1. Introduction

-Anemonia viridis as food item and restrictions in Andalusia and Spain. -> Main introduction point, but possibly not the first paragraph. What’s needed before this?

-Reproduction modes in A. viridis, birradial symmetry and anatomy. -> Anatomy is considered somewhat basic to describe in depth, but reproduction modes and specifically asexual reproduction is a major driving point of the article and links directly into the research questions.

- Integrated multitrophic aquaculture. Sustainability. Do not focus on this unless the journal calls for it. -> Is it even necessary to devote much space to this? Again, depends on the journal. Surely not for comparative.

- Oxidative stress as a marker of wellbeing in aquaculture -> If we’re doing comparative again, it’s interesting to highlight. Talk about physiology to justify the methodology of choice. Mention particularities of the antioxidant system in A. viridis.

Research questions:

* Can asexual reproduction be induced artificially in the snakelocks anemone? – Is this a research question or should I just merge it into the introduction a context.
* Does this induction affect the wellbeing of the animals negatively and irreversibly?

The snakelocks anemone (*Anemonia viridis*) is a species of Cnidarian widely distributed in the Northeast Atlantic Ocean and Mediterranean Sea. It inhabits rocky bottoms ranging from the lower intertidal zone to subtidal, down to around 15-20 m deep (referencia). The snakelocks anemone has been traditionally exploited for human consumption in Spain, particularly in Andalusia (referencia). 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 subjected to illegal gathering, there is great interest in the development of alternative production methodologies. Aquaculture of the snakelocks anemone is not fully established, but can function as a major engine in the production of this species in Andalusia.

*A. viridis* can reproduce either sexually, by releasing gametes into the water column, or asexually, typically by longitudinal fission of the animal and regeneration of two complete individuals (referencia). Although anemones generally have the potential to carry out both reproduction modes, some colour morphs of snakelocks anemones have been found to predominantly or exclusively reproduce sexually or asexually (referencia). Detallar más?

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, particularly in an aquaculture context. In order to achieve that, asexual reproduction must 1) be artificially inducible and 2) not affect the wellbeing of the animals severely nor irreversibly. Oxidative status analysis is a reliable way to assess the overall wellbeing state of marine invertebrates. Stress responses often converge into oxidative stress pathways, where a few key antioxidant enzymes may have their activity levels upregulated to face the increased influx of reactive oxygen species (ROS) (referencias). Coupled with determinations of oxidative damage to cellular components, this type of assessment can quickly provide an overall picture of the condition of the animals at a cellular level (referencia).

* As many organisms inhabiting the intertidal, Anemonia viridis is exposed to large variations in temperature, salinity, humidity and UV exposure, among others (referencia). Furthermore, because A. viridis is a symbiotic anthozoans, hosting photosynthetic microalgae in their endoderm, they are adapted to deal with swings in oxygen partial pressure between day and night time (referencias). Their antioxidant systems are thus considered to be quite efficient, and these anemones have been proven to deal with anoxia better than other, non-symbiotic anthozoans (referencia).
* Therefore, artificial induction of asexual reproduction via sectioning of the animal through the column arises as an option that could allow for quick increases of stock sizes and have a non-lasting impact on their overall wellbeing.

In the present study, we assessed the impact of artificial sectioning of snakelocks anemones on their oxidative state at two different timescales, within an Integrated Multitrophic Aquaculture setting.

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. 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, centred around 34.8 ‰ and pH 8. Water temperature varied with ambient temperature, with an overall mean of 17.5 º C through the study.

Anemones were distributed in 11.8 L floating baskets at a density of 20 individuals per basket, making a total of 15 baskets. An IMTA set up was established by introducing selected organisms in the tank: beadlet anemones (*Actinia equina*), sea urchins (*Paracentrotus lividus*, *Arbacia lixula*), sea snails (Monodonta turbinata), limpets (*Patella caerulela*), sea cucumbers (*Holothuria tubulosa*), mussels (*Mytillus edulis*), and macroalgae (*Ulva rigida*, *Cystoseira mediterranea*) (Coll et al., 2025). Table 1 summarizes the composition and initial stock density of the multi-trophic environment.

Table 1

After acclimation to the aquaculture conditions for one month, (Nº) anemones from different baskets were selected as experimental group, and they 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.

Samplings were carried out at two different temporal stages. The first sampling (T0) took place in January 2021, once the anemones had had time to heal completely. 9 sectioned anemones from different baskets 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 (T1) took place in April 2021, when 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 ºC. 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 4ºC (Sigma 3 K30), and the supernatant was collected and stored at -80 ºC for posterior analysis.

