

# Effects of Seawater Acidification on Early Development of Clam *Cyclina sinensis*

SUI Yanming<sup>\*</sup>, ZHOU Kai, LAI Qifang<sup>\*</sup>, YAO Zongli, and GAO Pengchen

Engineering Research Center of Saline-alkaline Water Fisheries, East China Sea Fisheries Research Institute,  
Chinese Academy of Fishery Sciences, Shanghai 200090, China

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**Abstract** Anthropogenic emission of atmospheric carbon dioxide (CO<sub>2</sub>) has led to a rapid increase in atmospheric CO<sub>2</sub> concentration. Increasing atmospheric CO<sub>2</sub> can reduce seawater pH and carbonate ions, which may adversely affect the survival of the larvae of calcareous animals. *Cyclina sinensis* is a commercially and ecologically important species in several Asian countries. Living in coast shallow waters, this species has experienced the coastal environmental changes frequently throughout its life cycle. In this study, we simulated possible future seawater pH values including 8.2, 7.8 and 7.4 and examined the effects of ocean acidification on the early development of *C. sinensis*. Clam embryos were incubated for 48 h (2 d) in control and high-CO<sub>2</sub> seawater to compare embryogenesis, larval growth and swimming behavior. Fertilization rate was quite sensitive to pH, and moderate acidification could induce a significant decrease in fertilization rate. However, only extreme acidification could bring significant negative effect to hatching rate, body size, and average path velocity of trochophora. Moreover, with seawater acidification, *C. sinensis* needs much more time to reach the same developmental stage, which increases the risk of larva survival. Together with recent studies demonstrating negative impacts of high CO<sub>2</sub> on fertilization and larva swimming behavior, the results imply a future decrease of *C. sinensis* populations in oceans if its acclimation to the predicted environmental alteration does not occur.

**Key words** seawater acidification; *Cyclina sinensis*; fertilization; hatching rate; development; average path velocity

## 1 Introduction

Because of human activities, the extensive use of fossil fuels has released a large amount of CO<sub>2</sub> into atmosphere. It is estimated that about one third of the carbon dioxide released by humans into the atmosphere has dissolved into oceans, rivers and lakes (Millero, 1995; Feely *et al.*, 2004). The pH of the ocean might drop by 0.15–0.4 units at the end of the 21st century. This process is named as ocean acidification (OA) and is expected to affect various marine ecosystems (Hofmann *et al.*, 2010). In particular, organisms that use calcium carbonate to build their shells or skeletons are estimated to be much more negatively impacted (Dupont and Pörtner, 2013; Kroeker *et al.*, 2013).

Compared to the adult stage, calcifying organisms at their early developmental stages are much more susceptible to elevated *p*CO<sub>2</sub> because larvae cannot keep the acid-base balance well (Dupont *et al.*, 2008; Kurihara, 2008; Byrne, 2012; Andersen *et al.*, 2017). Additionally, the shell in larvae contains a thin layer of amorphous CaCO<sub>3</sub> which is relatively easy to dissolve (Gazeau *et al.*, 2013). Recently, numerous researches have focused on

the early development of marine calcifying organisms under OA (Gazeau *et al.*, 2010; Kroeker *et al.*, 2013; Szalaj *et al.*, 2017). Basically, fertilization, development, metamorphosis, shell growth and survival rate are all significantly reduced at low pH for most species whereas marine calcifying organisms also perform different degrees of susceptibility to OA during their early developmental stages (Kroeker *et al.*, 2013). For instance, in *Saccostrea glomerata* and *Crassostrea gigas*, both D-veliger percentage and D-veliger normality decline with *p*CO<sub>2</sub> increment and significant effects appeared under a moderate acidic condition; however, *C. gigas* exhibits more tolerance to OA than *S. glomerata* (Parker *et al.*, 2010). Guo *et al.* (2015) compared the effects of OA on the early development of 3 species including *Haliotis diversicolor*, *H. discus hannai* and *C. angulata*, and found that OA negatively affects the 3 species, and the resistance of *C. angulata* to OA is stronger than the other two. These studies clearly showed that the response to OA in calcifying organisms is species specific.

