

Graphical Abstract

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Abstract

Anemonia viridis is a species of sea anemone (Cnidaria:Anthozoa) currently experiencing environmental impacts on their natural populations in Southern Spain. Their regeneration capacity allows for inducing asexual reproduction in captivity via longitudinal sections, but there is no information about the impact of this technique on the animal's well-being after regeneration. The goal of this work was to assess the viability of this reproduction technique in *A. viridis*, using oxidative status as a marker of well-being, and characterizing the early wound healing process through histological analysis.

We measured enzymatic and non-enzymatic antioxidants, lipid peroxidation, phosphatases activity and myeloperoxidase activity in regenerated and control anemones, at 4 weeks post-injury and 20 weeks post-injury, and performed Principal Component Analysis to identify trends at the multivariate level. Additionally, we carried out a descriptive histological study of the wound healing process during the first 7 days post-injury.

Results and Discussion: We found higher antioxidant enzymatic activity (mainly detectable in catalase, glutathione peroxidase and glutathione-S-transferase) in regenerated anemones. This response was often reverted by

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20 weeks post-injury in tentacular measurements. No differences in lipid peroxidation were observed, indicating that the antioxidant response was effective containing oxidative damage

Keywords:

1. Introduction

The snakelocks anemone (*Anemonia viridis*) is a species of Cnidarian widely distributed in the Northeast Atlantic Ocean and the Mediterranean Sea. It inhabits rocky bottoms ranging from the lower intertidal zone to the subtidal, down to around 15-20 m deep (Calvín Calvo and Eisman Valdés, 2020). The snakelocks anemone has been traditionally exploited for human consumption in Spain, particularly in Andalusia (Daza Cordero et al., 2002; Utrilla et al., 2019). However, the poor ecological state of stocks in the Andalusian coast led to an indefinite ban on its capture in the region, in effect since October 2023. As current demand of this species is not being met, and populations still are vulnerable to illegal gathering, there is great interest in the development of alternative production methods. Aquaculture of the snakelocks anemone is not yet fully established, but can function as a major engine in the production of this species in the region.

Like most sea anemones, *A. viridis* is capable of both sexual and asexual reproduction (Bocharova, 2016; Utrilla et al., 2019). Concerning sexual reproduction, *A. viridis* is a dioic species with external fertilization, and spawning usually takes place in spring, around the months of April and May in the Andalusian coast (Utrilla et al., 2019). Asexual reproduction is widespread among sea anemones and can happen through different mechanisms. In the case of *A. viridis*, asexual reproduction primarily takes place through longitudinal fission of the animal, in a plane that is perpendicular to their pharynx plane (Bocharova, 2016; Macias-Muñoz, 2025). Other mechanisms, such as budding, have been recorded previously but seem to be sporadic (Utrilla et al., 2019). In cnidarians, asexual reproduction can also occur as a way of regeneration after an injury, and sea anemones are able to regenerate missing body parts with ease (Amiel et al., 2015; Bocharova, 2016; Holstein et al., 2003). While sexual reproduction is vital for the long-term success of an aquaculture stock, asexual cloning of anemones represents a reliable way of quickly increasing stock size. However, in order to achieve that, asexual reproduction must 1) be artificially inducible and 2) not affect the well-being

of the animals severely or irreversibly.

Artificially sectioning the animals to induce regeneration of the missing body parts and obtain two clonal anemones is possible in *A. viridis*, yet it is also an invasive procedure that involves manipulation of the animals, wound healing and tissue regeneration. Generation of reactive oxygen species (ROS) occurs both after injuries and during regeneration in sea anemones and other anthozoans (Corte et al., 2023; Mydlarz and Jacobs, 2006; Parisi et al., 2020; Vullein, 2024), and while ROS signaling and production perform important roles during these processes, imbalances between ROS generation and scavenging can result in damage to cellular components such as poly-unsaturated fatty acids (PUFAs), proteins, or DNA binding, potentially impacting the organism's health even after the regeneration process is over (Lesser, 2006; Lushchak, 2011).

However, *A. viridis* possesses a combination of traits that makes it a good candidate-species to reproduce asexually in captivity without severe impacts on its health after regeneration . First, as an intertidal-dwelling organism, *A. viridis* polyps are often exposed to large variations in temperature, salinity, humidity and UV exposure, and to other environmental stressors such as wave action (Casado-Amezúa et al., 2016; Richier et al., 2005). Their wide tolerance limits and ability to recover from challenging environmental situations could mitigate stress due to handling and manipulation of the animal. Secondly, like most cnidarians, they possess a great tissue regeneration capability, as they are able to heal wounds in 24 h and regenerate missing body parts with ease (Corte et al., 2023; Parisi et al., 2020). Finally, *A. viridis* is a symbiotic anthozoan that hosts photosynthetic microalgae or zooxanthellae of the genre *Philozoon* (previously *Symbiodinium* clade A temperate) (LaJeunesse et al., 2022) in their gastrodermis. As a result of this trophic mutualism, they are adapted to deal with the wide variations in oxygen partial pressure that take place between daytime and nighttime due to zooxanthellae photosynthesis (Casado-Amezúa et al., 2016; Richier et al., 2005). These adaptations to symbiosis involve a more robust antioxidant system than found in non-symbiotic sea anemones (Cotinat et al., 2022; Merle et al., 2007; Pey et al., 2017; Richier et al., 2003, 2005, 2006), that could prove beneficial in dealing with the surge in ROS production expected during regeneration.

