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The role of copper and zinc accumulation in defense against bacterial pathogen in the fujian oyster (*Crassostrea angulata*)

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ABSTRACT

Cu and Zn are hyper-accumulated in oysters, and the accumulation of these metals increases host resistance to pathogens. However, the role of Cu/Zn in oyster immune defense remains unclear. In this study, *Crassostrea angulata* with different levels of Cu and Zn were obtained through metal exposure or selective breeding. Both *in vivo* and *in vitro* experiments showed that oysters accumulating more Cu/Zn exhibited stronger antibacterial abilities. *Vibrio harveyi* infection significantly promoted the metal redistribution in oysters: Cu and Zn concentrations decreased in the mantle, but increased in the plasma and hemocytes. This redistribution was accompanied by changes in the expression levels of Cu and Zn transporter genes (CTR1, ATP7A, ZIP1, and ZNT2), suggesting that the Cu/Zn burst observed in the hemocytes was likely due to the transfer of heavy metals from plasma (mediated by the metal importer proteins) or released from intracellular stores. The degree to which Cu/Zn concentration increased in the plasma and hemocytes was more dramatic in oysters with high levels of Cu/Zn accumulation. *In vitro*, Cu and Zn both inhibited the growth of *V. harveyi*, while Cu plus H_2O_2 was lethal to the bacteria. The strength of the growth-inhibition and lethal effects depended on the metal dose. In addition to these effects, increases in Cu concentration increased the activity levels of PO in the oyster plasma and hemocytes *in vivo* and *in vitro*. However, SOD activity was not affected by Cu or Zn accumulation. Thus, our results suggested that the Cu/Zn burst in the hemolymph was an important factor in the oyster immune reaction, creating a toxic internal environment for the pathogen, as well as catalyzing inorganic or enzymatic reactions to strengthen bacteriostasis. By determining the extent of Cu/Zn burst in the immune response, Cu/Zn accumulated levels could affect the resistance of oysters to pathogens.

1. Introduction

Oysters live in the intertidal zone, where the environment fluctuates drastically. Uniquely, oysters assimilate and retain high levels of copper (Cu) and zinc (Zn) despite low ambient concentrations, accumulating these elements against chemical gradients [1,2]. In fact, the oysters are one of the strongest marine accumulators of Cu and Zn. Sun et al. [3] found that the bodily concentrations of Zn in *Crassostrea gigas* and *C. rivularis* were 140 times greater than that in other organisms from the same coastal area. Pan & Wang [4] studied five bivalves from Clearwater Bay, Hong Kong, showing that the Cu concentration in the soft body of *C. hongkongensis* was 6–65 times greater than the Cu concentration in the other bivalves. Biokinetic quantifications have suggested that oysters assimilate Cu and Zn more efficiently and have lower elimination rates than other bivalves, thus exhibiting low rates of Cu and Zn turnover [4,5]. Therefore, the accumulation of Cu and Zn is not merely a passive adaptation to metal pollution, but is an inherent "preference" in oysters.

Cu and Zn are essential micronutrients for organisms and they are both integral to biological metabolism [6,7]. However, the concentrations of Cu and Zn in oysters seems far exceeding what is typically required by other organisms [8]. In organisms, the absorption and transport of Cu and Zn are absorbed and transported accomplished by unique binding proteins (CTRs, ZNTs, and ZIPs), which consume energy [9,10]. Oysters are highly adaptable organisms, inhabiting a complex environment. It is therefore unclear why oysters consume so much energy absorbing Cu and Zn from the environment and storing these elements in tissues and cells. These elements might be key factors in oyster survival and distribution because their critical functions in nutrition and immunization. In mammals, Cu and Zn are required for

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proper immune system function. The concept of "nutritional immunity" has emerged in the context of host defense against pathogens [11–16]. In some aquatic organisms, appropriate increases in dietary Cu or Zn increase pathogen resistance [17,18]. Considering these, what role do Cu and Zn play in the innate immunity of oysters? Is Cu and Zn accumulation derived from the immune needs of oysters? Investigations of this topic would increase our understanding of the biological roles of Cu and Zn accumulation, as well as our knowledge of oyster innate immunity.

However, few related studies are available. Brown [19] showed that Cu and Zn released from oyster hemocytes formed clots in plasma by precipitating proteins, an inorganic and non-enzymatic reaction. It is possible that these clots prevent pathogenic infection by trapping invading exogenous microorganisms. In addition, oyster Cu/Zn body concentration is significantly positively correlated with total hemocyte count (THC) and antibacterial activity [20]. Fisher [21] showed that oysters grown for 12 weeks in seas with high concentrations of Cu and Zn had elevated levels of THC and antibacterial ability in the hemolymph. Indeed, accumulated Cu greatly reduced *Perkinsus marinus* infection levels in hemocytes *in vivo*, presumably via direct toxic effects on the parasite [22]. Although these studies identified a link between Cu/Zn accumulation and oyster innate immunity, the underlying mechanism remains uncharacterized. For example, it is unknown whether Cu and Zn are redistributed in the oyster body after pathogenic invasion, and it is unclear how oysters use Cu and Zn to combat bacterial infections. Therefore, studies of the immune response to pathogenic infection in oysters with different levels of Cu or Zn accumulation are urgently needed.

The Fujian oyster, *C. angulata,* is the most-commonly cultivated oyster species in southern China. In this study, *C. angulata* specimens with different bodily Cu/Zn concentrations were obtained through metal exposure or genetic selection. *Vibrio harveyi* was used to infect these specimens. We then determined the effects of Cu and Zn accumulation on resistance to pathogenic infection by testing the antibacterial activity of the hemolymph *in vitro* and *V. harveyi* growth *in vivo*. We also assessed Cu and Zn homeostasis in the hemocytes, plasma, and mantle during infection, as indicated by Cu/Zn content, relative free Zn ion concentration, and the gene expression levels of metal transporter proteins (CTR1, ATP7A, ZNT2 and ZIP1). We also measured the activity levels of Cu/Zn superoxide dismutase (Cu/Zn-SOD) and phenoloxidase (PO) in hemolymph. Finally, the effects of Cu^{2+}/Zn^{2+} on vibrio growth and enzymatic activity were examined to explore the antibacterial function of Cu and Zn. This study provides new insights into the role of Cu and Zn in innate immunity of oysters. Our results will improve the biological understanding of Cu/Zn accumulations and its immunological role in the oyster, to combat pathogenic invasion.

2. Materials and methods

2.1. Oyster rearing and metal exposure

We designed a long-term, low-dose metal exposure protocol to obtain oysters with different levels of Cu and Zn accumulation. Fujian oysters (10 months old; mean weight: 33.42 ± 3.03 g) were collected from an oyster-farming site in Gangkou Village (23° 42′ N, 117° 19′ E), Fujian Province, China. Before the exposure, oysters were brushed to remove attachments. Seawater was filtered through a 0.45-μm filter (salinity: 28–30; 25 °C). Stock solutions of 10 mg/L Cu ($CuCl_2$; Sigma, USA) and 10 mg/L Zn ($ZnSO_4$•H_2O; Sigma, USA) were prepared with Milli-Q-filtered water. We divided the oysters into four groups (200 individuals per group). Oysters in each group were cultured in a 300 L tank and exposed to either 20 μg/L Cu (Cu group), 20 μg/L Zn (Zn group), 20 μg/L Cu + 20 μg/L Zn (CZ group), or pure seawater (control group) for 28 days. During the exposure period, seawater was renewed daily. One day before the replaced, seawater was pumped and filtered into a reservoir which was in the same room as the experimental tank. When changing water, removed oysters, cleaned tanks, added seawater and metal storage liquid, then put oysters back. The whole process was controlled within 15 min. Oysters were fed commercial *Chlorella* spp. powder at a rate of approximately 2% of their soft tissue dry weight per day.

