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Effects of ocean acidification on toxicity of two trace metals in two marine molluscs in their early life stages

Xiaoyu Guo[1,2,4], Miaoqin Huang[3,4], Bo Shi[3,4], Weiwei You[3,4,5], Caihuan Ke[3,4,5,*]

[1]College of Oceanology and Food Science, Quanzhou Normal University, Quanzhou 362000, PR China
[2]Fujian Province Key Laboratory for the Development of Bioactive Material from Marine Algae, Quanzhou Normal University, Quanzhou 362000, PR China
[3]State Key Laboratory of Marine Environmental Sciences, Xiamen University, Xiamen 361102, PR China
[4]College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, PR China
[5]State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen University, Xiamen 361102, PR China

ABSTRACT: Ocean acidification (OA) is usually thought to change the speciation of trace metals and increase the concentration of free metal ions, hence elevating metal bioavailability. In this study, embryos of the oyster *Crassostrea angulata* and abalone *Haliotis discus hannai* were cultured under 4 pCO_2 conditions (400, 800, 1500 and 2000 µatm) with Cu and Zn added. Fertilization rate was measured 2 h post-fertilization (hpf), while larval deformation and larval shell length were measured 24 hpf. Our results show that OA can alleviate Cu and Zn inhibition of *C. angulata* fertilization by 86.1 and 26.4% respectively, and Zn inhibition of *H. discus hannai* fertilization by 43.7%. However, OA enhanced the inhibitory effect of Cu on fertilization of *H. discus hannai* by 34.7%. OA enhanced the toxic effect of Cu on larval normality of *C. angulata* by 22.0% and the effect of Cu and Zn on larval normality of *H. discus hannai* by 71.4 and 37.2%, respectively. OA also enhanced the inhibitory effects of Cu and Zn on larval calcification in *H. discus hannai* by 8.8 and 8.6%, respectively. However, OA did not change the effect of Cu on the calcification of *C. angulata* larvae. OA decreased Zn inhibition of oyster larval calcification from 3.1 to 1.5%. Based on our results, the toxic effects of metal on early development of molluscs are not always increased by rising pCO_2 and differ across developmental stages, egg structure and species. This complexity suggests that caution should be taken when carrying out multiple environmental stressor tests on molluscan embryos.

KEY WORDS: Oyster · Abalone · Ocean acidification · Trace metal · Fertilization · Larvae

1. INTRODUCTION

Carbon dioxide (CO_2) discharged by anthropogenic activities has caused a sharp increase in atmospheric CO_2. In 2004, atmospheric pCO_2 had risen from the preindustrial concentration of 280 µatm to 380 µatm (Sabine et al. 2004); by 2017 it had risen to 405.0 µatm (Lindsey 2020). Approximately one third of anthropogenic CO_2 is absorbed by the ocean, thereby causing reduced pH and calcium carbonate saturation, and it is predicted that 69% of the surface ocean will acidify by more than 0.2 pH units relative to preindustrial levels by the end of the 21st century (Gattuso et al. 2015). In addition, in coastal seawater, eutrophication due to anthropogenic activities could promote the production of algae, whose decomposition and remineralization would release large amounts of CO_2, leading to the acidification of subsurface sea-

*Corresponding author: chke@xmu.edu.cn

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water. Therefore, coastal water which receives massive terrestrial input may be affected by both ocean acidification (OA) and local acidification, and is predicted to suffer further pH reduction compared with offshore seawater in the future (Cai et al. 2011). In a marginal sea of China (Yellow Sea), seasonal acidification has been observed in bottom seawater with a pH value of 7.90 (pH is expressed in total scale) and is predicted to further reduce to a pH of 7.80–7.85 by 2050 (Zhai 2018).

Increased pCO_2 can directly affect physiological functions controlling gas exchange in marine organisms. Elevated ambient CO_2 could reduce the pCO_2 gradient from aquatic animals to their environment, hence inhibiting CO_2 excretion and causing hypercapnia which leads to acidosis (Heuer & Grosell 2014). In addition, decreased calcium carbonate saturation may hinder calcification of marine organisms, resulting in thinner and smaller shells, making them more vulnerable to predators (Gazeau et al. 2013). The effects of OA on embryos and larvae of calcareous marine organisms have been extensively reviewed (Byrne 2010, 2011, 2012, Dupont et al. 2010, Kroeker et al. 2013). There is compelling evidence that OA can adversely affect early life stages of these organisms, with biological effects including reduced fertilization, hatching, growth and calcification, and increased larval deformation.

In addition to OA, trace metals are another environmental threat, and toxicity of trace metals to marine molluscan embryos and larvae has been studied in oysters *Crassostrea virginica* (Calabrese et al. 1977), mussels *Mytilus edulis* (Johnson 1988) and abalone *Haliotis rubra* (Gorski & Nugegoda 2006). Toxicity of trace metals occurs through non-specific binding with enzymes and transcription factors or through metal-induced oxidative damage to proteins and critical cellular components (Stohs & Bagchi 1995, Ringwood et al. 1998, Geret et al. 2002, Dailianis et al. 2005, Valko et al. 2005, Martelli et al. 2006). Coastal regions may be affected by both OA and trace metal pollution from various sources, including wastewater discharge, antifouling paints and coastal engineering. After decades of rapid industrial expansion and ineffective pollution management, trace metal contamination has frequently been observed in coastal environments in China (Pan & Wang 2012).

OA likely changes the speciation of metals by increasing concentrations of free ions such as Cu and Zn (Millero et al. 2009), thereby increasing bioavailability of trace metals and aggravating their toxicity in estuaries. The combined effects of OA and trace metals have been studied in polychaetes (Lewis et al. 2013), molluscs (Lacoue-Labarthe et al. 2009, Ivanina et al. 2013, Götze et al. 2014, Belivermiş et al. 2016, Shi et al. 2016) and copepods (Pascal et al. 2010, Fitzer et al. 2013, Li et al. 2017). In contrast to predictions that OA would increase the toxicity of some trace metals (Millero et al. 2009), the results of these studies showed complex responses of marine organisms to the combined influence of OA and trace metals. For example, fertilization of the polychaete *Pomatoceros lamarckii* was unaffected by adding Cu to ambient or acidified seawater, while larval survival significantly decreased when Cu and OA co-occurred relative to OA only (Lewis et al. 2013). OA enhanced Cu and Cd accumulation in mantle tissue of oysters *C. virginica* and hard clams *Mercenaria mercenaria*, and proteasome responses to metals in those species were also modulated by OA (Götze et al. 2014). Cd accumulation in 3 bivalve species (*Mytilus edulis*, *Tegillarca granosa* and *Meretrix meretrix*) was significantly higher in CO_2-acidified seawater (Shi et al. 2016). Greater decline in copepod naupliar production was observed in seawater with decreasing pH when Cu was added than in seawater with decreasing pH alone; however, Cu addition enhanced copepod growth regardless of pH level (Fitzer et al. 2013). OA could even alleviate the toxic effects of Hg on copepod reproduction by reducing its accumulation (Li et al. 2017). To date, however, research concerning the combined effects of OA and trace metals on molluscan embryos and larvae remains scarce.

Oysters are an important ecosystem component in controlling primary production, maintaining water clarity, providing habitats for benthic organisms and transferring particles from water to sediment (Dame et al. 1989, Ulanowicz & Tuttle 1992, Dame & Libes 1993, Dame 1996). In addition, oysters are an economically important aquaculture species and as such are extensively cultured in China. In 2017, 4 879 422 t, with a value of 5.26 billion USD were harvested in China (www.fao.org). Abalone is also an important aquaculture species, and China accounted for 88.2% of global output in 2017 (www.fao.org).

In the present study, the combined effects of OA and trace metals (Cu and Zn) on embryos and larvae of oysters *C. angulata* and abalone *H. discus hannai* were studied to test whether OA could aggravate trace metal toxicity in molluscs. Oysters and the abalone were selected as study organisms because they are the most widely cultured species along the southern coast of Fujian Province, China. Cu and Zn were used as test trace elements because the 2 metals are the main pollutants in the Jiulong River estu-

ary on the southern coast of Fujian Province (Wang et al. 2011).