The duality of antioxidant defenses and oxidative damage makes oxidative stress a unique and valuable physiological marker to assess both the responses that organisms develop in other to face stressors and the adverse effects that

they experience when doing so (Beaulieu and Costantini, 2014). Furthermore, biomarkers of oxidative stress are not confined to a certain taxonomic range: they can be assessed in any organism that has aerobic metabolism or is exposed to oxygen. The universality of these markers makes them highly valuable when studying cnidarians, where other stress markers would be non-applicable (Beaulieu and Costantini, 2014; Casado-Amezúa et al., 2016; Valavanidis et al., 2006).

Cnidarian immunity is fundamentally innate, comprised of processes of pattern recognition and intracellular signaling followed by effector responses and lastly, tissue repair (Parisi et al., 2020). Alkaline phosphatases (ALP) are one of the first enzymes to act in inflammatory processes, and high expression levels of this enzyme have also been reported during regenerative processes in *A. viridis* (Abe et al., 2001; Mauro et al., 2021; Parisi et al., 2021). Lysosome hydrolases (such as acid phosphatase (AP) are considered regeneration markers in invertebrates, and both acid and alkaline phosphatases have been linked to epithelial cell and nematocyst differentiation in *Hydra* (Konada et al., 2020; Lentz and Barrnett, 1962; Orlando et al., 1991; Trapani et al., 2016). Regarding cellular responses, mass recruitment and proliferation of amoebocytes, the immune effector cells in anthozoans, has been described both in *A. viridis* and other hexacoral anthozoans during wound healing (Corte et al., 2023; Mydlarz et al., 2008; Parisi et al., 2020; Snyder et al., 2021; Vargas-Ángel et al., 2007). Amoebocytes also undergo degranulation during wound healing, may differentiate into fibroblast-like collagen-producing cells, and, in some cnidarian species, have been reported to exhibit respiratory burst as an immune mechanism (Hutton and Smith, 1996; Corte et al., 2023; Mydlarz and Jacobs, 2004, 2006; Parisi et al., 2014; Snyder et al., 2021).

In the present study, we examined the viability of artificially sectioning snakelocks anemones (*Anemonia viridis*) as a method for inducing asexual reproduction in captivity. We recorded mortality during the process and assessed the impact of this process on their well-being, at two different time scales after manipulation. We evaluated oxidative status and activity levels of immune enzymes as well-being markers. We then performed a histological study of regenerating (*Anemonia viridis*) anemones to characterize the regeneration process after this type of injury and determine the temporal scale at which operates on.

2. Materials and Methods

2.1. Experimental design and sampling

300 wild anemones were obtained from natural environments off the coast of Granada (Andalusia, Spain) in November 2020 with the pertinent permits from local authorities. Animals were transferred to facilities of Andalmar Biotech S.L., where they were distributed in floating baskets in a concrete 16 m³ outdoor tank, set under a 0.5 mm white nylon mesh cover. The tank was equipped with a recirculation system consisting of a mechanic sand filter, biological filter with 300 m²/m³ bio-balls and protein skimmer. Natural seawater was pumped from a well in the facilities and then filtered to be used in the circuit. Salinity and pH were monitored during the experimental period, centered around 34.8 ‰ and pH 8. Water temperature varied with ambient temperature, with an overall mean of 17.5° C through the study.

Anemones were distributed in 11.8 L floating baskets at a density of 16-17 individuals per basket, making a total of 18 baskets. An Integrated Multitrophic Aquaculture (IMTA) culture environment was established, where other organisms were co-cultured in the same system: beadlet anemones (*Actinia equina*), sea urchins (*Paracentrotus lividus*, *Arbacia lixula*), sea snails (*Monodonta turbinata*), limpets (*Patella caerulela*), sea cucumbers (*Holothuria tubulosa*), mussels (*Mytilus edulis*), and macroalgae (*Ulva rigida*, *Cystoseira mediterranea*) (Casado-Amezúa et al., 2016).

After acclimation to the aquaculture conditions for one month, nine of the baskets were selected as experimental group, and anemones in them were sectioned in two through their pharyngeal biradial axis. Each of the resulting halves was separated and left to heal, so as to generate two different clonal individuals. Mortality during the healing phase was recorded.

The first sampling (T1) took place 4 weeks post-injury, once the anemones had had time to heal completely. 9 sectioned anemones (1 per basket) were selected, as well as 9 control anemones which had not undergone the procedure. Sectioned anemones were identified at all times to ensure that sampled individuals would not include both clones obtained from the same parent anemone. The second sampling (T2) took place 20 weeks post-injury, and again 9 control anemones and 9 sectioned anemones were collected with the same regards over clonal anemones.

At both sampling events, each anemone was snap-frozen in liquid nitrogen and stored at -80° C. The column and tentacles of each individual was homogenized separately (Heidolph Instruments) in 100 mM Tris, 0.1 EDTA

and 0.1% Triton buffer (pH 7.8) at a 1:4 ratio (w/v). Extracts were then centrifuged at 16 000 rpm for 30 minutes at 4° C (Sigma 3 K30), and the supernatant was collected and stored at -80° C for posterior analysis.

2.2. Oxidative status assays

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined according to McCord and Fridovich (1969) method, consisting on an indirect measurement as the degree of inhibition of cytochrome c reduction. Determination of catalase (CAT) (EC 1.11.1.6) activity was performed using Aebi (1984) method, based on the decrease in absorbance produced by H_2O_2 consumption by this enzyme.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity of the samples was determined following Flohé and Günzler (1984) method, based on an indirect measurement of NADPH oxidation, generated by its coupling with a standard glutathione reductase (GR) (EC 1.8.1.7) reaction. GR activity was measured according to Carlberg and Mannervik (1975) method, consisting on a measure of the absorbance decrement caused by NADPH oxidation. Glucose 6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) activity of the samples was obtained using a modified method of Löhr and Waller (1965), based on recording the change in absorbance due to NADPH production by the enzyme.