2.2. Experimental infection

V. harveyi is a serious pathogen of marine vertebrates and invertebrates, and many strains are pathogenic to oysters [23,24]. The strain of *V. harveyi* used in this study was isolated from diseased oyster. After metal exposure, 90 oysters from each of the four groups (control, Cu, Zn, and CZ) were randomly selected for bacterial injection. The shells of the selected oysters were drilled near the adductor muscle using a microbit (1–2 mm) to facilitate bacterial injection. The injection volume was 100 μL of filtered seawater (FSW) containing 10^8 CFU/ml of *V. harveyi*. We randomly selected 25 oysters from each group just before the infection (at 0 h), and then at 12 h and 36 h after infection. All of the infected oysters were kept in membrane-filtered (0.22 μm) seawater (salinity: 28–30; 25 °C), without metal exposure.

After carefully opening the shells, the mantle was removed and stored at −80 °C for metal assays. Approximately 1.5 mL of hemolymph was withdrawn directly from the pericardial cavity of each oyster with a 1 ml syringe. The pooled hemolymph of five oysters was considered a single biological replicate to reduce technical and biological variation within treatments. Each pooled 7.5 ml hemolymph sample was filtered (80 μm) and divided into seven parts (the samples taken at 0 h) or six parts (the samples taken at 12 h and 36 h): 1 ml was used for the assessment of antimicrobial activity in the hemolymph (for the 0 h sample only); 1 ml was stored at −80 °C for *V. harveyi* quantification; 1 ml was kept on ice for cellular analysis; 1 ml was used for total metal measurement; and 1 ml was used for the quantification of free Zn ions in the hemocytes; 1 ml was centrifuged (800 g, 10 min, 4 °C) and the hemocytes were stored at −80 °C for gene expression analysis; and 1 ml was used for enzymatic assays.

2.2.1. Total hemocyte count using flow cytometry

A CytoFLEX Flow Cytometer (Beckman, USA) was used analyze the hemocytes. We determined the total hemocyte count (THC) as previously described [25] with minor modifications. In brief, 200 μL hemolymph samples were fixed with equal volumes of 3% formalin antiaggregant solution (AASH; 1.5% EDTA, 2.5% NaCl, and 0.1 M PBS; pH 7.4). Samples were then incubated for 120 min in the dark at room temperature with SYBR Green I (Solarbio, China), at a final dilution of 10× (1/1000 of the commercial stock solution). After incubation, samples were analyzed with the flow cytometer.

2.2.2. Antimicrobial activity of the hemolymph in vitro

Each 1 mL hemolymph sample was centrifuged (800 g, 10 min, 4 °C) to separate the hemocytes. The supernatant (HS) was retained. The hemocyte pellet was washed once with Tris buffer (0.1 M Tris-HCl, 0.45 M NaCl, 26 mM $MgCl_2$, and 10 mM $CaCl_2$; pH 7.4) and suspended. The hemocyte suspension was adjusted to a final concentration of 10^6 cells/ml, and lysed using ultrasonication. After centrifugation (10 000 g, 10 min, 4 °C), the hemocyte lysate supernatant (HLS) was collected.

The antimicrobial assay was carried out as described previously [26], with modifications. In brief, 500 μl of *V. harveyi* (at 10^6 CFU/ml) and 1000 μl of marine 2216E medium (0.5% tryptone, 0.1% yeast extract, 3.4% NaCl, and 0.01% FePO4; pH 7.6–7.8) were added to 500 μl of HS or HLS. All of the samples were incubated at 25 °C in a rotor (40 rpm) for the remainder of the experiment. We measured the A_{600} of all samples at 0 h, and then at 1 h intervals for 4–6 h. The *V. harveyi* growth rate was expressed as $\triangle A_{600}$ (i.e., sample A_{600} - sample A_{600} at 0 h).

2.2.3. V. harveyi quantification

We used a 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and TaqMan Universal Master Mix (Life Technologies Corporation, USA) to quantify *V. harveyi,* as previously described [27]. Briefly, 1 ml of hemolymph was centrifuged (7000 g, 10 min, 4 °C), and total DNA was extracted from the precipitate. We diluted *V. harveyi,* using a tenfold gradient, from 10^9 CFU/ml to 10^5 CFU/ml in FSW, then extracted total DNA to generate a standard curve. We used a primer pair specific to the *V. harveyi* tox-R gene (CCA-CTG-CTG-AGA-CAA-AAG-CA; GTGATT-CTG-CAG-GGT-TGG-TT) for RT-qPCR amplification. The TaqMan probe sequence was CAG-CCG-TCG-AAC-AAG-CAC-CG. Reactions were performed in a 20 μL volume, containing 4 μL DNA template, 12.5 μL 1X premix, 300 nM each of specific forward and reverse primers, 200 nM of probe, and supplementary pure water. The PCR program was as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.2.4. Determination of Cu and Zn concentrations

We measured alterations in total Cu concentration, total Zn concentration, and relative free Zn ion concentration in response to bacterial infection. The total concentrations of Cu and Zn in the mantle, HS, and HLS were measured using inductively coupled plasma-mass spectrometry (ICP-MS-7700; Agilent, USA), as previously described [28]. Briefly, mantle samples were freeze-dried, weighed, and digested with 2 ml 70% ultrapure HNO_3 (CNW, Germany). The hemolymph was separated into HS and HLS. Next, 500 μL aliquots of HS or HLS were digested with 3 ml 70% HNO_3. The completely digested samples were diluted with 2% HNO_3 (V/V) before analysis with the ICP-MS. The Cu and Zn concentrations in the HLS were calibrated against the THC (Supplementary Table 1).

To visualize the free Zn ions, we used FluoZin-3 (FZ3; Invitrogen, USA) as a fluorescent indicator. Cell staining and fluorescence analysis were performed following a previous study [14], with some modifications. Briefly, 500 μl aliquots of hemolymph were mixed with equal volumes of 8% paraformaldehyde (in 0.1 M PBS) to fix the hemocytes. After 20 min, each sample was centrifuged (800 g, 10 min, 4 °C) to separate the hemocytes. The separated hemocytes were washed once and then suspended in 200 μl PBS. The hemocyte -PBS suspensions were incubated for 20 min at 25 °C with 10 μM FZ3. As controls, cells were incubated with an ion chelating agent and FZ3, such as 10 μM FZ3 + 40 μM N,N,N′,N′-tetrakis (2 pyridylmethyl) ethylenediamine (TPEN). Hemocytes were then examined under a microscope (Zeiss, Germany). The relative free ion concentration in the hemocytes was expressed as the fluorescence intensity (measured using an ELISA) at an excitation wavelength of 494 nm and an emission wavelength of 516 nm.

2.2.5. Gene expression after bacterial infection

We studied differential expression levels of four genes in response to *V. harveyi* infection: *CTR1*, *ATP7A*, *ZIP1*, and *ZnT2*. These genes encode Cu or Zn transporters [10,29]. Thus, changes in the expression levels of these genes, in conjunction with the concentrations of Cu and Zn, effectively reflect the effects of *V. harveyi* infection on Cu/Zn homeostasis. Hemocyte RNA isolation and cDNA synthesis were performed as described by Shi et al. [30]. Primers were designed using the Primer Premier 5.0, based on the EST sequences obtained from transcriptome sequencing (unpublished data). Ubiquitin (UBQ) and elongation factor 1α gene (EF1α) were used as internal controls [31]. All of the primer pairs (Supplementary Table 2) were tested to ensure that PCR efficiency was 95–100% ($R^2 > 0.99$). Real-time PCR was performed on a 7500 fast qPCR system (Applied Biosystems, USA), using Thermo DyNAmo Flash SYBR Green qPCR Kits (Thermo Scientific, USA). The PCR program was as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s. The fold change in target gene expression relative to the control was calculated using the $2^{-\triangle\triangle Ct}$ method [32].

