

## Article

# Effects of Enhanced $p\text{CO}_2$ and Temperature on Reproduction and Survival of the Copepod *Calanus sinicus*

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**Abstract :** We tested the combined effects of increased partial pressure of  $\text{CO}_2$  ( $p\text{CO}_2$ ) and temperature on the reproduction and survival of the copepod *Calanus sinicus* from Asan Bay, the Yellow Sea under laboratory conditions to understand the impact of acidification on copepods. Egg production rate, survival rate, and fecal pellet production of *C. sinicus* were not affected by 1305 ppm  $p\text{CO}_2$  or with combined treatments of temperature and  $p\text{CO}_2$ , including 8°C and 289 ppm  $p\text{CO}_2$  (ambient), 8°C and 753 ppm  $p\text{CO}_2$  (high  $p\text{CO}_2$ ), 12°C and 289 ppm  $p\text{CO}_2$  (high temperature), and 12°C and 753 ppm  $p\text{CO}_2$  (greenhouse), for 5 or 10 d of exposure. However, egg hatching success of *C. sinicus* decreased significantly in the greenhouse treatment compared with the ambient or the high  $p\text{CO}_2$  treatments. These results suggest that a combined treatment ( $p\text{CO}_2$  and temperature) affected egg viability more than a single treatment ( $p\text{CO}_2$ ).

**Key words :**  $p\text{CO}_2$ , temperature, copepods, reproduction, survival

## 1. Introduction

The recent increase in atmospheric  $\text{CO}_2$  concentration is inducing global warming and acidifying the ocean. Global warming and reduced ocean pH can affect marine ecosystems directly and indirectly (Caldeira and Wickett 2003; Feely et al. 2004, 2009; Orr et al. 2005). Considerable attention has been paid to understand the potential effects of ocean acidification on marine animals and ecosystems (Kleypas et al. 2006; Fabry et al. 2008; Doney et al. 2009).

Ocean acidification can affect calcifying and non-calcifying marine animals. Calcifying animals, such as coccolithophores, foraminiferans, gastropods, bivalves, corals, and echinoderms, have a calcium carbonate shell

that dissolves or calcium carbonate formation can be reduced (Caldeira and Wickett 2003; Kurihara et al. 2004; Kleypas et al. 2006; Fabry et al. 2008; Kurihara 2008; Lischka et al. 2010; Kim et al. 2013b; Sung et al. 2014; Lee and Kim 2016). A decrease in seawater pH can negatively affect reproduction and survival of non-calcifying animals, such as copepods and other crustacean zooplankton (Kurihara et al. 2004; Mayor et al. 2007; Fabry et al. 2008; Kawaguchi et al. 2010; Zhang et al. 2011; Weydmann et al. 2012; McConville et al. 2013; Pedersen et al. 2013; Zervoudake et al. 2013). Research on the impact of ocean acidification on non-calcifiers, such as copepods, has increased (Mayor et al. 2007; Kurihara and Ishimatsu 2008; Zhang et al. 2011; McConville et al. 2012; Zervoudaki et al. 2013; Isari et al. 2015).

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Copepods dominate marine zooplankton in terms of biomass in most of the world's oceans (Mauchline 1998), and the effects of increasing partial pressure of CO<sub>2</sub> (*p*CO<sub>2</sub>) on the response of marine copepods have been studied (Kurihara et al. 2004; Mayor et al. 2007, 2012; Kurihara and Ishimatsu 2008; Zhang et al. 2011; Weydmann et al. 2012; McConville et al. 2013; Pedersen et al. 2013). However, most studies have focused on the effects of higher *p*CO<sub>2</sub> seawater on reproduction and survival rates of copepods, which is not realistic for the near-future *p*CO<sub>2</sub> scenario (e.g., ~900 ppm in 2100; RCP8.5 scenario, IPCC 2013). Although upwelling events (Feely et al. 2008; Kim et al. 2013b) and eutrophication (Kim et al. 2013a; Lee and Kim 2016) in nearshore may reduce pH of seawater, such higher *p*CO<sub>2</sub> may be valid only around the area of marine CO<sub>2</sub> sequestration (Zhang et al. 2011; Mayor et al. 2012; Weydmann et al. 2012; McConville et al. 2013; Pedersen et al. 2013). Thus, it is necessary to understand the impact of low *p*CO<sub>2</sub> comparable to expected *p*CO<sub>2</sub> scenarios in 2100 on marine copepods.