Determination of glutathione S-transferase (GST) (EC 2.5.1.18) activity was performed following the method of Frasco and Guilhermino (2002), based on the formation of a conjugate between glutathione and 2,4-dinitrochlorobenzene that increases absorbance. NQO1 (EC 1.6.99.2) activity was determined using a modified method of Lemaire et al. (1996), based on measurement of the decrease in absorbance caused by reduction of 2,6-dichlorophenol indophenol.

Soluble protein content was quantified following Bocharova (2016) method in order to express enzymatic specific activity. One 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, units of activity had a different definition, as the amount of enzyme required to generate a 50% inhibition in the reduction of cytochrome c.

Total antioxidant activity was measured according to Erel (2004) method, based on the change of absorbance due to reduction of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), to determine Trolox-equivalent antioxidant capacity (TEAC) of the extracts. Last, oxidative damage to lipids

was assayed as thiobarbituric acid reactive substances (TBARS) content, following a modified method from Buege and Aust (1978) and using malondialdehyde (MDA) as a standard.

2.3. Immune status parameters

Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases (AP and ALP) activity were measured following the method of Huang et al. (2011), based on the change of absorbance produced by the activity of the enzyme on p-nitrophenyl at different pH. Determination of myeloperoxidase (MPO) activity (EC 1.11.1.7) was carried out following a modified method of Mohanty and Sahoo (2010), based on the increase of absorbance due to oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by the products of MPO activity.

Both oxidative status and immune parameters measurements were carried out with a PowerWave microplate spectrophotometer (Bio-Tek Instrument, Inc.) at a stable temperature of 25 °C for enzymatic determinations.

2.4. Histology

For histological characterization, a different subset of snakelocks anemones were kept in 3 polyester fiber 500 L tanks, with a common filtration tank, provided with mechanical filtration media (industrial foam) and 10 L of bio-balls (Kaldnes). Recirculation was established using a pump (Sicce Multi, 2500 L/h 50W). Natural illumination was complemented with 15W-6400K white LED lights programmed to match natural photoperiod.

12 individual anemones were subjected to the sectioning procedure. Samples were taken immediately after the section, 6 hours after, 24 hours after, and a week after the section, resulting in 3 replicates per time point. All anemones displayed the *vulgaris* colour morph (Porro et al., 2019) and had similar sizes to reduce possible sources of variability in regeneration time.

From one of the two halves of the sectioned anemones, a small portion which included the original injury was extracted and fixated in PFA 4% for 24 h. After fixation they were washed and transferred to PBS for storage prior to histological processing. Paraffin-embedded tissue blocks were sectioned at 5 micrometers using a rotary microtome (Leica RM 2135). Sections were deparaffinised and stained with Masson-Goldner trichrome stain (Sigma-Aldrich) for light microscopy observation and descriptive analysis of the injured body wall.

2.5. Statistical analysis

All statistical analysis and data processing was carried out using R. 4.4.3 and Rstudio 2024.12.1. Results were expressed as mean \pm standard error of the mean (SEM). A confidence level of 95% ($\alpha = 0.05$) was established for all statistical tests. For each variable, a two-way ANOVA was conducted to examine the effect of the reproduction procedure at short and long term on the oxidative status of *A. viridis*. When interaction between both variables was found to be significant, a t-test was performed at each level of the variable time (T1 and T2) to test for differences between sectioned and control animals. Obtained p-values were adjusted using Benjamini-Hochberg correction for multiple testing. Normality of residuals was assessed via Shapiro-Wilk's normality test, while homoscedasticity was tested using Levene's test. All residuals were normally distributed ($p > 0.05$) and all variables had homogeneity of variances ($p > 0.05$).

Additionally, principal component analysis (PCA) was performed separately on columnar and tentacular variables to identify correlational structure of the data. Mardia's test was used to check multivariate normality. Significance of the PCA, loadings and principal components was assessed via permutation tests proposed by Vieira (2012), using the R package PCAtest (Camargo, 2022).

3. Results

Overall, the sectioning procedure registered an 8% mortality during healing of the anemones. The rest of the anemones were able to heal and kept growing during the experimental period.

3.1. Oxidative status assays

The ANOVA test for SOD activity in sectioned and control individuals of *A. viridis*, at different sampling times (Figure 1.A), did not reveal a significant interaction effect between the two variables on columnar or tentacular samples. There was also no significant main effect of the reproduction procedure or the time variable on either tissue. CAT activity (Figure 1.B) showed different responses in tentacular and columnar samples. Columnar activity was increased by regeneration, while there was no effect of time or interaction between both variables. Tentacular CAT activity, however, featured a significant interaction effect. At T1, sectioned anemones featured a significantly higher CAT activity than control anemones ($p < 0.001$). However, a

T2, this relationship became inverted and sectioned individuals exhibited a lower activity ($p < 0.01$).