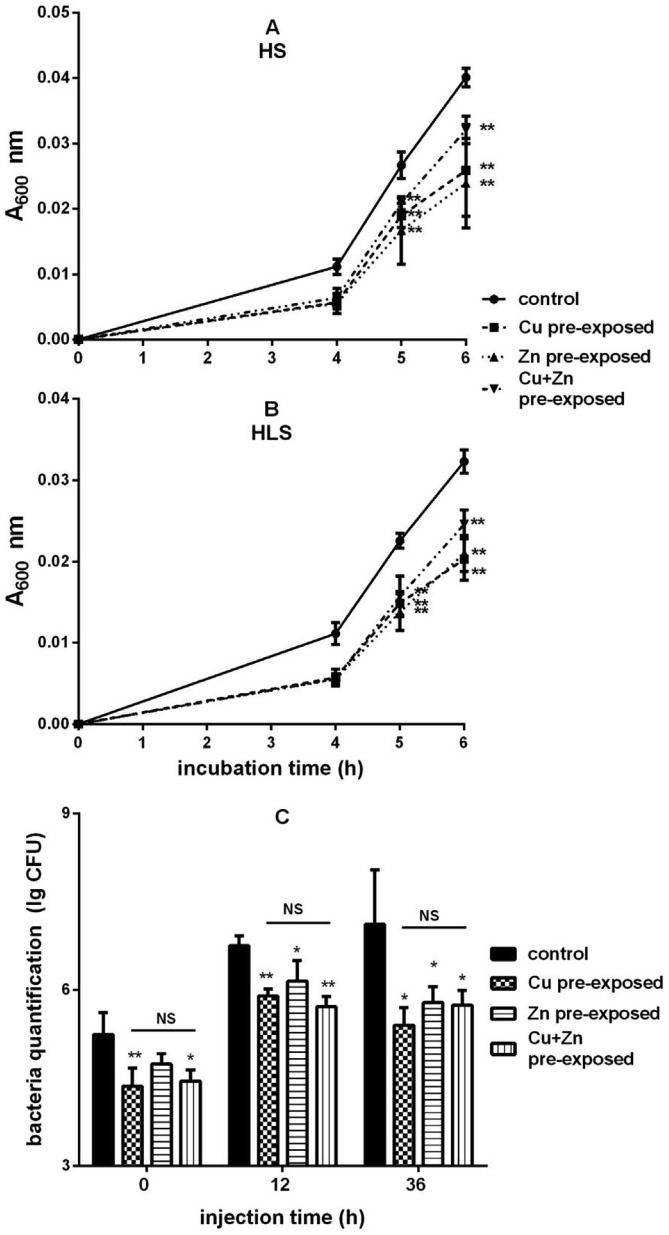

Fig. 1. Effect of metal exposure on the growth of vibrio in oyster hemolymph. HS (A) and HLS (B) were incubated with *V. harveyi in vitro*. Absorbance readings were taken from the cultures at same intervals and plotted against time. (C) *V. harveyi* concentration in oyster hemolymph after 0 h, 12 h, 36 h *in vivo* injection. The measures were made by quantitative PCR. N = 5 replicates, Mean ± SD. Differences between metal exposure and control groups were considered statistically significant (*) at $P \leq 0.05$ and very significant (**) at $P \leq 0.01$.

2.2.6. The toxic effects of Cu and Zn on V. harveyi

Our preliminary experiments indicated that *V. harveyi* infection resulted in greatly increased concentrations of total Cu and Zn in the HS and HLS, as well as increased concentrations of free Zn ions in the hemocytes. To determine the roles of Cu and Zn in innate immunity, we measured how these elements affected *V. harveyi* growth. We cultured 500 μl of *V. harveyi* (10^6 CFU/ml) in 1500 μl marine 2216E medium, supplemented with different concentrations of Zn and Cu: 200, 500, or 1000 ppb Cu^{2+}; 1000, 2000, or 5000 ppb Zn^{2+}; 250 ppb Cu^{2+} + 1000 ppb Zn^{2+}, 500 ppb Cu^{2+} + 2000 ppb Zn^{2+}, or 1000 ppb Cu^{2+} + 5000 ppb Zn^{2+}. *V. harveyi* was cultured in unsupplemented marine 2216E medium as a control. The Cu/Zn concentrations used were selected based on the concentrations measured in the oyster

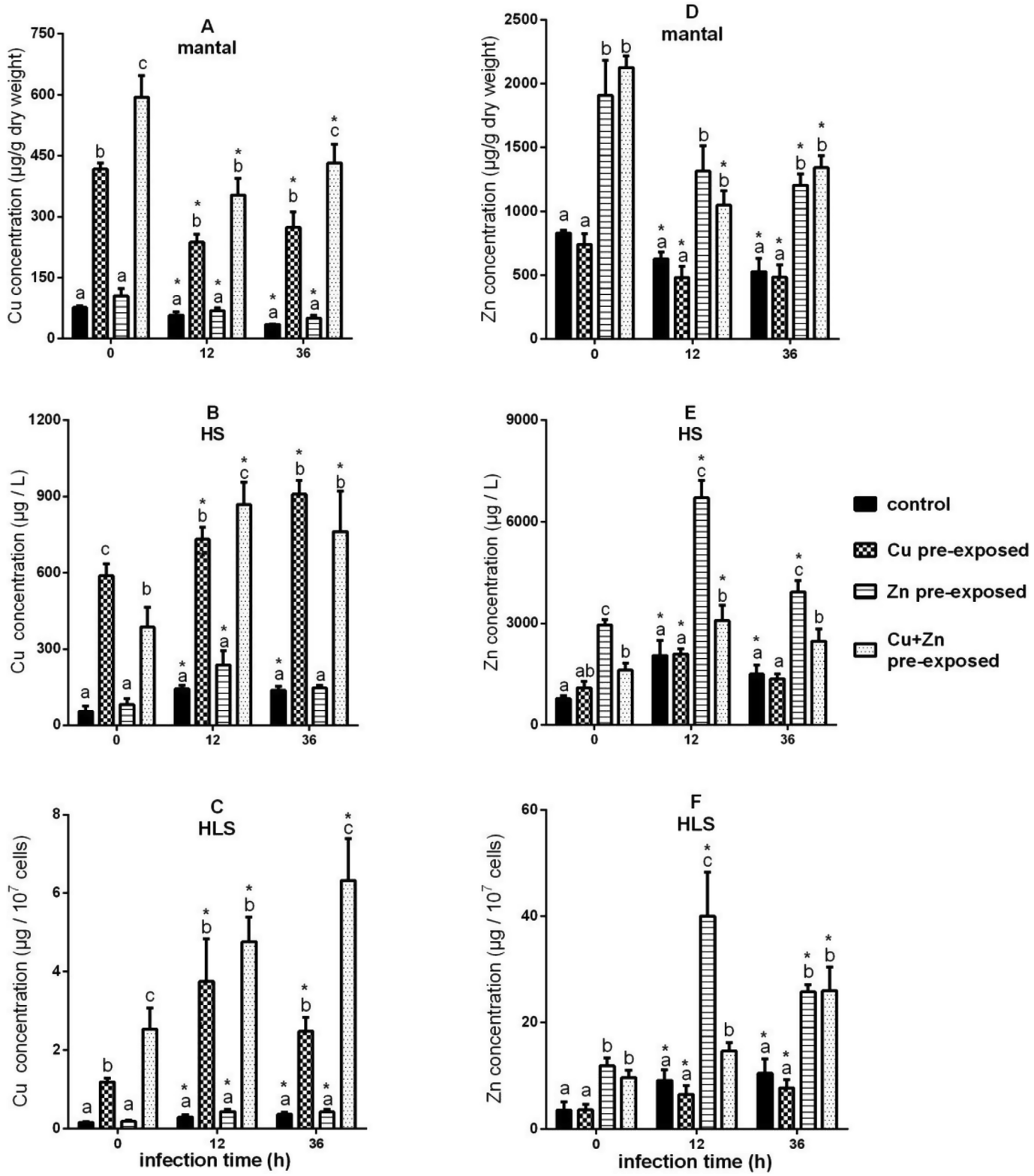

Fig. 2. Changes of Cu and Zn content in oysters mantle, HS and HLS during vibrio infection in four metal pre-exposed groups. Five replicates, Mean ± SD. Different lowercase letters in the same time point represent significant differences among metal pre-exposed groups ($P \leq 0.05$). Significant differences of metal content before (0 h) and after (12 h, 36 h) *V. harveyi* infection were indicated in * at $P \leq 0.05$ (in the same metal exposed group).

hemolymph during the infection experiment. Readings were taken at A_{600} nm after the culturing if 7 h.

