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Note

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Effects of pH Change by CO_2 Induction and Salinity on the Hatching Rate of *Artemia franciscana*

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Abstract

To understand the effects of lower pH levels due to elevated CO_2 and salinity, we designed and constructed a pH-control system that included automatic CO_2 infusion and measured the hatching rate of a crustacean model species, *Artemia franciscana*. The pH-control system was cost-effective and capable of performing animal tests in which pH fluctuated around 8.0 ± 0.1, with the temperature around 27 ± 0.5°C. Hatching rate was observed under four different pH levels (7.0, 7.3, 7.6, and untreated control) combined with three salinity ranges (15, 25, and 35 ppt). The results demonstrated that lower pH levels led to decreased hatching rates regardless of salinity, and the minimum hatching rate was detected at pH 7.0 compared to the control (pH 8.0 ± 0.1), supporting the idea that OA has adverse effects on hatching rates and increases the risk of juveniles being introduced in the ecosystem. In contrast, salinity changes exhibited no synergistic effects with pH and had independent effects.

Key words: Ocean acidification, Hatching rate, *Artemia franciscana*, Carbon dioxide (CO_2), pH, Salinity

Introduction

The world oceans are vast reservoirs of carbon dioxide (CO_2) (Feely et al., 2004; Sabine et al., 2004). The atmospheric CO_2 concentration has increased rapidly over past decades due to the burning of fossil fuels and other anthropogenic activities (Intergovernmental Panel on Climate Change, 2007). Dissolution of CO_2 in seawater shifts the carbonate equilibrium, increasing the H^+ ion concentration (i.e., pH) and decreasing the CO_3^{2-} concentration. Increased pCO_2 has led to a 0.1 unit decrease in surface ocean water pH over the past 200 years (Caldeira and Wickett, 2003) and it is projected to decrease by 0.3 to 0.4 units by 2100 (Caldeira and Wickett, 2003; Raven et al., 2005). Reductions in seawater pH have detrimental effects on the development and reproductive processes of many marine organisms (Pörtner et al., 2004, 2005; Raven et al., 2005). Also, increases in CO_2 have been suggested to not only affect individuals but also the entire living ecosystem (Widdicombe and Spicer, 2008).

Mimicking ocean acidification (OA) has been difficult to study its effects on ecosystems and animals, although several experimental systems have been designed to estimate the effects of OA. Some groups have designed microcosm systems in which concentrated CO_2 was manipulated by air-CO_2 gas mixing systems (Findlay et al., 2008; Arnold et al., 2009; Egilsdottir et al., 2009). Other groups have used different molecular acids (H_2SO_4) to control pH manually, with pH being monitored once a day (Zalizniak et al., 2009). The designs of the pH-controlling systems noted above either require large budgets and space or well-trained workers to obtain reliable data. In the present study, we constructed a pH controller that was a simple, cost-effective, and automatically operated system for maintaining water pH accurately.

Until now, studies on the effects of OA have mainly focused on the growth and development of calcareous marine organisms, including corals (Reynaud et al., 2003; Langdon

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and Atkinson, 2005), mollusks (Michaelidis et al., 2005; Ellis et al., 2009), echinoderms (Dupont et al., 2008; Havenhand et al., 2008), copepods (Kurihara et al., 2004), and amphipods (Egilsdottir et al., 2009). Since crustaceans are considered one of the most vulnerable groups because of their dependence on the availability of calcium and bicarbonate ions for the mineralization of their exoskeleton. The effects of lower pH (either from direct exposure to HCl or CO_2) have been noted on the survival and growth of shrimps *Penaeus monodon* (Allan and Maguire, 1992), *Penaeus borealis* (Bechmann et al., 2011), *Palaemon pacificus* (Kurihara et al., 2008), and *Homarus americanus* (Arnold et al., 2009). Little attention has been paid to the synergistic effects of salinity and CO_2-mediated pH on hatching rates for elucidating the effects of CO_2 on coastal and estuarine ecosystems.

