Curcumin improves antioxidant efficiency in oysters *Crassostrea gigas*: a potential approach to support bivalve aquaculture?

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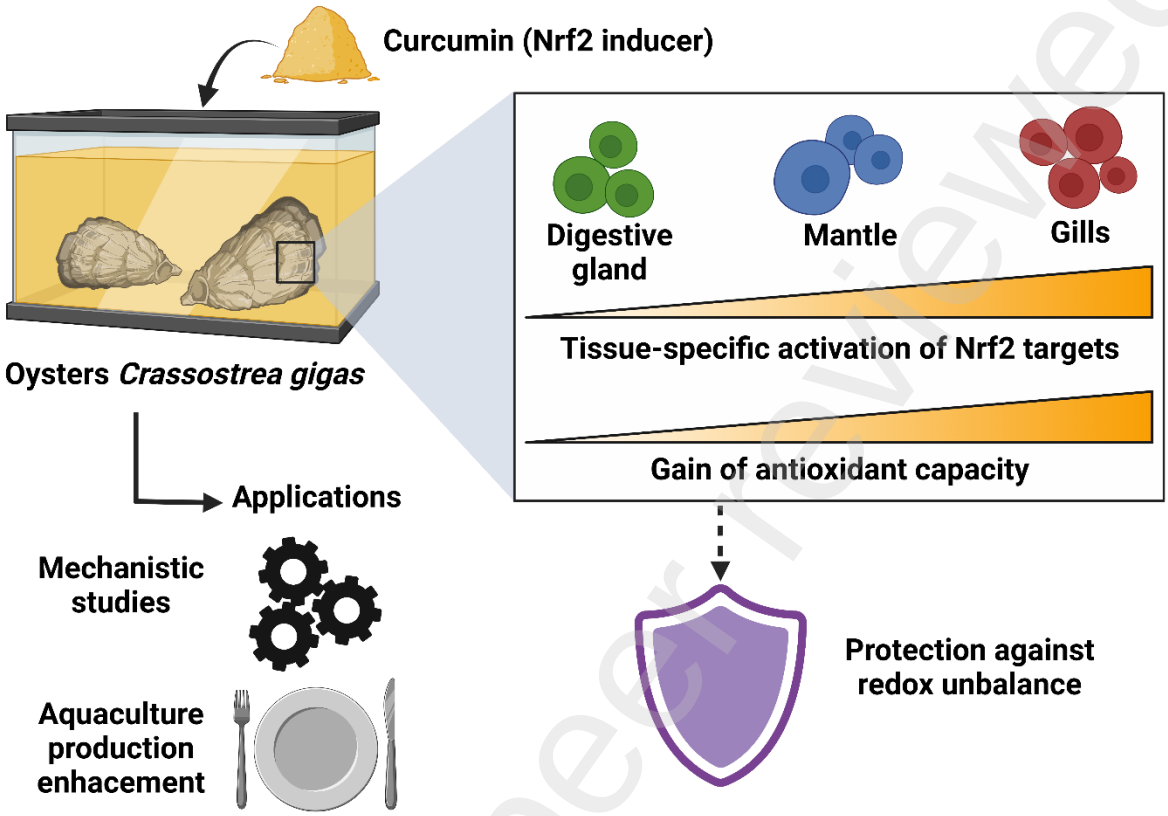
Abstract

Aquatic animals inhabiting marine coastal environments are highly susceptible to environmental fluctuations and pollution, exemplified by widespread mass mortalities induced by marine bacteria or viruses. Enhancing antioxidant defenses presents a promising strategy to mitigate such environmental stressors. We postulated that supplementation of oysters with natural compounds such as flavonoids, exemplified by curcumin (CUR), could effectively bolster their antioxidant protection. Adult Pacific oysters were supplemented with CUR (30 µM) in seawater for 2, 4, 8, and 16 days. CUR metabolites progressively accumulated in gills, mantle, and digestive glands. Notably, oyster antioxidant response was significantly augmented, as evidenced by elevated glutathione (GSH) levels, and enhanced activities of glutathione reductase (GR), thioredoxin reductase (TrxR), and glutathione S-transferase (GST) after 4, 8, and 16 days of CUR supplementation. This response was tissue-specific, with the most pronounced increase in gills, followed by mantle, whereas digestive gland exhibited minimal response. After being supplemented with CUR for 8 days, oysters were subjected to antioxidant-disrupting agents such as N-ethylmaleimide (NEM), 1-chloro-2,4-dinitrobenzene (CDNB). Both chemicals reduced antioxidant protection in untreated animals. However, CUR supplementation prevented these redox-disrupting effects, suggesting the potential ability of CUR to counteract antioxidant stressors. The effects of 8 days of CUR supplementation were also tested against the lethal effects of the pathogens *V. tapetis, V, alginolyticus,* and *V. anguillarum,* but CUR failed to induce immunological protection. The antioxidant protection induced by CUR holds promise for application in aquaculture to bolster animal health and resilience against abiotic stressors. Further research is needed to investigate the long-term impact of CUR supplementation and its role against biotic stressors, such as bacterial and viral infections.

Keywords: curcumin; Pacific oysters; glutathione; resilience; oxidative stress; Vibrio; pathogens

41 **Graphical abstract**



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1 Introduction

Seawater covers around 70% of the Eart's surface and contains numerous marine creatures, each adapted to specific regions and depths. Marine bivalves, for example, are essential engineer species and shellfish reef builders that play a vital role in the coastal ecosystem. They are also significant species for aquaculture in many countries. For instance, the Pacific oyster *Crassostrea gigas* has been introduced worldwide for aquaculture and is currently one of our main aquaculture species (Stiger and Thouzeau, 2015; Suplicy, 2022). However, as filter-feeder organisms, bivalves are sensitive to seawater quality, and environmental stressors such as climate change, water pollutants, and pathogens can lead to production losses in these aquaculture species (Segarra et al., 2010; Trevisan and Mello, 2024). Despite these challenges, bivalves have defense mechanisms, such as the antioxidant system, to protect themselves from environmental stress and hazards.

The antioxidant system is a complex set of biochemical components that includes vitamins, small peptides, and enzymes. These molecules are essential for controlling cellular activity and promoting animal health by regulating the levels of oxidative species and cellular signaling processes, and by protecting DNA, lipids, and proteins from excessive oxidation (Sies et al., 2017). For instance, the antioxidant system is critical for helping marine bivalves adapt to the highly dynamic intertidal environment, which experiences significant fluctuations in temperature, salinity, oxygen supply, and UV radiation (Trevisan and Mello, 2024). Additionally, environmental pollution, harmful algal blooms, diseases, and even the spawning season are factors that influence oxidative metabolism (Canesi, 2015). Thus, marine bivalves have developed dynamic protection mechanisms that enable them to respond to and cope with changes in environmental variables on a daily, seasonal, and long-term basis (Trevisan and Mello, 2024). Indeed, the genome of Pacific oyster presents a high number of genes related to defense pathways, including genes encoding antioxidant molecules (Zhang et al., 2016).

In our research, we have found various protection strategies for the Pacific oyster. When under attack from electrophilic chemicals (*i.e.,* chemicals reacting with molecules containing an electron pair available), Pacific oysters can quickly neutralize the threat by using a detoxification system that relies on glutathione (GSH) and

glutathione S-transferase (GST) (Trevisan et al., 2016b). This indicates that the gills act as a chemical barrier to protect against some types of environmental toxins. However, if the two key enzymes responsible for eliminating peroxides, namely glutathione peroxidase (Gpx) and peroxiredoxin, are compromised, the oysters become more susceptible to oxidative stress (Trevisan et al., 2012a, 2014a). The antioxidant system is also essential for supporting other defense mechanisms, such as the immune system. For instance, Pacific oysters (*C. gigas*) that survive Vibrio challenges display increased expression of various antioxidant genes, including different glutathione S-transferase (GST) isoforms (Lorgeril et al., 2011). Exposure of mussels *Mytilus coruscus* to lipopolysaccharides, components of the bacterial wall of gram-negative bacteria, results in the expression of antioxidant genes (Qu et al., 2019). Conversely, Pacific oysters deficient in GSH are prone to Vibrio infections and the resulting lethality caused by *V. anguillarum*, *V. alginolyticus*, or *V. harveyi* (Mello et al., 2020a). Overall, bivalves have a strong antioxidant system that plays a crucial role in abiotic and biotic stress conditions and for their adaptation to highly dynamic changes in their natural environment.

