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Effects of environmental factors on the oxidative status of *Anemonia viridis* in aquaculture systems

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ABSTRACT

Due to its depletion in natural settings, the potential for aquaculture of the cnidarian *Anemonia viridis* is currently attracting research interest. Knowledge about the physiology of this species is necessary to ensure optimal development of, and well-being in, aquaculture. This study tested the effects of different abiotic (limited sunlight, brackish water) and biotic (integrated multitrophic aquaculture or IMTA) conditions on *A. viridis* in captivity. Growth and reproduction were measured, and antioxidant status was evaluated in tentacular and columnar tissues as antioxidant enzymatic activity (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose 6-phosphate dehydrogenase, glutathione S-transferase and DT-diaphorase), Trolox-equivalent antioxidant capacity (TEAC) and tissue lipid peroxidation (MDA). Animals in the brackish water and IMTA treatments displayed significant changes in glutathione peroxidase, glucose 6-phospate dehydrogenase and TEAC compared to control anemones, with these effects noted primarily in columnar tissue. These results support the relevance of enzymatic pathways involving glutathione as antioxidant mechanisms under osmotic disturbances or ecological interactions. Limited light intensity was not found to be detrimental to the oxidative status of the anemones, despite *A. viridis* harbouring photosynthetic symbionts, and enhanced growth performance parameters suggested a higher individual weight increase than in control conditions. Lipid peroxidation was not significantly affected in any experimental condition. Principal Component Analysis (PCA) suggested that similar antioxidant status parameters can correlate positively (tentacular parameters) or negatively (columnar parameters) with MDA concentration. In conclusion, aquaculture of *Anemonia viridis* can be improved under suitable environmental conditions supported by the evaluation of welfare markers based on antioxidant status.

1. Introduction

Aquaculture and fisheries are key economic activities in the establishment of food production systems that are ecologically, socially and economically sustainable. Failure to achieve this sustainability entails serious long-term consequences stemming from overexploitation, habitat degradation and disparities in access to the produced resources (FAO, 2022).

Integrated multitrophic aquaculture (IMTA) is a promising approach to sustainable development in aquaculture (Buck et al., 2018; Gamito et al., 2020; FAO, 2022). IMTA systems involve the integrated co-culture of several species with different trophic niches, so that the metabolic waste of one species can serve as a resource for another species in the system. This integrated system achieves highly efficient nutrient cycling, thus reducing the release of organic contaminants into the environment and increasing productivity simultaneously (Buck et al., 2018; FAO, 2022; Nissar et al., 2023). Moreover, the concept of IMTA is extremely flexible, with applications in both land-based and marine off-shore

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aquaculture, and is easily adaptable to the use of native species (Chopin et al., 2012; Nissar et al., 2023).

In Spain, the snakelocks anemone (*Anemonia viridis*) (Forsskål, 1775) is a highly valued seafood product (Daza Cordero et al., 2002) and in recent years it has gained relevance due to its significant biotechnological potential as a source of diverse bioactive compounds (Cabeza et al., 2021; Ciccone et al., 2019; Piccialli et al., 2021). However, increased exploitation pressure and expansion of the invasive algae *Rugulpteryx okamurae* had left local stocks of this species in a critical state, leading to authorities closing the fishery indefinitely in June 2023 (BOJA, 2023). Knowledge of the physiological processes of the snakelocks anemone and standardization of techniques for optimal culture of this species, can function as a tool for the conservation of its natural populations, and as a starting point for developing aquacultural methods for other anemone and coral species (Fraser et al., 2021; Watson and Younger, 2022).

A. viridis is a solitary marine anemone that inhabits intertidal and shallow environments both in the Mediterranean Sea and the northern Atlantic Ocean. It is a heterotrophic organism that captures particles and small prey, which it immobilizes using its tentacles and toxins from specialized cells known as cnidocytes, unique to the phylum Cnidaria (Rodríguez et al., 2023; Schick, 1991). Just like many others anthozoan species, *A. viridis* features a symbiotic relationship with dinoflagellates of the genus *Symbiodinium* (recently proposed as *Philozoon actiniaria)* (Casado-Amezúa et al., 2016; LaJeunesse et al., 2022). These microalgae, also known as zooxanthellae, supplement the heterotrophic nutrition of their animal host with products from their own photosynthetic activity (Davy et al., 2012; Schick, 1991). This mutualistic relationship is of profound relevance in oligotrophic ecosystems, such as the Mediterranean Sea, and the co-evolution of anthozoans and zooxanthella profoundly shaped this organism's physiology, particularly in regard to their antioxidant metabolism and defences (Casado-Amezúa et al., 2016; Davy et al., 2012). For these reasons, this species has been used a model organism for studying physiological processes such as anthozoan bleaching or antioxidant metabolism (Merle et al., 2007; Pey et al., 2017; Richier et al., 2006; Richier et al., 2003).

Salinity, light intensity, dissolved oxygen (O_2), pH, nitrogen compounds, and even interactions with other organisms present in the environment can easily become stressful stimuli for the cultured animals, and trigger a stress response. Metabolic disruptions resulting from a stressful situation may lead to an increase in the production rate of reactive oxygen species (ROS) (Lesser, 2006; Lushchak, 2011). ROS are typically generated in chloroplasts and mitochondria as a consequence of aerobic metabolism, and all organisms are equipped with various antioxidant systems that maintain a balance between ROS synthesis and degradation processes (Lesser, 2006; Lushchak, 2011; Rosset et al., 2021). In regard to enzymatic responses, superoxide dismutase (SOD) promotes the conversion of superoxide radicals into hydrogen peroxide which will subsequently be degraded by the catalytic action of catalase (CAT). Glutathione peroxidase (GPx) is also involved in the reduction of hydrogen peroxide and other organic peroxides through the oxidation of glutathione. This peptide is regenerated by the action of the enzyme glutathione reductase (GR) requiring NADPH obtained by glucose-6-phosphate dehydrogenase (G6PDH) activity that participates in the pentose phosphate pathway. Enzymes such as glutathione transferase (GST), also dependent on glutathione, and DT-diaphorase (DTD) are also involved in the oxidative stress response, through their antioxidant activity (Do et al., 2024; Ross and Siegel, 2021). Finally, in addition to glutathione, there are other non-enzymatic molecules that can have an antioxidant action, such as uric acid, carotenes and vitamins E and C.

Despite organisms being equipped with different antioxidant defences, oxidative damage to various cellular components can occur in cases where this antioxidant response proves insufficient to cope with a high ROS production. Malondialdehyde (MDA) is an end product of lipid peroxidation, measured as thiobarbituric reactive substances (TBARS), and one of the most recognized oxidative stress markers. It is widely used to assess oxidative damage in aquatic organisms, which typically contain a large amount of polyunsaturated fatty acids, highly susceptible to oxidation (Lesser, 2006; Lushchak, 2011; Valavanidis et al., 2006). Nevertheless, evaluation of TBARS must be performed in a context that assesses whether these changes are correlated with the antioxidant enzymatic and molecular response to obtain a thorough picture of the welfare state of the animal (Lushchak, 2011). In this regard, multivariate analysis techniques can be of interest in identifying influential or non-influential factors on oxidative status (Lushchak, 2011; Ringnér, 2008). Principal Component Analysis (PCA) is achieved by extracting new principal components from linear combinations of the original variables in a dataset (Goodman, 1972; Ringnér, 2008). It is a valuable technique for identifying influential variables and potential markers for different biological processes, such as oxidative stress (Onwosi et al., 2019; Rodríguez-Piñeiro et al., 2007; Schueth and Frank, 2008; Staniszewska-Slezak et al., 2015; Taguchi and Murakami, 2013).