Bacteria are highly sensitive to Cu^{2+} in the presence of H_2O_2, which can be formed during the respiratory redox chain [33]. The *in vitro* survival assay was performed as previously described [34]. Briefly, exponential phase cultures of *V. harveyi* were pelleted and resuspended (10^9 CFU/ml) in 2 ml of assay buffer (filtered seawater and 0.5 mM ascorbic acid as a reducing agent), then incubated with 0.5 mM H_2O_2, plus 0, 250, 500, or 1000 ppb Cu^{2+} respectively for 120 min at 25 °C. The control group was *V. harveyi* just incubated with assay buffer. The

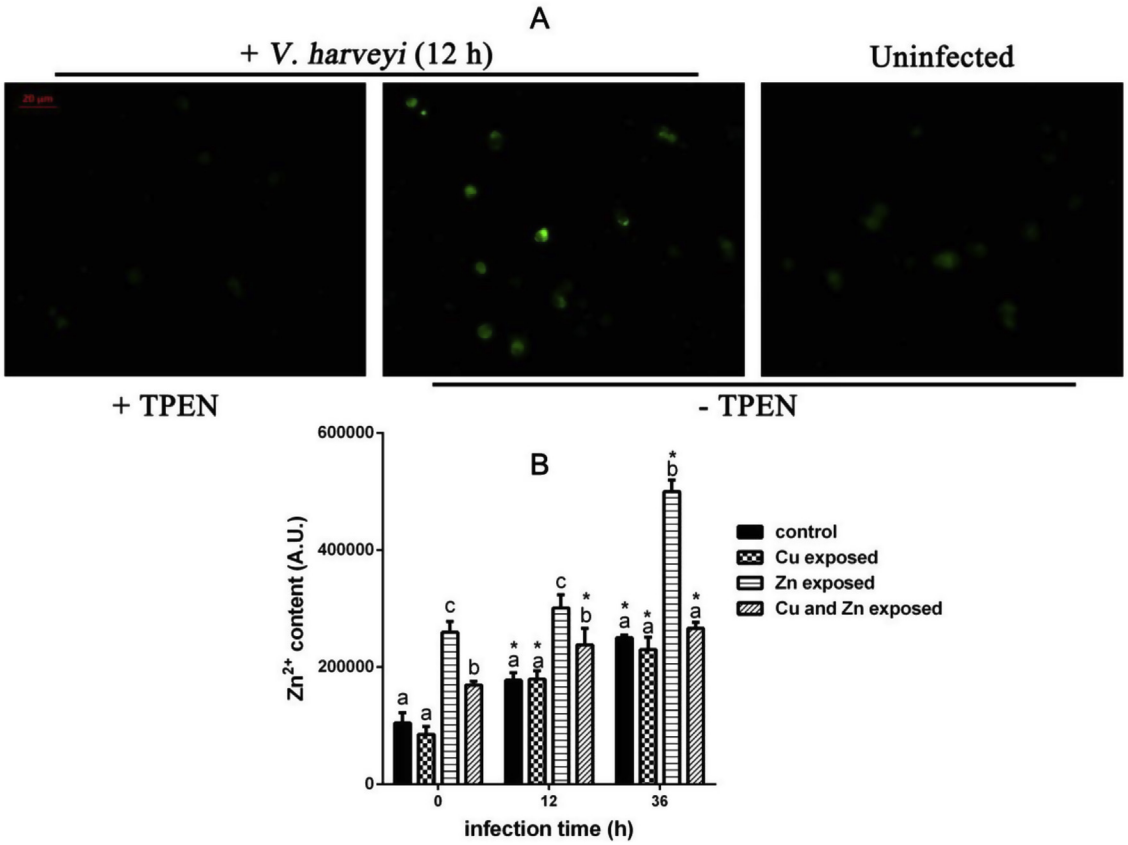

Fig. 3. Free Zn ion increased during *V. harveyi* infection. (A) Free zinc labeling in hemocytes. Oyster hemocytes were uninfected or infected for 12 h, in the absence or presence of the zinc-chelating agent TPEN. Cells were fixed and stained with the free zinc-specific fluorescent probe FluoZin-3 (FZ3, green). (B) Changes of Zn ion content in hemoctyes during vibrio infection in four metal pre-exposed groups. FZ3 signal quantification (in arbitrary units) was expressed as fluorescence level measured by ELIASA. N = 5, Mean ± SD, different lowercase letters in the same time point represent significant differences among metal pre-exposed groups ($P \leq 0.05$). Significant differences of Zn ion before (0 h) and after (12 h, 36 h) *V. harveyi* infection were indicated in * at $P \leq 0.05$ (in the same metal exposed group). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dead bacteria were counted using PI fluorescence staining (20 ppb, 20 min).

2.2.7. Enzyme activity assays

Cu/Zn-SOD and PO are important immune-related enzymes that are associated with the oxidative killing of invasive organisms and the elimination of excess reactive oxygen species [26,35]. Cu/Zn-SOD and PO are also metal proteins, which combine Cu or Zn with their structural and active sites. HS and HLS for each sample were prepared as described above, and stored at −80 °C until the enzyme activity assays. The total protein concentration in each HLS sample was determined using the Bradford method [36].

SOD activity was measured using Cu/Zn-SOD Assay Kid (Beyotime, China). One unit of SOD activity was defined as the amount of enzyme causing a 50% inhibition in a 1 ml reaction system. HS SOD activity was expressed as one SOD unit per ml HS, while HLS SOD activity was calculated as sample activity per mg protein.

PO activity was measured by measuring the amount of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA; Sigma-Aldrich, USA) [37]. The dopachrome concentration was measured at 490 nm every 3 min for 30 min at 25 °C using a spectrophotometer. One unit of PO activity was defined as an increase in absorbance min^{-1}. HS PO activity was expressed as one PO unit per ml HS, while HLS PO activity was calculated as sample activity divided by total protein.

To further analyze the effects of Cu^{2+} and Zn^{2+} on enzyme activity in the HS and HLS, we performed an *in vitro* experiment. We mixed the hemolymph from 30 oysters (collected from Gangkou Village), and then separated the pooled hemolymph into HS and HLS. We added Cu and Zn in the HS/HLS and measured the SOD and PO activities. The final concentrations of metal ions in the nine experimental groups (three repeats per group) were set as follows: 250 ppb Cu^{2+}, 500 ppb Cu^{2+}, 1000 ppb Cu^{2+}, 1000 ppb Zn^{2+}, 2000 ppb Zn^{2+}, 5000 ppb Zn^{2+}, 250 ppb Cu^{2+} + 1000 ppb Zn^{2+}, 500 ppb Cu^{2+} + 2000 ppb Zn^{2+}, 1000 ppb Cu^{2+} + 5000 ppb Zn^{2+}. The control was set as HS/HLS without metal ions.