In the present study, we developed a cost-effective pH-control system that can maintain preset seawater pH by automatically regulating CO_2 injections. This system was reliable for various animal experiments related to OA. Once the reliability of the system had been confirmed, four different pH treatments (7.0, 7.3, 7.6, and control) combined with three salinity levels (15, 25, and 35 ppt) were maintained and the hatching rate of *Artemia* was measured.

Materials and Methods

Artemia

Commercially available *Artemia franciscana* cysts (Red Fish brand; Golden Sea Aquatic Products Co., Ltd ., China) were purchased and stored at 4°C until use. *A. franciscana* is considered the ideal crustacean model due to the ease of culture and maintenance and its short life cycle of 3-4 weeks.

pH-control system using automatic CO_2 injection

A CO_2 control system was constructed by assembling a CO_2 gas tank, pH sensors, a pH monitor, and an automatic pH controller. Fig. 1 depicts how different pH levels were maintained over the experimental period by the automatic pH controller. The pH level was set at the onset of an experiment and a pH sensor was placed in each hatching cylinder (1 L). Another sensor from the pH controller was linked to the CO_2 container and regulated the amount of CO_2 injected into the water. A solenoid valve connected to the pH controller allowed the CO_2 input to be switched on and off automatically to achieve a constant pH, which could be checked with the pH monitor.

Measurement of the hatching rate

An experiment was conducted to investigate the effects of pH changes that were induced by CO_2 and salinity on *Artemia* hatchings. For this purpose, three pH treatments were chosen: 7.0, 7.3, and 7.6. Untreated seawater was used as a control (pH 8.0 ± 0.1). Three replicates were conducted for each experimental treatment. One gram of *Artemia* cyst was used for each pH treatment. Cysts were incubated in 500 mL of seawater in a 1,000 mL glass cylinder with continuous vigorous aeration. Salinity was measured by a temperature corrected refractometer (YSI Inc., Swedesboro, New Jersey, USA) and salinity was adjusted by adding deionized water to autoclaved stock seawater solution. Temperature was maintained at 27 ± 0.5°C by a thermostat and a light intensity of 2000 Lux was supplied. The hatching rate was assessed after 24 h using the counting method of the Laboratory of Aquaculture & Artemia Reference Center, Ghent, Belgium. With a Pasteur's pipette, 50-mL aliquots were taken from each cylinder. Hatching rate was determined as the number of hatched cysts versus unhatched cysts or partially hatched cysts × 100 (Clegg and Conte, 1980). Hatching rates were evaluated statistically by t-tests using the Sigma plot program and values of $P < 0.05$ were considered statistically significant.

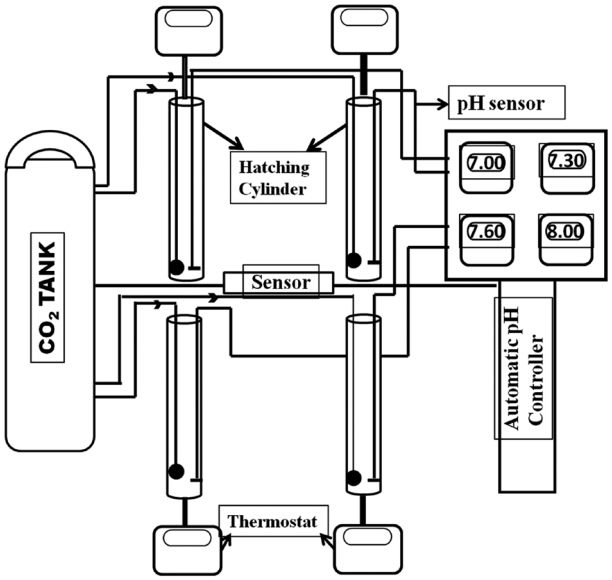

Fig. 1. The flowchart of a CO_2 controlling system. Location of sensor, thermostat and pH controller is indicated.