The aim of the present study was to evaluate the physiological response of snakelocks anemone (*Anemonia viridis*) to different biotic and abiotic factors under aquaculture conditions by measuring oxidative status parameters.

2. Materials and methods

2.1. Animal husbandry and sample collection

Approximately 270 wild specimens of *A. viridis* were collected from the natural environment with authorization of the competent local authorities. The origin population was located in Salobreña (36°44′12.2" N, 03°35′35.1" W) (Granada, Andalusia, Spain), and after collection, they were transferred to the facilities of Andalmar Biotech S.L (Carchuna, Granada, Andalusia, Spain). Specimens of *A. viridis* were weighed and acclimated in an outdoor concrete tank (8 m^3) for four months (natural temperature and photoperiod) and were fed twice a week with wet feed based on various low commercial value fishes.

After acclimation, specimens were randomly distributed into four outdoors concrete tanks (8 m^3), each equipped with an independent closed recirculation circuit and identical, previously maturated, filtering systems. All the tanks used natural seawater, which was pumped from a well and filtered before flowing into the circuits. Four experimental conditions were established: control (C), limited sunlight (LS), brackish water (BW) and integrated multitrophic aquaculture (IMTA).

For each treatment tank, approximately 65–70 specimens were distributed in five different partitions (about 13–14 individuals each), therefore establishing five replicates per experimental condition. During the experimental period, all tanks were maintained under equal temperature (21 ± 2 °C). Water quality parameters such as dissolved oxygen (6–7 ppm) and pH (7.8–8.2) were monitored daily and remained within optimal ranges and homogenous across treatments.

The LS condition consisted of a dense black mesh covering, which blocked most of the incidental sunlight on the organisms. The mean daily illuminance measured at the centre of the tank at surface level was 279 ± (standard deviation; SD) lux, which represented around 1 % of the mean illuminance measured at the other 3 tanks, which averaged 25,720 (± 15,676) lux. All measurements were taken on a sunny day using a lux meter (Delta HD 9221).

The BW condition featured a reduced salinity of approximately 30 g/L, achieved by replacement of the evaporated water with freshwater, twice a week. Salinity was monitored daily and thoroughly in all tanks using a refractometer, and it was maintained consistently at 34 g/L in the rest of the tanks, where water replacement was carried out using natural seawater.

Finally, the IMTA condition involved co-culturing anemones with sea urchins, sea snails, sea cucumbers, and macroalgae; which acted as herbivores, detritivores and primary producers, respectively. Table 1 displays the exact species composition of the tank and their density at the beginning of the assay.

Table 1
Initial species composition of the IMTA condition.

Phylum	Species	Density
Mollusca (Gastropoda)	*Monodonta turbinata*	3.375 individuals/m^3
	Stramonita haemastoma	0.500 individuals/m^3
Echinodermata (Echinoidea)	*Paracentrotus lividus*	4.375 individuals/m^3
(Holothuroidea)	*Holothuria arguinensis*	0.125 individuals/m^3
	Holothuria sanctori	0.125 individuals/m^3
	Holothuria tubulosa	1.875 individuals/m^3
Chlorophyta (Ulvophyceae)	*Ulva rigida*	23.250 g/m^3
Ochrophyta (Phaeophyceae)	*Cystoseira mediterranea*	12.500 g/m^3

2.2. Macroalgae mass refers to wet weight

Once the experiment began, the specimens remained in the tanks for four weeks during May 2023 with natural photoperiod, and were fed as described previously for the acclimatation period.

At the beginning of the trial, all specimens were weighed using a precision scale. This procedure was repeated at the end of the experimental period to calculate growth performance parameters. Since *A. viridis* is prone to asexual reproduction via longitudinal fission, growth and reproduction were measured as increase in total weight per replicate and change in total number of individuals per replicate, respectively.

Sample collection was performed subsequently, and five individuals per experimental condition (1 per replicate) were collected and dissected, separating the column from the crown of tentacles. Each body region was immediately frozen in liquid nitrogen and kept at −80 °C for oxidative status analysis.

2.3. Oxidative status analysis

Samples were homogenized (Heidolph Intruments) in 100 mM Tris, 0.1 mM EDTA and 0.1 % Triton buffer (pH 7.8), at a 1:4 *w/v* ratio. Homogenates were centrifuged at 30,000*g* for 25 min at 4° C (Sigma 3 K30), and the supernatant was collected and distributed in aliquots kept at −80 °C.

Determination of superoxide dismutase (SOD) activity was performed using the McCord and Fridovich (1969) method, based on an indirect measurement of activity according to the degree of inhibition of a control reaction consisting of cytochrome *c* reduction. Catalase (CAT) activity was determined using the method originally described by Aebi (1984), based on the decrease in absorbance resulting from the reduction in H_2O_2 concentration generated by the activity of this enzyme. Glutathione peroxidase (GPx) activity was measured following the method from Flohé and Günzler (1984), based on an indirect measure of NADPH oxidation, obtained by coupling it with a standard glutathione reductase (GR) reaction. GR activity was determined using the method from Carlberg and Mannervik (1975), based on the absorbance decrement caused by NADPH oxidation. Glutathione S-transferase (GST) activity of the samples was determined following the method of Frasco and Guilhermino (2002), by measuring the increase in absorbance due to the formation of a conjugate between glutathione and 2,4-dinitrochlorobenzene. DT-diaphorase (DTD) activity was measured using a modified method of Lemaire et al. (1996), based on the decrease in absorbance resulting from the reduction of 2,6-dichlorophenol indophenol. Glucose 6-phosphate dehydrogenase (G6PDH) activity was measured following a modified method of Löhr and Waller (1965), measuring the change in absorbance due to NADPH production.

These enzymatic activities were expressed as specific activity, for which the soluble protein content in the samples was quantified using Bradford (1976) method. A unit of activity was defined as the amount of enzyme required to transform one μmol of substrate per minute under the measurement conditions. For SOD, a unit of activity was defined as the amount of enzyme required to generate a 50 % inhibition in the reduction of cytochrome *c*.

Total antioxidant capacity of each extract was also determined as Trolox-equivalent antioxidant capacity (TEAC), a measure of non-enzymatic or low-molecular weight antioxidants including glutathione, vitamins and protein -SH radicals. For this, the method described by Erel (2004) was used, in which reduction of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) was used to determine TEAC of the samples. Simultaneously, the absorbance of a Trolox standard, an antioxidant analogous to vitamin E, was measured to create a standard curve for interpolating the results. Finally, thiobarbituric acid reactive substances (TBARS) content was determined as a marker of oxidative damage to lipids using a modified method of Buege and Aust (1978). For determination of TBARS, malondiadehyde (MDA) was used as standard.