2.3. Immune system analysis of two oyster lines

To further investigate the role played by Cu and Zn accumulation in oyster innate immunity, we compared the immune performance of two Fujian oyster lines (cultivated by our laboratory). Although the grow rates of the two lines are similar, there are significant differences in Cu and Zn accumulation level between these lines. Both oyster lines are farmed under the same environmental conditions in the same aquaculture area (Qixia Village, Fujian; 23°36′44.6″ N 117°20′31.3″ E). Using the experimental methodologies described above, we compared hemolymph antimicrobial activity, changes in Cu/Zn homeostasis, and the immune response to bacterial infection between the two oyster lines.

2.4. Statistical analysis

Basic statistical analyses of all of the data were performed with SPSS 22.0 (IBM, USA). Data were checked for normality and homogeneity of variance before analysis. One-way ANOVAs were performed to evaluate whether metal content, gene expression level, enzyme activity, and *V.*

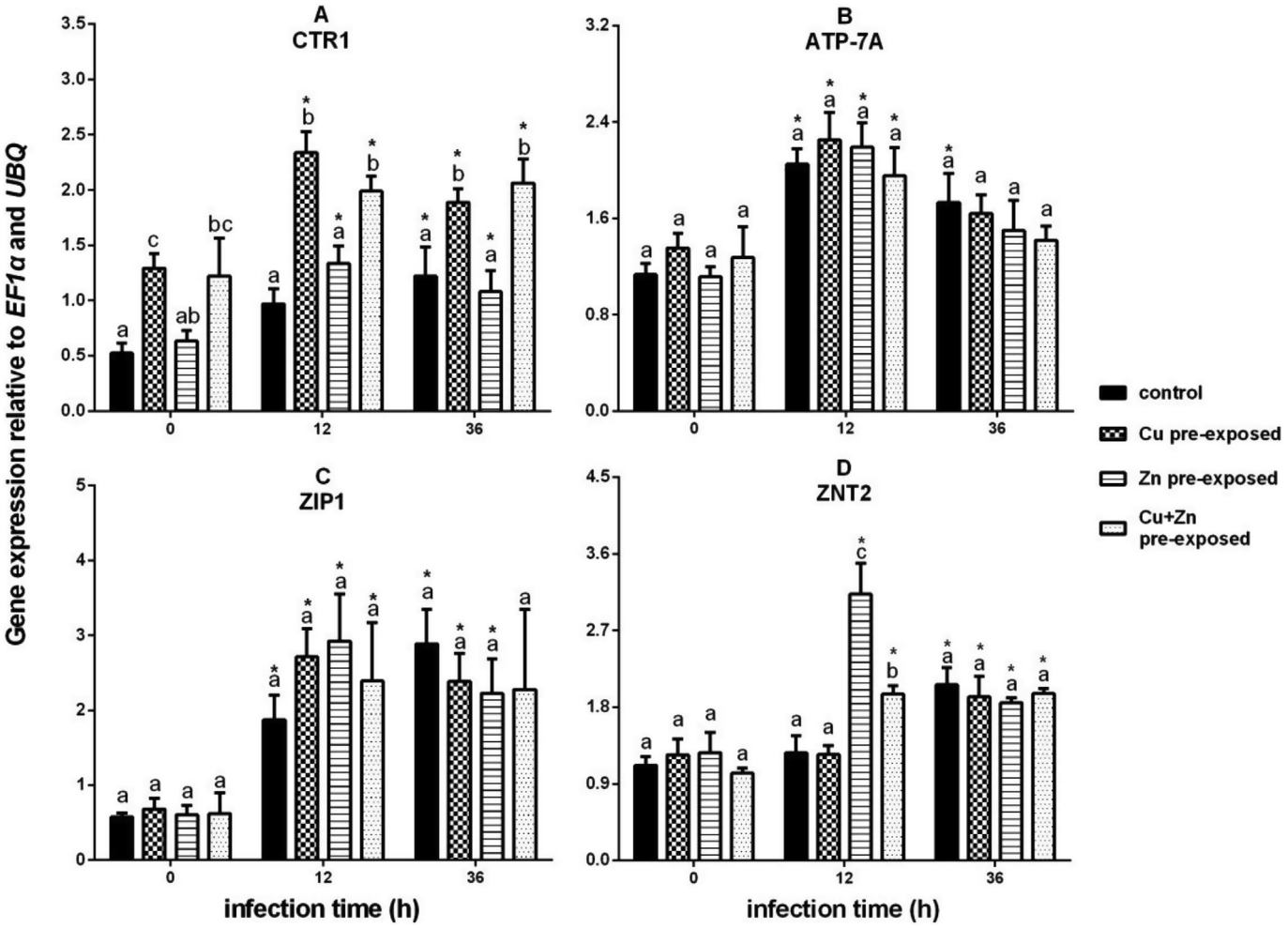

Fig. 4. Gene expression of Cu and Zn transporters after *V. harveyi* infection. N = 5, Mean ± SD, different lowercase letters in the same time point represent significant differences among metal pre-exposed groups ($P \leq 0.05$). Significant differences of gene expression level before (0 h) and after (12 h, 36 h) infection were indicated in * at $P \leq 0.05$ (in the same metal exposed group).

harveyi growth differed among experimental groups. We used Scheffe's test to verify significant differences between groups. We considered $P \leq 0.05$ statistically significant. All of the plots were drawn using GraphPad Prism 6.

3. Results

3.1. Effects of Cu and Zn accumulation on antimicrobial activity in the hemolymph (in vivo and in vitro)

After 28 d of metal exposure, the groups exposed to Cu and Cu + Zn had significantly higher levels of Cu in the mantle, HS, and HLS than the control and Zn-exposed groups ($P \leq 0.05$, Fig. 2). Similarly, the groups exposed to Zn and Cu + Zn had significantly higher levels of Zn in the mantle, HS, and HLS than the control and Cu-exposed groups ($P \leq 0.05$; Fig. 2). Oysters with varying levels of Cu and Zn accumulation were used for subsequent experiments. When *V. harveyi* was incubated with HS and HLS *in vitro*, the measured spectrophotometric values of pre-exposed group were significantly lower than those measured in control ($P \leq 0.05$; Fig. 1A and B), indicating an inhibition of bacterial growth. Bacteria injection *in vivo* significantly increased *Vibrio* concentrations in the hemolymph; *Vibrio* concentrations were significantly lower in the metal pre-exposed groups as compared to the control group (Fig. 1C). However, there were no significant differences in *Vibrio* concentration among the Cu, Zn, and Cu + Zn pre-exposed groups. In *vivo* and *in vitro* experiments showed that oysters with higher levels of Cu or Zn accumulation exhibited stronger antimicrobial activity.

3.2. Changes of in Cu/Zn homeostasis in the mantle and hemolymph after vibrio infection

To better understand the role of Cu and Zn in the resisting of *Vibrio* infection, we measured how Cu and Zn concentration fluctuated in oysters with different levels of Cu and Zn accumulation after *Vibrio* infection. After *V. harveyi* injection, the Cu concentration in the mantle decreased significantly, but increased substantially in the HS and HLS across all of the experimental groups (Fig. 2A, B, C). The Cu concentration changes were even greater in the oysters with high levels of Cu accumulation (i.e., the oysters pre-exposed to Cu or Cu + Zn). The changes of Zn concentration in oysters after *V. harveyi* infection were similar to that of Cu (Fig. 2D, E, F). Staining hemocytes with a fluorescent probe for free Zn showed that *V. harveyi* infection led to a rapid increase in Zn^{2+} concentration. In addition, the hemocyte Zn^{2+} concentration was higher in the oysters with higher levels of Zn accumulation (Fig. 3). Therefore, Cu and Zn were redistributed among tissues in response to *Vibrio* infection, rapidly accumulating in the plasma and hemocytes.