*Cyclina sinensis* is a commercially and ecologically important species in several Asian countries, especially in the coast shallow waters of these countries (Ren *et al.*, 2016). Thus, this species experiences the coastal environmental change frequently throughout its life cycle. So far, little has been known about the effect of OA on *C.*

<sup>\*</sup> Corresponding authors. E-mail: yanmingsui@foxmail.com  
E-mail: qifanglai@163.com

*sinensis*, especially on its early development. In this research, we measured the fertilization, hatching rate, and proportion of larvae at various developmental stages, as well as the larva size and average path velocity to assess the effect of possible OA on the early life stages of *C. sinensis* in the future.

## 2 Materials and Methods

### 2.1 Experimental Animals

Mature *C. sinensis* were obtained from Haining City, Zhejiang Province, China. About 1 kg of *C. sinensis* (in average,  $31.5\text{ mm} \pm 6.0\text{ mm}$  in shell length and  $13.0\text{ g} \pm 2.5\text{ g}$  in wet weight) were selected and transported to an aquaculture farm where the clams were acclimated for 72 h at seawater temperature  $25^\circ\text{C}$ , salinity 18, dissolved oxygen (DO) concentration  $>7.0\text{ mg L}^{-1}$  and pH 8.1. The clams were fed with *Isochrysis* spp. twice a day ( $<10^5$  cells  $\text{mL}^{-1}$ ).

### 2.2 Seawater for Culture and Experiments

The experiment was done in nine 50 L tanks (3 replicate each  $p\text{CO}_2$ ). Three pH levels were designed as pH 8.2

representing the current seawater hydron concentration; pH 7.8 mimicking the fluctuation state in the sampling waters (8.2–7.7, Li *et al.*, 2014) and other areas (Caldeira and Wickett, 2005; Melzner *et al.*, 2013) and pH 7.4, an extreme pH especially in hypoxic zones (Cai *et al.*, 2011; Chou *et al.*, 2013). All the *C. sinensis* were transferred to the tanks, respectively, with the same handling condition. The pH was controlled by bubbling pure  $\text{CO}_2$  using a  $p\text{CO}_2/\text{pH}$  feedback STAT system (DAQ-M) associated with WTW pH 3310 meters and SenTix 41 pH electrodes (Loligo Systems Inc.), and was manipulated by CapCTRL software (Loligo Systems Inc.). The salinity was guarded throughout the experiment using a multi-parameter meter (model 5200 A, YSI, USA). Total alkalinity ( $A_T$ ) was measured by titration. Other parameters of the seawater carbonate chemistry, including  $p\text{CO}_2$ , calcite saturation state ( $\Omega_{\text{ca}}$ ), and aragonite saturation state ( $\Omega_{\text{ar}}$ ) were calculated from  $A_T$  and  $\text{pH}_{\text{NBS}}$  using CO2SYS (Lewis *et al.*, 1998). Calculations depended upon a series of constants, K1 and K2, which were brought from Millero (2010). Seawater was sampled twice a day during the experiment, and the pH, total alkalinity, temperature, salinity and other calculated data are listed in Table 1.

Table 1 Seawater carbonate chemistry variables (mean  $\pm$  SD,  $n=5$ ) over the experimental period

Treatments	Temperature ( $^\circ\text{C}$ )	Salinity	$\text{pH}_{\text{NBS}}$	$A_T$ ( $\mu\text{mol kg}^{-1}$ )	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$\Omega_{\text{ca}}$	$\Omega_{\text{ar}}$
pH 8.2	$25.10 \pm 0.07$	$18.12 \pm 0.08$	$8.16 \pm 0.04$	$2263 \pm 26$	$368 \pm 45$	$4.91 \pm 0.52$	$2.95 \pm 0.31$
7.8	$25.08 \pm 0.04$	$18.12 \pm 0.17$	$7.83 \pm 0.03$	$2257 \pm 30$	$872 \pm 79$	$2.49 \pm 0.21$	$1.50 \pm 0.12$
7.4	$25.06 \pm 0.05$	$18.16 \pm 0.08$	$7.37 \pm 0.04$	$2232 \pm 17$	$2631 \pm 311$	$0.91 \pm 0.09$	$0.55 \pm 0.05$

Notes: Seawater pH on the NBS scale ( $\text{pH}_{\text{NBS}}$ ), temperature ( $^\circ\text{C}$ ), salinity, and total alkalinity ( $A_T$ ;  $\mu\text{mol kg}^{-1}$ ) were used to calculate dissolved inorganic carbon (DIC),  $\text{CO}_2$  partial pressure ( $p\text{CO}_2$ ;  $\mu\text{atm}$ ), as well as aragonite ( $\Omega_{\text{ar}}$ ) and calcite ( $\Omega_{\text{ca}}$ ) saturation states.