All measurements were performed with a PowerWave microplate spectrophotometer (Bio-Tek Instrument, Inc.) setting a stable temperature of 25 °C for enzymatic determinations.

2.4. Statistical analysis

Data processing and statistical analysis were carried out using R 4.3.0 and RStudio 2023.12.0. All results were expressed as mean ± standard error of the mean (SEM). For all nine oxidative stress response variables, mean values measured on column and tentacle were compared using a paired *t*-test. In order to compare the means of the response variables between different treatments, a one-way ANOVA test was applied, using Tukey's Honest Significant Difference test as a post-hoc analysis when significant differences were detected. Homoscedasticity and normality of residuals were checked using Levene's test and Shapiro-Wilk test, respectively. A confidence level of 95 % ($p < 0.05$) was established for all the performed techniques. All *p*-values were adjusted using Benjamini-Hochberg correction to account for multiple testing. To reduce dimensionality and describe the dataset, examine variable correlations and identify relevant oxidative stress markers, principal component analysis (PCA) was performed on the oxidative stress parameters.

3. Results

3.1. Growth and reproduction

Fig. 1A shows the mean body weight gain for each experimental condition. While the control and limited sunlight (LS) groups experienced intense growth, the mean body weight gain was moderate and closer to 0 for brackish water (BW) and integrated multitrophic aquaculture (IMTA) treatment, with some replicates having experienced a decrease in weight. However, a one-way ANOVA did not report statistically significant differences across treatments for this parameter.

Asexual reproduction during the experimental period was measured as change in total number of individuals per replicate (Fig. 1B). Control conditions resulted in a significantly higher number of individuals than the brackish water and IMTA conditions, which in average decreased their total number of anemones. The change in number of anemones found in LS conditions was found to be similar to 0.

3.2. Oxidative state analysis

Most analysed parameters featured significant differences between columnar and tentacular samples (Fig. 2). SOD, CAT, GPx, GR, and G6PDH were all found to have a higher mean enzymatic activity in the column compared to tentacle ($p < 0.001$). GST and DTD also exhibited the same pattern ($p < 0.001$), and the difference was of greater magnitude in both cases: enzymatic activity was found to be 3.55 (GST) and 6.49 (DTD) times higher in column.

Trolox equivalent antioxidant capacity (TEAC) was the only parameter not to significantly differ between column and tentacle. Average MDA concentration was, however, higher in tentacles than in columns ($p = 0.007$).

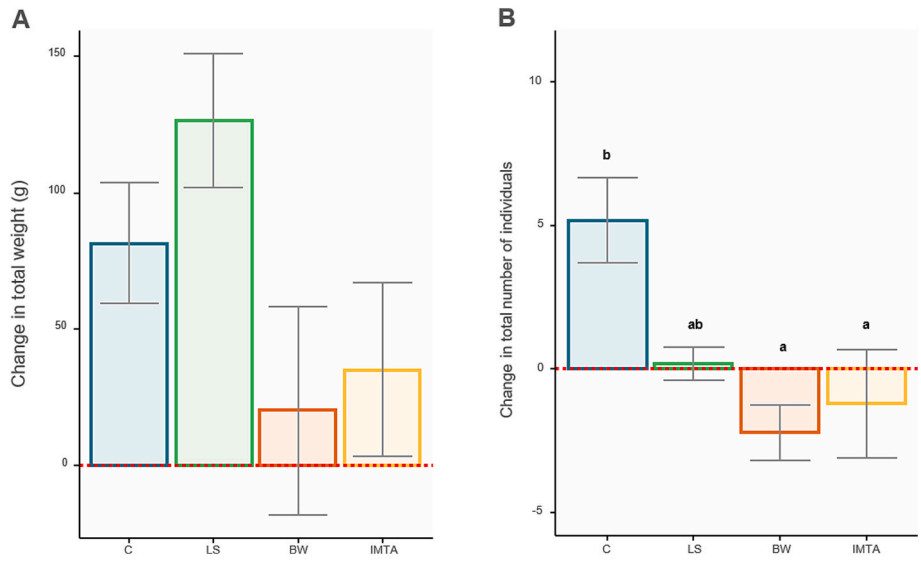

Fig. 1. Change in total weight (A) and change in total number of individuals (B) across different experimental conditions: C (Control), LS (Limited sunlight), BW (Brackish water) and IMTA (Integrated Multitrophic Aquaculture). Values correspond to mean ± standard error of the mean (SEM). a, b, c: significant difference across conditions. $n = 5$ replicates per experimental group.

