model including size, year, and their interaction. Subsequently, each factor was tested independently. When significant effects were detected, pairwise comparisons between groups were conducted individual PERMANOVA tests for each pair of groups. Furthermore, Permutational Analysis of Multivariate Dispersions (PERMDISP) was conducted to test for homogeneity of within-group variance for each main factor (size and year). Results were considered significant at $p < 0.05$.

The Integrated Biomarker Response (IBR) was calculated following Devin et al. (2014, 2023) modification to the Beliaeff and Burgeot (2002), and used as a simple qualitative method to visualize the different response degree in our targets between the smaller and larger specimens (Raftopoulou and Dimitriadis, 2010; Serafim et al., 2012).

3. Results

3.1. Morphometric data

The analyzed 100 specimens of *S. spinulosa* showed comparable sizes in both 2023 and 2024 samples (Fig. 2). The specimen size (length of the body) ranged from 0.438 to 1.96 mm in 2023 and from 0.448 to 1.74 mm in 2024. Mean sizes were consistent in both the sampling years (2023: 0.963 ± 0.370 mm; 2024: 0.917 ± 0.333 mm), with similar median values (0.892 and 0.866 mm, respectively). The dispersion patterns were also similar in both years, as indicated by their interquartile range values (0.502 and 0.398 mm, respectively), and confirmed by the Levene's test ($F = 0.407$, $p = 0.525$). Size distributions were non-normal in both years (Shapiro-Wilk test; 2023: $W = 0.931$, $p = 0.006$; 2024: $W = 0.952$, $p = 0.042$). The Kolmogorov-Smirnov test confirmed no significant differences between the size distributions of specimens collected in 2023 and in 2024 ($D = 0.12$, $p = 0.867$).

3.2. Biochemical data

Before investigating the primary effect of organism size, it was essential to determine if the measured target activities were consistent across the two sampling years. A comparative analysis between the 2023 and 2024 cohorts revealed no significant inter-annual differences for seven of the eight enzymes measured (Figs. S2, S3 available as Supplementary Materials). Only SOD showed a statistically significant inter-annual variation ($p < 0.05$) but of very low magnitude (mean 3.68 ± 0.23 vs. 3.03 ± 0.41 U mg^{-1} prot).

Biochemical analyses of the pooled data from both sampling years revealed distinct, size-dependent patterns in the enzymatic profiles of *S. spinulosa*. A consistent trend was observed across the antioxidant markers, with significantly higher activities recorded in the small (< 1 cm) specimens compared to the large (> 1 cm) ones (Fig. 3). The most pronounced differences were observed for CAT (3.77 ± 0.75 vs. 1.31 ± 0.18 U mg^{-1} prot; $p < 0.01$) and GSR (7.72 ± 0.42 vs. 3.70 ± 0.25 nmol mg^{-1} protein; $p < 0.01$). Similarly, GPx tot (17.60 ± 1.11 vs. 12.87 ± 1.10 nmol mg^{-1} protein; $p < 0.01$) and Se-GPx (3.79 ± 0.74 vs. 2.07 ± 0.44 nmol mg^{-1} protein; $p < 0.01$) activities were markedly elevated in