More importantly, the antioxidant protection of an organism can be further amplified by the activation of signaling pathways. For example, the transcription factor Nrf2 undergoes redox regulation to promote the fine-tuning of molecular and biochemical antioxidant responses in metazoa (Bellezza et al., 2018; Tonelli et al., 2018). The existence of numerous copies of antioxidant genes, as well as one copy of the *Nrf2* and kelch-like ECH-associated protein 1 (Keap1) genes in the Pacific oyster *C. gigas* highlight the relevance of this regulatory mechanism in marine bivalves (Zhang et al., 2016). Like in vertebrates, the Keap1 of the freshwater mussel *Clistaria plicata* interacts with Nrf2 under basal conditions, allowing access to a ubiquitin ligase enzyme that ubiquitinates Nrf2, directing the Nrf2 to proteasomal degradation (Wu et al., 2023). Under stress conditions, critical cysteine residues in Keap1 of metazoans are oxidized or alkylated, preventing Nrf2 binding. Thus, Nrf2 escapes proteasomal degradation, allowing migration to the nucleus and subsequent activation of transcription of Nrf2-target genes (Bellezza et al., 2018). This regulation of the Nr2 pathway has been demonstrated in *C. gigas* (Danielli et al., 2017b, 2017a) and other bivalve species such as the mussels *Perna viridis, Mytilus galloprovincialis*, and *M. coruscus* (Dou et al., 2020; Sendra et al., 2020; Qi and Tang, 2020). In addition, antioxidant protection in

bivalves can be induced by known mammalian Nrf2 inducers such as flavonoids, quinones, and itaconate (Danielli et al., 2017b, 2017a; Sendra et al., 2020), as well as by environmental relevant stressors such as toxic microalgae, cyanobacteria or their toxins (Dou et al., 2020; J. Lv et al., 2021; Wu et al., 2020; Ye et al., 2022).

The effects of Nrf2 regulation have been extensively studied in mammals. For example, the compounds curcumin (CUR), resveratrol, and fisetin, which modulate the Nrf2 system, are currently being tested in human clinical trials and showing promising results for disease protection (Hipólito-Reis et al., 2022; Wissler Gerdes et al., 2021). There is also a growing interest in applying similar principles to aquatic animals. For instance, Nrf2 inducers have been used to strengthen antioxidant defense and promote animal health and resilience to chemical stressors in fish such as the liver toxicant carbon tetrachloride and the metal chromium (Mohamed et al., 2020; Cao et al., 2015). However, only a few studies have investigated natural Nrf2 inducers in bivalves (Danielli et al., 2017b; Sendra et al., 2020).

CUR is a natural polyphenolic substance derived from the rhizome of *Curcuma longa*, with antioxidant and anti-inflammatory properties (Menon and Sudheer, 2007). Studies have shown that CUR and other flavonoids target Keap1 cysteine sites, disrupting the interaction between Keap1 and Nrf2. This results in Nrf2 nuclear translocation and antioxidant amplification (Bi et al., 2023; Jin et al., 2023; Maher, 2021; Shin et al., 2020). For instance, CUR protects fish hepatocytes against hydrogen peroxide (Li et al., 2020) and the liver and kidney of Nile tilapia exposed to hexavalent chromium (Mohamed et al., 2020). Moreover, CUR is an effective inducer of fish growth in Nile tilapia and rainbow trout, thereby increasing production and economic gain in aquaculture activities (Mohamed et al., 2020; Yonar et al., 2019a). Similar effects are also detected in marine invertebrates. CUR promotes improved growth and survival to pathogens (*Vibrio harveyi*) in the gastropod *Haliotis discus hannai* (Zou et al., 2022). In mussels of the species *Perna viridis*, CUR increases the clearance microalgal toxins from the digestive gland of the animals (Yuan et al., 2021).

In the first study investigating the beneficial impacts of CUR on bivalves, we found that supplementing *C. gigas* with 30 µM CUR for 4 days increased the expression of antioxidant genes (GCL, GR, GPx2, and GSTpi), the concentration of GSH, and the activity of the enzymes GST, GR, and GPx in the gills but not the digestive gland

(Danielli et al., 2017b). In the present study, we aimed to further explore the effects of Nrf2 regulation by 30 µM CUR by testing the efficiency of a prolonged 16-day supplementation with CUR on the levels and activities of antioxidant molecules. Additionally, we expanded the number of tissues analyzed (gills, digestive gland, and mantle) and tested if CUR-induced antioxidant improvements translate into functional phenotypic gains such as the protection of oysters against redox disrupting abiotic and biotic stressors. N-ethylmaleimide (NEM) and 1-chloro-2,4-dinitrobenzene (CDNB) were chosen as model abiotic stressors because of their pro-oxidative effects on *C. gigas* (Trevisan et al., 2016a; Trevisan et al., 2012b), and vibrios (*V. tapetis, V. anguillarum* and *V. alginolitycus*) were chosen as models of pathogenic stressors causing redox alterations (Mello et al., 2020b; Richard et al., 2016; Smits et al., 2020). The use of CUR supplementation is a strategy to use natural compounds to enhance animals' ability to resist stress. This is an increasingly recognized area of focus as an alternative approach to boosting aquaculture production gains (Jiang et al., 2016; Mohamed et al., 2020; Ling et al., 2010), yet to be tested with marine bivalves.

2 Materials and methods

2.1 Animals and acclimation

Adults of *C. gigas* (shell length = 11 ± 1.08 cm) were obtained from the Laboratory of Marine Mollusks of the Federal University of Santa Catarina, located in Florianópolis, Brazil. Oysters were acclimated for four days in static tanks with UV-treated and filtered seawater (1 L/animal) at 19-20°C and 35‰ salinity. Oysters were fed every other day with a commercial plankton diet (Aquavitro fuel, Madison, USA), followed by full water changes.

For the experiments with bacterial challenges, adult *C. gigas* oysters (shell length = 4 to 5 cm) were obtained from IFREMER, France. Animals were produced at the hatchery facility of Argenton, and further grown at the rearing facility of Bouin. Animals were then transported to the laboratory and acclimated for four days in static tanks with UV-treated and filtered seawater (0.150 L/animal) at 16°C, 35‰ and, feeding with a commercial plankton diet (Live Marin Phytoplankton, Sustainable Aquatics SA, Germany).

2.2 Supplementation with CUR

Oysters were transferred to glass aquaria (1 L/animal) after acclimation and supplemented with 30 µM CUR (Sigma-Aldrich). The concentration was based on previously published research from our lab, which demonstrated the activation of the antioxidant system in *C. gigas* gills after 4 days of supplementation (Danielli, et al., 2017b). CUR was freshly prepared at a concentration of 200 mM in 0.5 M NaOH. The stock solution was added to seawater to achieve a final concentration of 30 µM CUR, whereas the final concentration of NaOH was 75 µM and had no effect on the pH of the seawater during the treatment. Oysters were supplemented with 30 µM CUR for 2, 4, 8, or 16 days, with full water renewal and CUR addition every 48 hours. During this time, animals were fed for 1 hour before water renewal with the same commercial food described earlier (section 2.1). The control group was treated with CUR vehicle (NaOH 75 µM). Two independent experiments were carried out with six animals per group (n=12). At the indicated time points, the mantle, gills, and digestive gland were collected and stored at -80°C until further use, except for total glutathione (GSH-t) levels that were analyzed immediately, as described in section 2.5.

2.3 CUR uptake and tissue distribution

A second set of experiments was conducted to assess the rate of CUR uptake from the seawater by the oysters throughout the treatment. Oysters were supplemented with CUR for 2, 4, 8 or 16 days, as stated in section 2.2. Oysters were transferred to glass beakers (1 oyster/beaker) containing 1 L of aerated saltwater. Two independent experiments were performed in triplicate (n=6). Freshly prepared CUR was added to each beaker to a final concentration of 30 µM, and CUR levels in seawater were measured for 6 hours using a spectrophotometric assay (422 nm and 600 nm), as previously reported (Danielli, et al., 2017b). Because CUR degrades spontaneously in water (Kadam et al., 2013; Kadam et al., 2013), and the spectrophotometric method cannot discriminate between CUR and derivates, findings are reported as curcumin equivalents (CUR-eq), based on a CUR standard curve. The same experiment was repeated in beakers containing just seawater (no CUR) to evaluate any interferences from CUR supplemented animals in the spectrophotometric test (*e.g.,* excretion of previously bioaccumulated CUR-eq).

The presence of CUR and their potential metabolites (CUR-eq) was also analyzed in the gills, mantle and digestive gland of oysters following the same treatment conditions described in section 2.2 (n=6). Samples were homogenized (1:2; w:v) in phosphate-buffered saline (513 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4). Subsequently, ethyl acetate:methanol solution (95:5; v:v) was added, at the ratio 1:3 (v:v) and mixed by vortexing for 1 min before the liquid phase extraction. The samples were centrifuged at 10,000 x g, for 10 min, at room temperature. The absorbance of the supernatant was accessed at 422 nm (CUR-eq) in a plate reader, as previously described (Vareed, et al., 2008), and modified (Danielli, et al., 2017b). The absorbance at 600 nm (turbidity) was used to subtract any interference of particulate material.