Marine animals can be affected additively or synergistically by multiple stressors associated with global warming, such as ocean acidification, increased temperature, anoxia, and pollution (Invidia et al. 2004; Kurihara and Ishimatsu 2008; Vehmaa et al. 2012; Zervoudaki et al. 2013). Scientists have noted the need for studies on the combined effects of *p*CO<sub>2</sub> and temperature on marine organisms (Hare et al. 2007; Kurihara 2008; Feng et al. 2009; Rose et al. 2009). However, relatively little information is available on the combined impact of increased *p*CO<sub>2</sub> and temperature on copepod reproduction and survival (Invidia et al. 2004; Zervoudaki et al. 2013).

The copepod *Calanus sinicus* is an important component of zooplankton biomass and a key ecological species in Korean waters, the Yellow Sea, and the northern East China Sea (Uye 1988; Park 1997; Wang et al. 2003; Zhang et al. 2005; Kang et al. 2011). *Calanus sinicus* is important prey for anchovy, sand eel, and sardine in the continental shelf ecosystem (Uye 2000). Although some biological studies of *C. sinicus*, including feeding, fecundity, growth, and their spatiotemporal distribution have been conducted (Uye 1988; Kang et al. 2007; Huo et al. 2008; Wang et al. 2009; Kang et al. 2011), little is known about the impact of increased *p*CO<sub>2</sub> and temperature on survival and fecundity of *C. sinicus* in Korean waters.

The purpose of this study was to understand the combined effects of increased *p*CO<sub>2</sub> and temperature on survival rate, egg production rate (EPR), and egg hatching

success (EHS) of the copepod *C. sinicus* under laboratory conditions.

## 2. Materials and Methods

### Sampling of live copepods

*Calanus sinicus* were collected from Asan Bay, the Yellow Sea, Korea on 8 June 2009 and 30 April 2010 using a conical plankton net (200 µm mesh size, 50 cm diameter) and held in an insulated box. The samples were transported to the laboratory within 1.5 h. At the sampling site, water temperature was measured using a thermometer (YSI 60; YSI Inc., Yellow Springs, OH, USA). In the laboratory, healthy adult *C. sinicus* females were sorted out and incubated in 800 mL glass beakers filled with GF/C (Whatman, Maidstone, UK)-filtered seawater and cultured phytoplankton as food ( $4 \times 10^4$  cells mL<sup>-1</sup> *Isochrysis galbana* and  $1 \times 10^3$  cells mL<sup>-1</sup> *Tetraselmis suecica*) for 24 h with 14 h dim light and 10 h darkness at either 8 or 17°C depending on the experimental set up to acclimate under the laboratory conditions.

### Seawater treatment

GF/C-filtered seawater was acidified in a 20 L carboy bottle by bubbling with compressed air containing 5000–6000 ppm *p*CO<sub>2</sub>; flow rate, 15 mL min<sup>-1</sup>) until the seawater *p*CO<sub>2</sub> was adjusted to the various experimental values (Table 1). Air and CO<sub>2</sub> gas flow rates were adjusted using a flow meter (KO-FLOC; Kojima Instruments Inc., Kyoto, Japan).

To determine the correct bubbling time for a given seawater *p*CO<sub>2</sub>, duplicate seawater subsamples were collected regularly into a 500 mL biological oxygen demand bottle, and seawater *p*CO<sub>2</sub> was estimated. Seawater *p*CO<sub>2</sub> was calculated from pH and dissolved inorganic carbon (DIC)/total alkalinity (TA) data using the CO2SYS software program (Lewis and Wallace 1998). pH was determined by the pH electrode (Orion A211; Thermo Scientific, Waltham, USA). DIC was analyzed using a highly precise gas extraction/coulometric detection system, consisting of an instrument for detecting TA (VINDTA; www.Marianda.com) coupled with a CO<sub>2</sub> coulometer (model 5012; UIC Coulometrics Joliet, IL, USA). TA was measured by potentiometric titration using a TA Gran titration system (model AS-ALK2; Apollo SciTech, Inc., Newark, DE, USA). We used the same air + CO<sub>2</sub> bubbling time to make all given acidified seawater once the correct bubbling time for a given acidified seawater (Table 1) was determined before starting the experiment. For example,

**Table 1. Experimental conditions for Experiment-I and Experiment-II, including temperature,  $p\text{CO}_2$ , pH, dissolved inorganic carbon (DIC), and total alkalinity (TA)**