We also compared the expression levels of genes associated with Cu and Zn transport in the hemocytes. CTR1 is a high-affinity Cu importer, which efficiently moves Cu into the interior of the cell [9]. In oysters infected with *V. harveyi, CTR1* gene expression increased as compared

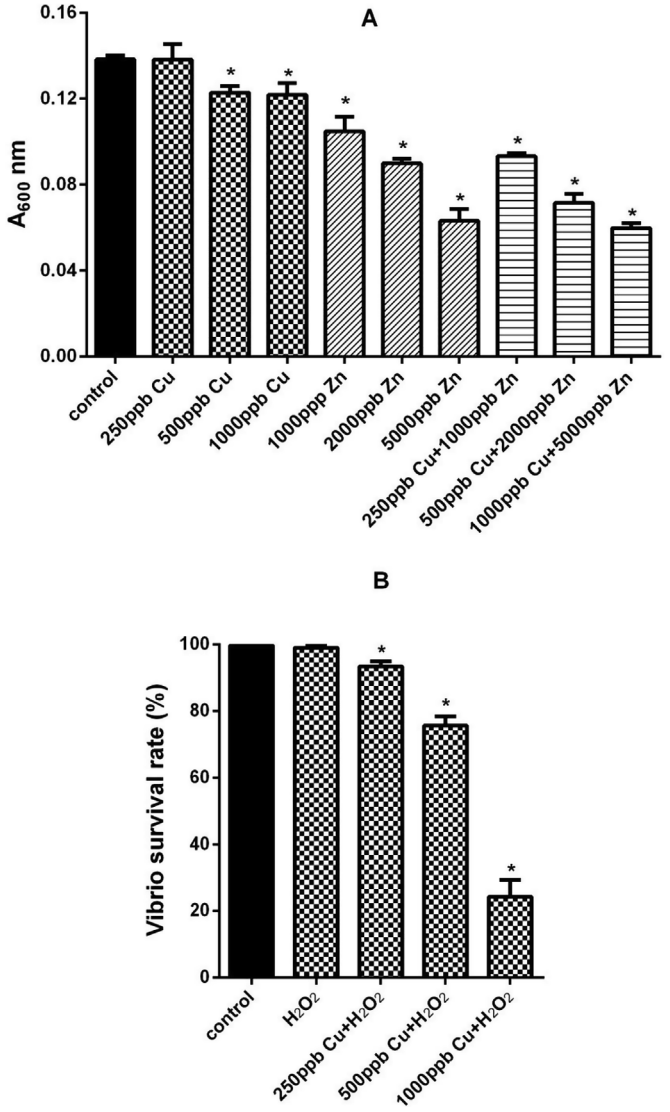

Fig. 5. The toxic action of Cu and Zn on *V. harveyi*. (A) Vibrio was incubated with Cu/Zn at different concentrations *in vitro*. Absorbance readings were taken from the cultures at 7 h (N = 3, Mean ± SD). (B) *in vitro* bacterial survival was assessed following exposure of *V. harveyi* to 0.5 mM H_2O_2, or H_2O_2 plus $CuCl_2$ (N = 3, Mean ± SD). Significant differences between treatment group and the control were represented by * at $P \leq 0.05$.

to uninfected oysters. In addition, *CTR1* gene expression was significantly greater in infected oysters pre-exposed to Cu or Cu + Zn, as compared to those unexposed (Fig. 4A). We then quantified changes in the gene expression of *ATP7A*, a copper-transporting P-type ATPase that is responsible for delivering Cu from the cytoplasm into secretory compartments or excretory vesicles [9]. Our results indicated that *Vibrio* infection increased the gene expression of *ATP7A* in the hemoctytes. However, there were no differences in relative *ATP7A* gene expression among oysters with different levels of Cu accumulation (Fig. 4B). In conjunction with the observed alterations in Cu content (Fig. 2C), our results suggested that *Vibrio* infection stimulates CTR1-mediated Cu uptake and ATP7A-mediated Cu trafficking in hemocytes.

We also determined the relative expression levels of the Zn importer and exporter. ZIP1 transport Zn from the extracellular space into the cytoplasm, while ZNT2 transports Zn from the cytoplasm to the vesicles and secretory granules [10]. After *Vibrio* injection, the gene expression levels of *ZIP1* and *ZNT2* increased significantly (Fig. 4C and D). Therefore, *Vibrio* infection promoted Zn uptake and trafficking in the hemocytes. There were no differences in *ZIP1* and *ZNT2* gene expression levels among the groups pre-exposed to the heavy metals, despite differences in the levels of Zn accumulation among groups (Figs. 2F and 3). Thus, our results indicated that other transporters might be involved in Zn absorption, or that additional Zn/Zn^{2+} was released from intracellular stores.

3.3. The toxic effects of Cu and Zn on V. harveyi

As Cu and Zn concentrations increased greatly in the plasma and cytoplasm in response to *V. harveyi* infection, and these increases were more dramatic in the oysters with higher levels of metal accumulation, we hypothesized that the excess Cu and Zn would enhance bacteriostatic activity in the hemolymph. Indeed, it might be possible to use excess Cu and Zn, especially in free ion form, directly as antibacterial agents due to their bacteriotoxicity. *V. harveyi* was cultured in media with different concentrations of Cu and Zn, which were designed based on the levels of Cu/Zn accumulation in oysters. It showed that Cu^{2+} somewhat inhibited the growth of *Vibrio*, while Zn exhibited significant, dose-dependent bacteriostatic effects (Fig. 5A). Interestingly, in the presence of H_2O_2, Cu^{2+} was highly toxic to *V. harveyi* (Fig. 5B). Thus, our results indicated that rapid increases in Cu and Zn accumulation in the hemolymph had a significant toxic effect on oyster-invading *Vibrio*.

3.4. Effects of Cu and Zn on immune-related enzymatic activity

In addition to the direct antibacterial effects of Cu and Zn, these metals might also participate in the resistance to pathogenic invasion by altering the activity levels of some metalloenzymes. After *Vibrio* infection, SOD levels increased significantly in the HS and HLS (Fig. 6), but no differences in SOD activity levels were observed among the groups pre-exposed to Cu and Zn (Fig. 6A and B). This suggested that SOD activity was induced by the *Vibrio* infection itself, and was not affected by the level of Cu or Zn accumulation. We tested this result *in vitro*, and found that the SOD activity levels in HS and HLS were unaffected by different levels of Cu and Zn (Fig. 7A and B).

PO activity levels increased after *Vibrio* infection, and were significantly higher in the groups pre-exposed to Cu and Cu + Zn, as compared to the unexposed groups (Fig. 6B and C). In our *in vitro* experiments, PO activity levels in the HS and HLS increased in a dose-dependent manner after the addition of Cu (Fig. 7B and C). These results indicated that an increase in Cu concentration in the hemolymph increased PO activity even up to 40-fold.

3.5. Verification of results in different oyster lines

Through selective breeding, we obtained two oyster lines, one with high levels of Cu/Zn accumulation, and one with low levels of Cu/Zn. To further verify our hypothesis, we compared antimicrobial activity in the hemolymph, the levels of Cu and Zn, immune-related gene expression, and enzyme activity levels between the two lines in response to *Vibrio* infection. The immune responses were similar to the oysters with different Cu/Zn enrichment level obtained by metal exposure (Supplementary Figs. 1, 2, 3, 4), with the exception of SOD activity, which was significantly increased in the line with high levels of heavy metal accumulation (Supplementary Fig. 4).

4. Discussion

In this study, Cu and Zn levels in *C. angulata* were increased through heavy metal exposure or selective breeding. Increased concentrations of Cu, Zn, or both significantly increased antibacterial activity in the hemolymph *in vitro*, consistent with previous studies [20,21]. In addition, higher levels of Cu or Zn accumulation reduced the severity of *V. harveyi* infection *in vivo*. These results indicated that Cu and Zn accumulation was important for oyster innate immune function.