2.4 *CUR supplementation to test the protection against electrophilic agents*

Two further sets of experiments were done with animals supplemented with 30 µM CUR or exposed to vehicle (75 µM NaOH) for 8 days (same protocol from section 2.2) to test if CUR-induced activation may confer protection against thiol-depleting electrophilic compounds. Each set of experiments was composed of two independent tests, resulting in 6-10 animals per group (n=6-10). In the first set of experiments, CUR supplemented oysters were further exposed to the thiol-depleting chemical NEM at 1 mM for one hour. In the second set of experiments, CUR supplemented oysters were further exposed to the electrophilic agent CDNB at 10 µM for 24 h. The time and concentrations of NEM and CDNB were based on previous works from our group (Trevisan et al., 2016; Trevisan et al., 2014). The mantle, gill, and digestive gland were collected and stored at -80°C until further use, except for GSH-t levels that were analyzed immediately.

2.5 *CUR supplementation to test the protection against Vibrio infections*

The animals used in this experiment were obtained from a different source (IFREMER, France). A first experiment was carried out to confirm that CUR supplementation also caused significant upregulation of antioxidant defences in these animals. Oysters were supplemented with CUR for 8 days at 30 µM as described in section 2.2, except that only 0.15 L of CUR was used per animal due to the smaller size of the organism. At the end of this period, 16 oysters per group (control and supplemented) were dissected for the collection of the gill and mantle for analysis of GST and GSH (see section 2.6).

For the initial *Vibrio* challenges tests to determine bacterial loads for infection*, V. tapetis CECT 4600, V, anguillarum 4437T and V. alginolyticus 4409T* were reactivated from storage at -80°C and cultured on Zobell medium for 24 hours at 18°C and checked for cellular density at 492 nm (Richard et al., 2015). Oysters were first anesthetized with 50 g/L $MgCl_2$ for 16 h to provide easy access to their adductor muscle, and then infected with suspensions of Vibrio species (diluted in 0.2 µm filtered seawater). A total of 100 µL of suspension was injected in the adductor muscle per animal, to achieve the bacterial loads of $5x10^7$ and $5x10^8$ cfu per animal. These values are based on previous studies from our research group (Mello et al., 2020b; Allam et al., 2002). The control group received the same bacteria, but they were attenuated by prior heating (*V. anguillarum* and *V. tapetis* at 50°C for 1h, *V. alginolyticus* at 70°C for 1h). Each experimental group had 20 animals infected, and mortality was checked daily for 4 days. The animals were not fed during the experiment.

After conducting tests for bacterial infections, animals that were either supplemented with CUR (30 µM for 8 days) or not, were infected with *V. tapetis (*$5x10^7$ cfu)*, V, anguillarum (*$5x10^7$ cfu) and *V. alginolyticus (1*$x10^9$ cfu) as previously described. A total of 24 animals were used per exposure condition, and mortality was monitored daily for 4 days. The water temperature was maintained at 18 °C during the infection.

2.6 *Biochemical assays*

To perform the total GSH assay (GSH-t, the sum of reduced and oxidized GSH), samples of gills, mantle, and digestive gland were freshly homogenized in a solution of 0.5 M perchloric acid with 1mM ethylenediamine tetraacetic acid at 1:9 (weight: volume). The homogenate was centrifuged at 15,000 × g for 2 minutes at 4 °C. The acid extract was neutralized with 0.5 M potassium phosphate buffer at pH 7.0 and then subjected to the GR/5,5'-dithiol-bis-(2-nitrobenzoic acid) (DTNB) recycling assay (Akerboom and Sies, 1981), as previously employed (Trevisan et al., 2014a).

For the enzymatic analyses of GR, thioredoxin reductase (TrxR), and GST, tissues of gills, mantle, and digestive gland were homogenized at a ratio of 1:9 (weight: volume) in 20 mM Hepes buffer at pH 7. The tissue extract was centrifuged twice at 20,000 × g, first for 10 minutes, followed by a second centrifugation for 20 minutes at 4 °C. The two-step centrifugation improved the removal of interfering substances from the supernatant in the digestive gland. The supernatant was stored at -80°C until further

use. GR activity was determined by monitoring NADPH consumption at 340 nm during the reduction of glutathione disulfide (GSSG) to GSH (Carlberg and Mannervik, 1985). TrxR activity was measured at 412 nm by reducing DTNB in the presence of NADPH as an electron donor (Arnér et al., 1999). GST activity was measured at 340 nm based on the conjugation of GSH and CDNB (Habig et al. 1974). Enzymatic activities were normalized by the protein concentration, determined using Coomassie Brilliant Blue G-250 (Bradford, 1976).

2.7 *Integrated biomarker response version 2 (IBRv2)*

The IBRv2 was calculated using the method described by Beliaeff and Burgeot (Beliaeff and Burgeot, 2002) and modified by (Sanchez et al., 2013). This method involves integrating and comparing data from different conditions, such as time and action of biomarkers in different tissues of an organism. In this study, the antioxidant biomarkers (GSH-t levels and activities of GR and GST) were used to calculate the IBRv2 at each CUR treatment period (2, 4, 8, and 16 days) in the gills, mantle, and digestive gland of *C. gigas*. This method provided a single set of metrics that incorporated the multiple and temporal antioxidant responses to CUR for each tissue studied.

2.8 Statistical analysis

The data obtained in sections 2.2 (showing the temporal profile of biochemical responses) and 2.3 (showing the temporal profile of CUR bioaccumulation) were analyzed using a one-way ANOVA followed by Dunnett's post-hoc test. The data from section 2.4 (CUR + NEM or CUR + CDNB) were analyzed using a two-way ANOVA followed by a Fisher's post-hoc test. The test was corrected for the analysis of selected multiple comparisons. Data from section 2.5 (were analyzed by the Student t-test (CUR effects on antioxidants before bacterial challenge) or the Gehan-Breslow-Wilcoxon test (survival curves). A statistical probability of $p < 0.05$ was considered significant. The data are expressed as average ± SD, and sample sizes are indicated in the figure legends.

Results

297 *3.1 CUR disappearance from the sea water and distribution within oyster tissues*

298 The rate at which CUR disappeared from the water was faster when oysters were
299 present compared to when they were absent, indicating that the oysters were taking
300 up the CUR by filtration (Fig. 1A). The amount of CUR that cleared from the seawater
301 in the first 4 hours after adding CUR was consistent throughout the entire
302 supplementation period, indicating that the rate of CUR uptake remained constant over
303 the 16 days (Fig. 1B).

304 The levels of CUR or its metabolites increased overtime in the gills (Fig. 1C), mantle
305 (Fig. 1D), and digestive glands (Fig. 1E). After 8 days of CUR supplementation, there
306 was a tendency for the levels to level off in all the studied tissues.

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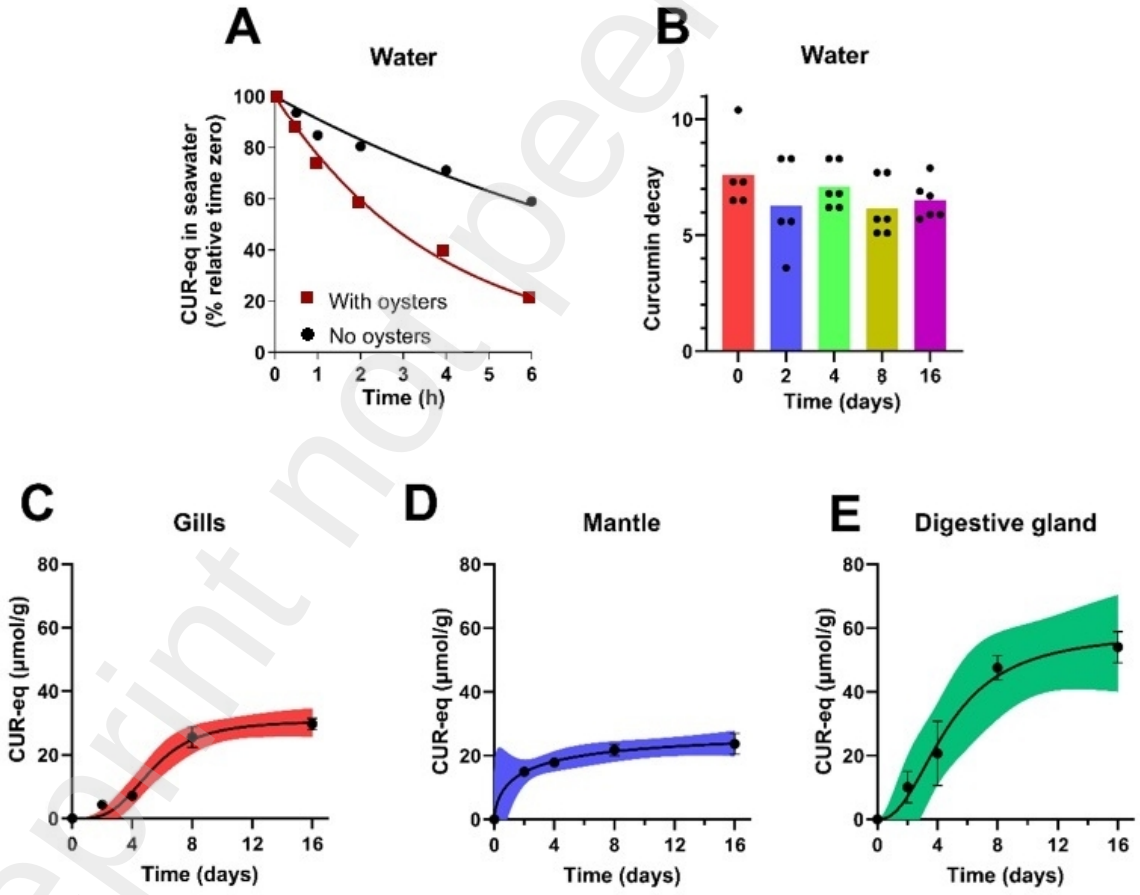