Treatment	Temperature (°C)	$p\text{CO}_2$ (ppm)	pH	DIC ( $\mu\text{mol kg}^{-1}$ )	TA ( $\mu\text{mol kg}^{-1}$ )
Experiment-I		(n = 10)	(n = 10)		(n = 10)
400 ppm	17	426 $\pm$ 30	8.137 $\pm$ 0.014	nd	2207 $\pm$ 120
1300 ppm	17	1305 $\pm$ 108	7.697 $\pm$ 0.033	nd	2170 $\pm$ 28
Experiment-II		(n = 8)	(n = 8)	(n = 8)	
Ambient	8	289 $\pm$ 20	8.285 $\pm$ 0.022	1969 $\pm$ 38	nd
High $p\text{CO}_2$	8	753 $\pm$ 80	7.916 $\pm$ 0.039	2013 $\pm$ 37	nd
High temperature	12	289 $\pm$ 20	8.285 $\pm$ 0.022	1969 $\pm$ 38	nd
Greenhouse	12	753 $\pm$ 80	7.916 $\pm$ 0.039	2013 $\pm$ 37	nd

nd: not determined

17 min of bubbling with air containing 5000 ppm  $\text{CO}_2$  gas was sufficient to make  $\sim 750$  ppm  $p\text{CO}_2$  seawater.

Control seawater was prepared by bubbling compressed air at a flow of 15 mL  $\text{min}^{-1}$  for 1 h to make  $\sim 400$  ppm  $p\text{CO}_2$  seawater. However, seawater  $p\text{CO}_2$  in the control treatment for Experiment-II was slightly lower than expected (e.g., 289 ppm; apparently similar to seawater  $p\text{CO}_2$  in the past century; Raven et al. 2005). We assumed that there would be no difference in the copepod response between 289 and  $\sim 400$  ppm, which is the current seawater  $p\text{CO}_2$  (IPCC 2013).

We measured seawater  $p\text{CO}_2$  in 20 L of stock seawater using the above-mentioned method to determine seawater  $p\text{CO}_2$  of the control and treatment seawater at the beginning of the experiments.

#### **Egg production, egg hatching success, survival, and fecal pellet production rates**

*Calanus sinicus* adult females acclimated under laboratory conditions were transferred to 500 mL incubation bottles filled with seawater of various  $p\text{CO}_2$  values and enriched with  $4 \times 10^4$  cells  $\text{mL}^{-1}$  *I. galbana* and  $1 \times 10^3$  cells  $\text{mL}^{-1}$  *T. suecica* phytoplankton as food. We used an egg production cylinder with 300  $\mu\text{m}$  mesh at the bottom of the cylinder in the incubation bottle to minimize egg cannibalism by adult females during the incubation.

Two to five replicates depending on the experiment were prepared for each condition (Table 1). Five *C. sinicus* females were kept in each bottle, and the bottles were sealed tightly with a cap. The control and experimental bottles were incubated for 5–10 d under 14 h dim-light and 10 h darkness.

Survival and fecal pellet production rates were measured

every day for 5 d in Experiment-I. The EPR, EHS, and survival rate were measured every 2 d for 10 d in Experiment-II. Both temperature and  $p\text{CO}_2$  increased simultaneously in the greenhouse treatment in Experiment-II (Table 1).

Eggs spawned in the egg production cylinder were collected gently by back washing with GF/C-filtered seawater and counted under a stereomicroscope (Stemi-2000C; Carl Zeiss Inc., Jena, Germany). Then, the eggs were transferred to 20 mL glass vials filled with GF/C-filtered seawater at various  $p\text{CO}_2$  values and incubated at the same temperature for 48–72 h depending on the temperature. EHS was calculated as the number of nauplii present after 72 h/the number of eggs added at the start of the egg incubation  $\times 100$  (Mayor et al. 2007). Abnormal nauplii and eggs were considered unhatched eggs (Kang and Poulet 2000).

Fecal pellet production was measured every day in Experiment-I to understand feeding activity on the artificial phytoplankton. The fecal pellets were collected using a Pasteur pipette and fixed in Lugol's solution. The volume of fecal pellets was measured under an inverted microscope (Olympus IX51; Tokyo, Japan) using an image analysis program (i-solution; IMTechnology, Taftville, CT, USA), and volume was converted to carbon content (Riebesell et al. 1995; Riser et al. 2001).

We also measured the *in situ* EPR of the same population of adult females in Experiment-II to understand whether the laboratory conditions biased the EPR pattern. Twelve *C. sinicus* adult females were reared individually in 50 mL glass jars filled with 8°C surface seawater from the field. The seawater was replaced every 2 d with fresh 8°C surface seawater.