2. MATERIALS AND METHODS

2.1. Construction of high-pCO_2 seawater system

The design of the experimental system followed that of a previous study (Guo et al. 2015), with several improvements. The principle of this system is mixing CO_2-enriched air with seawater to produce acidified seawater. Air was supplied from an air compressor (AT3000-120L FINE™) and passed through a freeze dryer (YD-3 YIYANG™), a pressure swing adsorption dryer (YS-3 YIYANG™) and an allochroic silica gel column. To avoid the disturbance of CO_2 produced by human activities in the laboratory air, a pipe was connected with the gas inlet of the air compressor on one end, and the other end was placed in the open air outside the lab. The 2 dryers were used to remove excess water vapor so that valves in the system could not be affected by moisture. The silica gel served as an indicator to ensure that all water vapor was removed. The dried air was conducted through a gas pressure regulator (GR300-08 AIRTAC™) and a filter (GF300-08 AIRTAC™) to stabilize its pressure and remove particles. After that, the air passed through another gas pressure regulator (GPR300-08 AIRTAC™) and a needle valve (LZB-6WB(F) SHUANGHUAN™) to obtain a fixed pressure and flow rate. CO_2 was supplied from a cylinder with a purity of 99.99% and manipulated similarly to air, so CO_2 with a fixed pressure and flow rate was obtained.

Both the air and the CO_2 were conducted into buffering bottles with a Venturi pipe and were well mixed. The mixture of air and CO_2 was divided into 2 parts, one for the experiment and the rest conducted through a bypass where a CO_2 sensor (K30FR SENSEAIR™) and a paperless recorder (NHR7100 HR™) were installed to detect the CO_2 concentration in the system. The paperless recorder received the analog signal from the CO_2 sensor, and the CO_2 concentration was displayed on its panel. When setting the pCO_2 level, a needle valve (TZF-1, Trieder) in the CO_2 line was adjusted to change the CO_2 flow rate, while that in the air line remained unchanged, until the CO_2 concentration in the recorder reached the target value. Finally, CO_2-enriched air was bubbled into 0.22 µm filtered seawater (FSW) to achieve the required pH level when the gas-liquid equilibration was obtained (Fig. 1).

Four pCO_2 conditions were established: 400 µatm, representing the current ambient atmospheric CO_2 concentration; 800 µatm, representing an intermediate IPCC case scenario of 747 µatm at the end of the 21st century; 1500 µatm, corresponding to the upper limit of the pCO_2 range observed in coastal regions affected by upwelling, including the Peruvian upwelling system (Friederich et al. 2008) and the California Current Ecosystem (Reum et al. 2016); and 2000 µatm, corresponding to the future pCO_2 level of coastal seawater in which acidification is amplified by anthropogenic effects (Melzner et al. 2013) and which has already been used in several studies (e.g. Lopes et al. 2016, Wang et al. 2016). Temperature and pH were measured with a pH meter (Thermo Scientific Orion Star Series™ Benchtop) with a probe (8157BNUMD) calibrated with NBS buffer (4.00, 7.00 and 10.00). A seawater sample (500 ml) was taken from the tank and immediately fixed using 200 µl of saturated mercuric chloride solution. Total alkalinity was determined by Gran acidimetric titration with an Apollo TA Analyzer (Cai & Wang 1998). Salinity was measured with an optical

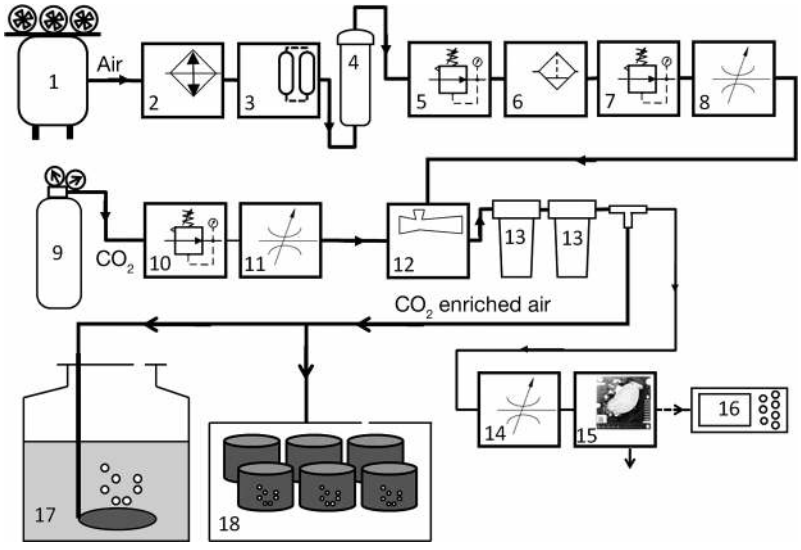

Fig. 1. Schematic of the instrument simulating ocean acidification (OA) for the molluscan larvae experiment. 1: air compressor, 2: freeze dryer, 3: pressure swing adsorption dryer, 4: allochroic silica gel column, 5: gas pressure regulator, 6: air filter, 7: gas pressure regulator, 8: needle valve, 9: CO_2 cylinder, 10: gas pressure regulator, 11: needle valve, 12: venturi pipe, 13: buffering bottles, 14: needle valve, 15: CO_2 sensor, 16: paperless recorder, 17: seawater tank, 18: chamber for larvae culture

salinity meter gauge. Carbonate system parameters were calculated using CO2SYS software (version 2.1). The pH, total alkalinity, temperature, salinity and other calculated data are listed in Table 1.

2.2. Test solutions of metals under different pCO_2 levels

Metals used in tests were from inorganic salts ($CuSO_4 \cdot 5H_2O$ and $ZnSO_4 \cdot 7H_2O$) and were all analytical grade. Stock solutions were made using deionized water and then diluted with filtered seawater of different pCO_2 levels to obtain test solutions with different metal concentrations. Background metal concentrations of Cu and Zn in seawater were measured by the co-precipitation method of Sawatari et al. (1995) and were 0.91 µg l^{-1} and 6.76 µg l^{-1}, respectively. Nominally, metal concentrations (above the seawater background levels) for the oyster fertilization tests were 0, 10, 20, 50, 80 and 100 µg l^{-1} for Cu and 0, 1, 2, 3 and 4 µg ml^{-1} for Zn. Metal concentrations for the oyster larvae tests were 0, 5 and 10 µg l^{-1} for Cu and 0, 25 and 50 µg l^{-1} for Zn. Metal concentrations for the abalone fertilization tests were 0, 30, 60, 150, 240 and 300 µg l^{-1} for Cu and 0, 0.2, 0.5, 1, 2 and 4 µg ml^{-1} for Zn. Metal concentrations for the abalone larvae tests were 0, 2.5 and 5 µg l^{-1} for Cu and 0, 20, 40 and 60 µg l^{-1} for Zn. Metal concentrations used in larvae tests were approximately the same magnitude as those in the polluted sites in the Jiulong River estuary (Cu: 2.84–12.54 µg l^{-1} and Zn: 4.81–19.92µg l^{-1}; Weng & Wang 2014). The short duration (~2 h) of fertilization tests allowed only transitory contact between gametes and metals, necessitating the use of higher metal concentrations than environmental levels to ensure an effect. Test solutions were prepared in 100 ml volumetric flasks, transferred into 100 ml polyethylene (PE) bottles with caps, sealed with parafilm, and stored for the biological tests. Metal concentrations in subsamples of test solutions were also measured, and the data are provided in Table A1 in the Appendix.

2.3. Fertilization and larvae tests for oysters and abalone

Adult oysters and abalone were obtained from FUDA aquaculture farm in Jinjiang, Fujian, China. The molluscs were transferred to the lab and acclimated for 7 d before the experiments, using the culturing method previously reported by Guo et al. (2015).