### 2.3 Ocean Acidification Effect on the Embryonic Development of *C. sinensis*

The air-exposure and flow stimulation methods were adopted for spawning (Guo *et al.*, 2016). Briefly, clams which experienced a 4 h air-exposure were transported to corresponding seawater with a flow generated by a submersible pump. Then the clams produced sperms and eggs. Based on the measured gamete concentrations, 10:1 was adopted as experimental sperm-egg ratio to avoid possible error caused by unreasonably high sperm-egg ratio (Shi *et al.*, 2017a). After 10 minutes, large tissue debris was removed from tank using a 200-mesh sieve and the eggs were collected with a 400-mesh sieve. The eggs were rinsed with corresponding seawater and then transferred to corresponding tanks (about  $15\text{ eggs mL}^{-1}$ ). Larvae were fed

with a mix of diet of *Chaetoceros* sp. and *Isochrysis galbana* from 8 hours post fertilization (hpf). At least 50 eggs or larvae were selected each tank and fixed with 4% paraformaldehyde at 2, 8, 16, 24, 48 hpf for further observation.

Samples for measuring fertilization were taken at 2 hpf and the fertilization rate (%) was calculated by checking the cleaved embryos among eggs:

$$\text{Fertilization rate} = \frac{\text{Number of cleaved embryos}}{\text{Number of total eggs}} \times 100.$$

Samples for hatching rate (%) were taken at 16 hpf. Hatching rate was quantified by observing the trochophore larvae and the D-shaped larvae that swam out of the fertilization eggs:

$$\text{Hatching rate} = \frac{\text{Number of Trochophores} + \text{Number of D-shaped larvae}}{\text{Total number of eggs} \times \text{Fertilization rate}} \times 100.$$

For developmental measurement of *C. sinensis*, the proportions of each developmental stage were calculated at 24, 48 hpf. Classification of deformed and normal larvae depended on morphological criteria set by Guo *et al.* (2016) for clam.

Larval size was measured at 16, 24, 48 hpf using Image-Pro Plus software. A total of 50 larvae were measured

each replicate each  $p\text{CO}_2$ .

### 2.4 Ocean Acidification Effects on Average Path Velocity of Trochophore in *C. sinensis*

Trochophore velocity was measured at 8 hpf using a dissecting microscope (Leica MZ 125, 4 objective) equipped with a camera Sony Exwave HAD (Suquet *et al.*,

2012). Average path velocity (VAP) was measured using a CASA plug for the Image J software. Calibration parameters adopted in this experiment were as following: frame rate, 25 frames<sup>-1</sup>; larval size range, 1 to 1000 pixels; minimum VAP for motile larvae, 30  $\mu\text{m s}^{-1}$ ; minimum number of larvae observed, 30; minimum track length, 25 frames.

## 2.5 Statistical Analysis

Percentage data were square-root and arcsine transformed before using the Shapiro-Wilks normality test and Levine's test for variance homogeneity. Larva size and average path velocity data were not transformed. One-way ANOVA and Duncan's multiple comparisons were used to compare differences among experimental groups when equal variances were assumed. The results are expressed as the means  $\pm$  standard deviation (SD).

## 3 Results

### 3.1 Seawater Chemistry

Three pH were maintained stable throughout the experiment (Fig.1). Salinity was controlled at  $15 \pm 0.4$ , and temperature was kept at  $25^\circ\text{C} \pm 0.3^\circ\text{C}$ . Seawater carbonate chemistry parameters measured and calculated for all treatments were reported in Table 1.