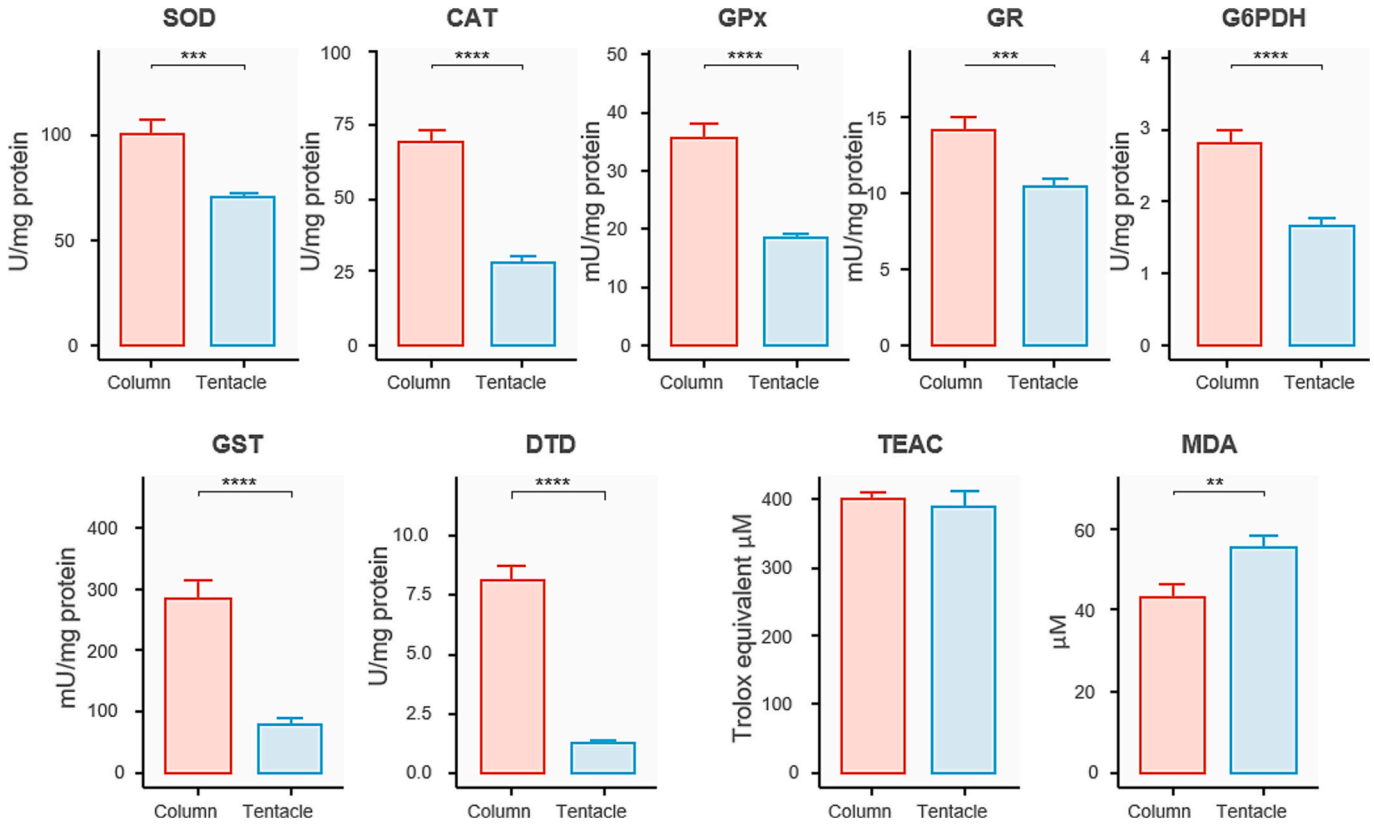

Fig. 2. Comparison between columnar and tentacular oxidative stress parameters for SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), GR (glutathione reductase), G6PDH (glucose-6-phosphate dehydrogenase), GST (glutathione transferase), DTD (DT-diaphorase) TEAC (Trolox-equivalent antioxidant capacity) and MDA (lipid peroxidation expressed as malondialdehyde). Values correspond to mean ± standard error of the mean (SEM). *: significant differences between body regions. $n = 20$ per body region.

Regarding differences between experimental conditions, a one-way ANOVA revealed that mean levels of SOD activity (Fig. 3A) did not significantly vary across conditions for either body region, although a non-significant ($p = 0.19$) trend can be observed in the column, where anemones under BW and IMTA conditions tended towards lower mean SOD activity. CAT activity measured in the column and tentacles also did not differ significantly across conditions (Fig. 3B).

Both body regions showed significant differences in GPx activity across experimental conditions (Fig. 4A). Anemones from the BW and IMTA treatments exhibited lower average columnar activity than control and LS groups ($p = 0.002$), and IMTA anemones also exhibited higher tentacular activity than BW ($p = 0.04$). A similar pattern, albeit

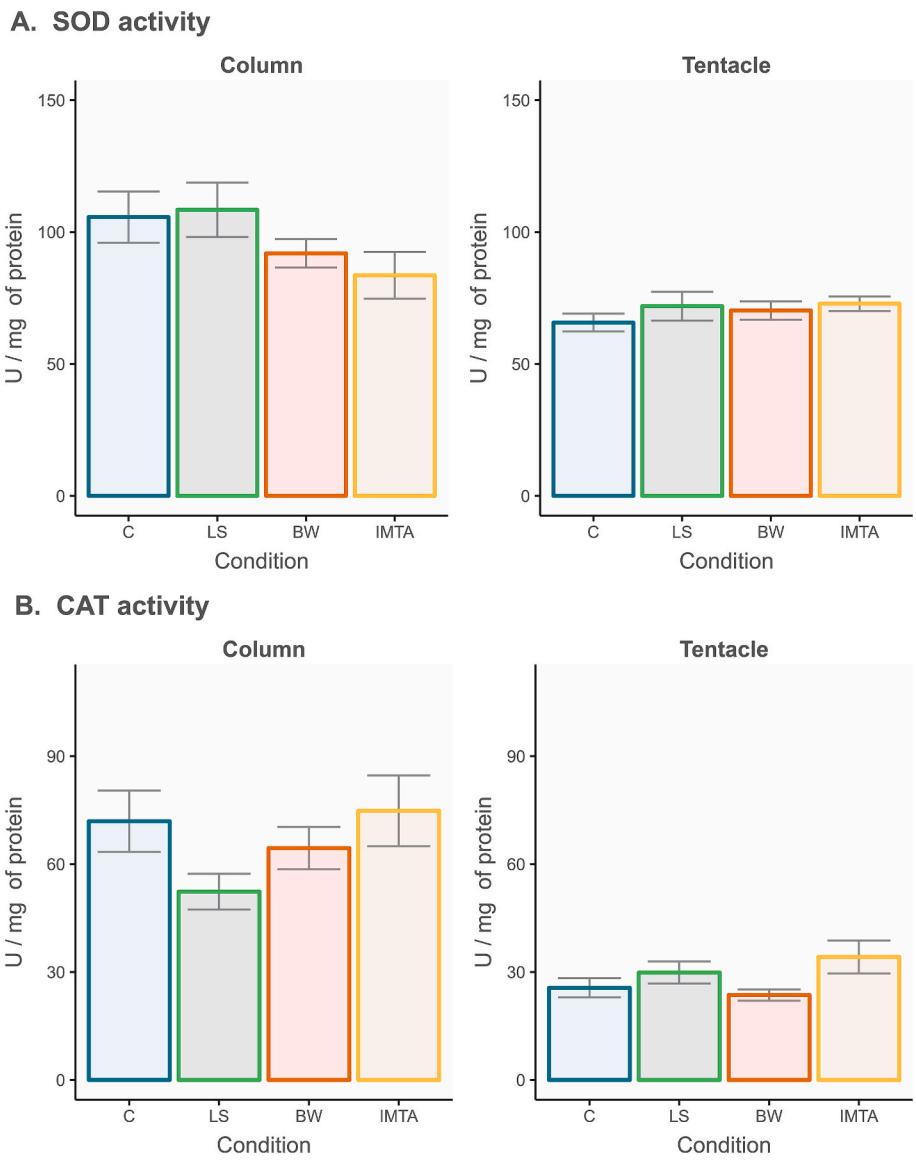

Fig. 3. Superoxide dismutase (SOD, A) and catalase (CAT, B) activity on column and tentacles, across different experimental conditions: C (Control), LS (Limited sunlight), BW (Brackish water) and IMTA (Integrated Multitrophic Aquaculture). Values correspond to mean ± standard error of the mean (SEM). $n = 5$ per experimental group.

non-significant, was observed for columnar GR activity (Fig. 3B). Regarding G6PDH, no significant differences were observed in the column across experimental conditions, but tentacular activity was significantly higher ($p = 0.02$) in animals from the IMTA treatment group compared to those of LS and BW treatments (Fig. 4C).

As for GST and DTD activity, no significant differences were found across experimental conditions (Fig. 5). TEAC levels in the column were significantly lower in the BW group ($p = 0.001$) relative to the other experimental conditions, while in the tentacles, there were no significant differences (Fig. 6A). MDA levels were not significantly different in any of the experimental conditions (Fig. 6B).

3.3. Principal component analysis (PCA)

PCA was performed on the data for all measured enzymes (SOD, CAT, GPx, GR, G6PDH, GST and DTD), TEAC and MDA. Discriminating between columnar and tentacular variables made for a total of 18 variables input into the model. Five principal components of relevance were selected, retaining 77 % of the original variance in the dataset. Fig. 7 plots the first two principal components, and the factor loadings of each variable for those two dimensions. PC1 accounted for around 30 % of the original variance in the dataset, and was found to divide the original parameters into tentacular or columnar variables. Columnar variables all contributed positively to PC1, and tentacular variables all contributed negatively, with the exception of MDA: both columnar and tentacle MDA contributed negatively to PC1. The most important contributing variables to PC1 were columnar SOD, GPx, GST and DTD activity.

