Mitigating oxidative stress in oyster larvae: curcumin as a Nrf2 activator for enhanced redox balance, antioxidant capacity, growth performance, and resistance to antifouling compounds

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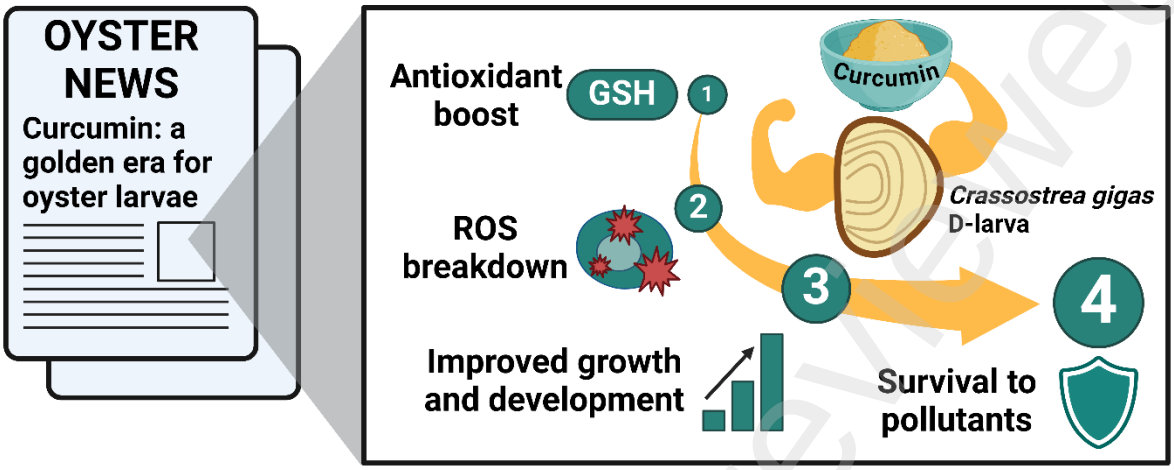
Abstract

Curcumin is a potent natural compound recognized for its antioxidant properties, attributed to its ability to activate the Nrf2 pathway and stimulate the expression of antioxidant genes. High antioxidant efficiency can improve resilience to stress, animal growth, and survival. Given the vulnerability of early life stages to environmental stressors, we hypothesized that supplementing early-developing *Crassostrea gigas* oysters with curcumin to enhance antioxidant capacity would improve growth and stress resistance. Our findings suggest that low curcumin concentrations (up to 1 µM) activate the Nrf2 pathway in *C. gigas* D-larvae, resulting in a significant threefold increase in glutathione levels and a remarkable 50% decrease in reactive oxygen species production. This antioxidant gain significantly improved larval antioxidant capacity and enhanced larval growth and survival under normal and oxidative stress conditions resulting from peroxides and the antifouling dichlorooctylisothiazolinone (DCOIT). Curcumin shows great promise in supporting larval development, but high concentrations were toxic (EC_{50} = 2.90 µM), probably due to excessive Nrf2 activation. Our results indicate that Nrf2 may play a role in controlling bivalve early development. Understanding how Nrf2 influences redox balance and gene expression during early life can enhance our knowledge of stress response mechanisms in marine organisms, offering insights into how they cope with pollutants and environmental challenges. Moreover, future research should focus on the molecular mechanisms of Nrf2 pathway control, its long-term effects on larval health, and the potential for breeding oysters with robust Nrf2 responses. Integrating curcumin and Nrf2-related approaches into aquaculture practices could boost productivity and sustainability in oyster aquaculture.

Keywords: Bivalve; Development; Glutathione; Reactive oxygen species; Antioxidants; Oxidative stress; SeaNine 211.

39 **Graphical abstract**



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1 Introduction

The Pacific oyster, *Crassostrea gigas* (also known as *Magallana gigas)*, is originally from the coastal waters of the western Pacific (Guo, 2009). It is highly prized for its fast growth, adaptability, and commercial value, which has led to its widespread farming across the globe, including Europe, North America, South America, and Oceania. However, due to its robust nature and ability to thrive in diverse environments, it has become an invasive species in many areas, displacing native oysters and disrupting local ecosystems (King et al., 2021). Given its widespread distribution and commercial interest, it is also a leading model for animal biology and ecotoxicology research (Zhang et al., 2012; Zhang et al., 2014; Trevisan and Mello, 2024).

C. gigas, like most other marine bivalves, release gametes into the water for fertilization. The fertilized eggs undergo rapid cell division and development, progressing through several stages. Initially, the embryo develops into a trochophore larva (12 to 24 hours). It later starts forming a shell, becoming a D-larvae (D-shaped shell) (20 to 48 hours). As organogenesis continues, the larvae will develop gills and a foot, transitioning to the pediveliger stage (15 to 20 days). Finally, after settling on a suitable substrate, the larvae undergo extensive metamorphosis (21 days) and transition from planktonic to the beginning of their benthonic phase, where they will stay for the rest of their lives (Wallace et al., 2008). These events involve highly regulated and dynamic changes in gene expression and tissue/organ formation, which are prone to environmental disturbances. In addition, embryos and larvae also have small size, large surface area for uptake of external molecules, and limited detoxification processes. All of that together makes the initial stages of life very sensitive to stress (Mohammed, 2013), both in the environment and in aquaculture conditions.

The redox biology, which involves the processes and mechanisms of reduction/oxidation reactions in living organisms, plays a crucial role in regulating cellular events that are essential for cellular proliferation, differentiation, and apoptosis (Covarrubias et al., 2008). As these events shape animal development, control of the dynamics of the cellular redox state during early development is critical. The nuclear factor erythroid-2-related factor 2 (Nrf2) pathway is the master

redox regulator of the cell (Vomund et al., 2017) and is also involved in controlling the development in mammals and aquatic species such as fish (Timme-Laragy et al., 2018; Sant et al., 2017). The understanding of the Nrf2 system in bivalves and the changes in antioxidants and cellular redox state during their early developmental stages remains limited despite its critical importance. Recently, the redox biology of *C. gigas* has been discussed in the context of animal ecophysiology, development, and environmental conditions, shedding light on crucial research areas (Trevisan and Mello, 2024).