To obtain eggs and sperm from oysters, oyster shells were opened using an oyster shucker. Individuals with fully developed gonads were selected and put on an enamel tray. A 24-well plate with FSW in each well was used for gamete selection. The gonad was pierced with a 200 µl pipette tip, and a 20 µl aliquot of gametes was removed and transferred into one well of the plate. A new pipette tip was used for each oyster and contact between tips was avoided to prevent undesired fertilization before the test. At 15 min post transfer, gametes in the wells were checked under an inverted microscope (DM IL LED Leica™). Eggs that were mature enough for fertilization were globular or pear-shaped, with a transparent nucleus in the center surrounded by densely packed granules. Suitable sperm were swimming rapidly. Eggs from 3 to 5 oysters were selected, pooled, and used to prepare a dense suspension in FSW. Eggs were then washed 2 to 3 times with FSW to remove undesired gonad debris. Sperm from 3 to 5 oysters were also selected, pooled, and made into a suspension with FSW. To obtain eggs and sperm from abalone, individuals with fully developed gonads were stimulated

Table 1. Mean ± SD of water chemistry measurements and calcite and aragonite saturation state (Ω) of seawater in control and high-pCO_2 groups. n = 3 for all treatments. Parameters such as pCO_2, concentration of CO_2, bicarbonate ions and carbonate ions and Ω were calculated by the CO2SYS software

Parameter	Control	800 µatm	1500 µatm	2000 µatm
Temperature (°C)	24.7 ± 0.1	25.0 ± 0.1	24.4 ± 0.2	24.7 ± 0.3
pH (NBS scale)	8.16 ± 0.02	7.93 ± 0.01	7.69 ± 0.01	7.60 ± 0.02
Alkalinity (µmol kg^{-1})	2294.1 ± 1.2	2242.1 ± 0.7	2248.1 ± 0.7	2286.8 ± 2.0
Salinity	30.3 ± 0.5	30.3 ± 0.5	30.3 ± 0.5	30.3 ± 0.5
pCO_2 (µatm)	423.3	808.3	1480.4	1884.4
$[CO_2]$ (µmol kg^{-1})	12.4	23.4	43.6	55.1
$[HCO^{3-}]$ (µmol kg^{-1})	1831.4	1960.2	2079.3	2144.5
$[CO_3^{2-}]$ (µmol kg^{-1})	191.6	116.4	69.7	59.0
Ωcal	4.78	2.91	1.74	1.47
Ωara	3.12	1.90	1.13	0.96

with UV-radiated seawater to produce gametes. Viable eggs and sperm were also selected, pooled and made into suspensions with FSW.

A complete 2 fixed factor factorial design was adopted in the biological tests: for example, there were 24 treatments (4 pCO_2 levels × 6 Cu concentrations) in the oyster fertilization test. Each treatment was replicated 4 times, and embryos or larvae were cultured in 40 ml polypropylene (PP) bottles. The bottles were placed in a chamber connected to a pipe that supplied CO_2-enriched air from the system described in Section 2.1 so that a micro-environment with high pCO_2 was maintained; this ensured that the water pH did not vary greatly during the experiment. Four chambers were used for each of the 4 pCO_2 levels, and each chamber contained a series of replicate bottles with different metal concentrations.

For both fertilization and larvae tests, a 20 µl volume of egg suspension and 20 µl of sperm suspension were added to each bottle to initiate fertilization. The volume of gamete suspension was small relative to the total volume of the test solution and had only a minor dilution effect on the metal concentration. Density of eggs in each was maintained at 10 eggs ml^{-1} for oysters and 2 eggs ml^{-1} for abalone to minimize the metabolic effects of embryos on the seawater pH, while sperm amounts were adjusted to achieve a concentration of 10^3 ml^{-1} in the test solution. After adding the sperm suspension, the bottles were gently shaken for 10 s and then transferred into the chambers. The chambers were put in a thermo-static incubator with a set temperature of 25°C for oyster and 22°C for abalone. At 30 min after fertilization, the FSW was refreshed several times to remove excess sperm.

At 2 h post-fertilization (hpf), the fertilization test was terminated by adding 20 µl buffered formalin to each bottle. Photos of the resulting embryos for fertilization rate and egg diameter measurements were taken using a CCD camera connected to a microscope (CX31 Olympus™). Fertilization rate (%) was calculated by counting the number of cleaved embryos and the total number of eggs:

$$\text{Fertilization rate} = (\text{no. cleaved embryos} / \text{no. total eggs}) \times 100 \quad (1)$$

Egg diameter was defined as the sum of the cytoplasm and vitelline layer according to Graham et al. (2006) and was measured with the software Image-Pro Plus 6.0. Egg diameter was observed only for abalone.

At 24 hpf, the larvae test was terminated by adding 20 µl buffered formalin to each bottle. Deformation and shell length were observed. Deformation rate (%) was calculated following the methods of His et al. (1997) for oysters and Hunt & Anderson (1990) for abalone.

$$\text{Deformation rate} = (\text{no. deformed larvae} / \text{no. total larvae}) \times 100 \quad (2)$$

Approximately 50 embryos/larvae were randomly sampled from each bottle and observed under the microscope.

All the containers used for solution preparation and biological tests were soaked in 1:10 (v:v) nitric acid for at least 24 h and rinsed with deionized water 3 times before the experiment.

2.4. Statistics

Data on fertilization rate and larval deformation were arcsine and square root transformed to meet the assumption of homogeneity of variance for ANOVA. A 2-way ANOVA was performed with pCO_2 and metal as fixed factors. Simple effects of pCO_2 or metal were analyzed using a 1-way ANOVA with a Student-Newman-Keuls (SNK) post hoc test when interactions of the 2 factors were found. A Kruskal-Wallis (KW) test was applied when data transformations failed to result in homogeneity of variance. Differences were considered significant if $p < 0.05$. All statistical analyses were performed using SPSS v17.0.

3. RESULTS

3.1. Combined effects of OA and trace metals on fertilization of *Crassostrea angulata*

There was an interaction between OA and the effect of Cu on fertilization of *C. angulata* (2-way ANOVA, $F_{15,72} = 95.5$, $p < 0.001$; Table 2). Fertilization was unaffected by rising pCO_2 when no Cu was added (1-way ANOVA, $F_{3,15} = 1.5$, $p = 0.27$); however, fertilization was significantly higher at high pCO_2 than low pCO_2 when Cu addition exceeded 10 µg l^{-1} (e.g. when 20 µg l^{-1} of Cu was added, fertilization rate increased 86.1% between 400 and 2000 µatm pCO_2: SNK, $p < 0.001$; Fig. 2a). In other words, the toxicity of Cu to oyster fertilization was alleviated by increasing pCO_2 in seawater.

An interaction also existed between OA and the effect of Zn on oyster fertilization (2-way ANOVA, $F_{12,60} = 12.7$, $p < 0.001$; Table 2), with similar effects to those of the combined OA and

Table 2. Results of 2-way ANOVA examining the combined effects of pCO_2 and trace metals on embryonic and larval development of the oyster *Crassostrea angulata* and abalone *Haliotis discus hannai*. *p < 0.05

Response variable	Source of variance	df	MS	*F*	p
Fertilization of *C. angulata*	CO_2	3	3088.5	439.4	<0.001*
	Cu	5	19046.8	2710.0	<0.001*
	CO_2 × Cu	15	671.3	95.5	<0.001*
	Residual	72	7.0		
	CO_2	3	858.9	120.1	<0.001*
	Zn	4	7905.6	1105.2	<0.001*
	CO_2 × Zn	12	90.9	12.7	<0.001*
	Residual	60	7.2		
Fertilization of *H. discus hannai*	CO_2	3	1144.1	42.1	<0.001*
	Cu	5	3980.1	146.6	<0.001*
	CO_2 × Cu	15	171.8	6.3	<0.001*
	Residual	72	27.2		
	CO_2	3	426.9	30.6	<0.001*
	Zn	5	21712.5	1556.9	<0.001*
	CO_2 × Zn	15	168.8	12.1	<0.001*
	Residual	72	13.9		
Egg diameter of *H. discus hannai*	CO_2	1	2940.7	87.0	<0.001*
	Cu	2	5345.6	158.2	<0.001*
	CO_2 × Cu	2	1432.6	42.4	<0.001*
	Residual	18	33.8		
	CO_2	1	23.6	13.3	<0.001*
	Zn	2	22.9	12.9	<0.001*
	CO_2 × Zn	2	24.9	14.1	<0.001*
	Residual	18	1.8		
Deformation of *C. angulata* larvae	CO_2	2	3719.3	1261.3	<0.001*
	Cu	2	6723.6	2280.1	<0.001*
	CO_2 × Cu	4	328.5	111.4	<0.001*
	Residual	27	2.9		
	CO_2	2	1853.3	164.9	<0.001*
	Zn	2	3619.1	322.0	<0.001*
	CO_2 × Zn	4	30.4	2.7	0.051
	Residual	27	11.2		
Deformation of *H. discus hannai* larvae	CO_2	2	1468.7	162.6	<0.001*
	Cu	2	2442.9	270.5	<0.001*
	CO_2 × Cu	4	441.4	48.9	<0.001*
	Residual	36	9.0		
	CO_2	2	7860.4	543.2	<0.001*
	Zn	3	3617.9	250.0	<0.001*
	CO_2 × Zn	6	579.3	40.0	<0.001*
Shell length of *C. angulata* larvae	CO_2	2	184.5	104.0	<0.001*
	Cu	2	226.5	127.7	<0.001*
	CO_2 × Cu	4	3.8	2.1	0.106
	Residual	27	1.8		
	CO_2	2	255.3	574.5	<0.001*
	Zn	2	35.8	80.6	<0.001*
	CO_2 × Zn	4	1.7	3.7	0.015*
	Residual	27	0.4		
Shell length of *H. discus hannai* larvae	CO_2	2	528.2	30.1	<0.001*
	Cu	2	301.1	17.1	<0.001*
	CO_2 × Cu	4	205.1	11.7	<0.001*
	Residual	27	17.6		
	CO_2	2	4183.5	110.2	<0.001*
	Zn	3	9920.3	261.2	<0.001*
	CO_2 × Zn	6	1098.9	28.9	<0.001*
	Residual	36	38.0		