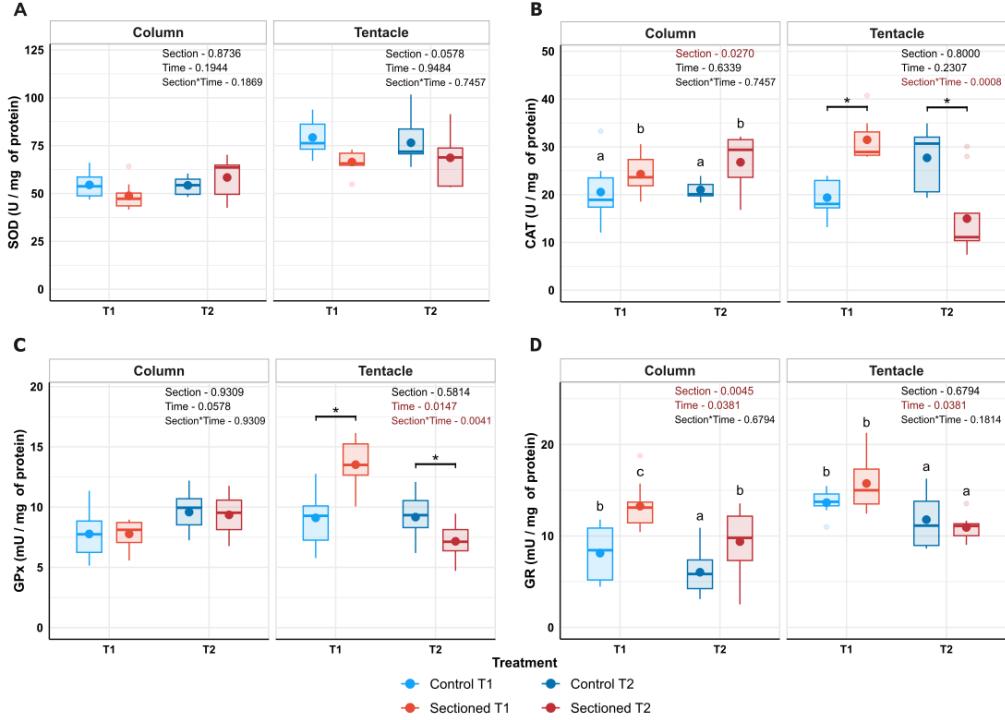


Figure 1: (A) Superoxide dismutase, (B) catalase, (C) glutathione-peroxidase and (D) glutathione reductase activity on column and tentacles of control and sectioned anemones, at T1 and T2. Coloured dot represents the mean activity. **a, b, c:** Significative differences due to sectioning and/or time. *****: Significative effects of sectioning at T1 or T2 when interaction is present

Columnar GPx activity (Figure 1.C) was not affected by either variable, although a nearly significant effect of time increasing the activity of this enzyme can be observed. Tentacular GPx activity featured interaction between both variables in a similar fashion to CAT activity. At T1, GPx activity was higher for sectioned animals ($p < 0.05$), while at T2, these anemones displayed lower GPx activity than control ($p < 0.05$). Time had a significant effect on both columnar and tentacular GR (Figure 1.D), as samples from T2 exhibited lower activity than T1 measurements. Furthermore, columnar GR activity was significantly increased for all sectioned individuals

There were no significant effects on columnar GST activity (Figure 2.A),

but tentacular samples featured significant interaction between the two variables. At T1, sectioned individuals had their GST activity significantly increased ($p < 0.001$). These differences were not reflected T2, where control and sectioned individuals were found to be similar ($p = 0.345$). Columnar NQO1 activity (Figure 2.B) increased significantly on sectioned anemones, while tentacular NQO1 activity featured a significant interaction effect. Anemones at T1 exhibited this same pattern of higher activity for sectioned individuals ($p < 0.001$) detected in columnar activity. At T2, however, no differences were found between control and sectioned samples ($p = 0.393$).

Total Antioxidant Capacity, measured as TEAC (Figure 2.C) showed no significant effects associated with regeneration or time, on neither columnar nor tentacular tissue. Lipid peroxidation, measured as MDA concentration (Figure 2.D), did not vary significantly in columnar samples, but tentacular MDA was found to be lower on sectioned individuals compared to control ones.

3.2. Immune status parameters

Neither AP (Figure 3.A) nor ALP (Figure 3.B) showed any significant effect or interaction between the variables. However, columnar ALP activity seemed to increase slightly in response to regeneration of the injury ($p = 0.088$). MPO activity showed significant interaction in columnar samples. At T1, columnar MPO activity (Figure 3.C) was higher in control individuals ($p < 0.001$), while there were no significant differences at T2 ($p = 0.674$). The effect of sectioning and time on tentacular activity also resulted non-significant.

3.3. Principal Component Analysis

Permutation tests for ψ and ϕ statistics were significant in both columnar variables and tentacular variables, indicating the presence of non-random correlation within the data and adequacy of PCA. In Figure 4, observations are grouped according to time point and section status, and 95% confidence ellipses were constructed, highlighting separation between experimental groups. Variables were overlaid as vectors.

For columnar variables, two principal components (PCs) were significant, and together they accounted for 51.4% of the original data variance. PC1 represented 33.1% (Confidence Interval-95%: 26.3-45.2) of this variation, and variables SOD, CAT, GPx, GST and MDA contributed significantly to it. PC2 explained 18.8% (C.I.-95%: 16.1-24.8), and only MPO