Notably, changes in the bodily biodistribution of trace metals

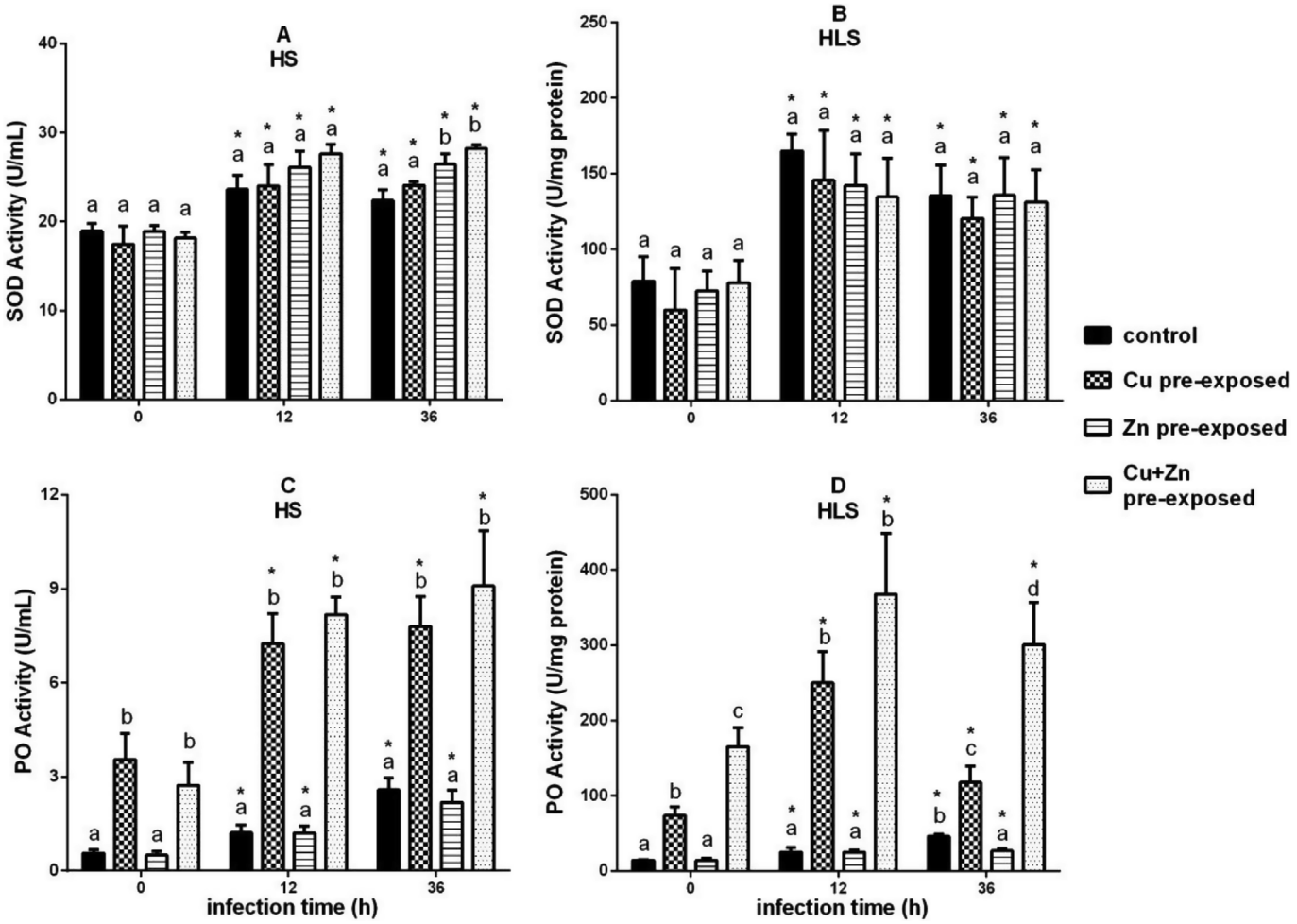

Fig. 6. Variations of SOD and PO activities in HS and HLS of *C. angulata* after *V. harveyi* injection, N = 5, Mean ± SD. Different lowercase letters in the same time point represent significant differences among metal pre-exposed groups ($P \leq 0.05$). Significant differences before (0 h) and after (12 h, 36 h) infection were indicated in * at $P \leq 0.05$ (in the same metal exposed group).

following infection are characteristic of the acute phase of the mammal immune response. In response to infection, Cu is mobilized to infected sites, markedly increasing Cu concentration in the serum and macrophages [34,38]. This trend was also observed in *C. angulata*: when infected with *V. harveyi*, Cu was transferred from tissues (e.g., the mantle) to the hemolymph. Indeed, the amount of Cu mobilized depended on the level of Cu accumulation (Fig. 2, Supplementary Fig. 2). CTR1 can efficiently and specifically transports Cu into cells [9]. *CTR1* expression combined with Cu changes of different groups after infection illustrated that the large increase in Cu concentration observed in the hemocytes might have been primarily due to the transfer of Cu from the plasma by CTR1. ATP7A is required for the cytoplasm trafficking of Cu and the biosynthesis of cuproenzymes [10]. *ATP7A* was also upregulated by *Vibrio* infection, suggesting an increase in intracellular Cu transport. However, cytoplasm Cu level did not affect *ATP7A* gene expression, this might because the increases of Cu-proenzymes biosynthesis or Cu efflux had certain limitations.

In mammalian inflammatory immune response, the host redistributes Zn as part of a strategy of nutrient immunity. That is, to prevent Zn acquisition by the pathogen, Zn is redistributed to the tissues, resulting in decreased serum Zn levels [12]. In contrast, mantle Zn levels in *C. angulata* decreased in response to *Vibrio* infection, while plasma Zn concentrations increased significantly and reached an extremely high level (3000–6000 ppb; Fig. 2). These results indicated that *C. angulata* did not adopt a nutritional-restricted immunity strategy with respect to Zn in response to *Vibrio* infection. In hemocytes, Zn accumulation increased markedly after *Vibrio* infection. We also identified a rapid increase in Zn^{2+} concentration using fluorescent staining. This was consistent with a study of human macrophages within a few hours of infection [14]. The expression pattern of the Zn importer gene (*ZIP1*), combined with observed alterations in Zn level, again suggested that immune response triggered an influx of Zn from the plasma to the hemocytes (Fig. 4, Supplementary Fig. 3). Although there were no significant differences in *ZIP1* expression levels among infected oysters with different initial Zn levels, the increase in Zn levels in the hemocytes differed significantly. This suggested, first, that other ZIP family members, besides ZIP1, were involved in transporting Zn into the cell. Indeed, a previous study showed an increase in the intracellular transport of Zn by ZIP2 within activated macrophages infected with a fungal pathogen [15]. Second, previous studies showed that Zn was primarily sequestered and stored in oyster hemocytes [39]. The Zn burst observed in the hemocytes after infection was likely due to the release of Zn from intracellular stores (e.g., metallothionein or vesicles). Hélène Botella et al. [14] showed that Zn^{2+} was released from metallothionein rather than imported from the extracellular space in macrophages infected with *M. tuberculosis*.