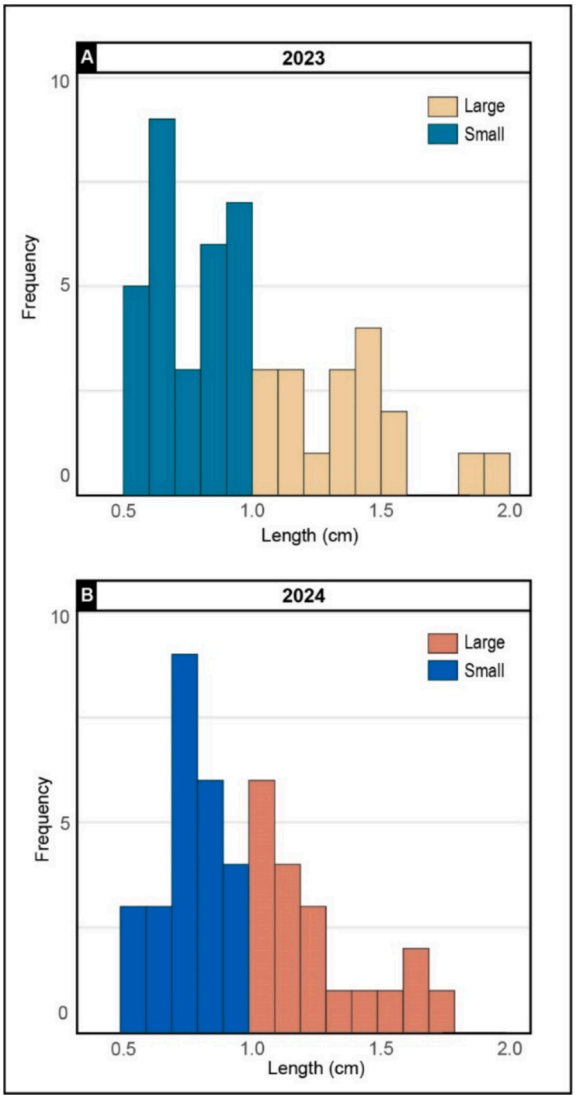

Fig. 2. Size/frequency histograms for the analyzed specimens of *S. spinulosa*: A) 2023 sampling event; B) 2024 sampling event.

small-sized individuals. GST (3.32 ± 0.22 vs. 2.96 ± 0.13 nmol mg^{-1} protein; $p < 0.05$) and SOD (3.62 ± 0.30 vs. 3.09 ± 0.46 U mg^{-1} prot; $p < 0.01$) followed the same pattern, with higher mean activities in the small size class.

In contrast, the markers for energy metabolism showed opposite trends (Fig. 4). PFK activity was significantly higher in large specimens (2.15 ± 0.14 mU mg^{-1} prot) compared to small ones (1.11 ± 0.14 mU mg^{-1} prot; $p < 0.01$). Conversely, PK activity was substantially greater in small individuals (0.28 ± 0.08 mU mg^{-1} prot) than in large ones (0.17 ± 0.01 mU mg^{-1} prot; $p < 0.01$).

To investigate the integrated response of the entire enzymatic suite, a PCA was performed. The first two principal components explained a cumulative 89.0 % of the total variance (PC1: 78.4 %; PC2: 10.6 %), confirming that the two-dimensional biplot is a robust representation of the data structure (Fig. 5). The PCA revealed a clear and strong separation of samples along the PC1 axis based on size. Small (< 1 cm) individuals clustered on the positive side of PC1, characterized by higher activities of CAT, GSR, GPx tot, Se-GPx, and GST, as indicated by the direction of their vectors. Conversely, large (> 1 cm) individuals grouped on the negative side of PC1, a separation primarily driven by higher PFK activity.

Multivariate analysis with PERMANOVA backed-up the observed differences between clusters, identifying size as the dominant factor shaping enzymatic profiles. It revealed that size was the dominant factor shaping enzymatic profiles (Pseudo-F: 56.2005; Df: 1; *P*(perm): 0.0001), explaining approximately 69 % of the total variance. A significant effect of year (Pseudo-F: 8.2928; Df: 1; *P*(perm): 0.0016) and size:year interaction (Pseudo-F: 9.0024; Df: 1; *P*(perm): 0.0021) were also detected, accounting for 10 % and 11 % of the variance, respectively. Pairwise comparisons revealed significant differences between size classes (Pseudo-F: 22.218; Df: 1; *P*(perm): 0.005), while comparisons between years showed no significant differences (Pseudo-F: 1.133; Df: 1; *P* (perm): 0.327), suggesting that variations between the two sampling efforts were primarily retained through their interaction with size rather than as independent yearly change. This result was supported by a non-significant PERMDISP test ($p = 0.191$), confirming that the observed separation was due to a true shift in the multivariate mean between size groups. Collectively, the multivariate analyses demonstrate that organism size is the dominant factor structuring the measured target profiles of *S. spinulosa*.

The IBR radar plots the overall integrated response (Fig. 6), suggesting distinct patterns in biomarker responses between size classes and sampling years. Although this represent a qualitative representation, small specimens (<1 cm) consistently showed higher IBR values in both years (2023: 5.60 ± 0.41; 2024: 6.16 ± 0.67) compared to larger specimens (>1 cm) (2023: 0.31 ± 0.18; 2024: 0.30 ± 0.30) with small specimens (A, B) displaying larger polygonal areas compared to the minimal areas observed in larger specimens (C, D). Moreover, small specimens showed notably higher contributions from antioxidant enzymes (i.e., CAT, GSR, and GPx), while larger specimens displayed minimal contributions from most enzymes, with only slight elevations in PFK. This pattern remained relatively consistent in both sampling years, supporting the statistical finding of non-significant temporal variation.