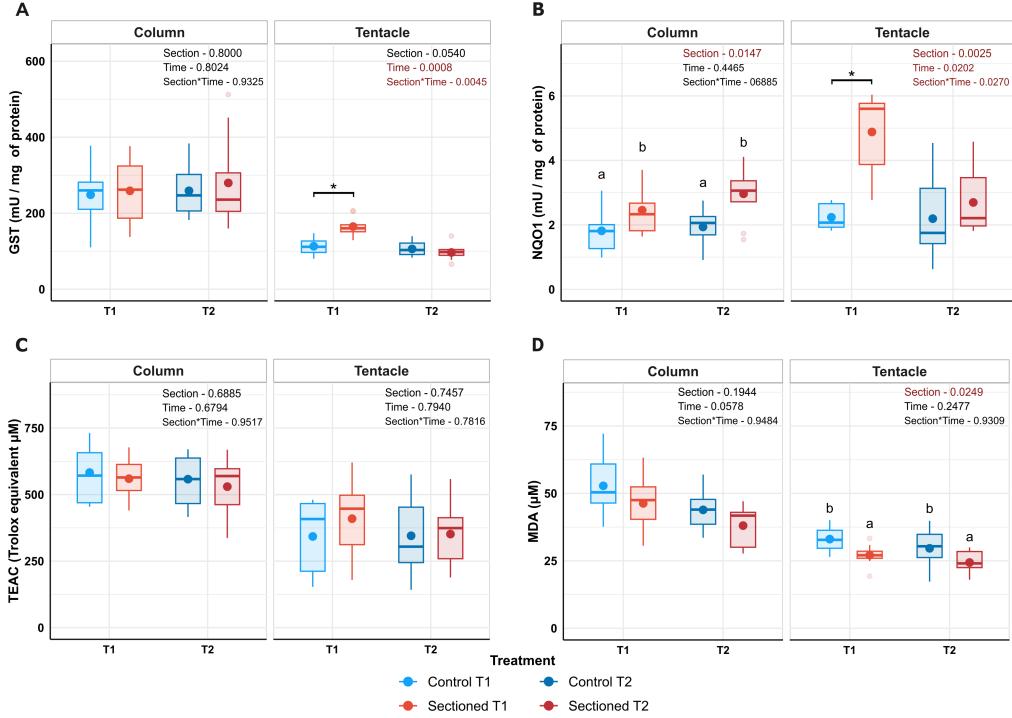


Figure 2: (A) Glutathione-S-transferase activity, (B) NAD(P)H quinone oxidoreductase 1 activity, (C) TEAC and (D) MDA on column and tentacles of control and sectioned anemones, at T1 and T2. Coloured dot represents the mean activity. **a, b:** Significative differences due to sectioning and/or time. *****: Significative effects of sectioning at T1 or T2 when interaction is present.

contributed more than expected by chance to this component. A slight separation can be observed between control observations, that tended to score positively for PC2, and sectioned observations, that mostly scored negatively for this principal component.

Regarding tentacular variables, only PC1 resulted significant according to permutation tests, and it accounted for 35% (C.I.-95%: 28.4-47.5) of original data variance. CAT, GR, GST, acid phosphatase, alkaline phosphatase and MPO contributed more than expected by chance to this component. PC2 was not significant according to permutation tests, meaning that it could reflect random correlations derived from sampling error. In this case, Sectioned-T1 samples were separated from other groups, including sectioned samples at T2 which overlapped with control observations.

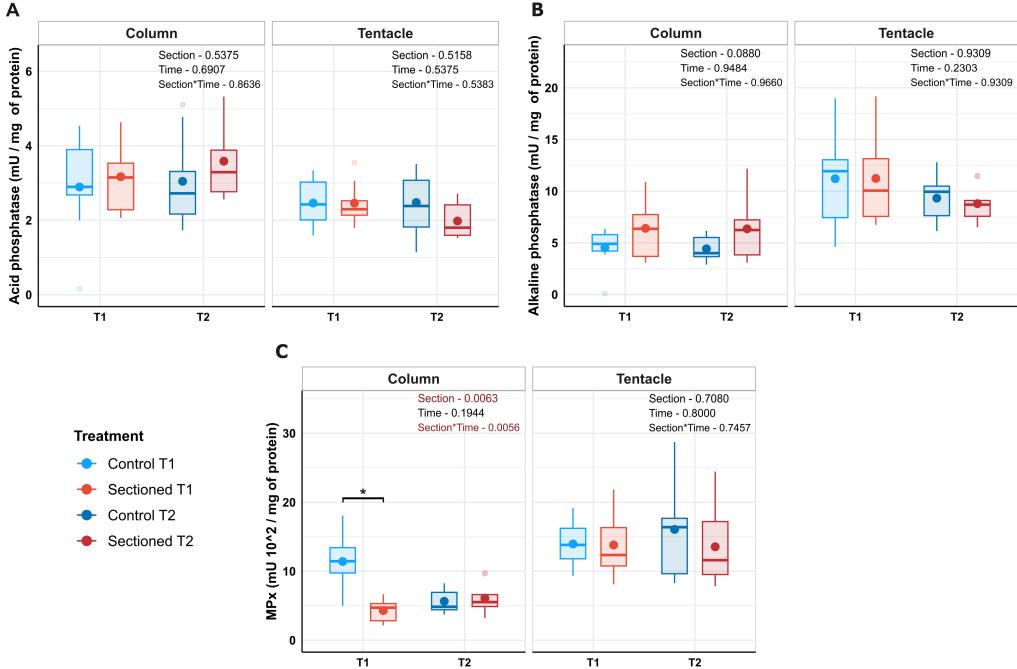


Figure 3: (A) Acid phosphatase, (B) Alkaline phosphatase and (C) myeloperoxidase activity on column and tentacles of control and sectioned anemones, at T1 and T2. Coloured dot represents the mean activity. *: Significative effects of sectioning at T1 or T2 when interaction is present.