Results and Discussion

To confirm the reliability of the pH-control system as described above, blanks without *Artemia* were used. After 2 days of experimental operation, salinity and pH were still being adjusted properly. pH and temperature were maintained at 8.00 ± 0.1 and 27 ± 0.5°C, respectively, indicating that the system could be used to perform animal tests of OA (Fig. 1).

After 24 h of cyst incubation, CO_2-mediated pH changes had significant effects on the hatching rate of *Artemia*. The

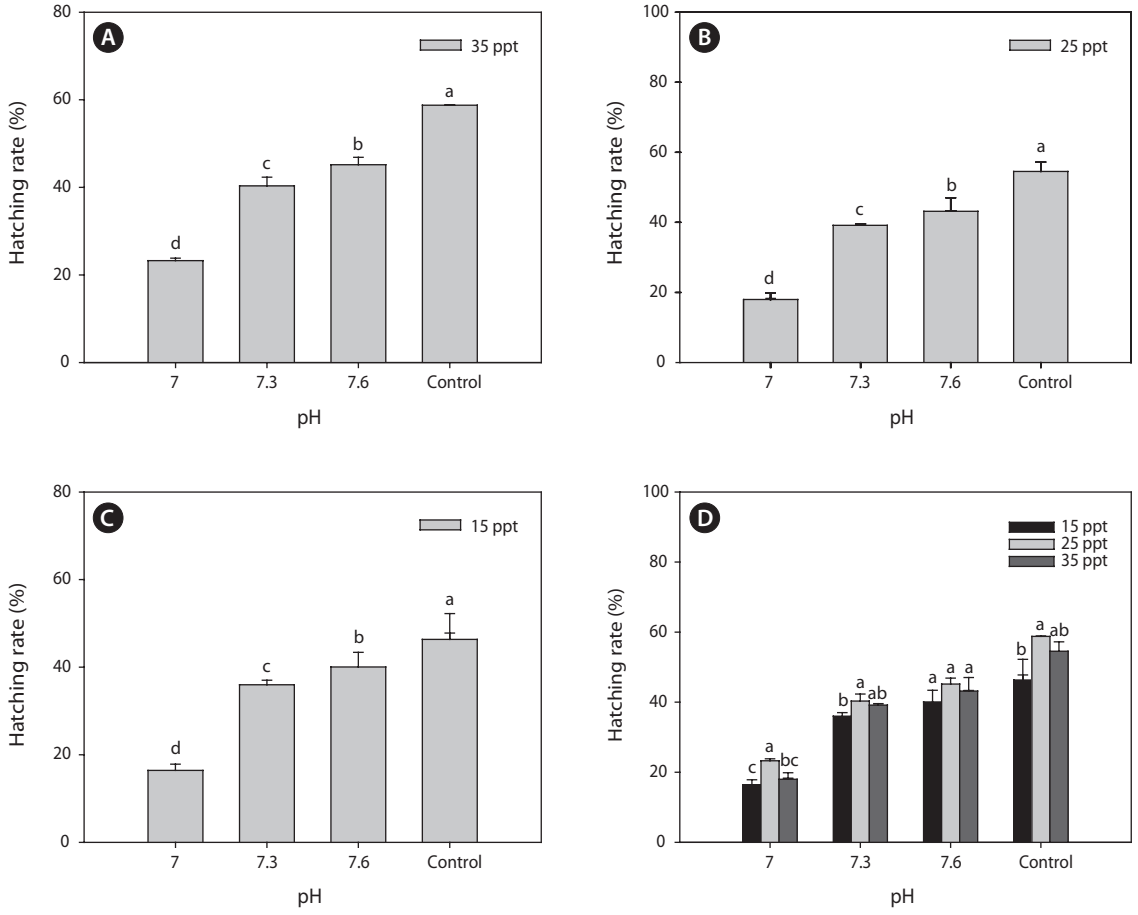

Fig. 2. Hatching rate of Artemia at different pH and salinity. (A) 35 ppt salinity, (B) 25 ppt salinity, (C) 15 ppt salinity, and (D) comparison of hatching rate at different pH and salinities Statistical difference is indicated by different letters on the top of the bar and taken as significant when *P*-value is <0.05.