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309 Figure 1: Curcumin (CUR) levels in sea water and tissues of oysters *Crassostrea gigas*. (A) CUR levels
310 in the seawater during the first 6 hours of supplementation with 30 µM CUR, in the presence (squares)
311 or absence (circles) of animals. (B) The rate of CUR disappearance from seawater was assessed on
312 each of the indicated days of the supplementation period and calculated based on the decay of CUR
313 levels during the first 4 h after CUR addition to the aquarium. Tissue distribution of CUR (µmol/g) in the

gills (C), mantle (D), and digestive gland (E) throughout the 16 days of treatment with 30 µM CUR. Data are presented as (A) average and non-linear regression (one phase decay), (B) average and scatter plot of individual values, or (C-E) average ± SD of the analyzed data (N = 4-6) and a non-linear fit analysis (presenting the 95% confidence interval as a shaded area). Given that CUR may decompose or be metabolized, and that the colorimetric method used for quantification cannot distinguish between these modifications, values are presented in curcumin-equivalents (Cur-eq).

3.2 Antioxidant response after supplementation with curcumin

We investigated the effects of CUR on the induction of components of the GSH systems in three primary detoxifying tissues of *C. gigas* oysters: gills, mantle, and digestive gland. The levels of GSH-t and the activity of the enzymes GR and GST were used as indicators of Nrf2 activation, given that they are highly responsive to short-term CUR supplementation in *C. gigas* (Danielli et al., 2017b).

GSH-t levels in the gills increased by 1.5-2-fold over 4-16 days of CUR supplementation (Fig. 2A), while this response was not observed in the mantle (Fig. 2B) and digestive gland (Fig. 2C).

Increased GR activity in the gills (Fig. 2D) and mantle (Fig. 2E) was only observed after 8 days of supplementation. While this increase persisted in the mantle, the activity returned to basal levels in the gills at the end of the exposure. No significant change was observed in the digestive gland (Fig. 2F).

The GST activity in the gills showed a progressive increase along CUR supplementation (Fig. 2G), starting with a 2.4-fold increase at day 4, reaching 4.1-fold at day 8, and 5.6-fold at the 16th day. The same 5.6-fold increase in GST activity was observed in the mantle at 8 days of CUR supplementation (Fig. 2H), while a lower but still significant increase (2.8-fold) was observed at the 16th day. Like GSH-t and GR, GST activity remained at control levels in the digestive gland (Fig. 2I).

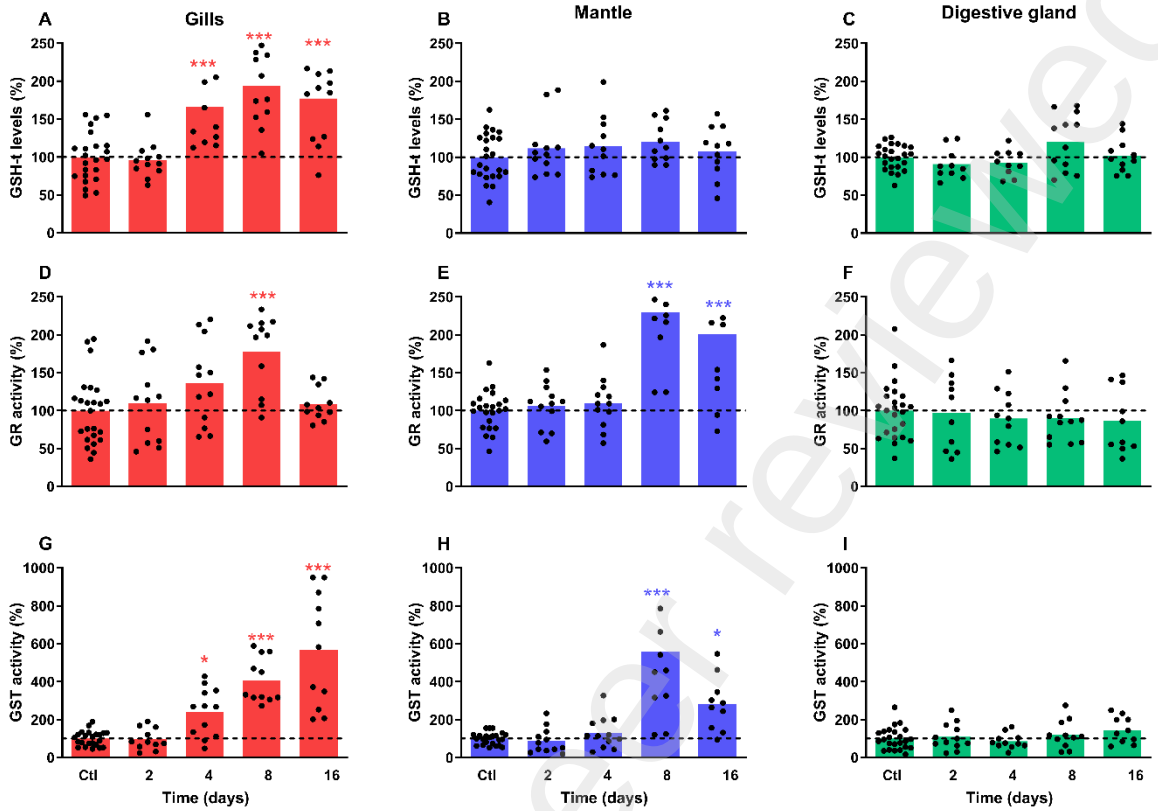

Figure 2: Curcumin treatment induces a robust antioxidant defense in the gills and mantle of oysters *Crassostrea gigas*. Animals were treated every 2 days to 30 µM curcumin (CUR), and (A-C) total glutathione (GSH-t), (D-F) glutathione reductase (GR), and (G-I) glutathione S-transferase (GST) were evaluated in the gills (left), mantle (middle), and digestive gland (right). Values are presented as percentages relative to the control group (Ctl) and scatter plots of the individual values. The basal levels of GSH-t (µmol/g) were: gills 0.85 ± 0.40; mantle 0.68 ± 0.27, and, digestive gland 1.25 ± 0.46. The basal GR activities (nmol/min/mg) were: gills 10.3 ± 4.63; mantle 5.90 ± 1.60, and, digestive gland 7.6 ± 2.05. The basal GST activities (nmol/min/mg) were: gills 70.52 ± 30.0; mantle 5.90 ± 9.37, and digestive gland 13.6 ± 6.31. Data were analyzed by one-way ANOVA, followed by Dunnnet's *post hoc* test when necessary (n=10-12). * $p < 0.05$, and *** $p < 0.001$ as compared to the control group.

3.3 IBRv2 analysis

The IBRv2 analysis presents a summary of the intensity, temporal profile, and tissue-specific amplification of the antioxidant systems in response to CUR supplementation (Fig. 3). These results could be used as a comprehensive indicator of the Nrf2 pathway's sensitivity and response to CUR in *C. gigas*, as well as provide insights into its function in marine bivalves. The gills showed the fastest antioxidant response, with IBRv2 levels increasing after four days and reaching their peak between 8 and 16 days (Fig. 3A). The mantle also exhibited significant antioxidant amplification between 8 and 16 days of supplementation (Fig. 3A), albeit at smaller values than the gills. On the

other hand, the antioxidant systems of the digestive gland remained largely unaffected by CUR (Fig. 3A). This highlights the responsiveness of gills in rapidly amplifying the antioxidant system, whereas the mantle can offer additional support over longer supplementation periods. The quick response in the gills is attributed to the responsiveness of GSH-t and GST, which are sustained until 16 days and reinforced by increased GR activity in the long term (Fig. 3B). In contrast, the mantle does not display the same GSH-t/GST acute response. Instead, the GST/GR pair is associated with the extended antioxidant response of this tissue (Fig. 3C). While not statistically confirmed, it seems that an antioxidant response in the digestive gland is initiated after 16 days of supplementation, primarily due to variations on GST activity (Fig. 3D).