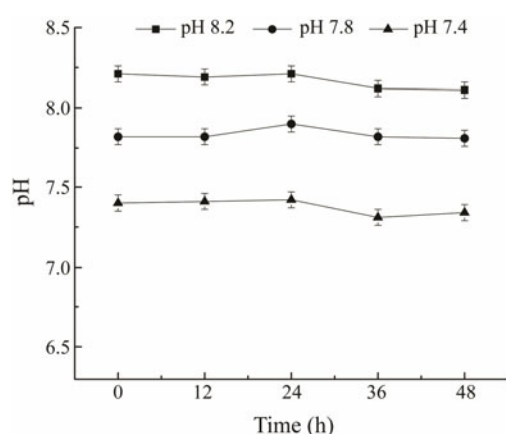


Fig.1 Daily pH (mean  $\pm$  SD) of seawater during a period of 48 h of different pH groups.

### 3.2 Ocean Acidification on Fertilization and Hatching Rate of *C. sinensis*

Both fertilization and hatching rate of *C. sinensis* were significantly affected by OA. They declined with seawater pH. Averagely, 56.5%  $\pm$  5.3% eggs were successfully fertilized at pH 8.2, but the percentage was 53.6%  $\pm$  2.4% at pH 7.8 and 53.2%  $\pm$  2.8% at pH 7.4, respectively. Similarly, the hatching rate at pH 8.2 was 67.0%  $\pm$  1.0%, the percentage at low pH was 65.7%  $\pm$  1.2% and 53.3%  $\pm$  2.5%, respectively (Fig.2).

### 3.3 Ocean Acidification on the Larval Development of *C. sinensis*

At 24 h after fertilization, 80.67% of *C. sinensis* larvae

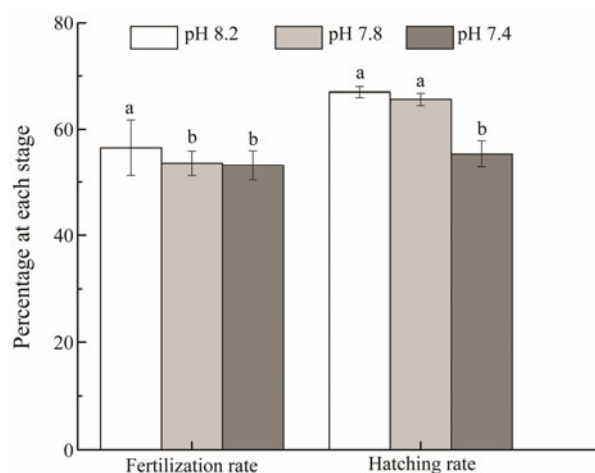


Fig.2 Fertilization and hatching rates of *C. sinensis* when it experiences OA. The error bars represent the standard deviation. Data are the averages ( $n=3$ ). Different lower-case means significant differences between fertilization rate or hatching rate at different pH ( $P < 0.05$ ).

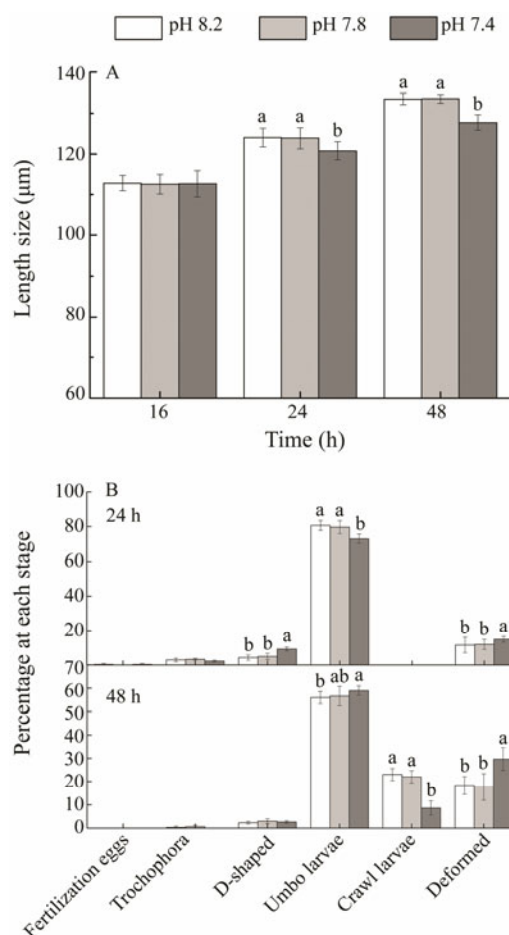


Fig.3 Percentage distribution at each developmental stage of *C. sinensis* embryos/larvae after 24, and 48 h when it experiences OA. The error bars represent the standard deviation. Data are averages ( $n=3$ ). Different lowercase means the significant difference between percentages at different pH but the same developmental stage ( $P < 0.05$ ).