Curcumin (CUR), a natural antioxidant found in turmeric, has strong antioxidant capacities by activating the Nrf2 system. CUR disrupts the interaction between Nrf2 and its inhibitor, Kelch-like ECH-associated protein 1 (Keap1), allowing Nrf2 to enter the nucleus and promote the expression of genes responsible for detoxification and antioxidant defense (Kobayashi et al., 2006; Ngo and Duennwald, 2022). These genes, which include those that regulate glutathione (GSH) synthesis and detoxify reactive oxygen species (ROS), are vital for maintaining redox balance and protecting cells from oxidative damage. Research has demonstrated that CUR amplifies antioxidants and activates the Nrf2 pathway across various models (Alagawany et al., 2021), providing valuable insights into how this pathway modulates cellular responses to external stressors. For instance, we previously identified that CUR supplementation holds promise in upregulating the expression of antioxidant genes, boosting antioxidant capacity, and enhancing tolerance to oxidative stress by activating Nrf2 in adult *C. gigas* oysters (Danielli et al., 2017b). Thus, CUR serves as a potent tool in elucidating the mechanisms and outcomes of Nrf2 activation enha in various organisms, including bivalves.

In the present study, we hypothesized that supplementing CUR to *C. gigas* oyster embryos would boost their antioxidant capacity in the early stages of development. This enhancement would lead to better growth and resistance to stress, such as protection against oxidative conditions caused by pro-oxidant compounds and pollutants. We tested this hypothesis by assessing developmental success, levels or activities of key Nrf2-induced antioxidants and ROS, and overall antioxidant capacity. Additionally, we investigated whether the Nrf2-activated phenotype resulting from CUR supplementation protected embryos and larvae against the pro-oxidant compounds tert-butyl hydroperoxide (TBHP) and the antifouling

dichlorooctylisothiazolinone (DCOIT; the active compound of the antifouling SeaNine 211). Our results indicate that Nrf2 may play a role in controlling bivalve early development. They also confirm our hypothesis that CUR supplementation can confer antioxidant improvement and protection against oxidative stress. Given the high vulnerability of early life stages to exogenous stressors, these findings open the way to discussing approaches modulating Nrf2 efficacy in aquaculture, animal biology, and ecotoxicology research.

2 Materials and methods

2.1 Animal maturation for spawning condition

Diploid *C. gigas* oysters, 18 months old, were bred from genitors with standardized genetic backgrounds (Petton et al., 2015), were kept at an aquaculture site at the Aber Benoît, northwest France. In June 2023, they were moved to the IFREMER research hatchery station at Argenton for a 5-week maturation period (Fabioux et al., 2005). During this time, they were kept in 150L tanks with filtered (1 µm) and UV-treated running seawater at 16-18 °C and were fed a mixed diet of the microalgae *Chaetoceros gracilis* (UTEX LB2658) and *T-Isochrysis* (clone: T-iso; CCAP927/14). Following maturation, batches of mature oysters were transported to LEMAR and kept for a maximum of one week in 80L tanks with running filtered seawater at 18°C, receiving constant feeding with *T-Isochrysis*.

2.2 Gamete sampling and fertilization *in vitro*

Procedures were based on the French norms for bivalve embryotoxicity toxicity testing (AFNOR XP-T90-382). Gonads of mature oysters were stripped to collect spermatozoa and oocytes, which were then observed under an optical microscope to determine their gender and quality. Debris was removed by sieving oocytes through 100 µm and spermatozoids through 20 µm (Steele and Mulcahy, 1999). Gametes were then pooled to form 4 pools of 3 males and four pools of three females. Subsequently, gamete concentration was determined by flow cytometry (EasyCyte Plus; Millipore Corporation, USA) according to protocols from a previous study (Le Goïc et al., 2014). One million oocytes from each pool were placed in individual beakers with 1L of filtered (0.2 µm) seawater and kept at 24 ºC for 1 hour to allow them to adjust their shape. After this, each beaker of oocytes received spermatozoa from one of the pools at a

ratio of 1:100 (eggs: sperm) to start the fertilization process. After 30 minutes at 24 ºC, 800 ml of filtered seawater was added to reach a final volume of 1.8 L. After an additional 1 hour at 24 ºC (1h 30min from the start of fertilization), samples from each beaker were collected to determine the fertilization rate under a microscope. Only pools with a fertilization rate above 90% were used. The fertilized oocytes were filtered through a 20 µm sieve and washed with filtered seawater to remove the spermatozoa, then suspended in 1.8L of filtered seawater. Then embryos are transferred for further testing in 24-well, 96-well microplates, or 50 ml tubes, depending on the assay. The density was kept at 120 embryos per milliliter.

2.3 Supplementation of embryos with CUR and exposure to DCOIT.

The embryos were treated with different concentrations of CUR (Sigma Aldrich, France) - 0.03, 0.1, 1, 3, 10, and 30 µM. CUR was first dissolved in NaOH, which reached the final concentration of 75 µM in all groups (including the control). This concentration of NaOH did not affect the pH of filtered seawater.

Additionally, the embryos were exposed to the antifouling compound DCOIT (Sigma Aldrich, France) at concentrations of 2.5, 5, 25, and 75 µg/L. The control group was treated with DMSO at a concentration of 0.15%. This is an example of environmental pollutant linked to oxidative stress through GSH oxidation (Cima et al., 2008).

In a separate experiment, we evaluated the effects of CUR supplementation on the toxicity of DCOIT. Based on the previous data, embryos were co-exposed to CUR (0.1, 0.3, or 1 µM) and DCOIT (2.5, 5, 25, or 75 µg/L). The control group received both NaOH and DMSO as solvent controls.

All exposures took place at 24 °C, protected from light, for 24 hours. After this period, the plates were analyzed for embryotoxicity, growth, mitochondrial activity, production of reactive oxygen species, and antioxidant capacity, as described below.

2.4 Embryotoxicity and D-larvae growth assessment

This assay was performed in 24-wells (120 embryos in 1 ml). At the end of the 24 hours of supplementation with CUR or exposure to DCOIT, samples were fixed with 0.1% formaldehyde and analyzed using a digital microscope (EVOS XL Core Imaging System, ThermoFisher Scientific) with a 20× objective lens. A total of 100 individuals were examined per well and individually assessed for normal or abnormal D-larvae development. The later was determined as larvae presenting deformities in their shells

and mantle, developmental arrest, or signs of death. The results were expressed as the net percentage of normal development (NPNe) (Mottier et al., 2013). Images of normal D-larvae were taken and analyzed for total shell area (ImageJ software) as an indicator of growth.