### Statistical analysis

Survival and fecal pellet production rates were compared daily between 400 and 1300 ppm  $p\text{CO}_2$  for Experiment-I using the Mann-Whitney  $U$ -test, due to the small sample size. The survival rate and EPR were compared daily for Experiment-II, but EHS was compared with the mean value for 10 d, among the ambient, high  $p\text{CO}_2$ , high temperature, and greenhouse treatments (Table 1), using two-way analysis of variance (ANOVA). Tukey's post-hoc test was conducted when the difference in means among treatments was significant (i.e.,  $p < 0.05$ ). The survival rate and EHS data were arcsine transformed and the mean and standard deviation were calculated. The EPR was  $\log_{10}(x + 1)$  transformed before analysis. All statistical analyses were conducted using SYSTAT ver. 13 software (Systat Software Inc., Chicago, IL, USA).

## 3. Results

### Experiment-I

#### Fecal pellet production

In Experiment-I, we measured fecal pellet production and survival rate of *C. sinicus* adult females because most adult females did not spawn during the incubation. However, the adult females ingested the phytoplankton diet (i.e., *I. galbana* and *T. suecica*) during the experiment and produced fecal pellets under both  $p\text{CO}_2$  conditions. Fecal pellet production was  $0.35\text{--}0.47 \mu\text{gC female}^{-1} \text{d}^{-1}$  (mean,  $0.41 \pm 0.20 \mu\text{gC female}^{-1} \text{d}^{-1}$ ) for 400 ppm and  $0.38\text{--}0.71 \mu\text{gC female}^{-1} \text{d}^{-1}$  (mean,  $0.53 \pm 0.24 \mu\text{gC female}^{-1} \text{d}^{-1}$ ) for 1300 ppm at  $17^\circ\text{C}$ . Daily fecal pellet production of adult females did not differ between the 400

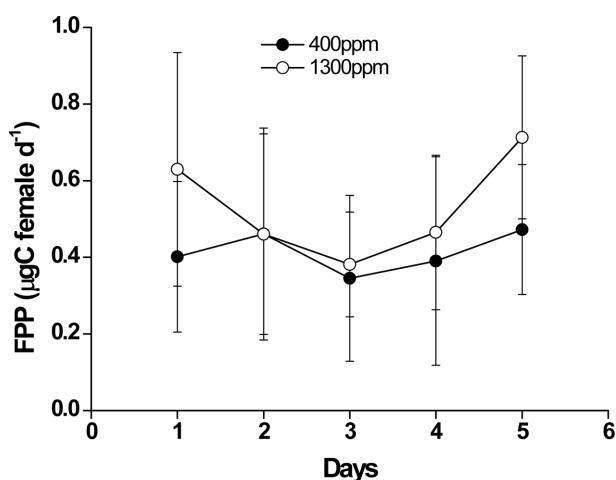


Fig. 1. Variations in fecal pellet production (FPP) of adult female *Calanus sinicus* in Experiment-I

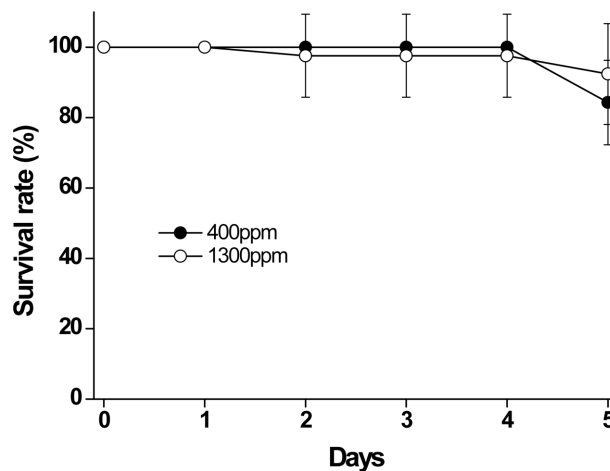


Fig. 2. Variations in survival rate of adult female *Calanus sinicus* in Experiment-I

and 1300 ppm  $p\text{CO}_2$  conditions over 5 d (Fig. 1; Mann-Whitney  $U$ -test,  $p > 0.05$ ).