PC2 explained 19.6 % of the original variance, and tentacular GPx, tentacular CAT and columnar GR were the most important contributing variables to this principal component. Only one variable (columnar MDA) was found to contribute negatively to this component, although this correlation was not strong.

PC3, PC4 and PC5 accounted for small portions of original variance, and the most important contributing variables to each of these components were tentacular G6PDH and columnar MDA for PC3 (positively correlated), tentacular SOD and MDA for PC4 (negatively correlated), and tentacular GST and TEAC for PC5 (negatively correlated).

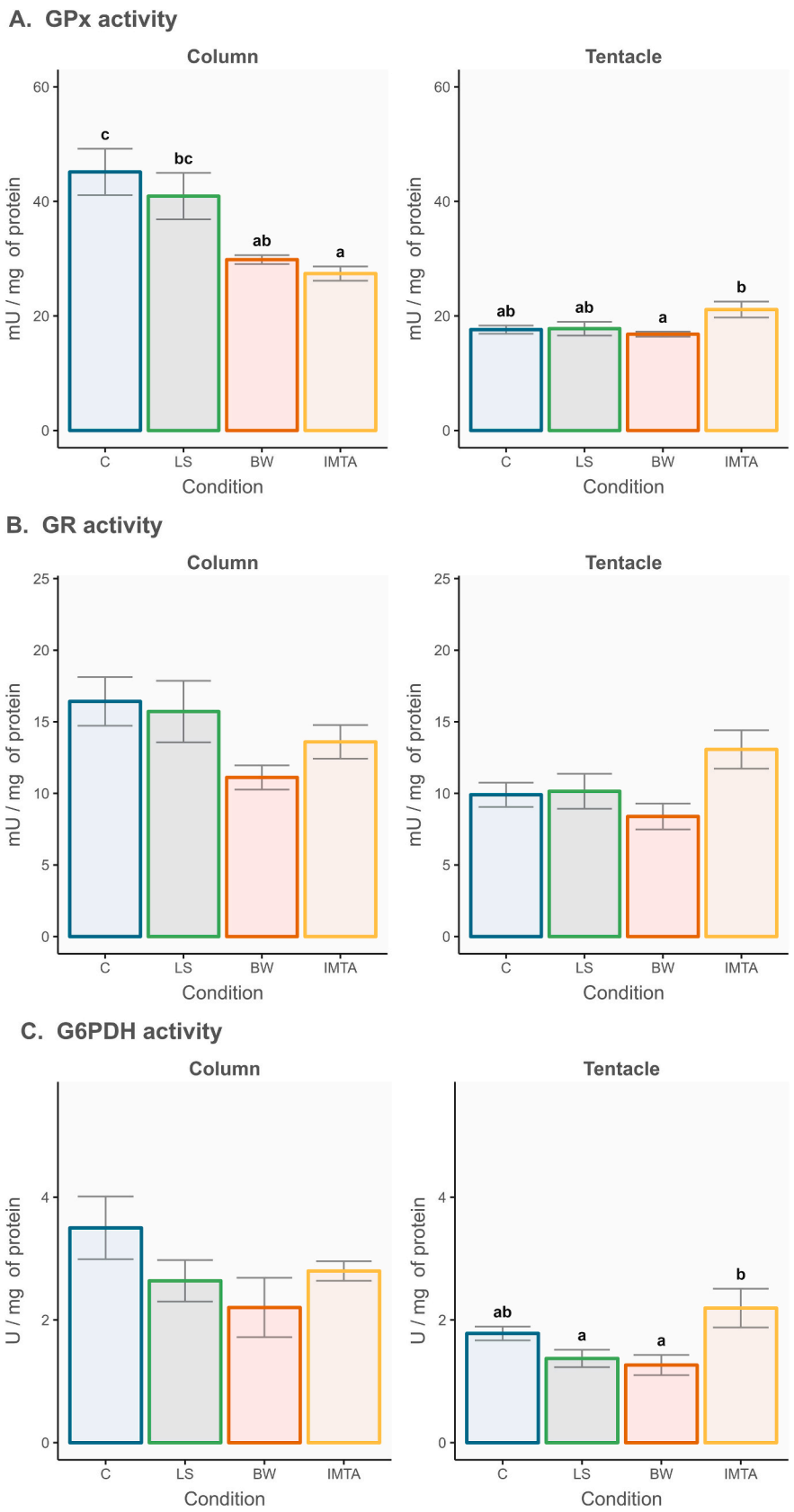

Fig. 4. Glutathione peroxidase (GPx, A), Glutathione reductase (GR, B) and glucose-6-phosphate dehydrogenase (G6PDH, C) activity on column and tentacles, across different experimental conditions: C (Control), LS (Limited sunlight), BW (Brackish water) and IMTA (Integrated Multitrophic Aquaculture). Values correspond to mean ± standard error of the mean (SEM). a, b, c: significant difference across conditions. $n = 5$ per experimental group.