2.5 Glutathione and Glutathione S-Transferase analysis

The glutathione (GSH) levels and glutathione S-transferase (GST) activity were measured in larvae supplemented with CUR in 50 ml tubes (6000 larvae in 50 ml). After 24 hours of CUR supplementation, the larvae were sieved through a 20 µm filter and washed three times with filtered seawater to get rid of CUR and debris that could affect the colorimetric assay. The larvae were then concentrated in 1 mL of filtered seawater. From this, 200 µL were transferred to a tube for GSH levels (approximately 1,200 D larvae) and 800 µL to a tube for GST activity (approximately 4,800 D larvae). Both tubes were kept on ice for further processing.

For GSH levels, the larvae were spun down, resuspended in 10 µL of 10% trichloroacetic acid, and lysed by 3 cycles of vortexing (5 seconds) and ice bath (1 min). The samples were then centrifuged at 15,000 × g for 2 minutes at 4 °C, and the supernatant was neutralized with 0.5 M potassium phosphate buffer at pH 7.0. GSH was quantified colorimetrically at 412 nm using the DTNB assay (Ellman, 1959) and a standard curve with known GSH concentrations.

For the GST activity, the larvae were resuspended in 60 µL of 20 mM Hepes buffer pH 7, followed by three cycles of freezing, thawing, and vortexing from homogenization. Samples were centrifuged at 20,000 × g for 30 minutes at 4 °C. The GST activity was measured at 340 nm based on the conjugation of GSH and 1-Chloro-2,4-dinitrobenzene (Habig et al., 1974). The protein concentration was determined in the same homogenate, using the Quick Start™ Bradford Protein Assay (Bio-Rad, France).

2.6 Reactive oxygen species production and antioxidant capacity against tert-butyl hydroperoxide

Production of intracellular reactive oxygen species (ROS) was determined in 96-wells (24 embryos in 200 µL), using the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma Aldrich, France). After DCFH-DA diffuses into the cell, the acetyl groups are cleaved by intracellular esterases to yield a non-fluorescent compound,

which is rapidly oxidized to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) by reactive oxygen species. Larvae within 96-well plates (see section 2.3) were incubated for 4 hours with DCFH-DA (10 µM) in the dark at 24 °C. Subsequently, the fluorescence was measured using an Infinite® 200 PRO plate reader (Tecan Life Sciences) at an emission/excitation wavelength of 485/530 nm (em/ex).

The antioxidant capacity was assessed using a previously described method (Amado et al., 2009), but with tert-butyl hydroperoxide (TBHP) as the oxidizing agent. We chose TBHP because it is a molecule that can be metabolized by GSH, GST, and the glutathione peroxidase/glutathione reductase (GPx/GR) system (Dringen et al., 1998), all of which are enhanced by CUR in *C. gigas* (Danielli et al., 2017b). This assay utilizes DCFH-DA to measure the levels of intracellular pro-oxidant molecules. These levels should increase in the presence of exogenous TBHP or show a smaller increase when TBHP is present alongside a higher antioxidant capacity, such as the one induced by Nrf2 activation. The larvae were treated with DCFH-DA and analyzed under the same conditions as previously described, but this time in the presence of 100 µM TBHP. The difference between DCF fluorescence with or without TBHP is used to estimate the antioxidant capacity, which is further normalized to the control group.

2.7 Mitochondrial activity

This assay was performed in 96-wells (24 embryos in 200 µL). At the end of the 24 hours of supplementation with CUR, overall mitochondrial activity was measured using the PrestoBlue™ HS (PB-HS) assay, a resazurin-based method. Resazurin is converted into its reduced form by mitochondrial enzymes that receive electrons from NADPH, $FADH_2$, $FMNH_2$, and NADH. This conversion produces the fluorescent compound resorufin at a rate that depends on mitochondrial metabolism (Trevisan et al., 2020). The HS designation indicates a high purity of resazurin, which reduces background fluorescence values. Larvae within 96-well plates (see section 2.3) were incubated for 4 hours with PB-HS (diluted 10x) in the dart at 24 °C. Subsequently, the fluorescence was measured using an Infinite® plate reader (Tecan Life Sciences) at an emission/excitation wavelength of 590/544 nm.

2.9 Statistical analysis

Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test. A statistical probability of $p < 0.05$ was considered significant. A total of 4 pools were

used in the experiments and analysis. EC50 values of CUR and DCOIT were determined by a four-parameter logistic curve (variable slope).

3 Results

Initial tests were conducted to assess the overall impact of CUR supplementation on the embryonic-larval development of *C. gigas* (Fig. 1). After 24 hours of exposure to CUR, no significant effects on developmental success were observed up to a concentration of 1 µM CUR (Fig. 1A). However, at higher concentrations, CUR led to an increase in developmental abnormalities and embryonic death (Fig. 1A). Specifically, at 3 µM CUR, developmental success decreased to approximately 50%, and these values dropped to 0% at both 10 µM and 30 µM. Analysis of CUR embryotoxicity by EC_{50} determination indicates a value of 2.90 µM CUR, with a 95% confidence interval between 2.39 µM CUR and 3.42 µM CUR (r^2=0.93, Fig. 1B).