Cu treatment. Fertilization was unaffected by pCO_2 levels when no Zn was added, while fertilization was significantly higher at high pCO_2 than at low pCO_2 with increasing Zn addition (e.g. when 1 µg ml^{-1} of Zn was added, fertilization rate increased 26.4% between 400 and 2000 µatm pCO_2: SNK, $p < 0.001$; Fig. 2b).

3.2. Combined effects of OA and trace metals on fertilization of *Haliotis discus hannai*

There was an interaction between OA and the effect of Cu on fertilization of *H. discus hannai* (2-way ANOVA, $F_{15,72} = 6.3$, $p < 0.001$; Table 2). For example, fertilization was not significantly different between pCO_2 400 and 2000 µatm when no Cu was added (1-way ANOVA, $F_{3,15} = 0.49$, $p = 0.70$; Fig. 3a). However, fertilization was reduced by 34.7% at 2000 µatm when 150 µg l^{-1} of Cu was added (SNK, $p < 0.001$; Fig. 3a). Similarly, fertilization was reduced by 33.0% at 1500 µatm when 300 µg l^{-1} of Cu was added (SNK, $p = 0.001$; Fig. 3a). In other words, the toxicity of Cu to abalone fertilization was aggravated by increasing pCO_2 in seawater.

In contrast, the toxicity of Zn to abalone fertilization was alleviated by increasing pCO_2, and the interaction of these 2 factors was significant (2-way ANOVA, $F_{15,72} = 12.1$, $p < 0.001$; Table 2). For example, fertilization was unaffected by pCO_2 level when no (1-way ANOVA, $F_{3,15} = 0.34$, $p = 0.80$) and 0.2 µg ml^{-1} (1-way ANOVA, $F_{3,15} = 1.1$, $p = 0.40$) Zn was added (Fig. 3b); however, fertilization was 43.7% lower at 400 µatm pCO_2 than at 1500 µatm when 1 µg ml^{-1} Zn was added (SNK, $p < 0.001$; Fig. 3b).

There were also interactive effects of pCO_2 and metals on egg diameter in abalone (Table 2). Cu addition caused a significant egg diameter in-

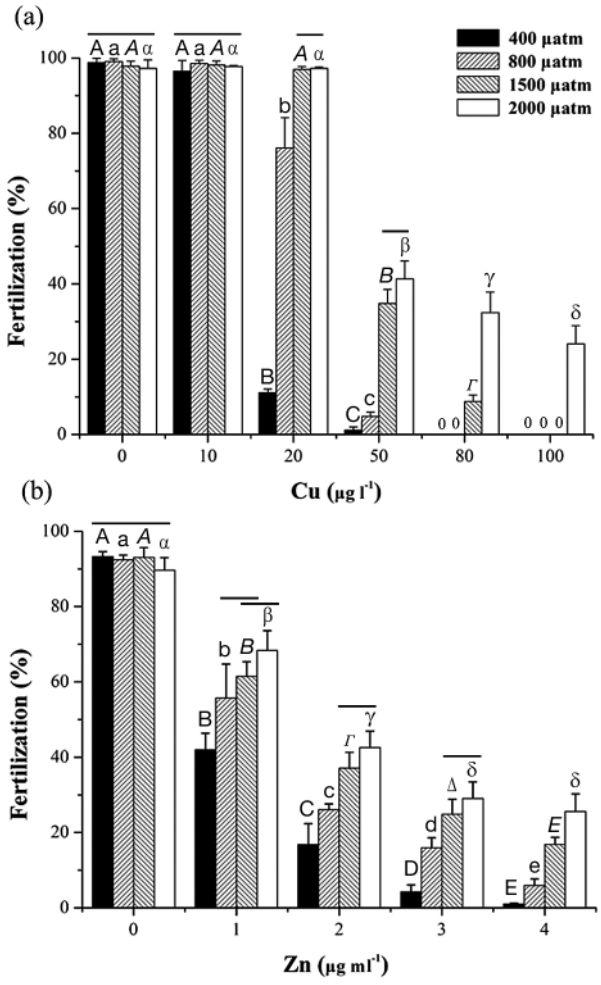

Fig. 2. Fertilization rate (mean + SD, n = 4) of *Crassostrea angulata* 2 h post fertilization at 4 levels of pCO_2 with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference across pCO_2 levels at the same trace metal concentrations. Columns not connected by lines are significantly different from each other ($p < 0.05$)

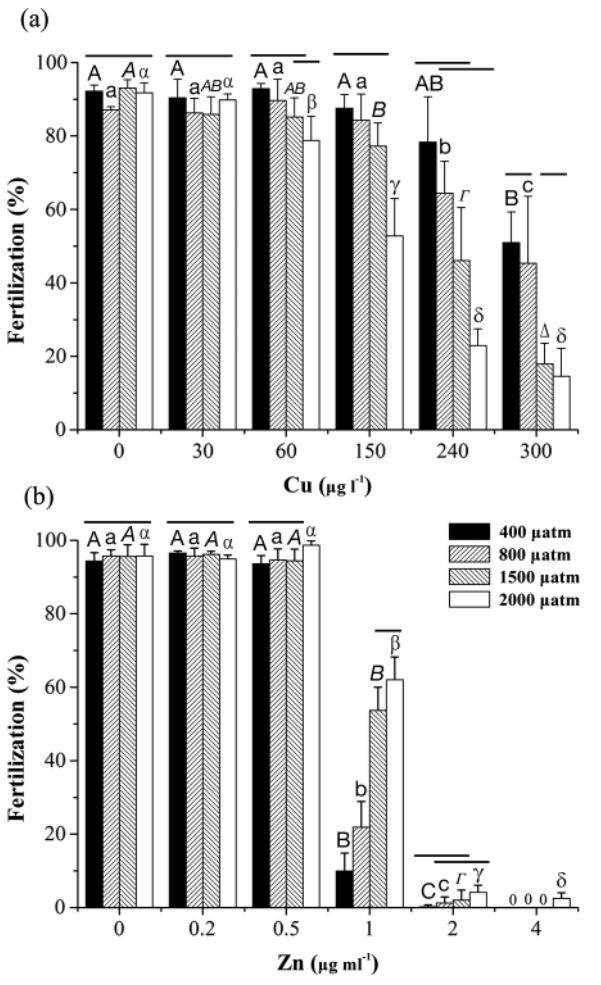

Fig. 3. Fertilization rate (mean + SD, n = 4) of *Haliotis discus hannai* 2 h post fertilization at 4 levels of pCO_2 with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference across pCO_2 levels at the same trace metal concentrations. Columns not connected by lines are significantly different from each other ($p < 0.05$)

crease at a pCO_2 level of 400 µatm, yet this effect decreased as pCO_2 increased. For example, at 400 µatm, egg diameter increased by 11.2% when 150 µg l^{-1} Cu was added (SNK, $p < 0.001$; Fig. 4a); however, at 1500 µatm, there was no significant difference in egg diameter between the 0 and 150 µg l^{-1} Cu treatments (SNK, $p = 0.16$; Fig. 4a). Similarly, the average egg diameter increased by 37.8% when 300 µg l^{-1} Cu was added at 400 µatm, but by only 11.6% at 1500 µatm (Fig. 4c). Zn addition had no effect on egg diameter at 400 µatm (1-way ANOVA, $F_{2,11} = 3.9$, $p = 0.061$); however, egg diameter decreased significantly when Zn was added at 1500 µatm (e.g. a 2.5% reduction in diameter with the addition of 1 µg ml^{-1} Zn: SNK, $p < 0.001$; Fig. 4b,d).