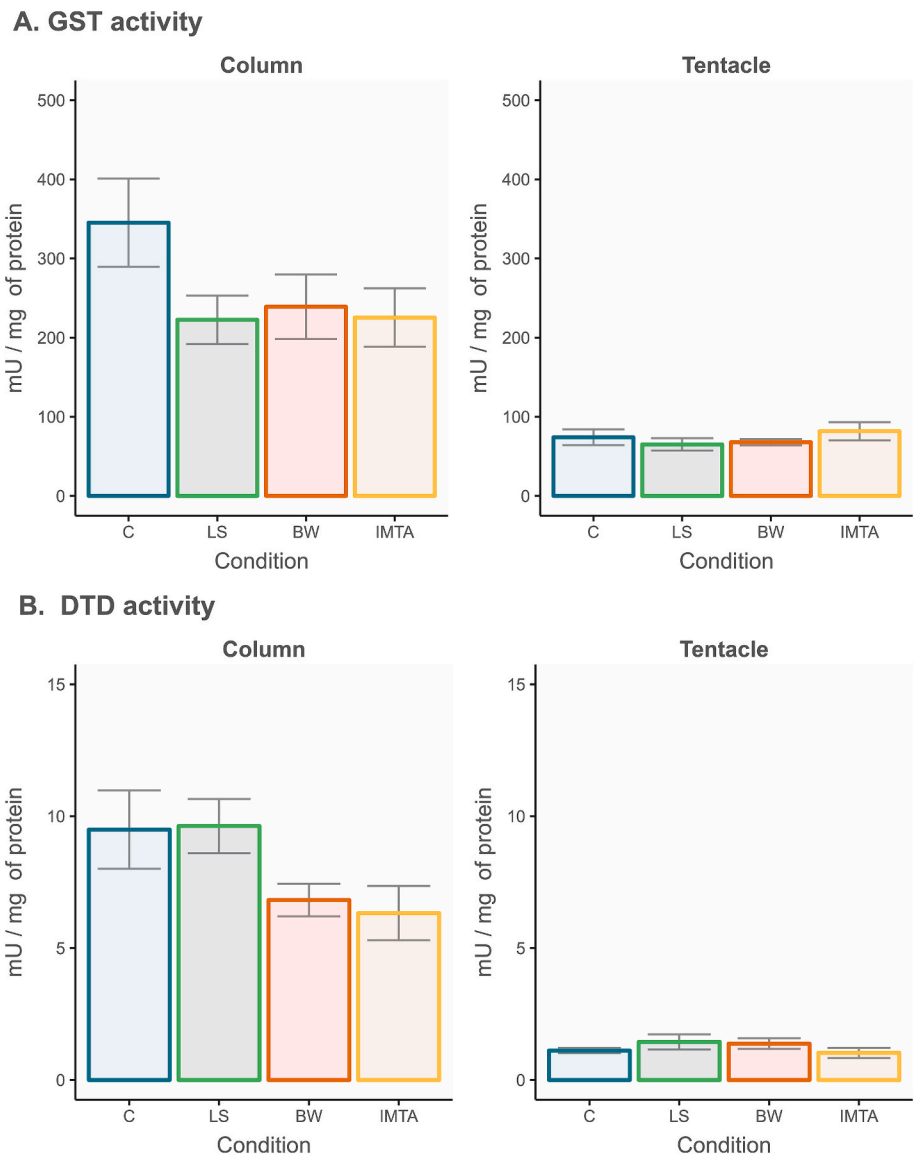

Fig. 5. Glutathione transferase (GST, A) and DT-Diaphorase (DTD, B) activity on column and tentacles, across different experimental conditions: C (Control), LS (Limited sunlight), BW (Brackish water) and IMTA (Integrated Multitrophic Aquaculture). Values correspond to mean ± standard error of the mean (SEM). n = 5 per experimental group.

4. Discussion

Several studies have highlighted the importance of oxidative status markers in assessing the effectiveness of the homeostatic response to compromised conditions (Chowdhury and Saikia, 2020; Jerez-Cepa and Ruiz-Jarabo, 2021; Sánchez-Muros et al., 2013; Sanz et al., 2012). Moreover, previous results from rainbow trout (*Oncorhynchus mykiss*) and sturgeon (*Acipenser naccarii*) reported changes to antioxidant capacity correlated with tissue function within the same organism. For instance, digestive tissues, which have a high metabolic rate, feature higher antioxidant capacity compared to gills, skin or red blood cells (Trenzado et al., 2006). In the present study, the evaluation of all enzymatic activities in different morphological sections of *A. viridis* were, in general, higher in the column compared to the tentacles. The magnitude of these differences was highly variable, ranging from average 1.68 times for G6PDH, to an average of 6.49 times for DTD. The mesenteries located in the column are where absorptive, digestive and reproductive processes, as well as major muscular contraction, take place, and therefore the centre of metabolic activity in sea anemones (Schick, 1991). As such, this region is expected to generate ROS at rates that would need scavenging systems to maintain oxidative balance.

The enzymes DTD and GST featured the highest differences in columnar and tentacular activity, and both have been reported to increase their activity in aquatic organisms under the effect of pollutants (Ahmad, 1995; Almar et al., 1998; Sturve et al., 2008; Sturve et al., 2005; Valavanidis et al., 2006). DTD is involved in quinone reduction at the mitochondrial membrane level, while GST is a second phase detoxification enzyme that uses glutathione (Blanchette et al., 2007; Board and Menon, 2013; Ernster, 1967). The natural seawater used in this study was obtained from an open natural environment with a limited likelihood of contamination. Similarly, freshwater used in the brackish water (BW) groups was not chlorinated public water for human consumption, but rather water for agricultural use, and so it was free of nitrates, nitrites and phosphates, with a pH of 8 and no indication of contaminants. The increased DTD and GST activity in columnar tissue is similar in nature to that observed for the other measured enzymes, and therefore is likely related to the greater metabolic activity of this anatomical section.

Anthozoan-zooxanthellae symbiosis can affect oxidative status of sea anemones (Richier et al., 2003). Tentacles are important sites of ROS

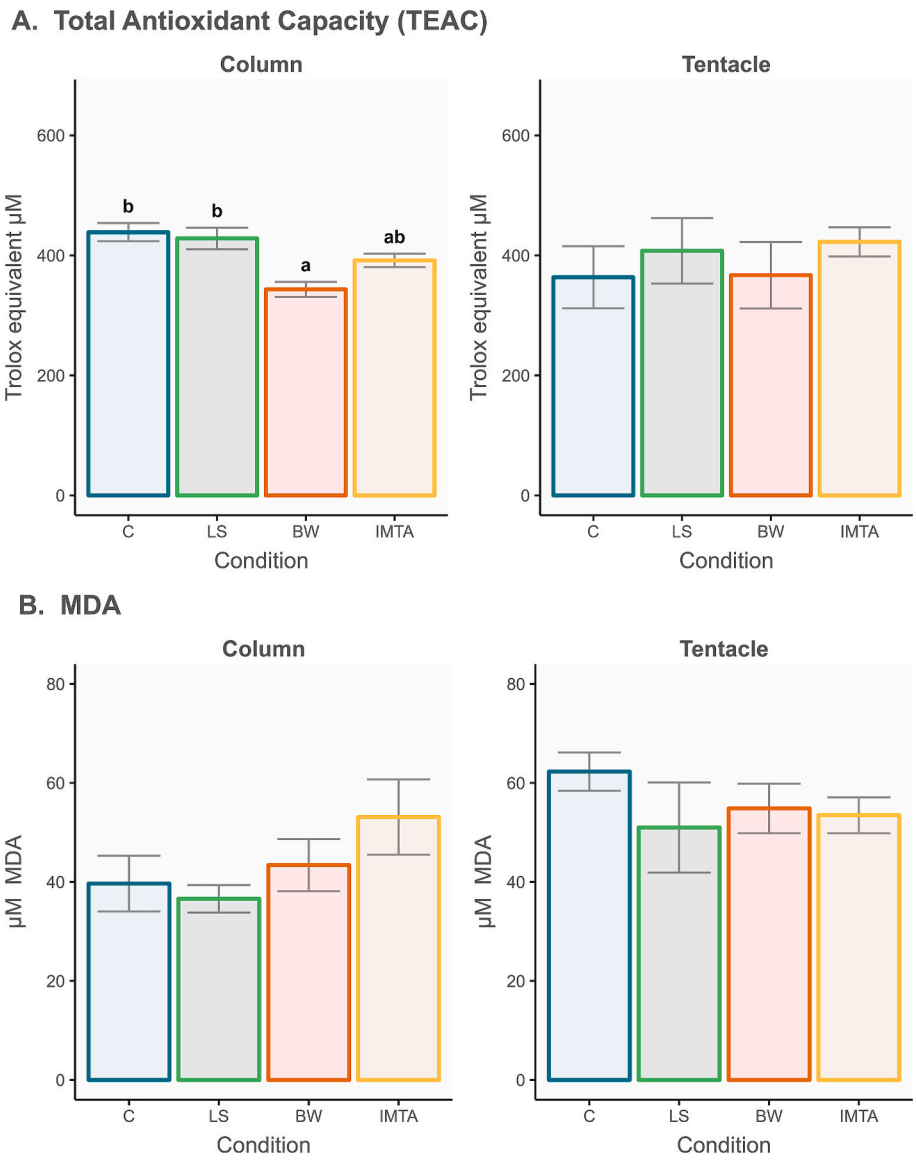

Fig. 6. Trolox-equivalent antioxidant capacity (TEAC, A) and lipid peroxidation (MDA, B) measures on column and tentacles, across different experimental conditions: C (Control), LS (Limited sunlight), BW (Brackish water) and IMTA (Integrated Multitrophic Aquaculture). Values correspond to mean ± standard error of the mean (SEM). a, b, c: significant difference across conditions. n = 5 per experimental group.