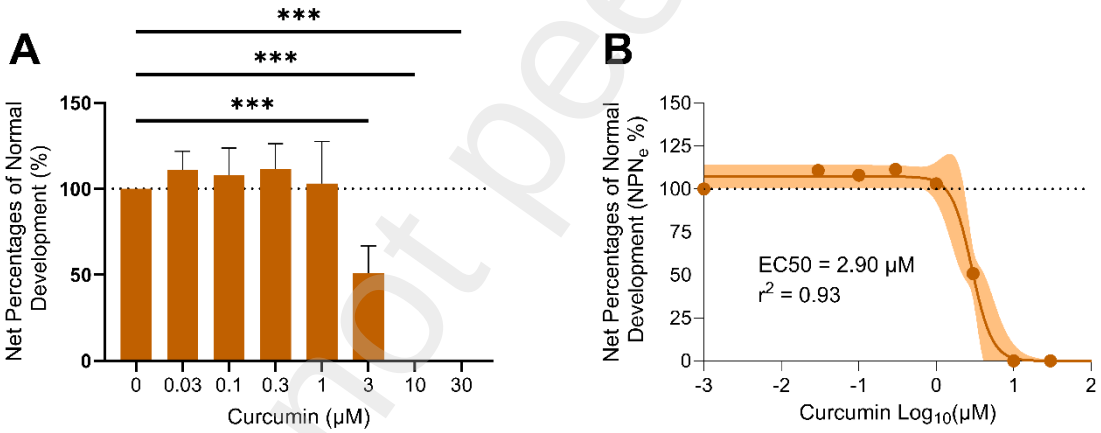


Figure 1: Embryotoxicity assessment of curcumin supplementation (CUR) on *Crassostrea gigas larvae*. The embryos were exposed to various concentrations of CUR for 24 hours, and their developmental success was measured as Net Percentages of Normal Development (NPN_e). (A) NPN_e values for each concentration and (B) non-linear regression of NPN_e and calculation of the EC_{50} value. The data is presented as (A) average and standard deviation (n=4) or (B) average (dots), non-linear regression (curve), and the 95% confidence interval of the curve (shaded area) (n=4). The data was analyzed using (A) one-way ANOVA followed by Dunnett's post-hoc test or (B) non-linear regression (sigmoidal, variable slope, bottom constrained to 0% NPN_e). Significant statistical differences are indicated as ***p<0.001.

In addition to the assessment of embryotoxicity, we also measured the size (total area) of D-larvae after 24 hours of supplementation with CUR. The total area was quantified only in D-larvae that showed no signs of abnormal development (healthy D-larvae). The analysis indicates a significant increase in the average total area of healthy D-

larvae supplemented with 1 µM CUR, while this value decreased at 3 µM CUR (Fig. 2A). It is not possible to determine D-larvae size at concentrations higher than 3 µM, given that all larvae presented abnormal development at 10 µM and 30 µM CUR. Furthermore, the analysis of the distribution frequency of D-larvae total area, according to a Gaussian model, indicated an increase in the predicted total area of D-larvae after supplementation with both 0.3 µM CUR and 1 µM CUR, while it confirmed the decrease in this value at 3 µM CUR (Fig. 2B).

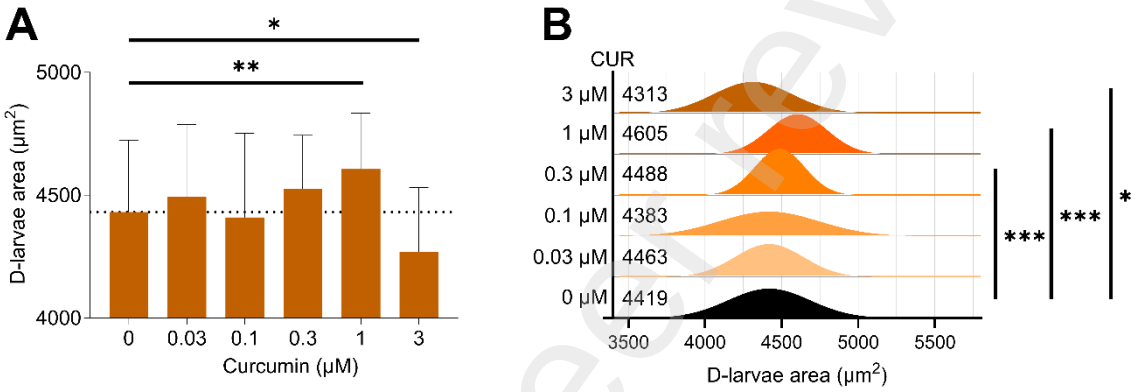


Figure 2: Size of *Crassostrea gigas* normal D-larvae supplemented with curcumin (CUR). The embryos were exposed to the indicated concentrations of CUR for 24 hours, and the total area of healthy, non-deformed D-larvae was determined by optical microscopy. (A) Values of D-larvae total area (average and standard deviation). (B) Frequency distribution of D-larvae total area (simulated average values of D-larvae area are indicated). The data was analyzed using (A) one-way ANOVA followed by Dunnett's post-hoc test (n=4) or (B) non-linear Gaussian distribution followed by Extra sum-of-squares F test (n=35-58). Significant statistical differences are indicated as * $p<0.05$, ** $p<0.01$, and ***$p<0.001$.

Based on the developmental analysis of *C. gigas* larvae, we selected the CUR concentrations of 0.03 µM, 0.1 µM, 0.3 µM, and 1 µM for subsequent experiments, as they did not affect the first 24 hours of larval development in *C. gigas* oysters.

The potential activation of the Nrf2 pathway during CUR supplementation was assessed by analyzing the levels of GSH and GST activity (Fig. 3). These are both markers that are highly responsive to CUR supplementation in adult *C. gigas*. GSH levels showed a significant response to CUR (Fig. 3A), more than doubling at the lowest concentration (0.03 µM CUR) and almost tripling at 0.1 µM CUR and 0.3 µM CUR. There was a slight decrease in GSH amplification at the highest concentration tested with CUR (1 µM), but it still remained at an impressing higher level, about 2.5-

fold higher. On the other hand, GST activity remained unchanged in all tested concentrations (Fig. 2B).