3.3. Combined effects of OA and trace metals on larval deformation of *C. angulata*

A significant interaction between the effects of OA and Cu on larval deformation in *C. angulata* was observed (2-way ANOVA, $F_{4,27} = 111.4$, $p < 0.001$; Table 2). For example, the larval deformation rate increased by 7.7% between the no Cu and 5 µg l^{-1} Cu treatments at 400 µatm (SNK, $p < 0.001$; Fig. 5a), but by 22.0% at 800 µatm (SNK, $p < 0.001$; Fig. 5a). Thus, OA increased Cu toxicity to oyster larval development.

In contrast, there was no interaction between the effects OA and Zn in larval deformation of *C. angu-*

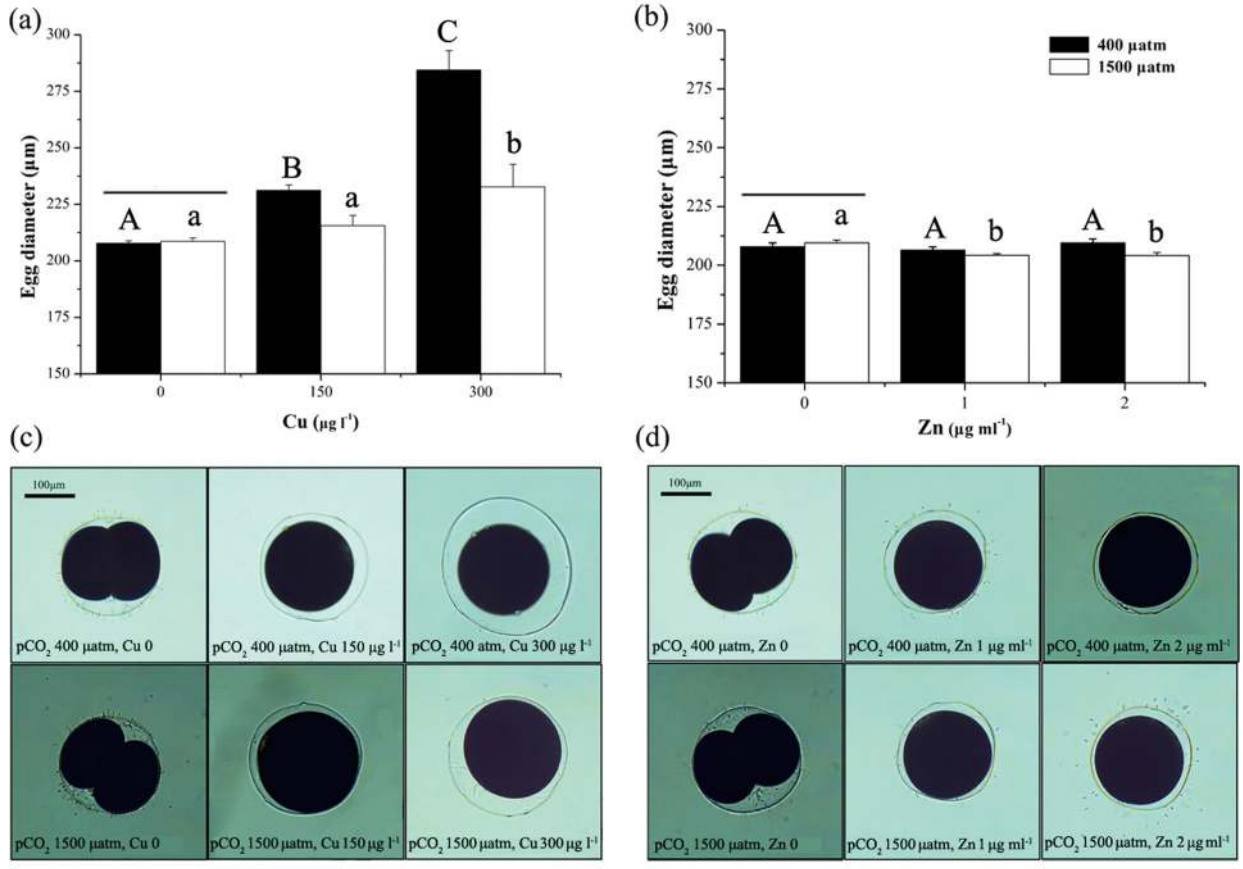

Fig. 4. Egg diameter (mean + SD; n = 4) of *Haliotis discus hannai* 2 h post fertilization at pCO_2 400 and 1500 µatm with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between different trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference between 400 and 1500 µatm at the same trace metal concentration. Columns not connected by lines are significantly different from each other ($p < 0.05$). Photos showing (c) significant expansion of egg membrane in high Cu concentration treatments at pCO_2 400 µatm but less evident expansion at pCO_2 1500 µatm, and (d) subtle but significant reduction of egg diameter in high Zn concentration treatments at pCO_2 1500 µatm

lata (2-way ANOVA, $F_{4,27} = 2.7$, $p = 0.051$; Table 2, Fig. 5b).

3.4. Combined effects of OA and trace metals on larval deformation of *H. discus hannai*

A significant interaction between the effects of OA and Cu on larval deformation in *H. discus hannai* was detected (2-way ANOVA, $F_{4,36} = 48.9$, $p < 0.001$; Table 2). For example, larval deformation increased by 10.7% between the no Cu and 5 µg l^{-1} Cu treatments at 400 µatm (SNK, $p = 0.001$; Fig. 6a), but by 71.4% at 1500 µatm (SNK, $p < 0.001$; Fig. 6a). OA thus increased Cu toxicity to abalone larval development.

An interaction also existed between the effects of OA and Zn on larval deformation of *H. discus hannai* (2-way ANOVA, $F_{6,36} = 40.0$, $p < 0.001$; Table 2). For example, at 400 µatm, larval deformation increased by 31.5% when 60 µg l^{-1} Zn was added (KW, $p = 0.032$; Fig. 6b); however, at 1500 µatm, deformation increased by 37.2% when only 20 µg l^{-1} Zn was added (SNK, $p < 0.001$; Fig. 6b). OA increased Zn toxicity to abalone larval development.

3.5. Combined effects of OA and trace metals on larval shell length of *C. angulata*

OA and Cu had no interactive effects on larval shell length in *C. angulata* (2-way ANOVA, $F_{4,27} = 2.1$, $p = 0.11$; Table 2, Fig. 7a). However, an interaction existed between the effects of OA and Zn on shell length (2-way ANOVA, $F_{4,27} = 3.7$, $p = 0.015$; Table 2). For example, shell length was reduced by 3.1% between 0 and 25 µg l^{-1} Zn at 400 µatm (SNK,