#### Survival rate of adult females

Survival rates were 84.3–100% (mean,  $99.5 \pm 3.9\%$ ) for 400 ppm  $p\text{CO}_2$  and 92.4–100% (mean,  $98.4 \pm 8.0\%$ ) for 1300 ppm  $p\text{CO}_2$  at  $17^\circ\text{C}$ . Daily survival rate of adult females did not differ between the 400 and 1300 ppm  $p\text{CO}_2$  conditions over 5 d (Fig. 2; Mann-Whitney  $U$ -test,  $p > 0.05$ ), indicating that the survival rate of copepods was not affected by increasing  $p\text{CO}_2$  to 1305 ppm.

### Experiment-II

#### Survival rate of adult females

Survival rate of the ambient treatment decreased to ca.

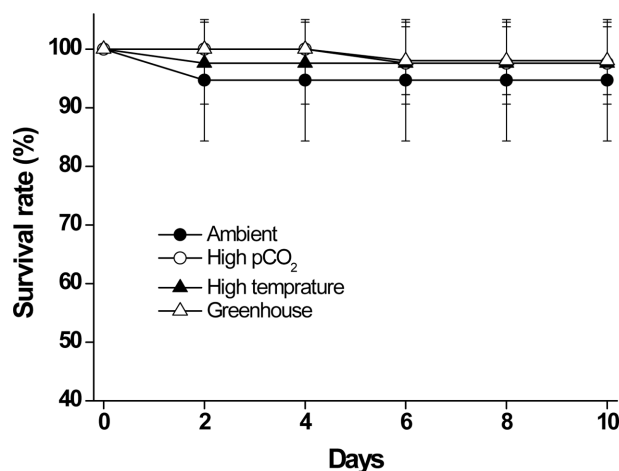


Fig. 3. Variations of survival rate of adult female *Calanus sinicus* in Experiment-II

90% on day 2 and then stabilized until the end of the experiment, due to individual variability between adult females (Fig. 3). Survival rates of the copepods were 94.7–100% (mean,  $96.3 \pm 5.6\%$ ) for the ambient, 97.6–100% (mean,  $99.4 \pm 3.1\%$ ) for the high  $p\text{CO}_2$ , 97.6–100% (mean,  $98.4 \pm 4.5\%$ ) for the high temperature, and 98.0–100% (mean,  $99.5 \pm 2.6\%$ ) for the greenhouse treatments. However, daily survival rate of adult females did not differ among the four treatments [Fig. 3; two-way ANOVA,  $p > 0.05$ ; daily survival rate of ambient ( $n = 2$ ), high  $p\text{CO}_2$ , high temperature, and greenhouse ( $n = 3$ )]. No significant interaction was detected between  $p\text{CO}_2$  and temperature for the daily survival rate ( $p > 0.05$ ).

#### Egg production rate

The EPR for the four treatments was highest on day 2 and then decreased sharply below ca. 5 eggs female  $2\text{ d}^{-1}$  (Fig. 2). The EPR was 0.6–17.7 eggs female  $2\text{ d}^{-1}$  (mean,  $4.3 \pm 7.4$  eggs female  $2\text{ d}^{-1}$ ) for the ambient, 0.5–20.7 eggs female  $2\text{ d}^{-1}$  (mean,  $5.4 \pm 9.0$  eggs female  $2\text{ d}^{-1}$ ) for the high  $p\text{CO}_2$ , 0.1–22.8 eggs female  $2\text{ d}^{-1}$  (mean,  $6.7 \pm 9.2$  eggs female  $2\text{ d}^{-1}$ ) for the high temperature, and 0.8–20.8 eggs female  $2\text{ d}^{-1}$  (mean,  $5.8 \pm 9.0$  eggs female  $2\text{ d}^{-1}$ )

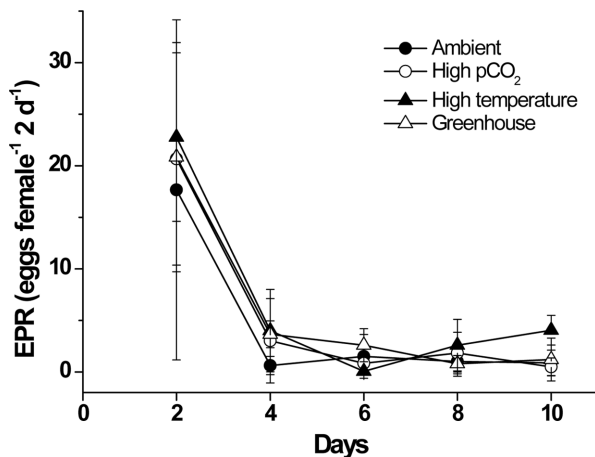


Fig. 4. Variations in egg production rate (EPR) of adult female *Calanus sinicus* in Experiment-II

for the greenhouse treatments. The mean daily EPR did not differ among the four treatments throughout the experiment [Fig. 4; two-way ANOVA,  $p > 0.05$ ; daily comparison of EPR for ambient ( $n = 2$ ), high  $p\text{CO}_2$ , high temperature, and greenhouse ( $n = 3$ )]. No significant interaction was detected between  $p\text{CO}_2$  and temperature in the daily EPR ( $p > 0.05$ ).