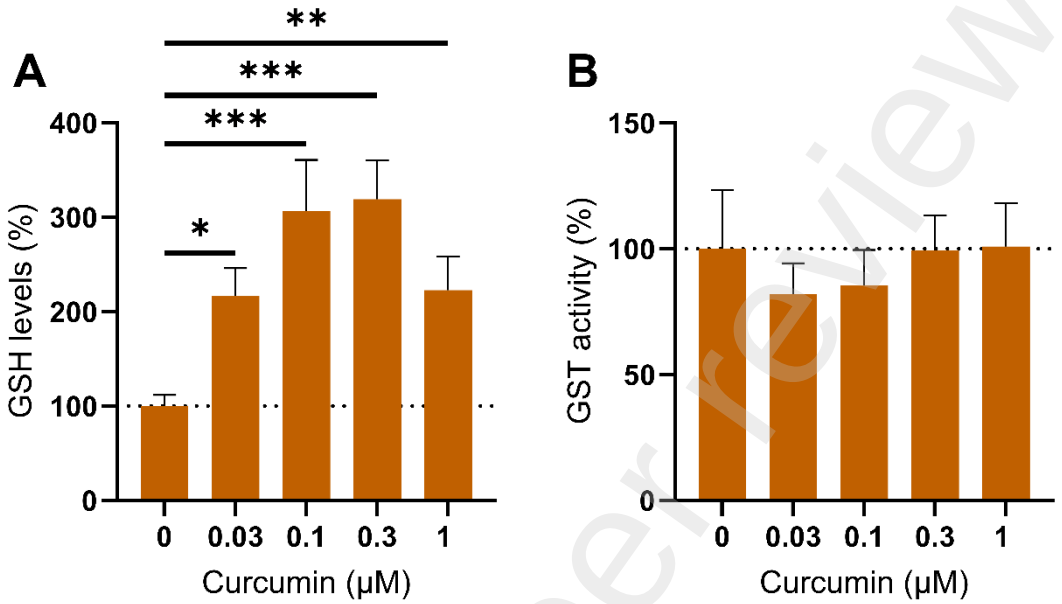
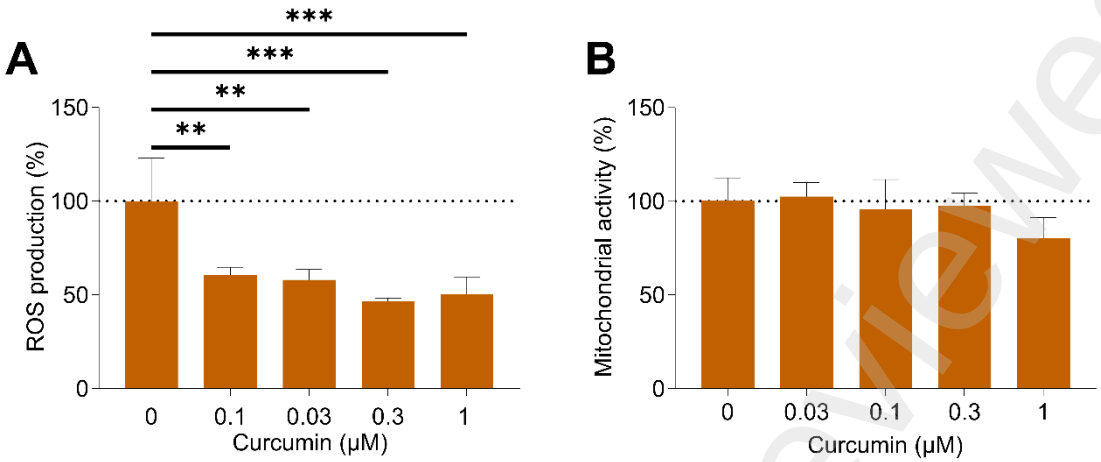


Figure 3: Antioxidant analysis of *Crassostrea gigas* D-larvae supplemented with curcumin (CUR). The embryos were exposed to various concentrations of CUR for 24 hours. (A) Glutathione (GSH) levels. (B) Glutathione S-transferase (GST) activity. The data is presented as average and standard deviation and was analyzed using one-way ANOVA followed by Dunnett's post-hoc test (n=4). Significant statistical differences against the control group are indicated as * $p<0.05$ and ***$p<0.001$.

The assessment of antioxidants was complemented with the analysis of the *in vivo* intracellular production of ROS and overall mitochondrial activity (Fig. 4). The supplementation with CUR reduced ROS production by approximately half at all tested concentrations (Fig. 4A). The overall mitochondrial activity, as determined by evaluating the production of NADH i*n vivo* using a resazurin-based assay, did not show any changes at the tested concentrations of CUR (Fig. 4B).



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304 Figure 4: Production of reactive oxygen species (ROS) and overall mitochondrial activity in *Crassostrea*
305 *gigas* D-larvae supplemented with curcumin (CUR). The embryos were exposed to various
306 concentrations of CUR for 24 hours, followed by fluorometric analyses *in vivo.* (A) ROS production. (B)
307 Mitochondrial overall activity. The data is presented as average and standard deviation and was
308 analyzed using one-way ANOVA followed by Dunnett's post-hoc test (n=4). Significant statistical
309 differences against the control group are indicated as ** $p<0.01$ and ***$p<0.001$.

310

311 We performed a functional assay to evaluate how the changes in antioxidants and
312 ROS production mediated by CUR can affect the antioxidant capacity of D-larvae. In
313 this assay, we used a pro-oxidant compound called TBHP, which is neutralized by
314 GSH and enzymes such as GST, GPx, and GR. When exogenous TBHP is added to
315 the medium, it should be taken up by the larvae, leading to an increase in intracellular
316 oxidation of the fluorescent probe DCFH-DA (Fig. 5A). In larvae with improved
317 antioxidant systems, such as the ones supplemented with CUR, this increase in DCFH-
318 DA oxidation caused by TBHP should be reduced (Fig. 5A).

319 Our results indicate that adding 100 µM of exogenous TBHP leads to a 50% increase
320 in DCFH-DA oxidation in the larvae, as expected (see Fig. 5B). Furthermore, the
321 intracellular pro-oxidative activity by TBHP increases at all tested CUR concentrations,
322 but this effect decreases as CUR concentrations increase (refer to Fig. 5B). We
323 observed an interactive effect between CUR and TBHP based on the results of the
324 two-way ANOVA ($F(4, 30) = 5.020$, $p=0.0032$), highlighting the role of CUR in reducing
325 the oxidation effects of TBHP. Analysis of the fluorescence gain (delta) in the presence

of TBHP (Fig. 5C) confirms that CUR supplementation between 0.1 and 1 µM significantly reduces in about 40-60% the oxidative condition caused by TBHP.