Thus, our results indicated that *C. angulata* redistributed Cu and Zn in response to *Vibrio* infection. Cu and Zn bursts were observed in both the plasma and the hemocytes in response to infection, and the level of Cu and Zn accumulation prior to infection affected the size of the burst. Considering the differences in antimicrobial activity among oysters at different levels of Cu/Zn enrichment, we hypothesize that the Cu/Zn

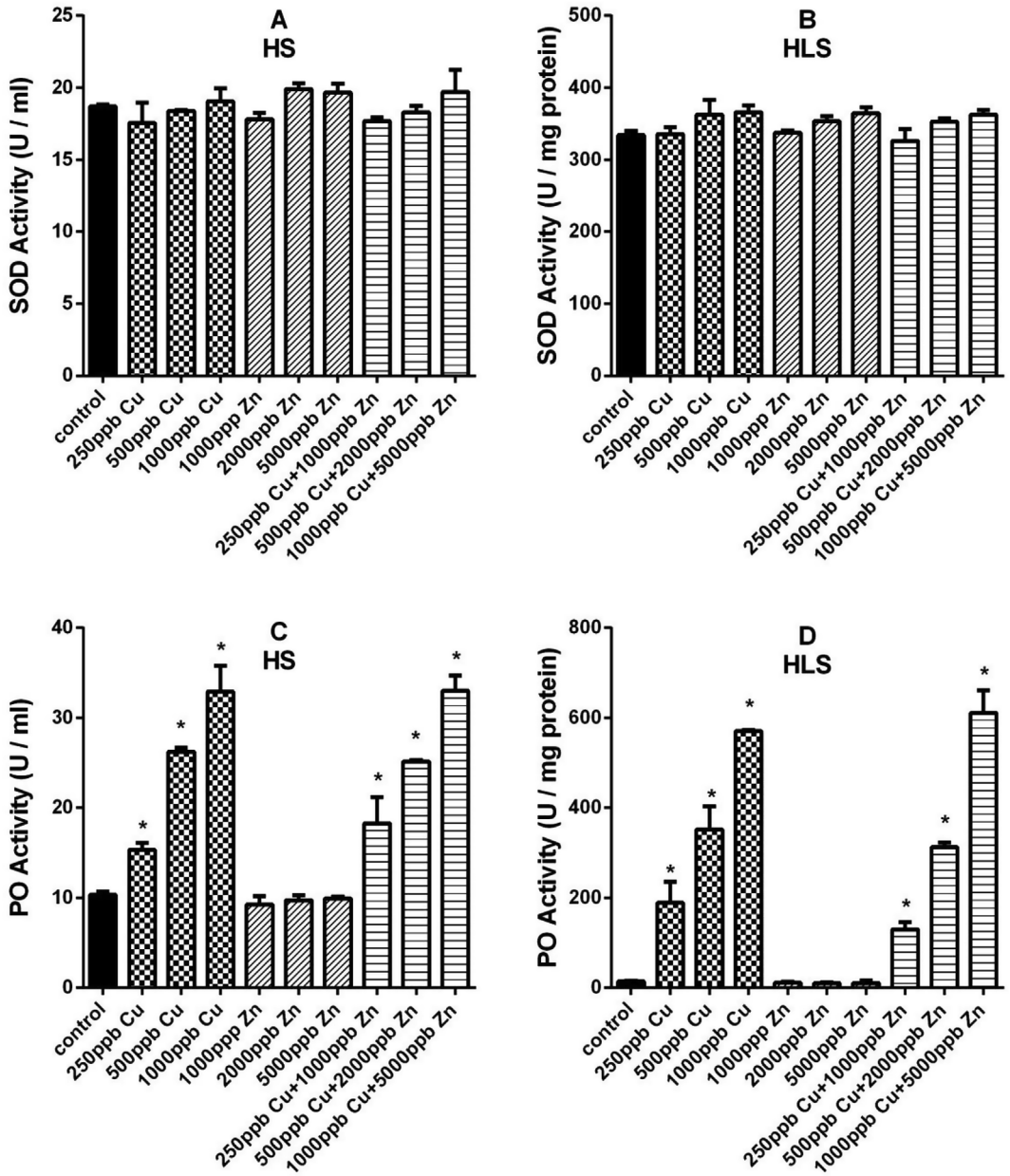

Fig. 7. *In vitro* analysis of SOD and PO activities in HS and HLS with different metal concentration, N = 3, Mean ± SD. Significant differences between control and metal addition groups were indicated in * at $P \leq 0.05$.

bursts may play an important role in pathogen resistance.

For Cu, how might its burst promote resistance to bacterial? Cu acts as a biocidal agent because high Cu concentrations are associated with the formation of reactive oxygen species (ROS), which lead to oxidative stress and cell damage [40]. *Vibrio* culture experiments *in vitro,* simulating the concentration of Cu in the hemolymph, showed that high concentrations of Cu inhibited the growth of *V. harveyi*. However, this inhibitory effect was not as dramatic as expected (Fig. 5A). In addition to direct toxicity, it was also possible that Cu catalyzed the production of the hydroxyl radical from H_2O_2, via Fenton-like processes [33]. Respiratory bursts, which increase H_2O_2 concentration in hemocytes, are an important immune response in oysters [41,42]. Alone, only supraphysiological millimolar concentrations of H_2O_2 are deadly to bacteria [43]. However, in the presence of Cu, small amounts of H_2O_2 are lethal to various microorganisms [33,34]. Here, Cu plus H_2O_2 was lethal to *V. harveyi*, and H_2O_2 toxicity increased proportionally to Cu dose (Fig. 5B). Thus, the combination of Cu and H_2O_2 might be toxic to *Vibrio*, especially in hemocytes. It was also possible that the Cu burst increased the activity levels of Cu enzymes, such as PO. The POs are a group of Cu proteins, including tyrosinase, catecholase, and laccase, that are directly involved in non-self-recognition and defense [44]. A positive correlation between PO activity level and disease resistance has been shown in oysters [45]. Luna-Acosta et al. [26] found that POs, especially laccase, acted as antibacterials in *C. gigas* hemocytes. Our *in vivo* and *in vitro* experiments showed that an increase in Cu concentration tremendously increased the activity level of PO in oyster plasma and hemocytes (up to 40 times). Such increases might improve the bacteriostatic functions of the hemolymph. Therefore, the Cu burst in the hemolymph acted as an antibacterial via various mechanisms.

Mammals limit the availability of Zn to pathogens by transporting Zn between tissues, or by producing large numbers of competitive binding proteins (e.g., calprotectin), which decrease the amount of available Zn [12]. However, our results suggested that oysters use a different Zn distribution strategy than mammals during the immune

response. Plasma Zn concentrations were increased three-fold (to 3000–6000 ppb), which was much higher than the plasma Zn concentrations reported in other organisms [46,47]. We thus hypothesized that, instead of limiting the intake of Zn by pathogens, oysters create a plasma environment unusually high in Zn, which then has a toxic effect on pathogens. This hypothesis was supported by our in vitro experiments: when Zn concentration exceeded 1000 ppb, the growth of *V. harveyi* was significantly inhibited. At a Zn concentration of 5000 ppb, the inhibition rate was up to 54.4% (Fig. 5A). Due to its obvious toxic effects, significant increases in Zn^{2+} concentration in hemocytes might increase the resistance of the oyster to invasive intracellular bacteria. A previous study showed that Zn released from intracellular stores was trafficked into mycobacterial-containing phagosomes after macrophages were infected with *M. tuberculosis* [14].

It has also been shown that dietary supplementation with Zn increased the activity levels of AKP, SOD, PA, and PO in aquatic organisms [18,48,49]. However, in *C. angulata*, the extent of the Zn burst did not affect SOD and PO activity levels in the infected oysters pre-exposed to heavy metals. The addition of Zn^{2+} *in vitro* also did not affect SOD and PO activity levels in the hemolymph. However, the oyster line with high levels of Zn accumulation had higher levels of enzymatic activity than did the line with low levels of Zn accumulation. This suggested that the effects of Zn on enzymatic activity might be a complex process, affected by Zn source, form, and concentration.

In conclusion, Cu and Zn were redistributed in *C. angulata* in response to *V. harveyi* infection, with a resultant rapid increase in concentration in the hemolymph. This increase in Cu and Zn concentration not only directly exposed the invading pathogen to a toxic environment, but also increased host resistance by catalyzing the production of the hydroxyl radical and increasing the activity of metalloenzymes. The level of Cu/Zn accumulation in the oyster affected the size of the Cu/Zn burst during the immune response. That is, oysters with higher accumulations of Cu/Zn had stronger Cu/Zn bursts, and, consequently, were better able to resist bacterial infection.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.049.

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