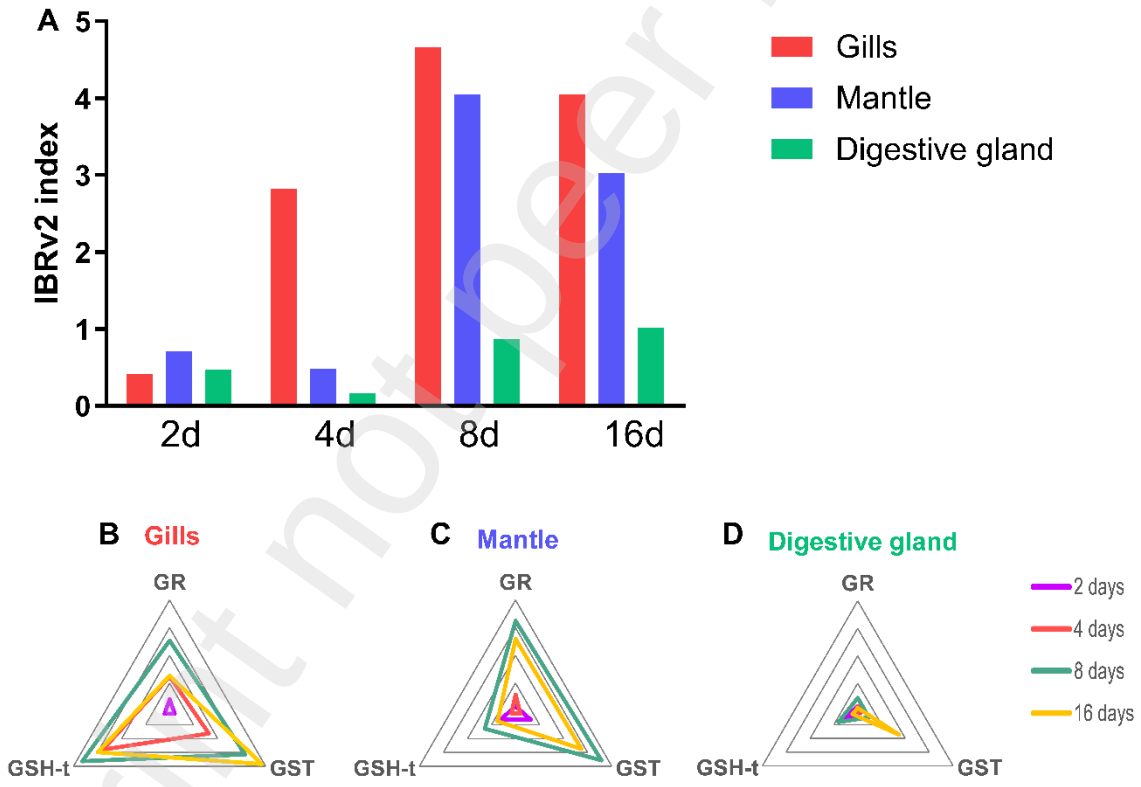

Figure 3: Integrated biomarker (IBRv2) response of antioxidant parameters (GSH-t, GR and GST) in oyster tissues in response to curcumin treatment. (A) IBRv2 index for each tissue along the 16 days of curcumin supplementation; (B-C-D) radar plots indicating the contribution of each biomarker to the IBRv2 index in each tissue and time point (scale from 0 to 2 at 0.5 intervals). The calculation was done using the IBRv2 formula with data from Figure 2.

3.4 Redox challenge in Pacific oysters supplemented with curcumin.

After analyzing the effects of antioxidants and CUR levels in tissues over time, we determined that an 8-day CUR supplementation protocol was the most efficient at the concentration of 30 µM. This protocol led to a significant increase in antioxidants such as GSH-t levels, as well as the activity of GR and GST enzymes in the gills and/or mantle. We then conducted physiological analyses at the level of whole organism to evaluate the effectiveness and strength of the antioxidant increase induced by CUR, with the goal of translating these biochemical changes into improved stress resilience. To test this, we exposed oyster *C. gigas* to the model antioxidant-disrupting compounds NEM (1 mM for 1 hour, as shown in Fig. 4) and CDNB (10 µM for 24 hours, as shown Fig. 5) to determine whether an increase in antioxidant defenses could protect them from subsequent stressors disrupting the antioxidant system. As expected, NEM rapidly depleted GSH in the gills (Fig. 4A) but not in the mantle (Fig. 4B) or digestive gland (Fig. 4C). GR and TrxR contain NADPH and FAD-binding domains in their active sites that are sensitive to redox dynamics, and so we investigated whether NEM exposure could inhibit these two enzymes. GR activity showed a tendency to decrease in the gills (Fig. 4D) and this decrease was also observed in the mantle (Fig. 4E) but not in the digestive gland (Fig. 4F). Similarly, TrxR activity tended to decrease in the gills (Fig. 4G) and mantle (Fig. 4H) but not in the digestive gland (Fig. 4I). NEM is unable to inhibit the GST activity in all tested tissues (Figs.4J, 4K, and 4L).

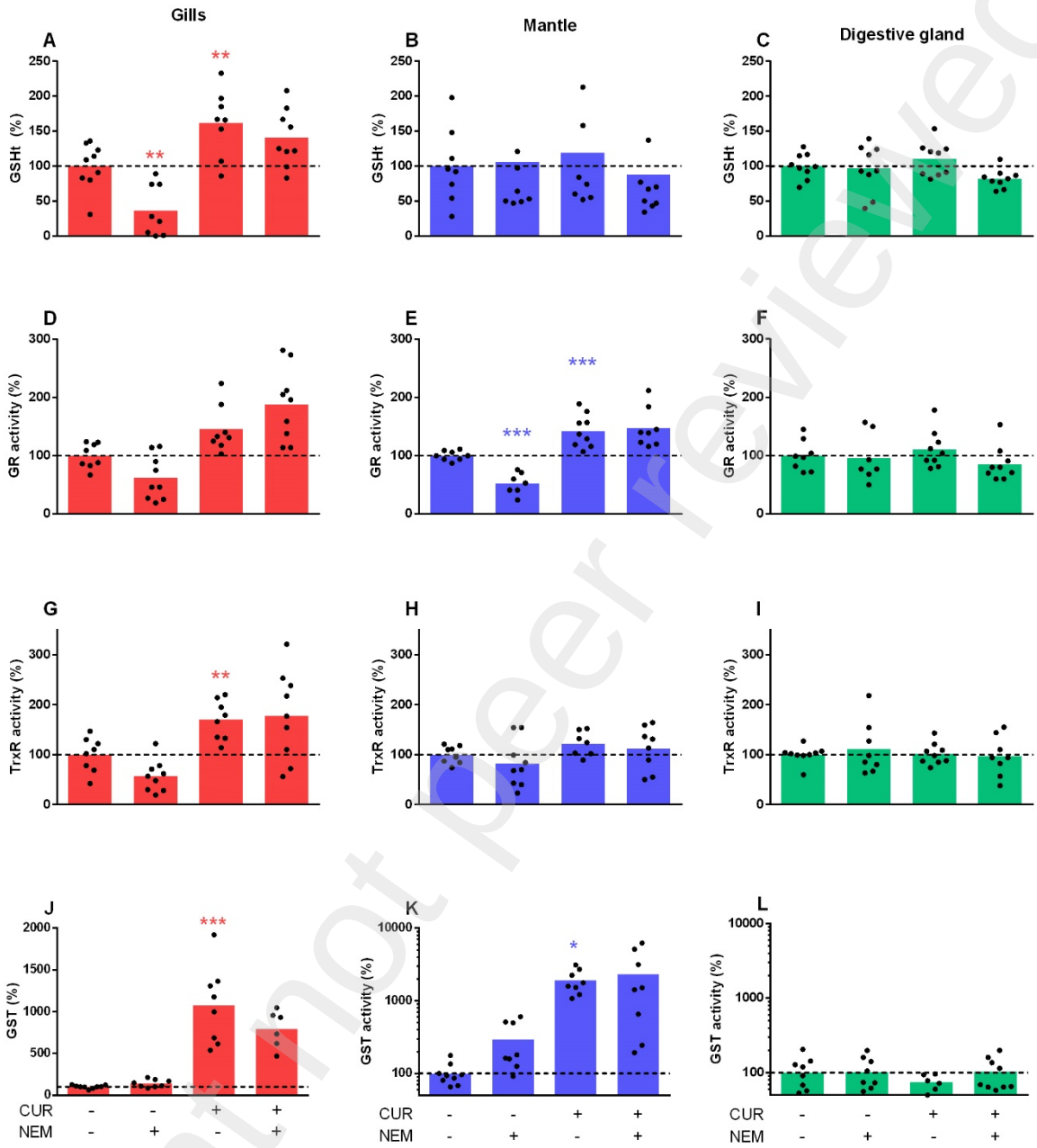

Figure 4: Pre-treatment with curcumin (CUR) protects antioxidants against the electrophilic attack induced by N-ethylmaleimide (NEM). Oysters *Crassostrea gigas* were pre-treated every other day with 30 µM CUR for 8 days to induce the antioxidant defenses and then treated with 1 mM NEM for 1 h. (A-C) Total glutathione levels (GSH-t), (D-F) glutathione reductase activity (GR), (G-I) thioredoxin reductase activity (TrxR), and (J-L) glutathione S-transferase activity (GST) were evaluated in the gills (left), mantle (middle), and digestive gland (right). Values are presented as percentages relative to the control group (Ctl) and scatter plots of the individual values. Data were analyzed by two-way ANOVA, followed by Fischer's post hoc test, with adjusted p-values. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared to the control group. The basal levels of activities of the enzymes TrxR (nmol/min/mg) for the Ctl group were: ± 0.5; 1.27 ± 0.26 and 2.68 ± 0.49, for the gills, mantle and digestive gland.