2.2. Oxidative status assays

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined according to McCord & 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 H2O2 consumption by this enzyme.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity of the samples was determined following Flohé & Günzler (1984), 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 & 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 & 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 & Guilhermino (2002), based on the formation of a conjugate between glutathione and 2,4-dinitrochlorobenzene that increases absorbance. DT-diaphorase (DTD) (EC 1.6.99.2) activity was determined using a modified method of Lemaire et al. (1996), based on the reduction of 2,6-dichlorophenol indophenol, that results in a decrease in absorbance.

Soluble protein content of the samples was quantified following Bradford (1976) method in order to express enzymatic activities as specific activity. Units of activity was defined as the amount of enzyme required to transform one μmol of substrate per minute under the measurement conditions (Coll et al., 2025). 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.

Erel (2004) method, based on the change of absorbance due to reduction of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), was used to determine Trolox-equivalent antioxidant capacity (TEAC) of the extracts, as a measure of Total Antioxidant Capacity. Lastly, thiobarbituric acid reactive substances (TBARS) content was measured as a marker of oxidative damage to lipids, following a modified method from Buege & Aust (1978) and using malondialdehyde (MDA) as a standard.

2.3. Non-specific immune parameters

Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases (AP and AlP) activity were measured following the method used by Easy & Ross (2010) and Huang et al. (2011), based on the change of absorbance at 405 nm produced by the activity of the enzyme on p-nitrophenyl at different pH. Lysozyme activity (EC 3.2.1.17) was determined according to Swain et al. (2007), by using a M*icrococcus lysodeikticus* suspension and an egg lysozyme standard. Determination of myeloperoxidase (MPx) activity (EC 1.11.1.7) was carried out following a modified method of Mohanty & Sahoo (2010), based on the increase of absorbance due to oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by the products of MPx 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. 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 ± standard error of the mean (SEM). A confidence level of 95% (α = 0.05) was established for all statistical tests. For each variable, a two-way ANOVA was conducted to examine the effect of the sectioning 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 (T0 and T1) 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).

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 keep growing in the next months.

3.1. Oxidative status parameters

SOD and CAT

Figure 1 shows the mean SOD activity per experimental group, as well as the ANOVA table. Interaction between the two variables was not significant, and there was no effect of the sectioning procedure or the time variable on SOD activity, neither on columnar nor tentacular samples. A light tendency was found on tentacular SOD activity, where sectioned individuals yielded a slightly smaller activity, but this pattern was not found to be statistically significant. CAT activity (Figure 2) showed different responses in tentacular and columnar samples. Columnar activity was increased by the sectioning protocol, while there was no effect of time or interaction between both variables. Tentacular CAT activity, however, featured a significant interaction effect. At T0, sectioned anemones featured a significantly higher CAT activity than control anemones (p = 0.000274, p < 0.001). However, a T1, this relationship became inverted and the sectioned individuals exhibited a lower activity (p = 0.00599, p < 0.01).

GPx, GR and G6PDH

GR activity is displayed in Figure 3. Time had a significant effect on both columnar and tentacular GR, as samples from T1 exhibited lower activity than T0 measurements. Furthermore, columnar GR activity was significantly increased for all sectioned individuals. Columnar GPx activity (Figure 4.A) 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 (Figure 4.B) featured interaction between both variables. At T0, there was no significant differences between control and sections animals (p = 0.0535); while, at T1, sectioned anemones displayed lower GPx activity (p = 0.0286, p < 0.05).

GST and DTD

Figure 5 shows GST activity on both body regions. While there were no significant differences in columnar GST activity, tentacular samples featured significant interaction between the two variables. At T0, sectioned individuals had their GST activity significantly increased (p = 0.000656, p < 0.001). These differences were not reflected T1, where control and sectioned individuals were found to be similar (p = 0.345). Columnar DTD activity (Figure 6. A) increased significantly on sectioned anemones. Tentacular DTD activity featured a significant interaction effect (Figure 5.B). Anemones at T0 exhibited this same pattern of higher activity for sectioned individuals (p = 0.000103, p < 0.001) detected in columnar activity. At T1, however, no differences were found between control and sectioned samples (p = 0.393).

TEAC and MDA

Total Antioxidant Capacity, measured as TEAC, is displayed on Figure 7. Neither columnar or tentacular TEAC showed any differences associated with sectioning or time. MDA concentration (Figure 8) did not vary significantly in columnar samples, but tentacular MDA was found to be lower on sectioned individuals compared to control ones.

3.2. Non-specific immune parameters

Neither acid phosphatase (Figure 9) nor alkaline phosphatase (Figure 10) showed any significant effect or interaction. MPx activity showed significant interaction in columnar samples. At T0, columnar MPx activity (Figure 11) was lower for sectioned individuals (p = 0.000895, p < 0.001), while there were no significant differences at T1 (p = 0.674). The effect of sectioning and time on tentacular activity also resulted non-significant.

4. Discussion

- Induction of asexual reproduction

- Impact on the oxidative state. Severe? Reversible? Use guidelines from that article about oxidative status analysis.

5. Conclusions

6. References

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