production since they are the anatomical section with the highest density of zooxanthellae, and they feature an increased photosynthetic activity during the day (Casado-Amezúa et al., 2016). This leads these tissues to experience large fluctuations in oxygen concentration originating from the symbionts' photosynthetic activity (Casado-Amezúa et al., 2016; Furla et al., 2005). Due to this exposure to oxygen and the fact that they tend to inhabit intertidal zones, symbiotic sea anemones exhibit a wide range of physiological adaptations in their antioxidant defences (Casado-Amezúa et al., 2016; Davy et al., 2012; Furla et al., 2005; Merle et al., 2007; Plantivaux et al., 2004; Richier et al., 2005; Richier et al., 2003). In this study, the results showed how all antioxidant enzymes measured had lower specific activity in the animals' tentacles. In accordance to this, a greater average concentration of MDA was also present in the tentacles compared to the column.

The oxidative status of the cultured anemones was not considered to be unfavourably influenced by any of the tested culture variables. However, certain experimental conditions generated differences in parameters related to glutathione metabolism and non-enzymatic antioxidants, which includes glutathione and other small molecular weight antioxidants (Erel, 2004).

The conditions of limited sunlight (LS), as established in this experiment, did not impose stress on the anemones, at least when combined with controlled feeding. None of the measured enzymes showed different levels of activity from the control, and there were likewise no differences in non-enzymatic antioxidants (TEAC) or MDA levels.

Symbiosis disruption in anthozoans (known as bleaching) is a phenomenon known to be linked to environmental stress, particularly thermal stress, and its absence is a sign of the animals' wellbeing (Hoegh-Guldberg, 1999; Richier et al., 2006). Since no aposymbiotic individuals were observed at the end of the experimental period, it is possible that photosynthetically active radiation (PAR) was sufficient to maintain activity in the zooxanthellae, and perhaps the lack of limitation in food availability was a key factor in maintaining symbiosis.

Moreover, LS conditions yielded little to no change in total number of individuals (Fig. 1B), indicating that asexual reproduction (longitudinal fission) was scarce. This fact, coupled with a high mean body weight gain (Fig. 1A), suggests that substantial individual growth was achieved in this experimental condition. Final body weight records support this idea, as individuals of greater size (> 50 g) were frequently

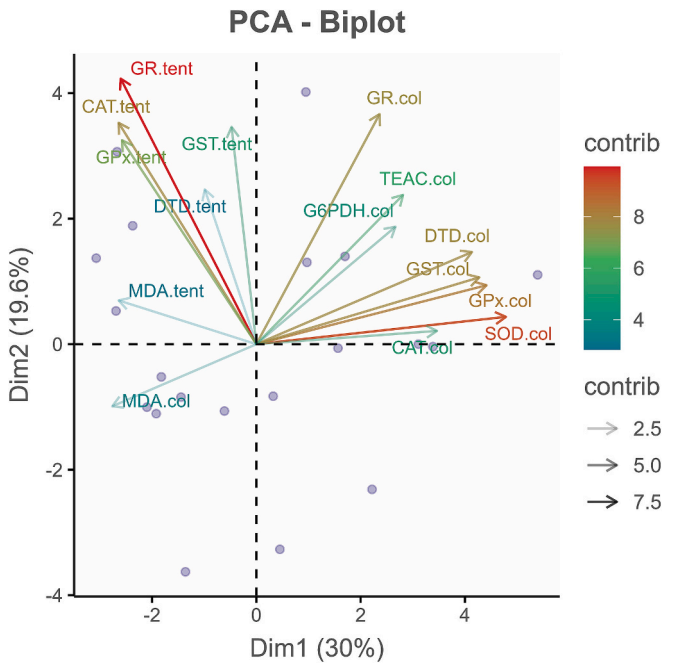

Fig. 7. Biplot of PC1 (horizontal axis) and PC2 (vertical axis). Observations are represented as points; variables are represented as arrow vectors. Colour represents variables contribution to PCs. Variables grouped closely together are positively correlated, variables in opposite quadrants are negatively correlated.

found in this experimental group. *A. viridis,* like other anemone species, features colour polymorphism, and some of the colour morphs of this species have been found to occur more frequently at certain depths and also feature a higher tendency to reproduce sexually rather than asexually (Mallien et al., 2017; Poding et al., 2024; Porro et al., 2019; Wiedemann, 1999). On the other hand, while animals kept under control conditions experienced a similar weight increase to LS animals, this condition resulted in more intense asexual reproduction, as shown by the increase in number of individuals (Fig. 1B) suggesting a lower body weight gain per animal. Although further exploration of this phenomena and its relationship with colour morphs is needed, the present results suggest that limiting the light intensity in the aquaculture environment could mimic deeper subtidal conditions and affect the reproductive behaviour of the anemones, without any apparent impact to the oxidative status of the animals.

The long-term exposure of snakelocks anemone specimens to brackish water (BW) revealed alterations in parameters related to growth, reproduction and oxidative status. In the present study, BW conditions yielded a slightly smaller change in body weight, though not significantly smaller than control conditions (Fig. 1A). Asexual reproduction, however, was not present, as shown by the mean change in total number of individuals (Fig. 1B). A similar response was shown by Podbielski et al. (2022) for the orange-stripped anemone (*Diadumene lineata*) in the Baltic Sea, where asexual reproduction was impacted as salinity declined. Regarding oxidative status, columnar TEAC levels and particularly GPx activity were significantly lower in BW than in the control, while no other significant effects were found associated with this condition.

Typically, oxidative stress induced by salinity is associated with increases in expression and activity of antioxidant enzymes in marine animals (Choi et al., 2008; Ren et al., 2015). Salinity-induced oxidative stress affects both osmoconforming and osmoregulating invertebrates, and other studies report different behaviours regarding the effects of salinity on the antioxidant response, depending of tolerance to salinity among species, specific tissue, experimental conditions and the adaptation period to the environmental stress (Bal et al., 2021; Liu et al., 2007; Mozanzadeh et al., 2021; Yin et al., 2011). Surprisingly, no increase of MDA level was found in the present study, suggesting that that oxidative damage did not occur.