As expected, CUR supplementation promoted antioxidant gain in the gills and mantle of *C. gigas.* In addition, we identified TrxR as another antioxidant molecule responsive to CUR supplementation, showing a significant increase in the gills. NEM exposure depleted antioxidants in CUR-untreated oysters, but this effect was not observed in CUR-supplemented oysters. These oysters, having retained the increased antioxidant gain induced by CUR, demonstrated a remarkable resilience to the influence of NEM on suppressing GSH-t levels, GR activity, and TrxR activity.

We tested the effects of CUR supplementation against CDNB in addition to NEM. CDNB is an electrophilic compound that quickly conjugates with GSH in oyster gills, causing redox alterations (Trevisan et al., 2016b) and inhibiting GR and TrxR (Tiwari et al., 2015). After CDNB exposure, GSH-t levels were almost non-existent in the gills (Fig. 5A) and mantle (Fig. 5B) and decreased by 60% in the digestive gland (Fig. 5C). As an electrophilic compound, CDNB can also interact with the FAD and NADPH-binding domains of GR and TrxR, potentially inhibiting the activity of these enzymes. Indeed, exposure to CDNB decreased the activity of GR in the gills (Fig. 5D) and showed a slight tendency for inhibition in the mantle (Fig. 5E) and digestive gland (Fig. 5G). While TrxR activity was not affected by CDNB, there was a tendency for lower values in the gills (Fig. 5F) and mantle (Fig. 5H). CDNB exposure did not alter the GST activity in the gills (Fig. 5J), mantle (Fig. 5K), and digestive gland (Fig. 5L).

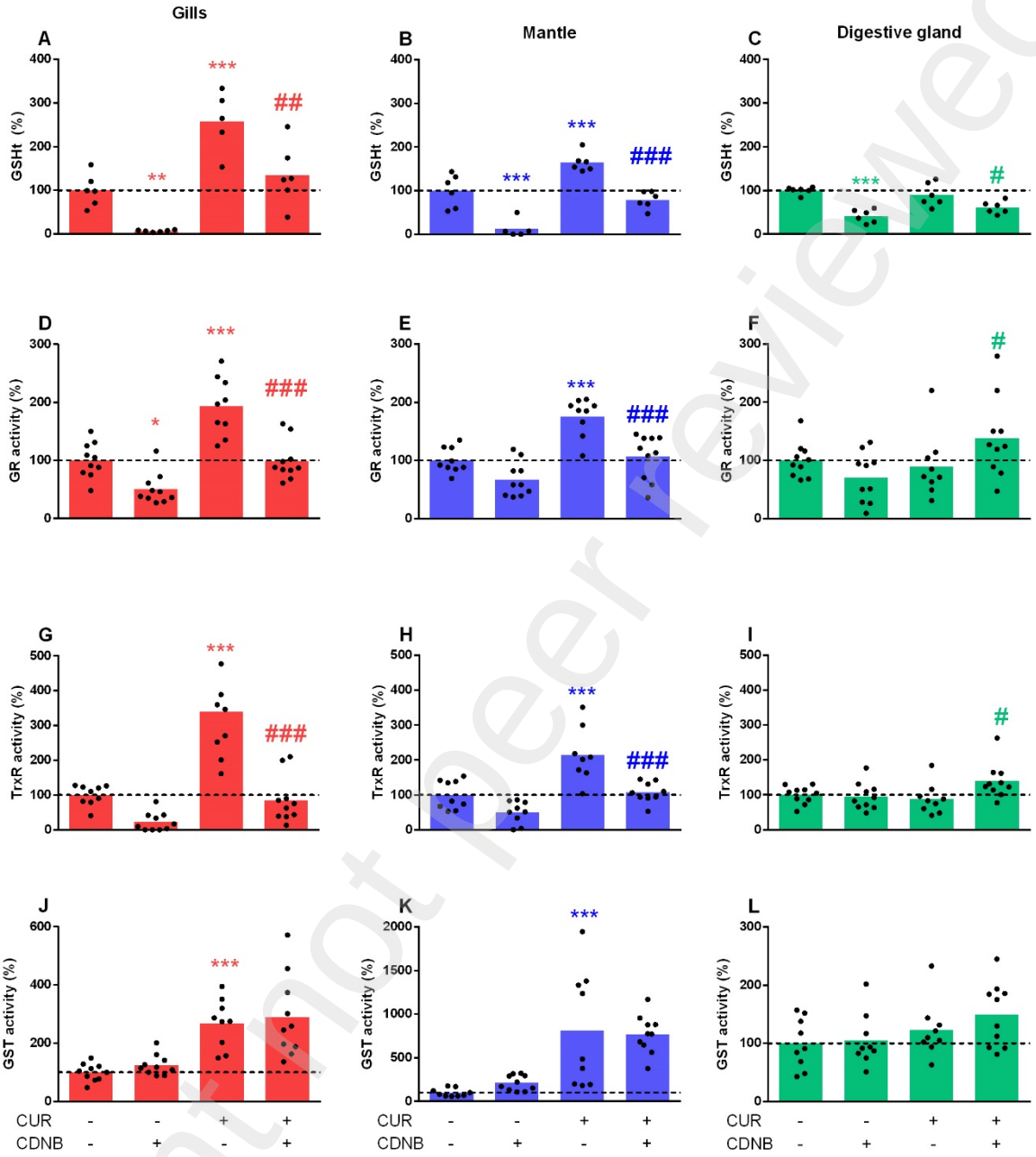

Figure 5: Pre-treatment with curcumin (CUR) protects antioxidants against the redox unbalance caused by 1-chloro-2,4-dinitrobenzene (CDNB). Oysters *Crassostrea gigas* were pre-treated every other day with 30 µM CUR for 8 days to induce the antioxidant defenses and then treated with 10 µM CDNB for 24 h. (A-C) Total glutathione levels (GSH-t), (D-F) glutathione reductase activity (GR), (G-I) thioredoxin reductase activity (TrxR), and (J-L) glutathione S-transferase activity (GST) were evaluated in the gills (red), mantle (blue), and digestive gland (green). Values are presented as percentages relative to the control group (Ctl) and scatter plots of the individual values. Data were analyzed by two-way ANOVA, followed by Fisher's post hoc test, with adjusted p-values. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared to the control group. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ as compared to the CUR group. The basal levels of GSH-t (µmol/g) and activities of the enzymes (nmol/min/mg) for the Ctl group were:

GSH-t 0.79 ± 0.41, GR 2.7 ± 0.5, TrxR 1.20 ± 0.39, and GST 258.6 ± 82.3 for the gills; GSH-t 0.56 ± 0.14, GR 2.1 ± 0.3, TrxR 1.11 ± 0.48, and GST 23.3 ± 12.3 for the mantle; GSH-t 0.77 ± 0.317, GR 11.0 ± 0.9, TrxR 1.26 ± 0.43, and GST 10 ± 6.09 for the digestive gland.

Once more, after 8 days of CUR supplementation, there was a tissue-specific increase in the antioxidants studied, as reflected by higher levels of GSH-t and increased activities of GR, TrxR, and GST in the gills and mantle but not in the digestive gland (Fig. 5). CUR could not completely prevent the antioxidant depletion activity of CDNB, since GSH-t levels (Fig. 5A-C), GR activity (Fig. 5D and E), and TrxR activity (Fig. 5G and H) of CUR+CDNB oysters were lower as compared to CUR alone. It's important to note that despite this antioxidant depletion in CUR+CDNB oysters, these values remained at levels similar to the control, indicating that CDNB exposure could only consume the excess antioxidants triggered by the CUR supplementation.

3.5 Testing the effect of CUR-induced Nrf2 activation on protection against Vibrio

We further investigated whether supplementing oysters with CUR, which improves their antioxidant system, could protect them from Vibrio challenges. We conducted the test using French oysters from a research hatchery facility (IFREMER, Argenton) due to the expertise of the French laboratory LEMAR in bivalve-pathogen interactions. We initially aimed to determine if we could replicate the antioxidant effects of CUR supplementation at a concentration of 30 µM over an 8-day period in a different laboratory using animals with a different genetic background. The results showed higher levels of GSH-t in the gills (Fig. 6A), along with increased GST activity in both the gills (Fig. 6C) and mantle (Fig. 6D), demonstrating the consistent efficacy of our CUR supplementation across different laboratory settings.