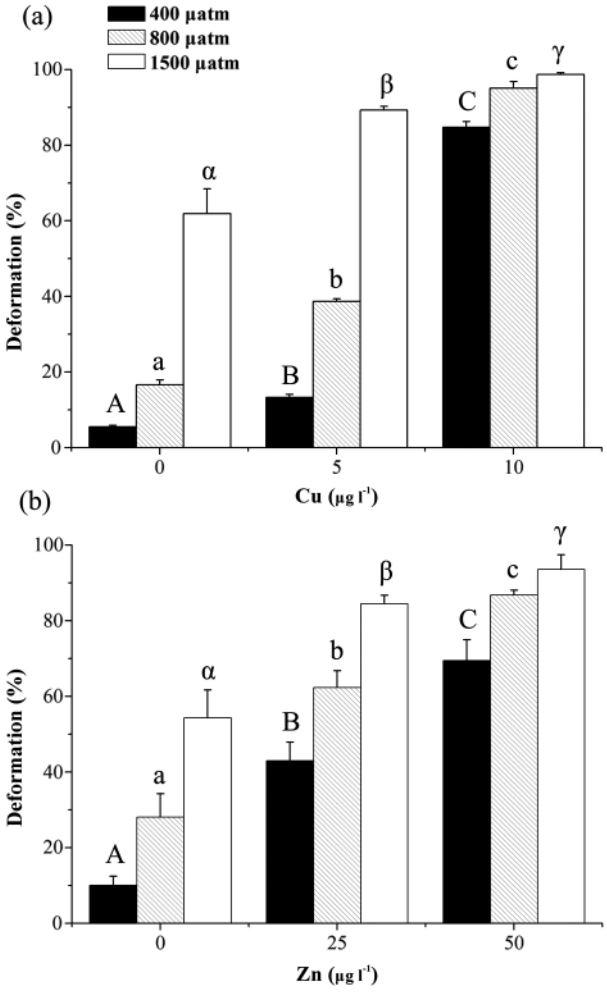

Fig. 5. Deformation rate (mean + SD; n = 4) of *Crassostrea angulata* larvae 24 h post fertilization at 3 pCO_2 levels with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference between pCO_2 levels at the same trace metal concentration. Columns not connected by lines are significantly different from each other ($p < 0.05$)

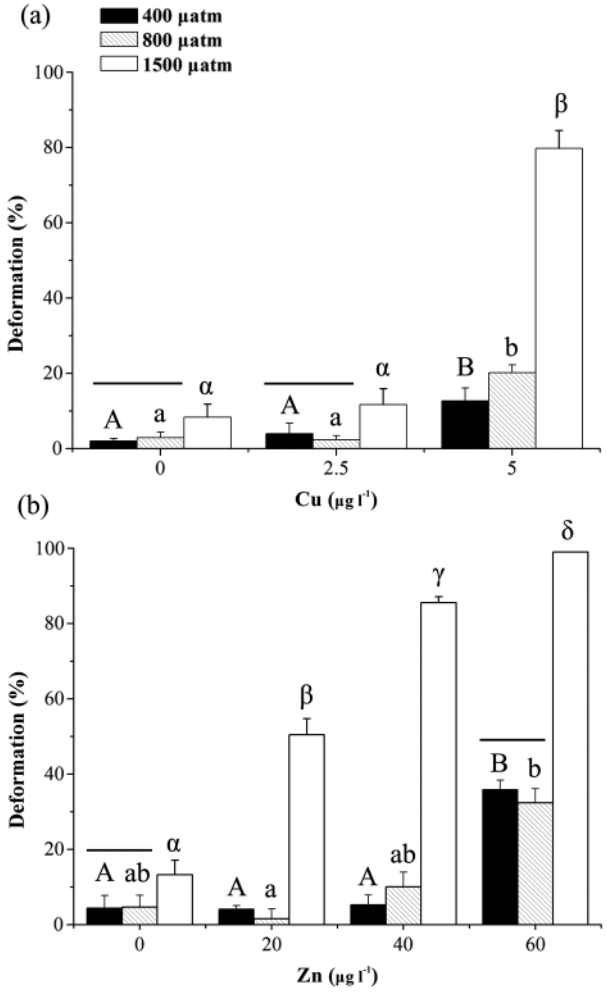

Fig. 6. Deformation rate (mean + SD; n = 4) of *Haliotis discus hannai* larvae 24 h post fertilization at 3 pCO_2 levels with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference between pCO_2 levels at the same trace metal concentration. Columns not connected by lines are significantly different from each other ($p < 0.05$)

$p < 0.001$; Fig. 7b), but only by 1.5% at 800 µatm (SNK, $p < 0.001$; Fig. 7b). Overall, OA alleviated Zn inhibition of oyster larval calcification.

3.6. Combined effects of OA and trace metals on larval shell length of *H. discus hannai*

There was an interaction between the effects of OA and Cu on larval shell length in *H. discus hannai* (2-way ANOVA, $F_{4,27} = 11.7$, $p < 0.001$; Table 2). For example, shell length did not change with rising Cu concentration at 400 µatm (1-way ANOVA, $F_{2,11} = 0.21$, $p = 0.82$; Fig. 8a) and 800 µatm (1-way ANOVA, $F_{2,11} = 1.9$, $p = 0.20$; Fig. 8a). However, at 1500 µatm, shell length was reduced by 8.8% when 5 µg l^{-1} Cu was added (SNK, $p = 0.001$, Fig. 8a).

An interaction also existed between the effects of OA and Zn on shell length in *H. discus hannai* (2-way ANOVA, $F_{6,36} = 28.9$, $p < 0.001$; Table 2). For example, at 400 and 800 µatm, shell length decreased by 8.1% (SNK, $p < 0.001$, Fig. 8b) and 6.6% (SNK, $p < 0.001$, Fig. 8b) respectively when 60 µg l^{-1} Zn was added. However, at 1500 µatm, shell length was reduced by 8.6% when only 20 µg l^{-1} Zn was added (SNK, $p < 0.001$, Fig. 8b).

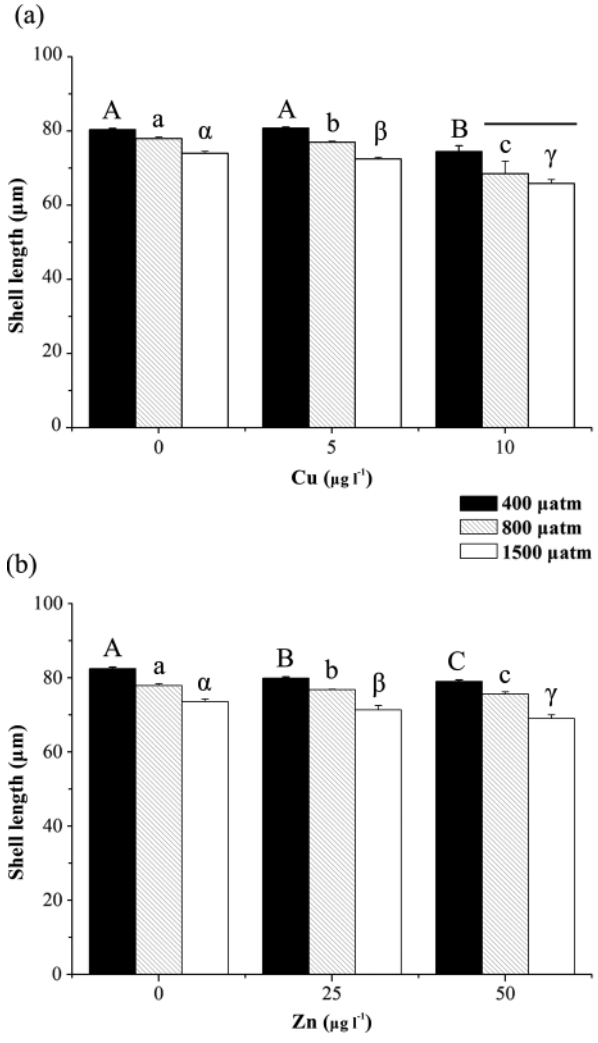

Fig. 7. Shell length (mean + SD; n = 4) of *Crassostrea angulata* larvae 24 h post fertilization at 3 pCO_2 levels with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference between pCO_2 levels at the same trace metal concentration. Columns not connected by lines are significantly different from each other ($p < 0.05$)