at pH 8.2 and 79.67% at pH 7.8 developed into umbo larvae. However, only 73% completed this process at pH 7.4, which was significantly lower than that of other two

pH. Conversely, deformation percentage at pH 7.4 was significantly higher than those at other two pH. At 48 h post fertilization, 23% larvae at pH 7.4 and 22% at pH 7.8 had entered the crawl larva stage, only 8.67% at pH 7.8 did so. Elevated deformation was found at all pH, and the number of deformations at pH 7.4 was still larger than those at the other two pH (Fig.3).

### 3.4 Effect of Ocean Acidification on Larval Size of *C. sinensis*

The size of *C. sinensis* larvae was measured at 16 hpf, 24 hpf and 48 hpf, respectively. There was no significant difference in larval length and height at 16 hpf. The larval length and height at pH 7.4 were significantly smaller than those at pH 7.8 and pH 8.2 since 24 hpf (Fig.4).

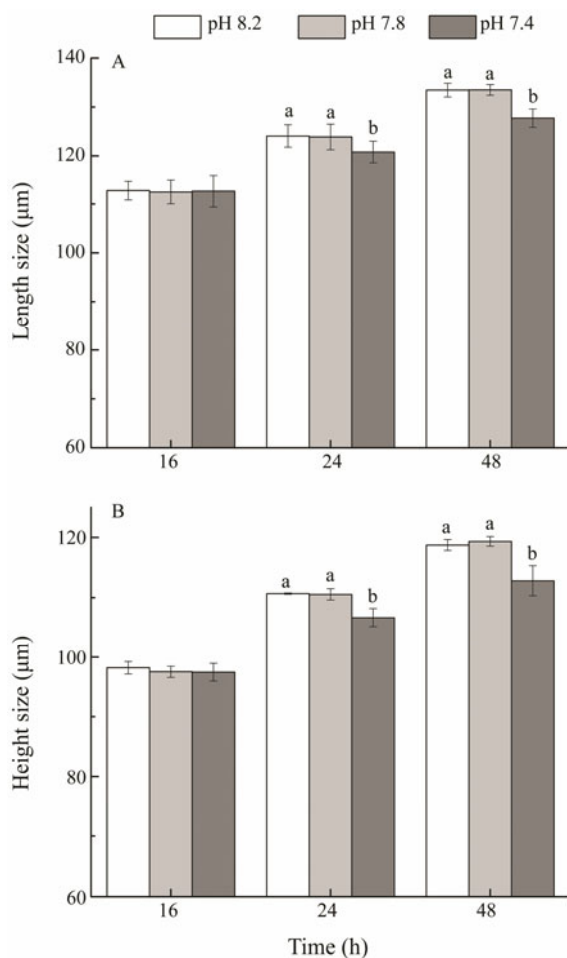


Fig.4 Larvae size of *C. sinensis* when it experiences OA. The error bars represent the standard deviation. Data are averages ( $n = 3$ ). Different lowercase means significant difference between larval size at different pH but the same sampling time ( $P < 0.05$ ).

### 3.5 Ocean Acidification Effect on Trochophores Average Path Velocity

Average path velocity (VAP) of trochophores at pH 7.4 was significantly depressed but not at pH 7.8 and 8.2. VAP at pH 8.2 reached up to  $103 \mu\text{m s}^{-1}$ . At pH 7.8, this was  $99 \mu\text{m s}^{-1}$ , and at pH 7.4, VAP was only  $62 \mu\text{m s}^{-1}$  (Fig.5).