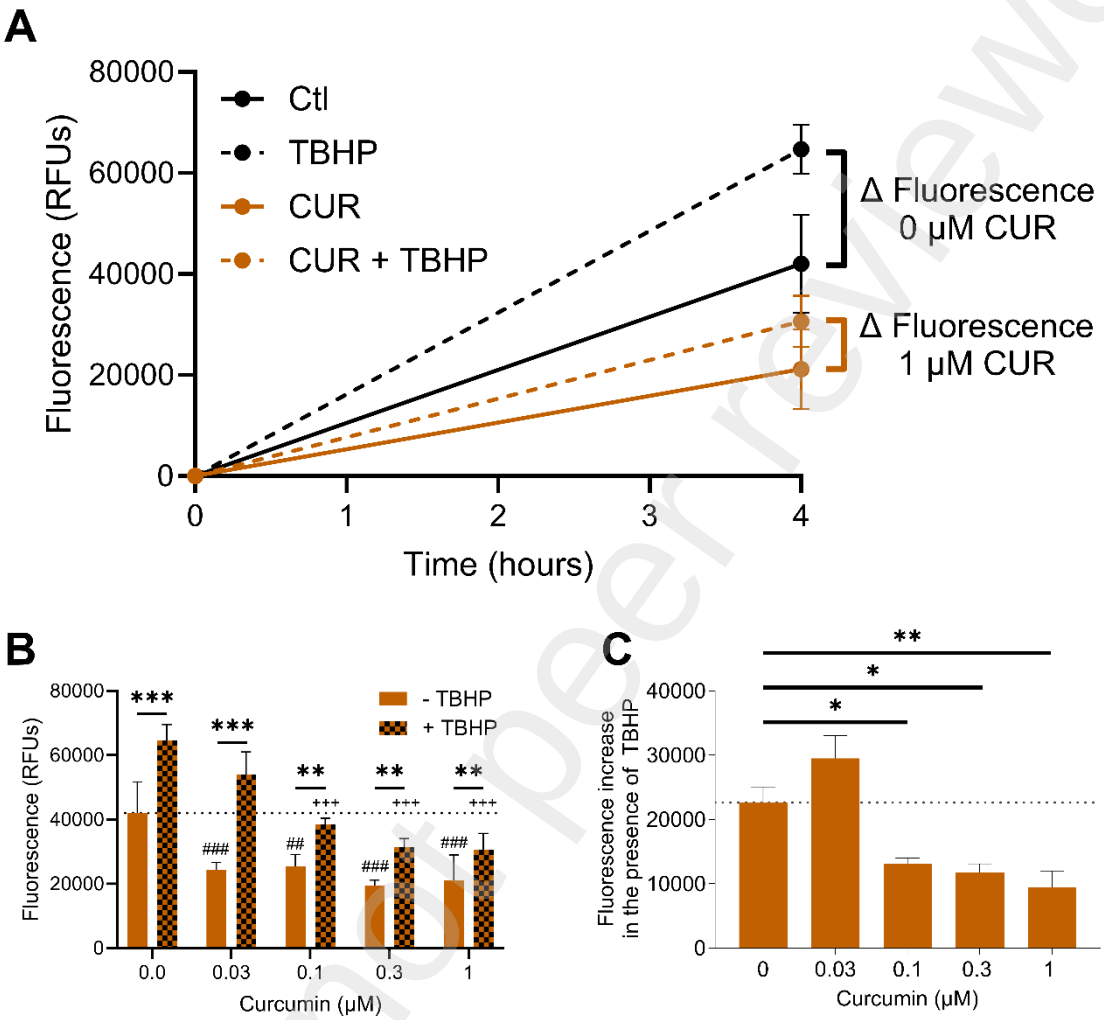

Figure 5: Estimation of antioxidant capacity in *Crassostrea gigas* D-larvae supplemented with curcumin (CUR). The embryos were exposed to various concentrations of CUR for 24 hours, followed by fluorometric analyses *in vivo* in the presence or absence of the oxidizing compound tert-butyl hydroperoxide (TBHP, 100 µM) for 4 hours. (A) Changes in fluorescence values during 4 hours in response to DCFH-DA oxidation over time in the control D-larvae, D-larvae exposed to TBHP, D-larvae supplemented with 1 µM CUR, and D-larvae supplemented with 1 µM CUR and further exposed to TBHP. The fluorescence difference (delta) between the presence and absence of TBHP can be used to estimate the antioxidant capacity against TBHP. (B) Fluorescence values in the presence and absence of TBHP for each CUR supplementation condition. (C) Fluorescence increase between the presence and absence of TBHP for each CUR supplementation. Data are presented as mean and standard deviation. Data was analyzed by (B) two-way ANOVA followed by Tukey's post hoc (n=4) or (C) one-way ANOVA followed by Dunnett's post-hoc test (n=4). Significant statistical differences are indicated as *p<0.05, **p<0.01, and ***p<0.001. Differences against the control group in the absence of TBHP

are shown as ##p<0.01 and ###p<0.001, while differences against the control group in the presence of TBHP are shown as +++ p<0.001. RFUs: relative fluorescence units.

After an initial characterization of developmental, biochemical, and functional effects of CUR supplementation on *C. gigas* D-larvae, we investigated whether CUR could offer protection against a common coastal pollutant that is highly toxic to bivalves. Our study focused on DCOIT, an antifouling and biocide compound containing the active ingredient dichlorooctylisothiazolinone. This compound is found in coastal waters worldwide (Chen and Lam, 2017) and has been linked to oxidative stress through GSH oxidation (Cima et al., 2008). Our findings confirmed the developmental toxicity of DCOIT on D-larvae, resulting in approximately 90% reduction in developmental success (Fig. 6A). Additionally, we determined the EC_{50} for DCOIT to be 47.6 µg/L (Fig. 6B).