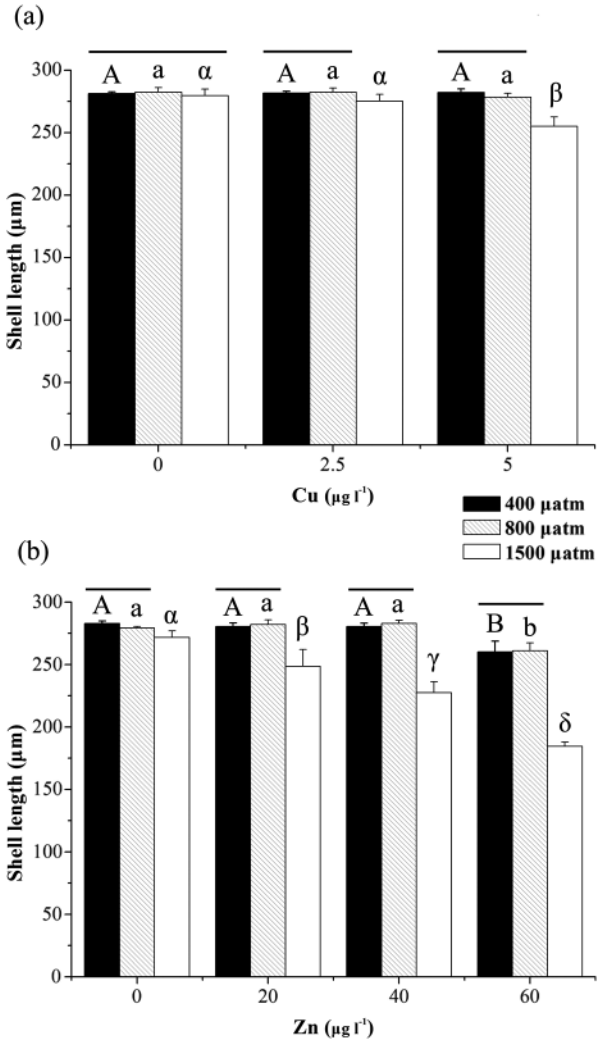

Fig. 8. Shell length (mean + SD; n = 4) of *Haliotis discus hannai* larvae 24 h post fertilization at 3 pCO_2 levels with the addition of (a) Cu or (b) Zn. Different upper- and lowercase letters represent significant differences between trace metal concentrations at the same pCO_2 level ($p < 0.05$). A line above columns indicates no significant difference between pCO_2 levels at the same trace metal concentration. Columns not connected by lines are significantly different from each other ($p < 0.05$)

4. DISCUSSION

Inhibition of fertilization under high-pCO_2 conditions occurs in different marine invertebrates (Morita et al. 2010, Moulin et al. 2011, Reuter et al. 2011, Barros et al. 2013, Foo et al. 2014). In the marine bivalve *Tegillarca granosa*, sperm motility, gamete fusion and ovular calcium oscillation in fertilization were all impeded by OA (Shi et al. 2017). Respiration of sperm was inhibited in the mussel *Mytilus edulis* at pH < 7.5 (Akberali et al. 1985). Similarly, the mitochondrial membrane potential (MMP) in sperm of the sea urchin *Centrostephanus rodgersii* was significantly reduced under OA conditions (pH reduced by 0.3 and 0.5; Schlegel et al. 2015). The main energy source for motility in sperm is mitochondrial respiration, for which MMP can be used as a proxy; sperm in ambient seawater usually have a higher MMP than sperm in high-pCO_2 seawater. Consequently, the reduced motility of sperm under OA conditions could be the result of inhibited respiration. The acrosome reaction and ovular calcium oscillation are crucial processes for gamete fusion and embryo development respectively, and both require an influx of cal-

cium into gametes. Since OA may lead to reduced intracellular pH (Gibbin et al. 2014), which prevents calcium uptake by the cell from seawater (Thomas & Meech 1982), the overall result may be failure of both the acrosome reaction and embryo cleavage.

Adverse effects of Cu and Zn on fertilization have often been reported (Reichelt-Brushett & Harrison 1999, Reichelt-Brushett & Michalek-Wagner 2005, Victor & Richmond 2005). Cu^{2+} is reduced to Cu^{+} after entering the cell, which binds with sulfhydryl groups (Viarengo et al. 1996) of enzymes in the electron transport chain (Ay et al. 1999). This can inhibit the ATP production of sperm and affect sperm motility. Cu accumulation in sperm mitochondria can decrease MMP and cause formation of reactive oxygen species (ROS), leading to oxidative damage (Krumschnabel et al. 2005). Similarly, ROS production is the main toxic effect caused by Zn, which has been observed in many species, including bivalves (Geret & Bebianno 2004). Trace metals may also induce the release of free calcium in eggs from intracellular calcium stores such as the endoplasmic reticulum, causing an increase of intracellular calcium concentration. Fertilized eggs of the sea urchin *Psammechinus miliaris* treated with Cu exhibited a significantly higher free calcium signal than control, untreated eggs (Schäfer et al. 2009). However, the abnormally high calcium concentration significantly inhibited fertilization because degradative enzymes that depended on excess calcium were activated, which compromised mitochondrial function and cytoskeletal organization, and ultimately resulted in developmental failure.

In contrast, Cu and Zn may also have positive effects on molluscan reproduction, e.g. by increasing the ionic permeability of organelle membranes. A significant decrease in calcium level was found in the acrosomes and mitochondria of *M. edulis* sperm after Cu and Zn exposure (Earnshaw et al. 1986). Both metals can induce the release of pre-loaded calcium from the matrix of *M. edulis* mitochondria, with Cu being more effective at this than Zn (Akberali & Earnshaw 1982). The result of this induction could be an increase in the concentration of free calcium in sperm cytoplasm. As intracellular free calcium plays an important role in activating sperm, Cu and Zn may cause an increase in sperm mobility. Likewise, exposure to Cu stimulated the respiration of unfertilized eggs of *M. edulis*, which was attributed to enhanced potassium influx and uncoupling of oxidative phosphorylation induced by Cu (Akberali et al. 1984). Considering respiration of fertilized eggs continues to increase throughout embryonic development, stimulation of respiration by Cu may benefit the development of molluscan embryos. Consequently, the effects of Cu and Zn on gametes of marine invertebrates are complex: toxicity to enzymes, production of ROS and excess calcium intake could hamper fertilization, while enhanced gamete respiration could benefit fertilization and embryonic development. Final effects of the 2 metals are likely exposure-dependent, both in terms of duration and concentration.

Under the combined OA and addition of Cu or Zn, metal ions promote calcium influx into the gametes, which could increase sperm velocity, stimulate the acrosomal reaction and also compensate for the insufficient uptake of calcium during fertilization caused by OA. These processes could reduce the adverse effects caused by OA and metals independently, which may be the reason for the antagonistic interaction between the effects of OA and Cu or Zn on fertilization seen in the present study. An alternative explanation is the competition between protons and metal ions for binding sites on the cell surface (Kerndorff & Schnitzer 1980, Schubauer-Berigan et al. 1993). When pCO_2 increases (inducing a higher seawater proton concentration), excess protons may bind to the sperm cell surface, increasing the positive charge on the outer cell membrane and inhibiting the entry of positively charged metal ions. Thus, low pH may represent a protection mechanism for invertebrate reproduction in metal-polluted sites.

In contrast to the antagonistic interaction observed in the combined treatments of OA and metals on oyster fertilization in the present study, rising pCO_2 could increase the toxicity of Cu to abalone fertilization, and this may be attributed to the structure of the abalone egg. Abalone eggs differ from those of oysters in that they are covered with an egg jelly coat and vitelline envelope, with the vitelline envelope beneath the jelly coat. The thickness of the egg jelly varies from 30 to 100 µm for different abalone species, while the vitelline envelope is thin (about 1 µm). The egg jelly of abalone *Haliotis asinina* mainly contains 2 glycoproteins at 107 kDa and 178 kDa, with glucose as the major sugar component, and the vitelline envelope also contains the 2 glycoproteins (Suphamungmee et al. 2010). The role of the egg jelly glycoproteins is to accelerate sperm motility (Suphamungmee et al. 2010), and the vitelline envelope binds with sperm and triggers the acrosome reaction (Vacquier et al. 1999).