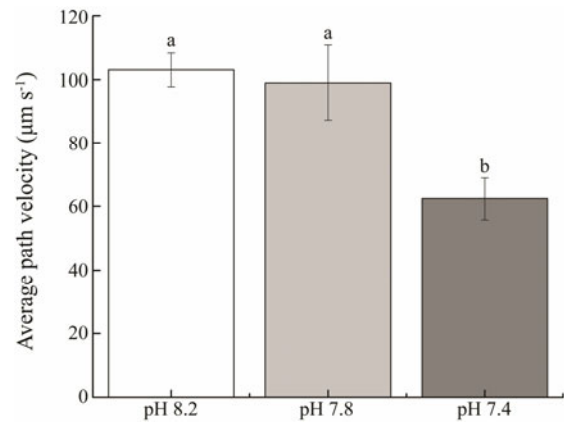


Fig.5 Average path velocity of trochophore of *C. sinensis* when it experiences OA. The error bars represent the standard deviation. Data are averages ( $n = 3$ ). Different lowercases mean a significant difference between average path velocity of trochophore at different pH ( $P < 0.05$ ).

## 4 Discussion

This study provided a scientific evidence for that OA brings a negative effect to calcifying organisms by affecting their development parameters at their early stages, such as fertilization, hatching success, size and average path velocity of larva.

The results of this study on fertilization revealed that decreased seawater pH only significantly inhibited the fertilization of *C. sinensis* when pH value dropped to 7.4, and moderate acidification brought a slight negative effect to this process. Similarly, Van Colen *et al.*, (2012) found that moderate seawater acidification brought a slight adverse effect on the fertilization of *Macoma balthica*, but under extreme acidic condition, fertilization was significantly reduced. Shi *et al.* (2017b) demonstrated that the fertilization increased with decreased seawater pH and attributed this phenomenon to the reductions in the opportunity of sperm-egg collisions by decreasing sperm velocity and the chance of gamete fusion for every gamete collision activity and the intracellular  $\text{Ca}^{2+}$  oscillation disturbance when the organisms experience OA. However, conclusions about the effect of OA on calcifying organism fertilization were different. Parker *et al.*, (2010) pointed out that OA brought a significant effect to the oyster from Port Stevens (Australia) whereas Kurihara *et al.* (2007) and Maggs and Samuela (2009) showed that there was no sign of decreased fertilization under OA in *C. gigas* obtained from the East China Sea and off Western Sweden. This implies that the effect of OA on organism fertilization may be species-specific.

The swimming velocity has been proposed as a larva quality indicator (Myrina *et al.*, 2015). In this experiment, the VAP of trochophora at 8 pfh was depressed at pH 7.4. Similar results were obtained in *Ahiura filiformis*. Chan *et al.* (2016) pointed out that the reduced pH brought significant negative effects on swimming velocity of larval brittle stars. However, researches in *Dendraster excentricus* demonstrated that seawater acidification had no significant effect on larva swimming (Chan *et al.*, 2011).

One possible explanation is that the three species showed various physiological responses to OA, *i.e.*, lowered swimming velocity for *C. sinensis* and *A. filiformis*, but stable swimming velocity for *D. excentricus*. Subsequently, larva body size was measured. At the initial moment, there was no significant difference among different groups. With the development going on, the body size of larvae at pH 7.3 was significantly depressed. The result was consistent with the findings documented previously (van Colen *et al.*, 2012). This may be attributed to energy allocations, *e.g.*, under OA, calcifying organisms need much more energy to maintain acid-base balance (O'Donnell *et al.*, 2010; Stumpp *et al.*, 2012; Lewis *et al.*, 2016). Alternatively, lowered swimming velocity may reduce the chance of larva initiation of feeding (Qiu *et al.*, 2015). As documented in other calcifying organisms, OA delayed *C. sinensis* larva development and increased the deformed individuals (Wang *et al.*, 2016). The delay in development implied that larvae should take more time to complete the same developmental stage, which might increase the probability of loss by predation in nature (Dupont and Thorndyke, 2009).

The results of our study showed that early development of *C. sinensis* was susceptible to OA. Reduced survival rate and delayed development would likely bring population scale impacts. The predicted OA might reduce the number of *C. sinensis* recruit and lead to a decline in fishery.

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