3.4. Histology

The wound area and surrounding mesoglea were located and monitored in sea anemones at the time of sectioning, at 6 hours, at 24 hours and at 7 days after the procedure (Figure 5 & 6). At 0 hours, the wound area was open, and all three layers of the body wall were completely disrupted. Mesenteric filaments and waste material were found outside the gastrovascular cavity (Figure 5.A). At 6 hours, the exposed mesoglea around the wound area was partially covered by epidermal tissue (Figure 5.B). This response was particularly notable in the external body wall, where epidermis is thinner. Some samples also feature aggregations of amoebocytes in the mesoglea (Figure 6.B).

At 24 hours, both gastrodermis and epidermis had stretched to cover the exposed mesoglea bud, in many cases surrounding it completely and connecting with each other (Figure 5.C). Near the injury areas, mesoglea

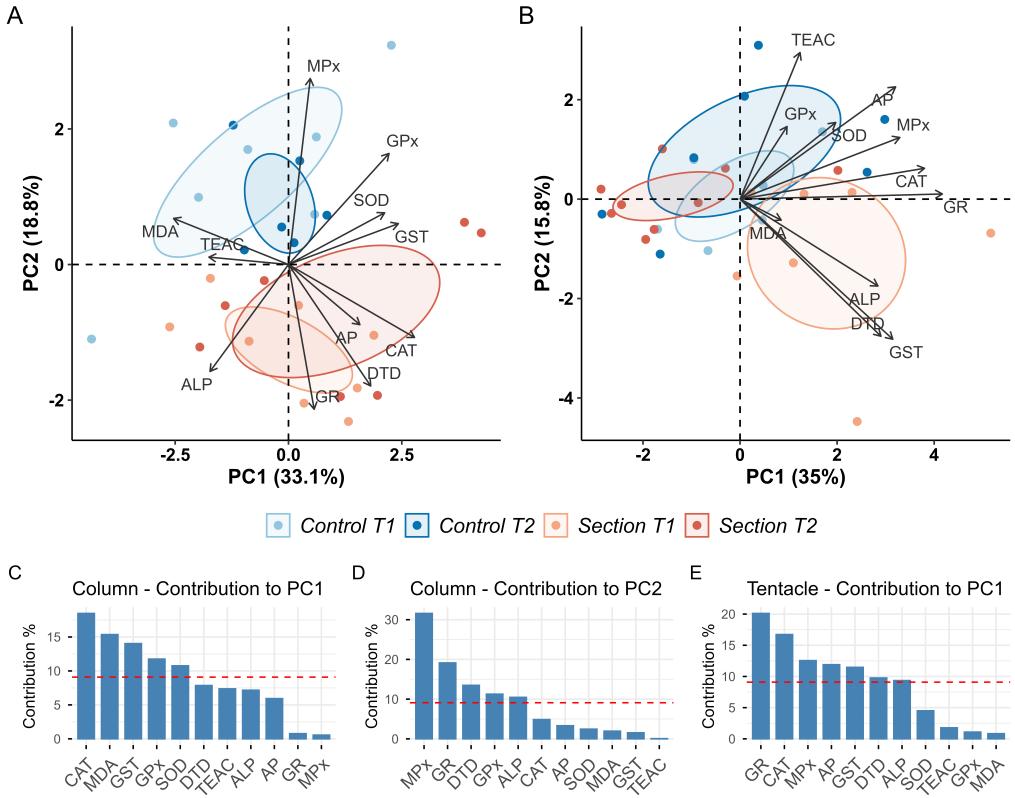


Figure 4: Principal Component Analysis (PCA) for examining correlation structure of the data. (A) Biplot of PC1 and PC2 for columnar variables. Samples are grouped according to treatment, with 95% confidence ellipses. (B) Biplot of PC1 and PC2 for tentacular variables. Samples are grouped according to treatment, with 95% confidence ellipses. (C) Percentage contribution of the twelve columnar variables to PC1. (D) Percentage contribution of the twelve columnar variables to PC2. (E) Percentage contribution of the twelve tentacular variables to PC1. Red dashed lines indicate threshold of expected average contribution.

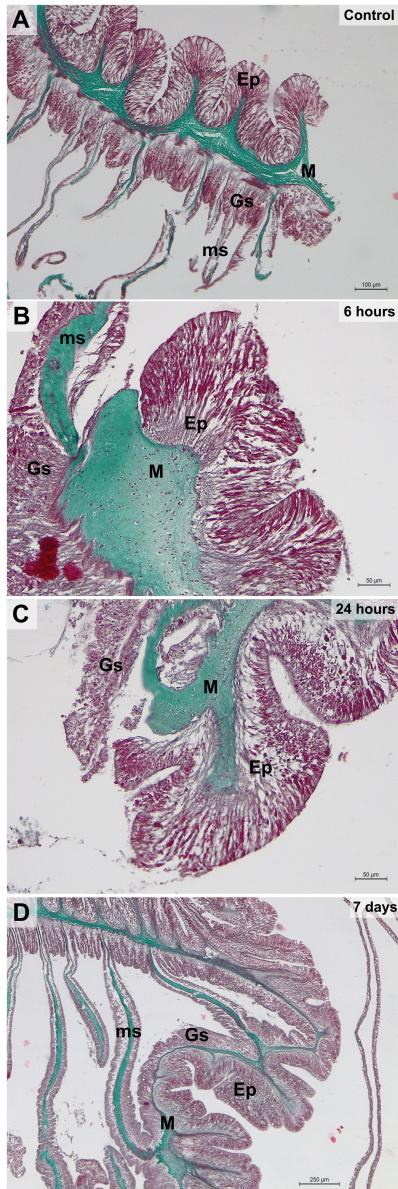


Figure 5: Evolution of the longitudinal section wound in the column of *A. viridis* immediately after the injury (A), 6 hours post-injury (B), 24 hours post-injury (C) and 7 days post-injury (D). 5 µm sections stained with Masson-Golder thrichrome, highlighting collagenous fibers and mesoglea in green. M: Mesoglea; Ep: Epidermis; Gs: Gastrodermis, ms: mesenteries. Scale bars—(A) 100 µm; (B-C) 50 µm; (D) 250 µm.