maximum hatching rate was observed with the control pH (~8.0) and decreased significantly to the lower pH (7.0), irrespective of salinity (Fig. 2A-2C). At a salinity of 15 ppt, the mean hatching rate decreased to 17% with pH 7.0, whereas it was 46% with the control pH (~8.0). Both the 25 and 35 ppt salinity treatments exhibited similar patterns of decreased hatching rates, dropping to 22% and 18%, respectively, in the lowest pH (7.00) treatment. Hatching rate decreased by 2.7, 2.5, and 2.9 times compared to the control pH with salinity levels of 15, 25, and 35 ppt, respectively (Fig. 2D). A study on the effects of H_2SO_4 acidified seawater on the hatching and survival of *A. franciscana* at 10 different pH levels (4 to 8.5) showed that the hatching rate was highest at pH 7.3 and decreased significantly at either lower or higher pH levels (Doyle and McMahon, 1995). In contrast, we found the highest hatching rate with the control pH (~8.0) and the rate decreased significantly as pH was reduced to 7.0. The difference between the two results may have been due to the different methods used to lower pH. Previous experiments used mineral acid (HCl), but in this study, we used molecular CO_2 to alter the pH. The effects of CO_2/HCl acidified seawater were observed on the fertilization rate of two sea urchins, *Echinometra mathaei* and *Hemicentrotus pulcherrimus* (Kurihara and Shirayama, 2004); the fertilization rate of eggs decreased linearly in high pCO_2 seawater, but it only decreased at pH < 7.0 in HCl acidified seawater. The reason for this difference was likely the diffusion capacity of CO_2 and protons. Molecular CO_2 diffuses directly through the biological cell membrane faster than protons (Gutknecht et al., 1977); hence it causes a faster decrease in the intracellular pH of eggs compared to HCl/H_2SO_4. The low intracellular pH may prevent fertilization and subsequent embryonic development (Kurihara, 2008). Both the hatching rate and nauplius survival in two marine copepods (*Acartia steueri* and *Acartia erythraea*) were significantly reduced at pH 6.8, which was the lowest level observed. Similarly, early development in the oyster *Crassostrea gigas* can be delayed because of exposure to acidified seawater with pH 7.4 (Kurihara et al., 2007). However, the survival, growth, morphology, and development of *Acartia tsuensis* eggs were unaffected at all stages when reared under 2000 uatm pCO_2, which is equivalent to pH 7.3 (Kurihara and Ishimatsu, 2008).

In this study, the hatching rate of *A. franciscana* was affect-

ed by reduced pH regardless of salinity and no notable synergistic effects were observed. Embryonic development in the amphipod *Echinogammarus marinus* was investigated using CO_2 acidified seawater with different salinity levels; low pH (7.5) resulted in prolonged embryonic development regardless of salinity, but reduced salinity, not lower pH, had a significant effect on the number and calcium content of hatchlings (Egilsdottir et al., 2009). The highest hatching percentage in horseshoe crab, *Tachypleus gigas*, was observed with salinity levels from 25 to 35 ppt, but no hatched nauplii were found at salinity levels of 15 and 20 ppt (Zaleha et al., 2011). Similar findings regarding hatching rate were observed in *A. urmiana* and parthenogenetic *Artemia* (Asem and Rastegar-Pouyani, 2010). These results support our findings that 25 ppt resulted in the highest hatching rate.

In conclusion, we constructed a cost-effective pH-control system involving automatic CO_2 injection that could be used to study the effects of CO_2-induced reductions in pH on marine animal physiology. We also demonstrated that pH and salinity changes had independent effects on the hatching rate of *A. franciscana.* This suggests that organisms spawned in coastal regions and eggs drifted from deep seas to coastal areas, where salinity and pH changes occur frequently, may be challenged by the adverse effects of OA. However, synergistic effects of salinity and pH may not occur and would be influenced more by species-specific traits of physiology.

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