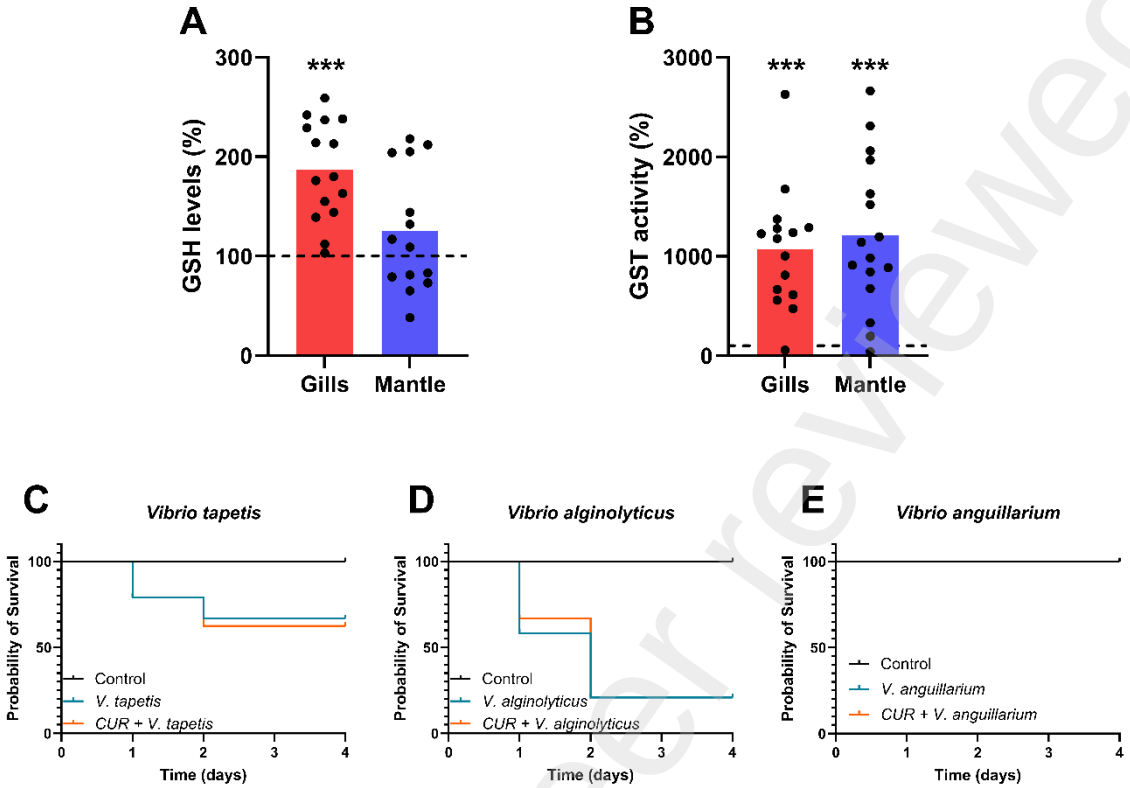

Figure 6: Effect of curcumin supplementation on the susceptibility of oysters to vibrio infection. Oysters *Crassostrea gigas* were pre-treated every other day with 30 µM CUR for 8 days (CUR) to induce the antioxidant defenses and then infected with *Vibrio tapetis* ($5x10^7$ cfu), *V. alginolyticus* ($1x10^9$ cfu), and *V. anguillarium* ($5x10^7$ cfu) for survival analyses. (A) Total glutathione levels (GSH-t) and (B) glutathione reductase activity (GR) in the gills and mantle before pathogen infection (n=16). Values are presented relative to the control group (100%, dashed line). (C-E) Survival curves of oysters supplemented or not with CUR and infected with the indicated vibrio species (n=20). Data was analyzed by Student t-test (GSH-t and GST) or by Gehan-Breslow-Wilcoxon test. Statistical differences against the control group for GSH-t and GST are shown as *** ($p<0.001$).

In a second step, we determined the lethality of two different bacterial loads to *C. gigas* not supplemented with CUR. The survival rate was 70%, 100% and 40% at the lower bacterial load concentration ($5x10^7$) in *V. tapetis, V, alginolyticus* and *V. anguillarum*, respectively (Suplementary table S1). These values decreased to 0%, 85%, and 0% at the higher bacterial load ($5x10^7$), respectively (Supplementary table S1).

We then tested the effects of CUR supplementation on the resistance to bacterial infection. However, we found that CUR did not change the oysters' susceptibility to bacterial infection. The survival rates remained similar in both the non-supplemented

and CUR-supplemented oysters when they were challenged with *V. tapetis* ($5x10^7$) (Fig. 6E) and *V. alginolyticus* ($1x10^9$) (Fig. 6F). Surprisingly, *V. anguillarum* ($5x10^7$) infection did not cause lethality in this experiment (Fig. 6G).

4 Discussion

Filter-feeding animals are susceptible to various stressors in the aquatic environment, such as changes caused by chemical agents of human origin and environmental factors like temperature and pH (Gabe et al., 2021; Pessatti et al., 2016; Peck et al., 2002; Abele et al., 1998; Bordalo et al., 2023; Figueiredo et al., 2022). These stressors can lead to oxidative stress, impacting animal health and decreasing the production of commercial aquatic species. To address this, researchers are exploring ways to enhance the antioxidant defense system in aquatic species, with natural substances like CUR being investigated as a potential solution. For example, studies involving fish have shown that CUR can improve growth rates and protect against stress and diseases (Yonar et al., 2019; Zou et al., 2022), leading to potential gain of production in aquaculture settings. As a result, the use of natural compounds to improve the health and enhance the production of commercial aquatic species, such as oysters, may emerge as a sustainable alternative, contributing to the development of a sustainable blue economy.

In our study, we investigated whether tissues in direct contact with seawater, such as the gills and mantle, showed strong and long-lasting (up to 16 days) antioxidant protection when oysters were supplemented with waterborne CUR (30 µM). We found that CUR disappeared more quickly from seawater when oysters were present, suggesting that it was being absorbed. Tissue analysis revealed a gradual increase in CUR levels (curcumin or its metabolites), indicating that CUR was being taken up by the tissues. Interestingly, the rate of CUR clearance from seawater remained constant throughout the 16-day supplementation period. Our data suggest that waterborne CUR can lead to potent and sustained antioxidant protection in tissues directly exposed to seawater, without evidence of saturation in the absorption rate over a 16-day period.

The concentrations of the antioxidants GSH-t, GR, and TrxR were approximately doubled in the gills, while GST activity increased around fourfold. This response was most notable on the 8th day of treatment, with GSH, GR, GST, and TRxR responding in all experiments in the gills, except once for GR. Similar patterns were observed in the mantle, with GSH-t increasing in 1 out of 4 experiments, GR in 3 out of 3 experiments, GST in 4 out of 4, and TrxR in 1 out of 2 experiments. The magnitude of these changes in the mantle varied, with GST activity showing increases of about 4 to 10-fold in the four sets of experiments. Nevertheless, the consistent increase in endogenous antioxidants in the gills and the mantle induced by CUR is a significant finding. This robust data strongly suggests the rapid adaptability of tissues in direct contact with seawater to regulate their redox biology, guided by master redox regulators such as the Nrf2 pathway. These findings expand the discussion of oxidative stress on the temporal and tissue-specific scale in marine organisms.

To better characterize the temporal and tissue-related profile of antioxidant response, we conducted a comprehensive analysis using IBRv2 (refer to Fig. 3A). This analysis provided an overall view of the variation in antioxidant amplification. The IBRv2 index showed that gills exhibited a rapid and strong ability to enhance the antioxidant system through the GSH/GST system. A slower but similar pattern of antioxidant enhancement was observed in the mantle, mainly relying on GST and GR activities. This supports our hypothesis that tissues in close contact with seawater can rapidly activate the Nrf2 pathway and adjust the response of antioxidant systems to external stress.

The activation of the Nrf2 by CUR can occur by the binding of CUR to Keap1 at Cys151, promoting Nrf2 release and stimulation of the expression of genes such as Gpx (Ruan et al., 2019), TrxR (Wu et al., 2021), and genes related to GSH synthesis (Shin et al., 2020). Activation of this pathway in bivalves, both marine and freshwater, appears to be associated to increased expression of GSH and Trx related genes such as Gpx, GST isoforms omega, pi, and sigma, as well as peroxiredoxins (Prx) (Wang et al., 2018; Wu et al., 2020; He et al., 2019). It has already been shown that CUR supplementation increases the mRNA levels of GCL (GSH synthesis), GR, and GST isoform pi in the gills of *C. gigas* (Danielli et al., 2017b), confirming the redox regulation at this species at the molecular level. Several recent reports present evidence of Nrf2 functionality in bivalves show similar antioxidant responses to Nrf2 in pro-oxidant conditions, such as after exposure to toxins (J.-J. Lv et al., 2021) and polycyclic

aromatic hydrocarbons (Wang et al., 2020; Wang et al., 2018). Thus, we believe that, similar to vertebrates, the gills and mantle of bivalves possess a functional Nrf2 pathway. This pathway involves downstream genes that target antioxidant and biotransformation systems, as well as a Keap1/Nrf2 interaction that is sensitive to redox alterations. This system has the potential to serve as a master regulator of redox biology and the response to chemical stress in these tissues.