The IBR visualizations support the statistically significant multivariate patterns observed in the PCA analysis, providing additional insight for size-dependent differences in metabolic and antioxidant responses in *S. spinulosa*, with smaller specimens showing consistently higher integrated stress responses regardless of sampling year.

4. Discussion

4.1. *S. spinulosa* population dynamics

Morphometric data show a remarkable consistence in size variations of specimens from samples collected in two different years (2023 vs. 2024), suggesting a substantially stable population at the study site. This consistency has been previously observed in other *S. spinulosa* bioconstructions (Lezzi et al., 2015). The co-occurrence of small and large adult specimens (0.438–1.96 mm size range) could be indicative of different stages of growth or individual intraspecific variability in both sampling years. We can cautiously hypothesize that the stable population sizes observed in both years is primarily driven by an intrinsic biological factor rather than by simply comparable environmental conditions between the two spring seasons (Gravina et al., 2018). Although further demographic studies would be needed to formally link size to age in this population, the reproductive strategy of *S. spinulosa*, which is characterized by a prolonged spring-summer period of continuous or "trickle" spawning, ensures a steady recruitment of new, smaller individuals into the established, multi-generational reef (Lezzi et al., 2015; Wilson, 1970).

4.2. Size-related variations in antioxidant defense and energy metabolism

The enzymatic profiles of small (< 1 cm) and large (> 1 cm) *S. spinulosa* are fundamentally different. This is well evident in the univariate analyses and strongly reinforced by the multivariate PCA, which shows a distinct separation of the two size classes along the primary axis of variation, explaining over 78 % of the total variance. The

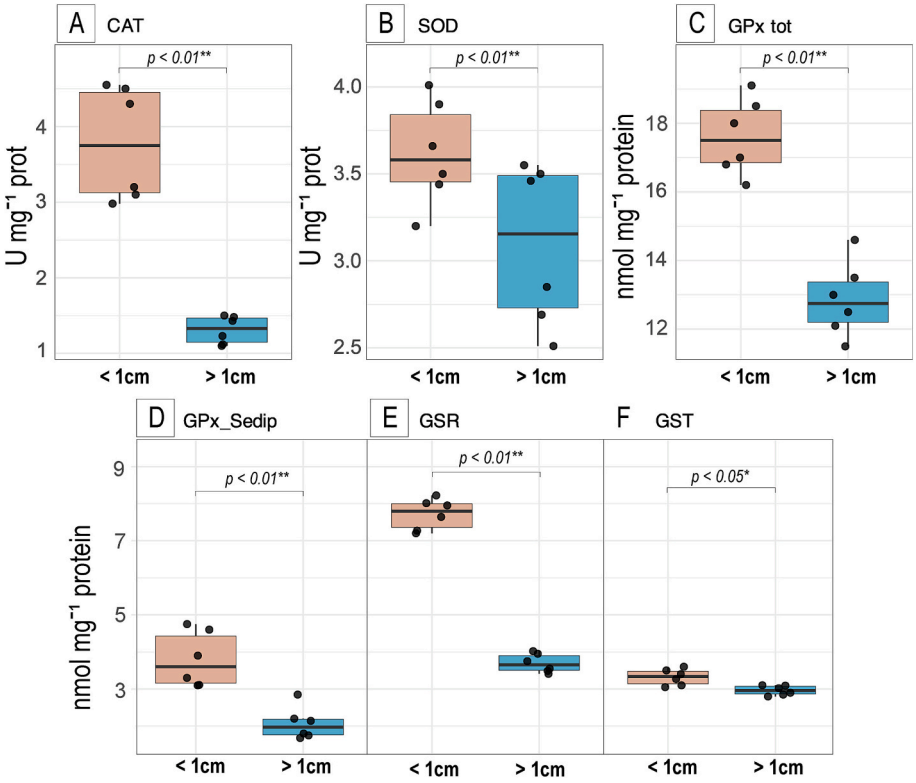

Fig. 3. Boxplots of antioxidant enzyme activities comparing size classes. Data for individuals smaller than 1 cm (< 1 cm; $n = 6$) and larger than 1 cm (> 1 cm; n = 6)) are pooled from both 2023 and 2024 sampling years. Panels show results for (A) Catalase (CAT), (B) Superoxide dismutase (SOD), (C) Total Glutathione peroxidase (GPx tot), (D) Selenium-dependent Glutathione peroxidase (Se-GPx), (E) Glutathione reductase (GSR), and (F) Glutathione S-transferase (GST). Statistical significance from Wilcoxon-Mann-Whitney tests is indicated above each comparison ($p < 0.05$*; $p < 0.01$**).