appeared to be laxer and acquired a reticular or fibrillar appearance (Figure 6.C). Furthermore, all samples exhibited intrusion of extracellular matrix components into the epidermis and gastrodermis of the injury area, as evidenced by light green staining. Some areas also featured amoebocyte recruitment in the mesoglea. 7 days after the injury, macroscopic observation of the specimens revealed that the wound was closed, and some of them had already regenerated part of their column. Microscopic examination revealed a new body wall, characterized by thin epidermal and gastrodermal layers and separated by a very narrow mesoglea (Figure 5.D). This new body wall ran parallel to pre-existing mesenteries, and some of the nearer mesenteries were connecting to the regenerated wall. Heavy amoebocyte recruitment was observed along the mesoglea of the new tissue (Figure 6.D).

4. Discussion

The aim of this study was to explore the viability of inducing asexual reproduction of the snakelocks anemone (*Anemonia viridis*) in captivity. Our results suggest that longitudinal sectioning of the anemones is an effective methodology for this purpose, as it resulted in a high survival and prompted fast wound healing, and the detected antioxidant response was effective protecting against lipid peroxidation.

Oxidative stress response is a complex interplay of ROS generation and ROS scavenging by antioxidant defenses. The present study detected increases in certain antioxidant enzymes activities in regenerating anemones at both time points (4 weeks and 20 weeks after the injury). The affected enzymes were mostly glutathione-dependent enzymes and/or enzymes involved in hydrogen peroxide detoxification. Increases in CAT, NQO1 and GR activity were detected in both columnar and tentacular extracts, while the enzymes GPx and GST only featured increases in their tentacular activity. The lack of changes in Trolox-equivalent antioxidant capacity (TEAC) suggests that, within the studied time range, non-enzymatic antioxidants are not the main pathway to scavenge ROS. However, La Corte et al. (2025) reported a peak in total oxyradical scavenging capacity at 24 hours after tentacle amputation in *A. viridis*, which supports the idea that low molecular weight antioxidants play an important role in the early wound healing response, but might be replaced by enzymatic antioxidants during and after the subsequent regeneration process.

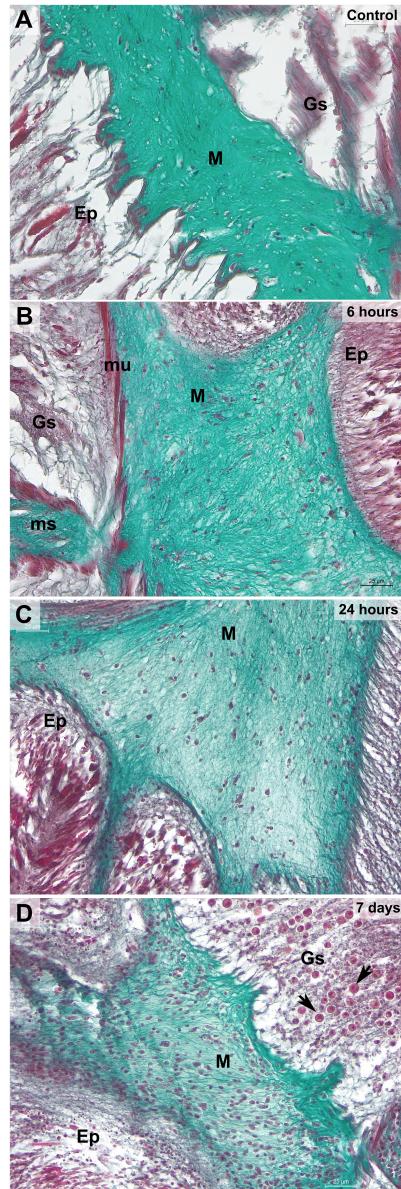


Figure 6: Evolution of the mesoglea in the wound area of *A. viridis* immediately after the injury (A), 6 hours post-injury (B), 24 hours post-injury (C) and 7 days post-injury (D). 5 μ m sections stained with Masson-Golder thichrome, highlighting collagen fibers and mesoglea in green. M: Mesoglea; Ep: Epidermis; Gs: Gastrodermis, ms: mesenteries; mu: muscle fibers; arrowheads: zoanthellae. Scale bars — 25 μ m.

Columnar lipid peroxidation was found to be similar for all experimental groups, and tentacles of regenerating anemones featured slightly lower levels of malondialdehyde than control samples. This lack of oxidative damage despite the detected increase in the antioxidant parameters suggests that antioxidant mobilization was able to prevent oxidative damage to cellular lipids, meaning that the sea anemones were able to protect themselves from the pro-oxidative conditions they faced during regeneration. PCA results for columnar data also further support this hypothesis, since antioxidant enzymes and MDA levels contributed significantly to PC1 in opposite senses, indicating negative correlation between these parameters (Figure 4). Previous works also report the robustness of this species and other symbiotic anthozoans to oxidative damage, likely linked to the evolution of symbiosis with zooxanthellae (Casado-Amezúa et al., 2016,?; Cotinat et al., 2022; Pey et al., 2017; Richier et al., 2003, 2005).