The IBRv2 indicates that a Nrf2-driven antioxidant response is not present in the digestive gland. Our group previously suggested this in a study where *C. gigas* was supplemented with CUR for a short period (Danielli et al., 2017b) or exposed to other Nrf2-inducers such as tert-butylhydroquinone (Danielli et al., 2017a). This is intriguing, as it is widely reported in literature that the digestive gland is an important detoxifying organ, capable of inducing antioxidants based on the nature of the challenge (Faggio et al., 2018). Despite the lack of response to CUR, the digestive gland significantly accumulates CUR or its metabolites. Before waterborne CUR reaches and accumulates in the digestive gland, it can be taken by external organs such as the gills and mantle, then carried through the circulatory system (hemolymph) until it finally reaches the digestive gland. During this process, CUR may be transformed into other compounds, either while in transit or after reaching the digestive gland. In mammals, CUR is mainly metabolized through reduction and conjugations reactions, leading to the creation of glucuronide or sulfate conjugates (Pandey et al., 2020). Some of the reductive metabolites of CUR can activate the Nrf2 system (*e.g.*, dihydrocurcumin, tetrahydrocurcumin, and octahydrocurcumin), but it's worth noting that others have a limited effect (Pandey et al., 2020). Furthermore, glucuronide and sulfate conjugates, which are produced readily after the reduction of CUR, do not possess pharmacological activity against the Nrf2 system (Stohs et al., 2018). Thus, additional research is necessary before further discussing the apparent inactivity of the Nrf2 pathway in the digestive system of *C. gigas* supplemented with CUR. This research should focus on the chemical composition of the compounds that accumulate during exposure to CUR, as well as the profile and response of CUR-metabolizing enzymes in the various analyzed tissues.

A significant goal of our research was translating the Nrf2-driven antioxidant amplification by CUR into functional gains. Therefore, our goal was to assess the potential of CUR supplementation in improving an animal's ability to endure stress. We

used NEM as a model for disrupting redox balance, a compound known for its ability to penetrate the plasma membrane and rapidly react with thiol groups, causing an immediate electrophilic assault (Rossi et al., 2001). Our previous research demonstrated that NEM depletes glutathione in the gills of *C. gigas* (Trevisan et al., 2016a), and is also known that NEM can decrease the activities of GR (Shi and Dalal, 1990) and TrxR (O'Donnell and Williams, 1985) by reacting with their Cys residues. The results of our study showed that the depletion of GSH-t and inhibition of GR induced by a rapid NEM exposure (1 mM for 1 hour) was prevented by a pre-treatment with CUR for 8 days. These results suggest that pre-conditioning the antioxidant defenses can be a useful strategy to mitigate environmental stress, especially against acute stressors impacting molecules containing reactive thiols in marine bivalves.

CUR supplementation also protected oyster from excessive redox alterations caused by CDNB. Nevertheless, such effect was partial, as CDNB decreased the antioxidant capacity of CUR-supplemented oysters, but to values similar to the control. Like NEM, CDNB is a molecule that can permeate the gills and has electrophilic properties. Once inside gill cells, it quickly combines with GSH, leading to a major depletion of GSH levels within 24 hours. This decrease occurs due to the strong GSH-conjugating activity in *C. gigas* gills, which is catalyzed by GST and supported by GSH levels. CDNB also inhibits both GR and TrxR activities in bivalves, likely through redox interactions within the active site of these enzymes (Trevisan et al., 2014b; Trevisan et al., 2012b). Unlike NEM, which immediately attacks as an electrophile, CDNB causes a relatively slower depletion of GSH followed by a prolonged period of oxidative stress, as the removal of peroxide will be impaired in inhibited antioxidant systems (Mitozo et al., 2011; Winterbourn, 2020). Thus, our findings indicate that the surplus of antioxidants induced by CUR through Nrf2 activation may offer an antioxidant buffering capacity, thereby enhancing the organism's resilience to oxidative disruptions that may arise over time.

Lastly, we conducted tests to study the effects of CUR supplementation on the survival against bacterial challenges, a promising area of research that could significantly impact the aquaculture industry. This topic lies at the intersection of redox biology, mediated by CUR supplementation, and immunology, mediated by pathogen infections. Redox biology directly impacts the immune system, for example by controlling inflammatory responses (Trevisan and Mello, 2024). In marine bivalves, pro-oxidative conditions also play a crucial role in clearing pathogens during infections

(Schmitt et al., 2011). At the same time, bivalves need a strong antioxidant response to protect themselves from the harmful side effects of such pathogen-induced oxidative responses (Lorgeril et al., 2011). Not surprisingly, improved antioxidant status was also correlated with increased *C. gigas* resistance against viral infections (Dupoué et al., 2023). Thus, as redox biology plays a central role in the immune response of marine bivalves, approaches modulating redox parameters can provide important mechanisms for controlling pathogen resistance and susceptibility in the aquaculture industry.

Despite confirming that CUR improved antioxidant levels and caused lethality in preliminary experiments, supplementation with CUR did not enhance oyster survival to *V. tapetis* and *V. anguillarum*. Additionally, no mortality was observed in animals infected with *V. alginolyticus*. These results contradict the literature, which showed that CUR supplementation improved resistance to bacterial infections in gastropods, shrimps, and fish (Zou et al., 2022; Bhoopathy et al., 2021; Mahmoud et al., 2017; Yonar et al., 2019a. These previous studies used dietary CUR and nano formulated CUR as a supplementation approach, which may explain the differing results compared to our research. Furthermore, the bacterial strains used in our study differed from the ones cited in that literature, which could have affected the interaction between CUR and infection outcomes. Lastly, the smaller size of the oysters used in these experiments compared to other assays we performed might have influenced the profile and intensity of antioxidant amplification. In summary, our findings do not support the hypothesis that CUR and the Nrf2 system affect the resistance of *C. gigas* to Vibrio infections. However, further research is necessary to investigate whether CUR supplementation can impact the tolerance to other pathogens. It is also important to explore if different life stages (*e.g.*, embryos, larvae, juveniles, adults) can influence this response, and whether other forms of CUR supplementation (*e.g.*, nanoencapsulated or combined with other natural compounds such as piperine) could yield better results for the aquaculture industry.

5 Concluding remarks

In this research, we have made significant progress in developing a natural protocol to enhance antioxidants in the marine bivalve species *C. gigas*. By analyzing molecular data from our previous studies on *C. gigas* and recent literature on other aquatic species, we have identified CUR as a compound with promising Nrf2 activation activity

in marine bivalves. This finding has important implications for aquaculture, as the antioxidant system plays a significant role in animal health, development, growth, and resistance to stress and disease. We studied the uptake and bioaccumulation kinetics of CUR and characterized the timeline of antioxidant amplification in three tissues strongly associated with responses to stress and diseases. The gills have the highest surface area in contact with seawater and showed the fastest and most significant antioxidant enhancement, mainly through the pair GSH/GST. The mantle is also in contact with seawater and displayed a significant Nrf2-like response, primarily through the pair GST/GR. In contrast, the digestive gland did not show an Nrf2-like antioxidant response. Nrf2 appears to serve as a crucial redox-sensing molecule within biological barriers, safeguarding against seawater pollutants and triggering an antioxidant response tailored to cellular stress levels.

While we have made significant progress in our understanding of the effects of CUR supplementation, there are still many questions that need to be answered. We have not thoroughly studied the molecular responses activated by Nrf2 when supplemented with CUR, nor have we fully examined the redox biology of animals with enhanced antioxidants. As a result, we still need to fully understand the range of cellular functions and pathways affected by CUR supplementation. We are also uncertain whether other major antioxidants respond through Nrf2 activation, and we have yet to determine the dynamics of reactive oxygen species and the oxidation of biomolecules in these organisms. These are limitations to the present study and the discussion on developing a natural protocol to enhance antioxidants in marine bivalve species.

Nevertheless, our prior observations point to functional benefits from CUR supplementation, such as improved peroxide detoxification rates *in vivo*. We have now expanded this to include the capacity of gills and mantle to provide effective protection against waterborne molecules that have the potential to deplete antioxidants. Yet, the lack of functional gain in the immune response against vibrio infection is a significant drawback of using CUR supplementation in aquaculture practices. We strongly believe further research is needed to explore this topic in more detail, CUR supplementation protocols, immunocompetence assays, and life stages with different susceptibility to pathogens. Other hos-pathogen interactions models can also be tested, such as *C. gigas* and the vibrios *Vibrio aestuarianus* and V*ibrio tasmaniensis* (Destoumieux-Garzón et al., 2020), the clam *Riditapes philippinaturam* and *V. tapetis* (Richard et al.,

2016), or the gastropod *H*aliotis *tuberculata* and *V.harveyi* (Zou et al., 2022a) as these pathogens are mainly involved in redox alterations.

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