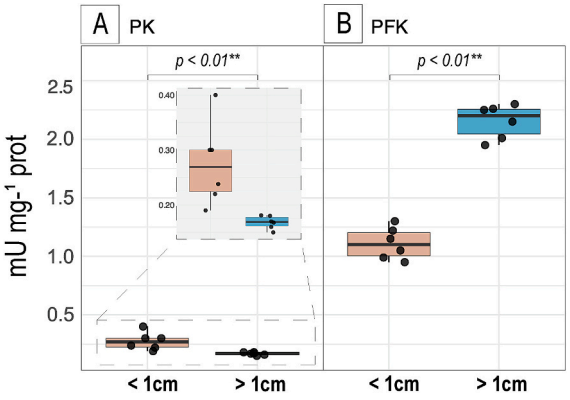

Fig. 4. Boxplots of energy metabolism proxies activities comparing size classes. Data for individuals smaller than 1 cm (< 1 cm; n = 6) and larger than 1 cm (> 1 cm; n = 6) are pooled from both 2023 and 2024 sampling years. Panels show results for (A) Pyruvate kinase (PK) and (B) Phosphofructokinase (PFK). An inset in panel A provides a magnified view of the PK data. Statistical significance from Wilcoxon-Mann-Whitney tests is indicated above each comparison ($p < 0.05$*; $p < 0.01$**).

PCA loadings reveal that this primary axis represents a functional trade-off between two key physiological systems. The small individuals, clustering on the positive side of PC1, are strongly associated with a suite of antioxidant enzymes (CAT, GPx_tot, Se-GPx, GSR, and GST), where all vectors point in this direction. While we did not directly measure reactive oxygen species (ROS), the enhanced enzymatic capacity—particularly for key enzymes like CAT, GPx, and SOD—is consistent with the high metabolic rates and rapid cell proliferation associated with somatic growth, processes known to increase endogenous ROS production (Brown et al., 2004). The coordinated upregulation of the primary ROS-detoxifying enzymes—CAT for hydrogen peroxide and SOD for the superoxide radical—in smaller worms supports this interpretation. The elevated GSR and GST activities in small specimens further support this hypothesis, suggesting a more robust glutathione-mediated defense system, which is essential for maintaining cellular redox balance during periods of high metabolic activity (Lesser, 2006).

The observed patterns in antioxidant enzyme activities align with findings from broader studies of other marine invertebrates. (Istomina et al., 2021) examined antioxidant enzyme activities (i.e., CAT, GPx, GSR, SOD) in 14 bivalve species, documenting that antioxidant activity well correlated with growth stages and habitat characteristics. Their observations, on bivalve species from shallower habitats and with a shorter lifespan characterized by higher antioxidant activity, are consistent with our findings of elevated values of antioxidant enzymes in smaller and therefore younger *S. spinulosa* specimens. This pattern might reflect a general adaptation strategy in some marine invertebrates (Annelida and Mollusca), where higher metabolic rates and natural environmental stressors in developing growth stages demand for enhanced antioxidant defenses (Bernard et al., 2015; Freire et al., 2011; Rivera-Ingraham and Lignot, 2017; Sokolova et al., 2012).

The consistency in enzyme activity patterns in both years of sampling gains additional significance when considered alongside studies of environmental stress responses. For instance, (Silva et al., 2020) demonstrated that marine polychaetes can rapidly modulate SOD activity in response to environmental stressors, such as the exposure to nanoplastics. (Leprêtre et al., 2022) established threshold values for antioxidant enzymes as pollution biomarkers in freshwater invertebrates as gammarids and mussels.