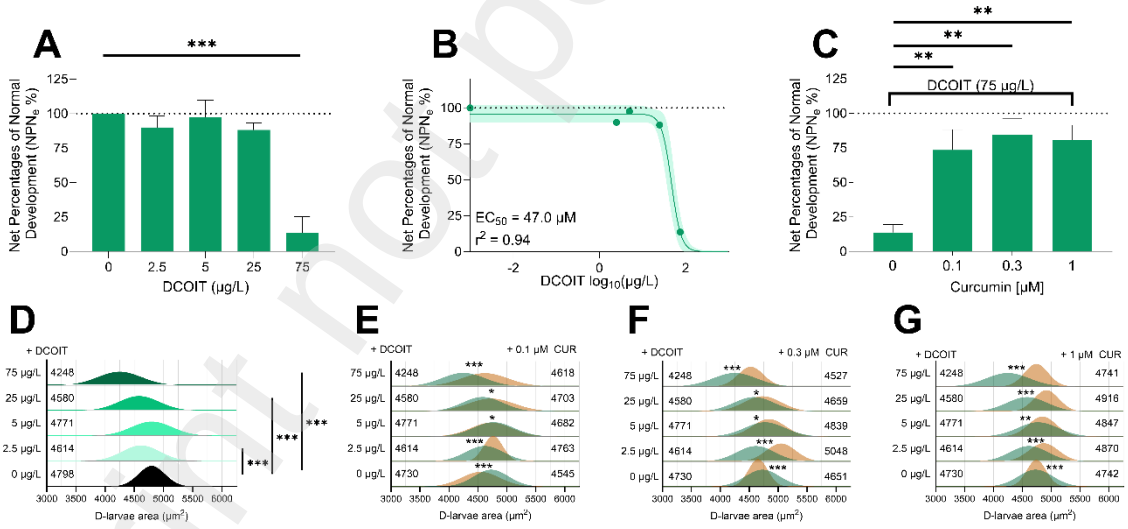

Figure 6: Effects of supplementing *Crassostrea gigas* embryos with curcumin (CUR) on the acute embryotoxicity of the antifouling compound DCOIT. Embryos were exposed to the indicated concentrations of DCOIT for 24 hours in the presence or absence of CUR (0.1 µM, 0.3 µM, or 1 µM). (A) Net percentages of normal development (NPNe) in the presence of DCOIT (average ± standard deviation). (B) Non-linear regression of NPNe and the calculation of the EC_{50} value (symbols=average, curve=non-linear regression, shaded area= 95% confidence interval). (C) NPNe values in animals exposed to DCOIT concurrently supplemented with CUR (average ± standard deviation). Gaussian distribution of the frequency of normal D-larvae area in the presence of DCOIT (D), as well as DCOIT in the presence or absence of 0.1 µM CUR (E), 0.3 µM CUR (F), or 1 µM CUR (G). Data analysis was performed using the following methods: (A and C) One-way ANOVA followed by Dunnett's post hoc test (n=4), (B) Non-linear regression (sigmoidal, variable slope, bottom constrained to 0% NPNe, n=4). (D-G) Non-linear Gaussian distribution followed by Extra sum-of-squares F test (n=37-72).

The compound DCOIT was found to be toxic to *C. gigas* embryos and larvae. However, when the embryos and larvae were exposed to DCOIT while being supplemented with CUR at concentrations of 0.1 µM, 0.3 µM, or 1 µM, the developmental toxicity effect was almost blocked (Fig. 6C). The NPNe values increased from 13% (with DCOIT alone) to above 73% (with DCOIT + CUR), representing a significant improvement due to the CUR supplementation.

The embryos that were exposed to DCOIT and developed into proper D-larva morphology showed a smaller size compared to those not exposed to DCOIT. The normal D-larvae size was 4% smaller at 2.5 µg/L, 5% smaller at 25 µg/L, and 12% smaller at 75 µg/L after exposure to DCOIT (Fig. 6D). However, the introduction of CUR played a crucial role in mitigating the developmental toxicity of DCOIT. At 0.1 µM CUR, the effects of DCOIT on larval size at 2.5 µg/L, 25 µg/L, 75 µg/L. Similarly, 0.3 µM and 1 µM CUR blocked the effects of DCOIT on normal D-larvae size at all tested concentrations, demonstrating the potential of CUR in counteracting the effects of DCOIT on *C. gigas* early growth.

4 Discussion

Research has consistently shown that the embryonic and larval stages of aquatic species are more vulnerable to environmental pollutants than juveniles, who in turn are more susceptible than adults (Hutchinson et al., 1998). This underscores the crucial role of early life stages in the face of external factors. As organisms mature, they develop an enhanced ability to respond to stress at the molecular to the physiological level (Trevisan and Mello, 2024). By enhancing defense mechanisms during these early stages, we could significantly improve growth performance and increase resistance to unfavorable conditions. Notably, CUR has been found to activate the Nrf2 pathway, a critical molecular mechanism of eukaryote cell resistance to oxidative stress, a common mode of action induced by many environmental stressors (Trevisan and Mello, 2024). The activation of the Nrf2 pathway has already been identified in adult *C. gigas* oysters (Danielli et al., 2017a; Danielli et al., 2017b) we hypothesize that CUR could similarly trigger antioxidant defenses in D-larvae of this species. This can support the opening of a new frontier in marine biology and aquaculture research with a potentially significant impact.

The initial testing of the developmental effects of CUR on *C. gigas* embryos and larvae suggests that it is highly toxic at concentrations of 3 µM or higher, with an estimated EC_{50} of 2.90 µM. This finding aligns with embryonic zebrafish studies, which examined the acute developmental toxicity of CUR and identified an LC50 ranging from 5 µM to 7.5 µM, depending on the developmental stage analyzed (Wu et al., 2007). Our results also highlight the susceptibility of oyster embryos to CUR compared to adult oysters, which can withstand exposures to CUR as high as 30 µM without signs of toxicity (Danielli et al., 2017b). This highlights the vulnerability of early-life stages to environmental stress. Since CUR activates the Nrf2 pathway and affects the cell's redox biology (Wu et al., 2019), it may lead to developmental toxicity due to the overactivation of the Nrf2 pathway and imbalanced redox parameters during a critical stage of life when redox reactions are much more tightly controlled (Trevisan and Mello, 2024; Marques et al., 2024).

Supplementation with sublethal concentrations of CUR significantly increased the size of D-larvae. Previous research has indicated that CUR has the potential to improve embryonic growth by protecting cells against oxidative stress in mammalian studies *in vitro* (Bari et al., 2021). Furthermore, the use of CUR and other natural compounds has demonstrated the ability to replace antibiotics for enhancing growth in fish, attributed to the increase in protein efficiency ratio and digestion (Alagawany et al., 2021). We do not have information on developmental and metabolically associated genes in *C. gigas* larvae supplemented with CUR. This could offer valuable insights into the observed enhancement of embryonic and larval growth at relatively low CUR concentrations.

Therefore, we conducted an initial assessment of the potential biochemical and physiological implications of CUR supplementation in oysters *C. gigas*. Our findings show that embryos supplemented with sub-teratogenic concentrations of CUR (up to 1 µM) exhibited higher GSH levels and lower rates of ROS production. This is consistent with the high GSH levels detected in adult oysters or mussels supplemented with CUR (Danielli et al., 2017b) or other Nrf2 activators such as tert-butyl hydroquinone (Danielli et al., 2017a), CDNB (Trevisan et al., 2016), and zinc (Trevisan et al., 2014a). On the other hand, GST activity remained unchanged, and we did not analyze the activity of other antioxidant enzymes due to the significant technical challenge of acquiring enough biological material for the tests. Thus, it is possible that

other important molecules, such as catalase, superoxide dismutase, glutathione peroxidase, and peroxiredoxins, are contributing to improved basal antioxidant levels. In addition, despite the potential of mitochondrial activity in modulating ROS production, it remained unchanged upon CUR supplementation. Lower ROS levels, decreased lipid peroxidation and inflammation, and improved antioxidant defenses are hallmarks of Nrf2 activation by CUR detected in multiple model organisms and cell types (Ashrafizadeh et al., 2020). Therefore, based on the current study, as well as previous (Danielli et al., 2017b) and ongoing research from our group on CUR and *C. gigas*, we propose that administering low levels of CUR during embryonic development stimulates the Nrf2 pathway, leading to an increase in antioxidant capacity and a decrease in ROS production.

