1. Introduction

-Anemonia viridis as food item and restrictions in Andalusia and Spain.

-Reproduction modes in A. viridis, birradial symmetry and anatomy.

- Integrated multitrophic aquaculture. Sustainability. Do not focus on this unless the journal calls for it.

- Oxidative stress as a marker of wellbeing in aquaculture.

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) from October 2020 to December 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.

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 multitrophic environment.

Table 1

After one month of acclimation to the IMTA environment, (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 then placed on a different calcareous disk and left to heal, so as to generate two different clonal individuals. Calcareous disks were left untreated, as they were meant not only to provide physical support during the healing process, but also serve as a medium to reintroduce the anemones into the natural environment and promote bio-fouling on them.

Sampling was carried out at two different temporal stages. The first sampling (T0) took place 4 weeks after the sectioning procedure, 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 16 weeks after, in month year, 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 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 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. 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). Obtained p-values were adjusted using Benjamini-Hochberg correction for multiple testing.

3. Results

By June 2020, the initial 300 anemones stock had triplicated to 906 individuals, of which 895 individuals were reintroduced to the natural environment. The healing procedure registered an 8% mortality.

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

5. Conclusions

6. References

Aebi, H. (1984). [13] Catalase in vitro. *Methods in Enzymology*, *105*(C), 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, *72*(1–2). https://doi.org/10.1006/abio.1976.9999

Buege, J. A., & Aust, S. D. (1978). Microsomal Lipid Peroxidation. *Methods in Enzymology*, *52*(C). https://doi.org/10.1016/S0076-6879(78)52032-6

Carlberg, I., & Mannervik, B. (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *Journal of Biological Chemistry*, *250*(14), 5475–5480. https://doi.org/10.1016/S0021-9258(19)41206-4

Coll, A., Rufino-Palomares, E. E., Ramos-Barbero, M., Ortiz-Maldonado, A. E., Pantoja-Echevarría, L. M., González-Ordóñez, I., Pérez-Jiménez, A., & Trenzado, C. E. (2025). Effects of environmental factors on the oxidative status of Anemonia viridis in aquaculture systems. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *275*, 111042. https://doi.org/10.1016/J.CBPB.2024.111042

Easy, R. H., & Ross, N. W. (2010). Changes in Atlantic salmon Salmo salar mucus components following short- and long-term handling stress. *Journal of Fish Biology*, *77*(7). https://doi.org/10.1111/j.1095-8649.2010.02796.x

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry*, *37*(4), 277–285. https://doi.org/10.1016/J.CLINBIOCHEM.2003.11.015

Flohé, L., & Günzler, W. A. (1984). [12] Assays of glutathione peroxidase. *Methods in Enzymology*, *105*(C), 114–120. https://doi.org/10.1016/S0076-6879(84)05015-1

Frasco, M. F., & Guilhermino, L. (2002). Effects of dimethoate and beta-naphthoflavone on selected biomarkers of Poecilia reticulata. *Fish Physiology and Biochemistry 2002 26:2*, *26*(2), 149–156. https://doi.org/10.1023/A:1025457831923

Huang, Z. H., Ma, A. J., & Wang, X. A. (2011). The immune response of turbot, Scophthalmus maximus (L.), skin to high water temperature. *Journal of Fish Diseases*, *34*(8). https://doi.org/10.1111/j.1365-2761.2011.01275.x

Lemaire, P., Sturve, J., Förlin, L., & Livingstone, D. R. (1996). Studies on aromatic hydrocarbon quinone metabolism and DT-Diaphorase function in liver of fish species. *Marine Environmental Research*, *42*(1–4), 317–321. https://doi.org/10.1016/0141-1136(95)00042-9

Löhr, G. W., & Waller, H. D. (1965). Glucose-6-phosphate Dehydrogenase: (Zwischenferment). *Methods of Enzymatic Analysis*, 744–751. https://doi.org/10.1016/B978-0-12-395630-9.50135-3

McCord, J. M., & Fridovich, I. (1969). Superoxide Dismutase: AN ENZYMIC FUNCTION FOR ERYTHROCUPREIN (HEMOCUPREIN). *Journal of Biological Chemistry*, *244*(22), 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5

Mohanty, B. R., & Sahoo, P. K. (2010). Immune responses and expression profiles of some immune-related genes in Indian major carp, Labeo rohita to Edwardsiella tarda infection. *Fish and Shellfish Immunology*, *28*(4). https://doi.org/10.1016/j.fsi.2009.12.025

Swain, P., Dash, S., Sahoo, P. K., Routray, P., Sahoo, S. K., Gupta, S. D., Meher, P. K., & Sarangi, N. (2007). Non-specific immune parameters of brood Indian major carp Labeo rohita and their seasonal variations. *Fish and Shellfish Immunology*, *22*(1). https://doi.org/10.1016/j.fsi.2006.03.010