In contrast, the analyzed key glycolytic enzymes reveal a divergent pattern, providing insight into potential size-dependent modulation of

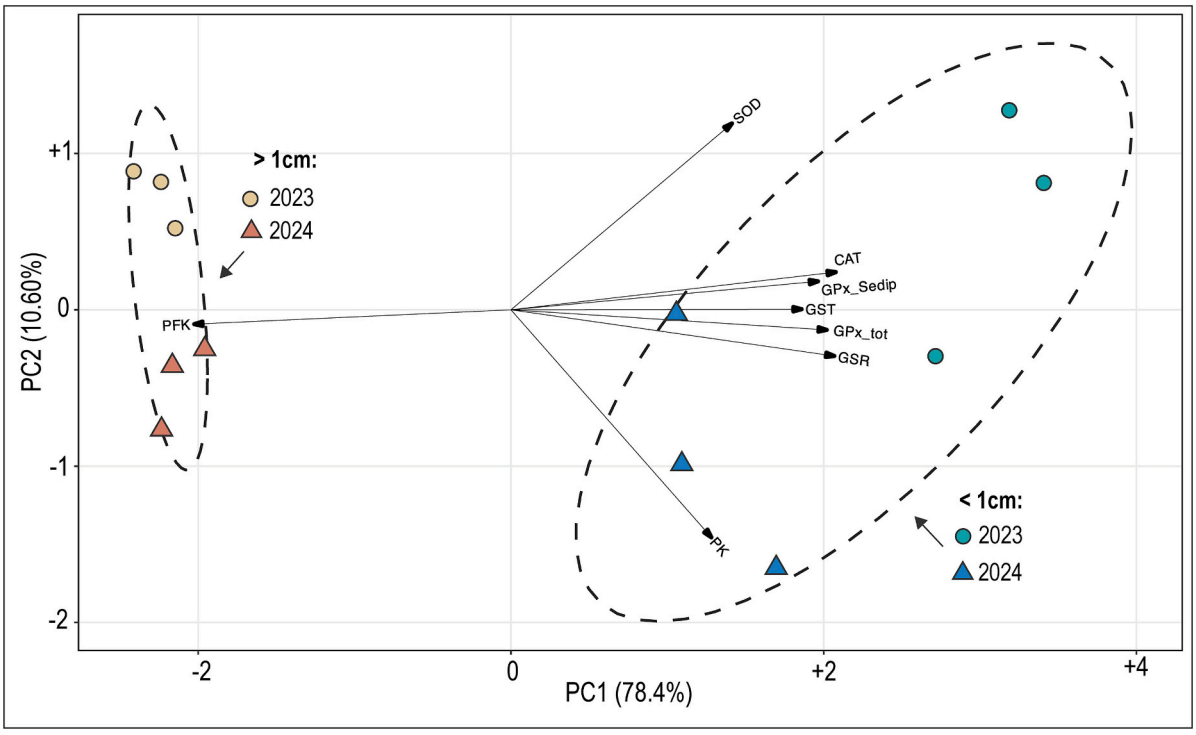

Fig. 5. Principal Component Analysis (PCA) biplot of enzyme activities. The plot is based on the seven target enzymes for all individuals, distinguished by size (< 1 cm and > 1 cm) and sampling year (2023 and 2024). The first two principal components (PC1 and PC2) explain 78.4 % and 10.6 % of the total variance, respectively. Vectors represent the loadings of each enzyme variable. Dashed ellipses represent the 95 % confidence interval for each size group.

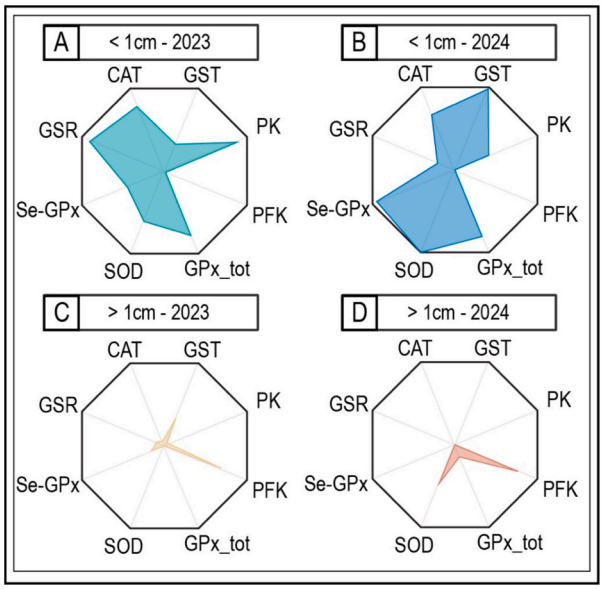

Fig. 6. Radar plots representing the integration of biomarkers used for the calculation of IBR measured for each combination of year and worm sizes.

energy production. It is worth to note that with only two glycolytic enzymes measured, we cannot define a complete energy metabolism strategy; however, the opposing activities of PFK and PK are informative (e.g., (Balbi et al., 2017). The large individuals are clustered on the negative side of PC1, a separation driven by the strong opposing vector of PFK, a key regulatory enzyme in glycolysis. This visual opposition in the PCA biplot provides compelling multivariate evidence for the divergent patterns observed in the markers of energy metabolism.