Influence of metal ions on the egg jelly and vitelline envelope of marine invertebrates has not been documented. However, a study on the plant cell wall led to the hypothesis that Cu^{2+} could be reduced to Cu^{+} by apoplastic electron donors when it complexes with cell wall glycoproteins and that the Cu^{+} can

then undergo a Fenton reaction with apoplastic hydrogen peroxide (H_2O_2) to generate hydroxyl radicals (·OH) (Fry et al. 2002). These radicals can cause non-enzymic scission of wall polysaccharides and may loosen the cell wall. Considering the high oxidative capacity of ·OH, the structure of the glycoproteins in the egg jelly and vitelline envelope could also be comprised under Cu exposure. Thus, both the egg jelly and the vitelline envelope may lose their integrity due to structural destruction, causing the vitelline envelope to expand, as indicated by the significant increase of egg diameter seen in our experiments. Fertilization success could be improved as a result of the larger surface area for sperm to come in contact with, thus increasing the chance that a sperm cell enters the egg. In other words, Cu may not only be toxic but may also promote abalone fertilization under ambient pCO_2 conditions. Thus, a nearly 50% fertilization rate was maintained in abalone at ambient pCO_2 when as much as 300 µg l^{-1} Cu was added, compared with no fertilization in oysters (where egg diameter was not affected) under a Cu addition of 100 µg l^{-1} at ambient pCO_2. In seawater with higher pCO_2, expansion of the egg membrane was significantly inhibited; therefore, the toxicity of Cu played a more important role. In other words, Cu toxicity to abalone fertilization was enhanced by OA. The subtle but significant reduction of abalone egg diameter under combined treatments of high pCO_2 and Zn addition also indicated that Zn ions might alter egg structure at higher pCO_2. Further studies are needed to reveal the mechanism of the combined effects of OA and metal ions on abalone egg structure.

Cu addition at high-pCO_2 levels significantly increased larval deformation in oysters in our study. Strong interactions between pCO_2 and Cu were also found by Lewis et al. (2013), where very high polychaete larval mortality occurred at low pH with Cu addition compared to low pH without Cu. This synergistic effect may be attributed to an increase of free Cu and Zn ions under OA, as predicted by Millero et al. (2009). Such a phenomenon was observed by Shi et al. (2016), who found that the ratio of Cd^{2+} to Ca^{2+} was significantly increased in high pCO_2 seawater. In addition, OA could reduce larval metabolic rate, so that the energy supply may become insufficient for larvae to expel trace metals extracellularly. Although OA temporarily inhibits metal ions from penetrating the embryo at the fertilization stage through higher proton concentration, it is highly likely that the metals could still accumulate inside the cell over longer exposure. A recent study found that Cu accumulation was significantly higher in adult oysters exposed to Cu for 28 d at pH 7.6 than in exposed individuals at pH 8.1 and 7.8 (Cao et al. 2019). Metallothionein (MT) plays an important part in the detoxification of many trace metals in molluscs, and transcription of MT-mRNA can be rapidly activated under metal exposure, leading to increased synthesis of MT which in turn binds metal ions (Isani et al. 2000). However, molluscan embryos might be relatively weak in dealing with metal toxicity. The oyster *Crassostrea virginica* was unable to produce metallothionein before 8 hpf (Roesijadi et al. 1996), and MT-mRNA expression was limited in the larval stage compared to adults (Unger & Roesijadi 1996). Consequently, higher metal accumulation induced by OA and limited detoxification ability during the larval stage could be the reasons for the synergistic effect of OA and metals on larval deformation.

Both Cu and Zn could adversely affect larval shell formation. Cu inhibits the activity of carbonic anhydrase (Vitale et al. 1999), a key enzyme in organism calcification, thus reducing shell growth. Decreased shell growth under Zn exposure might be caused by its competitive binding with calcium receptors when the shell is forming, as the Zn and Ca ions have similar atomic diameters and are both divalent ions. OA could also modulate gene expression of carbonic anhydrase. Todgham & Hofmann (2009) found an up-regulation of the carbonic anhydrase gene under high pCO_2, while Stumpp et al. (2011) reported a downward regulation in echinoderm larvae. Suppression of gene expression of carbonic anhydrase was also discovered in larvae of oysters *C. gigas* under high pCO_2 (De Wit et al. 2018). In the present study, the inhibition of abalone larval calcification induced by Cu and Zn was enhanced under OA conditions. OA may have inhibited carbonic anhydrase synthesis in abalone larvae so that more energy was diverted to detoxification of metals. Therefore, the energy was insufficient to sustain normal calcification, and larval shell growth was even more reduced when OA and metals co-occurred. In contrast to abalone, oyster larvae might allocate more energy to maintaining shell growth, and that is why their larval shell growth was not further inhibited under the combined treatment of OA and Cu. Zn ions are cofactors of carbonic anhydrase, and Zn addition might therefore induce the up-regulation of the carbonic anhydrase gene, therefore alleviating inhibition of larval shell growth caused by OA.

Acclimation to environmental changes can be assessed in transgenerational investigations. Exposing adult Sydney rock oysters *Saccostrea glomerata* to high pCO_2 conditions induced parental carryover ef-

fects in the next-generation larvae (Parker et al. 2012). Larvae from oysters undergoing conditioning within elevated pCO_2 environments grew faster and larger than larvae produced by adults raised in ambient pCO_2. Mussel *M. edulis* larvae originating from high pCO_2 environments exhibited increased tolerance to acidified environments (Thomsen et al. 2017). In another study on *M. edulis*, transgenerational exposure to high pCO_2 conditions induced the offspring to precipitate calcite — which is a less soluble form of calcium carbonate — instead of aragonite in their shells (Fitzer et al. 2014). Therefore, molluscs likely become tolerant to OA after long term and transgenerational exposure to low pH conditions. Our study species vary in their likelihood to adapt to OA. *C. angulata* is an estuarine intertidal species that lives in environments with large fluctuations in pH and trace metal concentrations, and has adapted to the unstable environmental conditions over generations of acclimation. Abalone *H. discus hannai* inhabit environmentally stable offshore regions and are thus more susceptible to a changing environment. This may be the reason why our results show *C. angulata* to be more tolerant to the combined effects of OA and metals than *H. discus hannai*. The larval stage is very important for population sustainability of molluscs, and impaired larvae would no doubt reduce the yield of molluscan aquaculture. It is very likely that culture of *H. discus hannai* would be threatened under the concurrence of OA and trace metal pollution at aquaculture sites. Larvae of *C. angulata* seem more resistant to OA and metal in their early developmental stage; however, chronic effects could emerge under longer exposure (e.g. during the entire larval stage or even to the adult stage). Therefore, culture of *C. angulata* could also be adversely affected by combined exposure to OA and metals at oyster farms.

In conclusion, this study has provided evidence that variation in pCO_2 levels, developmental stages, egg structure and species can all affect metal toxicity to the early development of molluscs. Future research should include additional parameters such as other environmental stressors, developmental stages and gamete properties of the tested organisms when assessing metal toxicity to molluscan embryos. Further biochemical and physiological studies are needed to clarify the biological mechanisms of the combined effects of OA and trace metals on molluscan embryonic and larval development. Based on the results of the present study, we propose that the production yield of molluscan aquaculture will decrease at metal polluted sites when seawater becomes more acidic as predicted by climate change models.

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APPENDIX.

Table A1. Cu and Zn concentrations of test solutions used in experiments testing larval deformation and shell length in *Crassostrea angulata*

	Replicate	pCO_2 (µatm)	Norminal concentration (µg l^{-1})	Tested concentration (µg l^{-1})
Cu	1	400	5	5.43
	2	400	5	5.49
	3	400	5	5.23
	4	400	5	5.80
	1	400	10	10.64
	2	400	10	10.37
	3	400	10	10.27
	4	400	10	10.36
	1	800	5	5.16
	2	800	5	5.82
	3	800	5	5.61
	4	800	5	5.83
	1	800	10	9.11
	2	800	10	10.90
	3	800	10	10.08
	4	800	10	10.78
	1	1500	5	5.12
	2	1500	5	5.23
	3	1500	5	5.16
	4	1500	5	4.73
	1	1500	10	9.66
	2	1500	10	9.23
	3	1500	10	9.66
	4	1500	10	10.20
Zn	1	400	25	28.98
	2	400	25	28.54
	3	400	25	30.01
	4	400	25	27.09
	1	400	50	51.64
	2	400	50	51.45
	3	400	50	52.07
	4	400	50	53.86
	1	800	25	26.41
	2	800	25	27.59
	3	800	25	27.69
	4	800	25	25.50
	1	800	50	53.99
	2	800	50	54.15
	3	800	50	51.33
	4	800	50	52.21
	1	1500	25	27.26
	2	1500	25	30.40
	3	1500	25	30.13
	4	1500	25	29.82
	1	1500	50	55.74
	2	1500	50	54.88
	3	1500	50	57.71
	4	1500	50	52.09

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