To further test the hypothesis of improved antioxidant capacity of D-larvae supplemented with CUR, we used TBHP as an exogenous oxidant primarily detoxified by the GSH system, namely GSH, glutathione peroxidase, glutathione reductase, and GST (Dringen et al., 1998). As expected, D-larvae supplemented with CUR demonstrated a significant 40-60% reduction in oxidative activity of TBHP. This finding is reassuring as it further suggests the efficacy of CUR supplementation in attenuating oxidative stress. It also establishes a direct correlation between the antioxidant capacity assay and the increased GSH levels observed in the CUR-supplemented larvae. Furthermore, our previous research has shown a similar increase in GSH levels and activity of GSH-related enzymes in adult oysters supplemented with CUR (Danielli et al., 2017b). On the other hand, a decrease in the efficiency of the GSH system results in a diminished antioxidant capacity to detoxify peroxides *in vivo* in bivalves (Trevisan et al., 2014b). Therefore, the higher GSH levels, lower ROS production, increased antioxidant capacity against TBHP, and improved growth in response to CUR supplementation indicate a phenotype of enhanced developmental success and resistance to stress.

The early development of oysters begins with fertilization, which happens externally in marine bivalves, leading to embryo formation. During organogenesis, bivalves transition from the gastrula stage to trochophore larvae and increase the expression of genes related to the GSH system (Zhang et al., 2022). In fish, low GSH levels are found during early development (up to the blastocyst stage), with synthesis beginning after gastrulation and doubling the GSH levels by the end of embryogenesis (Timme-

Laragy et al., 2013). Beyond its role as an antioxidant, GSH also mediates biological processes such as differentiation and cell death (Laborde, 2010), due to its reversible cysteine residue oxidation, which is crucial for animal development. Thus, modulating GSH levels during development may be essential to understanding the toxicity of CUR at high concentrations (excessively reducing redox state) and its protective effect at lower concentrations (moderately reducing redox state). Further studies are needed to understand the dynamics of GSH synthesis and its reduced/oxidized ratio during early bivalve development.

We conducted an additional set of experiments to investigate the Nrf2-like phenotype resulting from CUR supplementation. We used DCOIT (the active compound of the antifouling DCOIT), as an environmental pollutant with oxidizing activity against GSH (Cima et al., 2008; Gabe et al., 2021). Initially, we confirmed the developmental toxicity of DCOIT, with only 13% of the larvae appearing normal after exposure to 75 µg/L. This result is consistent with a prior study on the same oyster species and antifouling agent, where 80% of D-larvae exhibited developmental abnormalities after 24 hours at 50 µg/L of exposure (Tsunemasa and Okamura, 2011). Exposure to DCOIT also resulted in significantly smaller normal D-larvae. The ability of DCOIT to disrupt animal bioenergetics (Chen and Lam, 2017) and impact mitochondrial function (Bragadin et al., 2005) could alter cellular energy dynamics during development. As a result, normal D-larvae may experience a physiological cost due to decreased energy available for growth

Similar to TBHP, the mechanism of toxicity of DCOIT can assess the enhancements in the GSH system resulting from CUR supplementation. When GSH synthesis is induced, damage from DCOIT is reduced, as observed in the simultaneous exposure of CUR and DCOIT in *C. gigas* D-larvae. Dichlorooctylisothiazolinone, the active ingredient in DCOIT, is an isothiazolone that rapidly penetrates cell membranes (Collier et al., 1990) and oxidizes GSH (Cima et al., 2008). This biocide may also be associated with the formation of ROS (Chapman and Diehl, 1995). These features impair antioxidant defenses and biotransformation processes (Arning et al., 2008). Therefore, by improving antioxidant parameters, and potentially biotransformation pathways as suggested in the literature, CUR supplementation can mitigate some of the acute toxic effects of DCOIT. This set of data further points to a potential activation of Nrf2 by CUR in *C. gigas* and the resulting improved resilience to oxidative stressors.

5 Conclusion

Our research indicates that CUR has the potential to be a nature-based sustainable alternative for promoting antioxidant enhancement and developmental success in early-life *C. gigas*. By activating the Nrf2 pathway, CUR increases GSH levels, reduces ROS production, and improves antioxidant capacity and protection. These results are consistent with the known effects of CUR in adult oysters and other model organisms, demonstrating its role in strengthening cellular defenses against oxidative stress. Additionally, the enhanced growth and developmental success in larvae supplemented with CUR suggests an ability to improve physiological outcomes in aquaculture settings.

However, the toxicity of higher concentrations of CUR highlights the importance of dosage control. It remains necessary to investigate the molecular mechanisms through which CUR affects gene expression during early development, focusing on redox biology and the regulation of GSH synthesis. Additionally, studying the long-term impacts of CUR supplementation on larval survival, growth, and resistance to environmental stressors will provide valuable insights into its practical applications in aquaculture.

In summary, our research points to strategies to modulate the antioxidant system of developing bivalves. For example, integrating CUR into oyster hatchery protocols could produce robust oyster larvae with enhanced survival and resilience. Conversely, selective breeding programs could use oyster families with a robust Nrf2 response, leading to greater resilience and improved growth performance under stress. Thus, research on the Nrf2 system and other stress response pathways could pave the way for enhancing the health, productivity, and sustainability of oyster farming operations. They can also support increasing aquaculture production and making it more adaptable to environmental challenges.

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Author contributions statement

The following Contributor Roles Taxonomy (CrediT) are acknowledged for each author:

HBG: Conceptualization, data curation, formal analysis, funding acquisition, investigation, supervision (of KAT), visualization, and writing – original draft.

KAT: formal analysis and writing – review and editing.

FRQ: investigation and writing – review and editing.

RT: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision (of HBG), validation, visualization, and writing – original draft.

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