The significantly higher activity of PFK, a primary regulatory enzyme of glycolysis, in larger individuals, paired with their lower PK activity, might indicate a metabolic profile geared towards fine-tuned regulation rather than maximum glycolytic flux. Conversely, the markedly elevated PK activity in smaller individuals is consistent with a higher, more sustained glycolytic rate needed to generate the ATP necessary to fuel the energetic demands of biomass accumulation.

While we did not measure energy storage molecules like glycogen or lipids— which limits the reach of this study—this enzymatic profile is consistent with a potential life-history trade-off (Trestrail et al., 2020). The metabolic data from smaller individuals could be interpreted as being prioritized for immediate energy expenditure for growth. In contrast, the profile in larger, mature individuals may reflect a metabolic shift away from rapid somatic growth and towards resource allocation for other costly processes, such as reproduction and long-term homeostasis (Costantini, 2014). The potentially enhanced energy reserve capacity for large *S. spinulosa* individuals could provide the organism with advantages under stress conditions, including improved pathogen resistance (Lochmiller and Deerenberg, 2000; Pernet et al., 2014; Ravensdale et al., 2011). This has also been observed in other errant polychaetae species and tropical reef-building corals, where biochemical markers have been successfully used to assess metabolic responses to environmental stressors (Madeira et al., 2021; Rivest and Hofmann, 2014).

A direct comparison of our findings with the work of Curd et al. (2019) on the congener *S. alveolata* is particularly insightful. The most striking point of divergence between the two studies concerns the influence of organism size on CAT and SOD activities. In their study on *S. alveolata*, Curd et al. (2019) found that individual size (measured as wet weight) was not correlated with the activity of either CAT or SOD. For *S. alveolata*, these core antioxidant enzymes appear to be relatively stable andare not significantly influenced by the main biological factors tested, including size and sex. Our results for *S. spinulosa* present a stark contrast. We reveal that size is the dominant factor structuring the activity of these enzymes. Specifically, CAT activity in small (< 1 cm) individuals is nearly three-fold higher than in large (> 1 cm) individuals

(3.77 vs. 1.31 U mg^{-1} prot), a highly significant difference. Similarly, SOD activity is also significantly elevated in our smaller specimens.

Beyond this fundamental difference in physiological regulation, a quantitative comparison of the baseline values is also revealing. Curd et al. (2019) reported mean CAT activities of approximately 12.5 U mg^{-1} protein and SOD activities around 1.5 U mg^{-1} protein in *S. alveolata*. In contrast, our measurements for *S. spinulosa* show markedly lower baseline CAT activity across both size classes and substantially higher SOD activity (3.09 to 3.62 U mg^{-1} prot). It is important to interpret these quantitative differences cautiously. Such variations in baseline enzymatic activity between studies can arise from several factors, including 1) true inter-specific physiological differences, 2) variations in local environmental conditions between the respective study sites, or 3) subtle differences in laboratory assay methodologies that can influence measured rates. The geographical variations previously observed in *S. alveolata* (Curd et al., 2021) lend support to the hypothesis that local environmental context is a significant factor.

Ultimately, these two layers of divergence—both in the regulatory role of size and in the absolute baseline activities—underscore the danger of extrapolating physiological patterns from one species to another, even within the same genus. This strongly reinforces the central conclusion of our study: that establishing species-specific, size-dependent physiological baselines is an essential prerequisite for the development of robust monitoring programs or more experimental ecotoxicological testing. We acknowledge, however, that translating these baseline data into definitive thresholds for stress assessment is challenging. As highlighted in studies on other marine invertebrates (Porte et al., 2002; Viarengo et al., 1990), this would require extensive evaluation across multiple environmental contexts. Furthermore, there are significant practical limitations to consider; the laboratory procedures for a suite of enzymatic assays are inherently time-consuming, and the associated costs of reagents can be substantial. These challenges likely contribute to the research bias towards traditional model organisms and may explain the current lack of comparative physiological data for species like *S. spinulosa*.