While columnar antioxidant response was generally found to be sustained over time, enzymes that increased their activity in tentacular tissue all experienced a decline at T2, sometimes returning to levels similar to or under the control group. PCA for tentacular samples also highlighted this tendency at the multivariate level (Figure 4). A possible reason for this may be found in the different degree of complexity and organization of the cnidarian body wall between tentacles and column. Even though they're both composed of the same three layers, the tentacle body wall is thinner and lacks some of the more complex gastrodermal and epidermal specializations featured on the column, such as mesenteries, acrorhagi, and parietal and retractor muscles (Harrison and De Vos, 1991; Jahnel et al., 2014). Moreover, previous studies on tentacle regeneration show that wound healing after tentacle amputation occurs within 24 hours in *A. viridis* (Corte et al., 2023; Parisi et al., 2021), contrasting with our histological observations, where the wound was not fully closed until 7 days post-injury.

In contrast with the evident antioxidant response, we detected no alterations to ALP and AP within the studied time-frame, which could be due to an early initial inflammatory response before the first time point for colorimetric assays. Indeed, histological examination confirmed that wound healing occurred rapidly within the first week after the injury in the case of longitudinal sections. Parisi et al. (2021) reported increases to ALP activity in *A. viridis* after tentacle amputation within that temporal range and extending to 14 days post-injury. Our results complement this information and suggest that, at 28 days, phosphatase activity seems to be back to control

levels.

MPO is a peroxidase typically associated to neutrophils in vertebrates, as it is involved in the respiratory burst occurring during phagocytosis and phagosome digestion. MPO reacts with H_2O_2 and chloride to generate hydrochlorous acid, source of strong oxidants with microbicidal effect (Klebanoff, 2005; Lin et al., 2024). Phagocytic activity of granular amoebocytes has been described extensively in Hexacorallia. Hutton and Smith (1996) report ROS production *in vitro* by isolated amoebocytes in *Actinia equina*, a close relative of *A. viridis* within the family Actiniaria. Snyder et al. (2021) characterized two cell populations in *Pocillopora damicornis* and *Nematostella vectensis* exhibiting different phagocytic traits. Among these traits, they detected an increased ROS activity indicative of respiratory burst. In this study, we observed how columnar MPO activity decreases 4 weeks post-injury compared to control, uninjured anemones. Moreover, PCA analysis revealed how this parameter was the only significant contributing variable to PC2, which separated control and regenerated anemones at both sampling times. These results hint that *A. viridis* amoebocytes could use MPO in their phagosomal digestion to generate oxidant compounds, and that longitudinal section of the animals might impair this function. However, more research is needed to assess the implication of this enzyme during the early wound healing stage, prior to 4-weeks post-injury.

To complement colorimetric assays, we performed an histological descriptive analysis of the wound healing process in snakelocks anemone during the first week after a longitudinal section. As early as 6 hours, but more evidently at 24 hours, epidermis and gastrodermis started to cover the exposed mesoglea, probably as a result of cell contraction and re-adhesion to the mesoglea. At 6 hours and 24 hours post-injury, mesoglea took a fibillar. and lax appearance, which has been reported to be a sign of reorganization of the extracellular matrix during wound healing in this species (Parisi et al., 2021). We also detected the presence of collagen-fibers (potentially from mesogleal origin) infiltrating the epithelial layers at 24 hours, exclusive to areas adjacent to the wound. Amoebocyte recruitment seemed to increase progressively from 6 hours to 7 days post injury in the mesoglea of affected body wall. Matching macroscopic observations, the wounded body wall appeared closed at 7 days post-injury. Añadir articulo de regeneracion y curacion de herida en coral.

- Look more into the interaction between respiratory burst and oxidative stress. Could it have added to antioxidant demand? - SOD, CAT and GPx

results could be affected by cellular compartmentalization of these enzymes isoforms or post-transcriptional modifications that could affect enzymatic activity. These organisms possess a remarkable diversity of antioxidant enzymes isoforms between the animal host and the dinoflagellate symbionts, and according to previous studies by Richier, there are evidence of exchange of isoforms between them. For example, plant and algae based SOD is found within the animal host tissues and viceversa, the animal SOD isoform is found in the extracted microalgal component. - Not known if epidermal and gastrodermal alterations are due to cell proliferation or cell movement and epithelial reorganization.

The possibility of reproducing the snakelocks anemone in captivity via induction of their regeneration is a major step towards conservation of this species in the Mediterranean sea. The high demand of this product for human consumption currently clashes with the state of their natural populations. Moreover, illegal fishing of the species has increased in the last years and is thought to still be prevalent despite the current ban on their exploitation. Natural populations of snakelocks anemone then have to face competition with the invasive algae *Rugulopteryx okamurai*, while suffering the effects of marine heatwaves and strong exploitation pressure.

This method of production offers potential to increase stock sizes rapidly, and could be specially appropriate to supply individuals for human consumption. However, in order to apply this reproduction technique to carry out reintroduction into the natural environment, some limitations regarding genetic diversity of the stock need to be addressed first (Kitada, 2018).

- In vitro enzymatic activity can be affected by post-transcriptional regulation - First phase antioxidant enzymes in *A. viridis* have several isoforms and different intracellular location. - Compartmentalization can also be affected by post-transcriptional regulation. - In vitro activity would need to be backed up with gene expression results to further understand the complex interplay of antioxidant responses during regeneration.

- Limitations to experimental time-frame, as we're missing enzyme data during the first weeks post-injury - Limitations of descriptive histological analysis. Need to use quantification techniques, histochemical stains or immunohistochemical assays to detect enzymatic activity *in situ*. - Need to explore collagen synthesis and deposition during mesoglea re-organization.

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