In *A. viridis* and other sea anemones, the main mechanism regulating cell volume changes is the free amino-acid pool (FAA) (Podbielski et al., 2022; Schick, 1991). When exposed to hyposaline environments, intracellular FAA concentration is reduced either by increasing cellular membrane permeability to amino-acids or by increasing the oxidation rate of these compounds, which in turn increases oxygen consumption (Schick, 1991). Podbielski et al. (2022) found that organic osmolyte concentration decreased linearly in *D. lineata* with exposure to low salinity, while total osmolality of tissues remained stable with decreasing salinity until a threshold of 10 g/L was reached. The four-week duration of our experimental period and the use of brackish water (30 g/L) could mean that the initial short-term response of antioxidant enzymes to salinity was effective containing oxidative damage, and only after a long term stress the enzymatic activity of GPx decreased (by possible depleted expression) under the basal levels displayed by the control group.

Concerning the IMTA group, the lack of significant response in SOD and CAT levels is consistent with previous findings of a high constitutive activity of these antioxidant enzymes and high diversity of isoforms (Merle et al., 2007; Plantivaux et al., 2004; Richier et al., 2003). Instead, IMTA anemones were found to have a greater tentacular GPx and G6PDH activity than controls, suggesting activation of glutathione metabolism and NADPH generation via pentose phosphate pathway. GR activity showed a similar trend, as expected since its activity is coupled with GPx. However, this pattern did not manifest in column tissue of animals under IMTA, which displayed a significantly lower non-enzymatic antioxidant capacity (TEAC). Some direct observations of the water during the trial revealed a high turbidity due to phytoplankton growth, possibly because the system was not fully mature within the four-week experimental period. This could have influenced the photosynthetic activity and ROS production of zooxanthellae, which are more abundant in the tentacles (Casado-Amezúa et al., 2016). These results suggest the GPx/GR system is the main ROS scavenging pathway that was regulated in these animals under an ecological interaction generated by the IMTA treatment. In any case, this response was effective in preventing tissue damage, since MDA values were not increased in anemones under IMTA.

Oxidative status parameters can be difficult to interpret, since they can fluctuate for reasons other than stress, such as ongoing reproductive processes or natural variability during ontogeny (Beaulieu and Constantini, 2014). For instance, increases in antioxidant defences can have different biological meaning depending on physiological context. Evaluating antioxidant defences alongside markers of oxidative damage helps with interpretation of this context, but even then, common measures of MDA, such as TBARS, have relatively low-specificity that can hinder the interpretation of oxidative status (Lushchak, 2011). Furthermore, antioxidant status markers are functionally correlated among them, which means that there is a certain amount of redundant information in their evaluation (Beaulieu and Constantini, 2014; Hõrak and Cohen, 2010). In this type of scenarios, dimension reduction techniques are appropriate tools to unravel the underlaying structure of data and provide a clearer view of relationships between variables (Goodman, 1972; Hõrak and Cohen, 2010; Ringnér, 2008). In our study, PCA managed to reduce the number of dimensions in the dataset from 18 to 5, while retaining over 77 % of the original variance. Both tentacular and columnar MDA measures were negatively associated with Principal Component (PC) 1 along with all the measured tentacular parameters (Fig. 7). These results suggest that individuals with higher overall MDA tended to exhibit higher tentacular antioxidant enzyme activity and higher tentacular TEAC levels. On the other hand, individual anemones featuring higher columnar enzymatic activities tended to exhibit lower MDA in both tentacle and column. Since all the variables were standardized during PCA, this correlation exists independently from the

reported differences in magnitude between columnar and tentacular parameters. This information brings nuances to the results from the previous univariate analysis. For instance, the higher G6PDH activity found in anemones from the IMTA condition is re-contextualized as the likely result of metabolic activation due to oxidative stress, possibly to replenish NADPH used by GR.

PC2 separated columnar MDA from the rest of parameters, however, this pattern was of less explicative relevance than the one associated with PC1, and likely corresponds to the opposite relationship of oxidative damage end products with antioxidant defences. Columnar SOD and GPx activity, and both columnar and tentacular GR activity were the most relevant parameters contributing to the two first principal components. This means that despite not showing variation across experimental conditions, columnar SOD activity was a relevant parameter shaping the dataset at a multivariate level, as is expected since it is the first enzyme to act against ROS in antioxidant metabolism (Lesser, 2006; Lushchak, 2011). GPx and GR were once again highlighted as an important ROS scavenging pathway in *A. viridis*.

PCs 3, 4 and 5 explained a progressively lesser proportion of the total variance. PC3 showed a pattern of anemones with higher columnar MDA also having particularly high columnar G6PDH, which is consistent with the role of this enzyme in metabolic activation, either as the cause or effect of oxidative stress (Ho et al., 2007). In PC4, tentacular SOD and GPx were shown to be negatively correlated to MDA levels, highlighting the antioxidant function of these two enzymes. Principal components become more difficult to interpret and less biologically meaningful as they begin to explain smaller and smaller amounts of the data variance (Goodman, 1972; Ringnér, 2008).

Although PCA has some limitations, such as assumption of linearity between variables (Ringnér, 2008), it provided relevant information about the dataset that proved useful for interpreting the results of the experiment. Furthermore, due to their positive correlation to MDA, tentacular enzymatic activities and TEAC may be of interest as markers of oxidative stress in *A. viridis*, which could open the possibility of using less invasive techniques to evaluate the welfare of this sea anemone species.

5. Conclusions

In view of the results, both biotic and abiotic factors must be considered in an *A. viridis* culture system. Long-term exposure to brackish water caused alterations in some parameters related to the oxidative status, which could derive from physiological stress response mechanisms to osmotic changes. However, the lack of oxidative damage to lipids indicates small drops in salinity are unlikely to generate tissue damage and unduly affect the health of the cultured individuals. Regarding IMTA, anemones exhibited alterations in antioxidant enzymes possibly linked to the metabolic activity of zooxanthellae, modulated by short-term ecological interactions in a multitrophic system. Finally, the lack of direct solar radiation, under controlled feeding of the specimens, was not a limiting factor in *A. viridis* aquaculture, which seemed to be able to thrive in shaded aquaculture systems with no noticeable impact on their wellbeing. Results seem to indicate, according to MDA levels, that oxidative stress was not reached in any of the groups. However, LS condition seemed to have a positive impact on the performance of the culture, since the specimens cloned asexually less intensely (therefore achieving substantial individual growth) and their antioxidant response did not differ from that of the controls. This may be a point of interest to modulate the reproductive behaviour of the culture, in order to promote growth and/or yield individuals capable of sexual reproduction to preserve genetic variability.

In summary, aquaculture of *Anemonia viridis* can be carried out under controlled and suitable environmental conditions with a broad range of lighting conditions without affecting the wellbeing of the animals. Antioxidant mechanisms involving glutathione appear to be a suitable option as markers of welfare status.

CRediT authorship contribution statement

Alberto Coll: Writing – original draft, Investigation, Formal analysis. **Eva E. Rufino-Palomares:** Writing – review & editing, Methodology. **Marta Ramos-Barbero:** Investigation. **A. Esther Ortiz-Maldonado:** Resources. **Laura M. Pantoja-Echevarría:** Investigation. **Ismael González-Ordóñez:** Investigation. **Amalia Pérez-Jiménez:** Writing – review & editing, Methodology. **Cristina E. Trenzado:** Writing – review & editing, Supervision, Project administration.

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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