4.3. Baseline physiological information and future perspectives

We suggest that the combined analysis of antioxidant enzymes (CAT, GPx, GST, GSR, SOD) with energy metabolism markers (PFK, PK) could provide a robust physiological baseline, where significant deviations from the basal conditions might indicate stress responses. The relative stability of enzyme activities across the two sampling years, particularly for rapidly modulating enzymes like Se-GPx and GSR, allows us to hypothesize that the strong size-related differences are a primary feature of *S. spinulosa* physiology. An important avenue for future research is to test this hypothesis directly. For instance, controlled laboratory experiments could determine whether these size-specific profiles are fixed physiological setpoints or if they can be altered by specific environmental stressors. While our current in situ data are insufficient to deconvolve these factors, they strongly suggest that intrinsic, size-related physiology is the dominant driver of the observed patterns, illuminating a critical path for future ecotoxicological studies. Further studies are encouraged to understand the role of confounding factors, such as size (Kalman et al., 2010), or seasonality, in order to establish robust baselines. Future research should validate the discussed enzymatic target patterns by mesocosm experiments and by analyzing further samples collected in different seasons also investigating potential sex-specific variations in *S. spinulosa*. Such a comprehensive approach would strengthen our ability to distinguish natural physiological variations from stress responses due to specific pollutants such as microplastics in sabellariid polychaetes that could be used in the future as sentinel species for ecotoxicology. The applicability of these baseline values should be considered within the specific context of spring sampling on anthropogenic substrates. A key aspect of our experimental design was the use of two discrete size classes. This categorical approach was effective in demonstrating a strong, significant difference between smaller and larger individuals. However, a valuable future direction would be to investigate this relationship across a continuous size gradient. This could be achieved by analyzing pools of individuals and correlating their enzymatic activity with their average size, allowing for a more nuanced, regression-based analysis of how physiological profiles change progressively with individual sizes. The study has not accounted for possible differences between geographical locations, which could influence physiological patterns. Additionally, sex-specific physiological variations were not addressed, which could be an important factor in understanding population-level responses. These limitations highlight the need for further research to establish a more comprehensive understanding of *S. spinulosa* physiology across different environmental contexts and biological conditions. Future research directions should include experimental validation of these metabolic patterns, assessment of seasonal variations, and investigation of sex-specific differences. Such comprehensive characterization would enhance our ability to distinguish natural physiological variations from contaminant induced stress responses.

5. Conclusions

To the best of our knowledge, this study documents the first physiological assessment based on several biomarkers on *S. spinulosa* and provide reference values for both antioxidant and metabolic enzymes in different size classes. It is important to note that prior to this work, there was a significant lack of environmental physiological data for this species, which has hindered our understanding of its natural responses and stress tolerance. Our findings reveal distinct size-dependent enzymatic patterns in *S. spinulosa*, characterized by differential abundances of antioxidant defense and energy metabolism proxies. Small individuals exhibit enhanced antioxidant enzyme activities and higher PK levels. In contrast, larger specimens show increased PFK activity. Through an integrated, multidisciplinary approach, this study provides crucial insights into *S. spinulosa* physiology. While these enzymatic analyses have been well-established in other commercially important species, their application to *S. spinulosa* biochemical markers represents an important advancement in understanding this species' physiological characteristics. This comprehensive approach addresses the current scarcity of literature on *S. spinulosa* and establishes new analytical frameworks for future research.

CRediT authorship contribution statement

Giusto Lo Bue: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Caterina Ciacci:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sabrina Burattini:** Writing – review & editing, Methodology, Investigation. **Fabrizio Frontalini:** Writing – review & editing, Validation, Supervision, Conceptualization. **Mar Santos-Simón:** Writing – review & editing, Visualization, Formal analysis. **Nicoletta Mancin:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jembe.2025.152135.

Data availability

The datasets analyzed during the current study are fully available at Mendeley Data repository (DOI: 10.17632/fkn53ykdpx.1) and accessible at the following link: https://data.mendeley.com/preview/fkn53ykdpx?a=377ce282-2e97-478c-ae5b-ab35e390403f.

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