#### Egg hatching success

Because the EPR of adult female copepods was highest on day 2 and then decreased to almost zero at the end of the experiment (Fig. 4), it was not possible to compare daily EHS. Therefore, we only compared mean EHS over 10 d. Mean EHS rates were  $91.2 \pm 2.6\%$  ( $n = 6$ ) for the ambient,  $80.5 \pm 5.9\%$  ( $n = 10$ ) for the high  $p\text{CO}_2$ ,  $74.2 \pm 2.3\%$  ( $n = 10$ ) for the high temperature, and  $56.1 \pm 6.4\%$  ( $n = 10$ ) for the greenhouse treatments (Fig. 5).

Mean EHS rates were significantly different among the four treatments (two-way ANOVA,  $p\text{CO}_2$ ,  $p < 0.05$  and temperature,  $p < 0.01$ ; Table 2 and Tukey's post-hoc comparison; Table 3). Both temperature and  $p\text{CO}_2$  had significant effects on the mean EHS (Table 2;  $p < 0.01$  and  $p < 0.05$ , temperature and  $p\text{CO}_2$ , respectively). However, no significant interaction was detected between

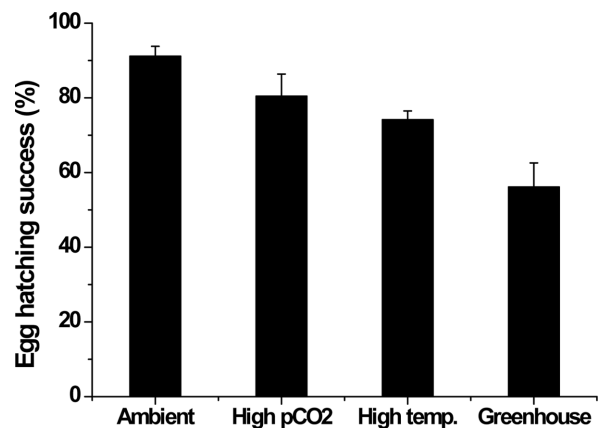


Fig. 5. Egg hatching success of adult female *Calanus sinicus* in Experiment-II

Table 2. Results of the two-way ANOVA for egg hatching success of *Calanus sinicus* for two different  $p\text{CO}_2$  and temperature treatments in Experiment-II

Source	d.f.	Sum of squares	Mean square	F-ratio	p-Value
$p\text{CO}_2$	1	0.2574	0.2574	5.5674	0.0246*
Temperature	1	0.5302	0.5302	11.4659	0.0019**
$p\text{CO}_2 \times \text{Temperature}$	1	0.0025	0.0025	0.0548	0.8163
Error	32	1.4796	0.0462		

\* $p < 0.05$ , \*\* $p < 0.01$



Fig. 6. Eggs, nauplius, and fecal pellets for the greenhouse treatment on day 10 in Experiment-II. Arrows indicate abnormal eggs and nauplius. Inside and outside diameter of the eggs are  $\sim 160 \mu\text{m}$  and  $\sim 214 \mu\text{m}$ , respectively

temperature and  $p\text{CO}_2$  in the mean EHS ( $p > 0.05$ ; Table 2), indicating that the effect of  $p\text{CO}_2$  on the mean EHS did not vary with temperature. The lowest mean EHS was observed in the greenhouse treatment.

The mean EHS in the greenhouse treatment was significantly lower than that in the ambient ( $\sim 35\%$ ) and high  $p\text{CO}_2$  ( $\sim 24\%$ ), but not in the high temperature treatment. No difference in the mean EHS was observed between  $p\text{CO}_2$  within the same temperature condition (i.e., ambient and high  $p\text{CO}_2$ ; high temperature and greenhouse). Within the lower  $p\text{CO}_2$  conditions (i.e., ambient and high temperature), temperature had no significant effect on the mean EHS, whereas temperature had a significant negative effect on the mean EHS within the higher  $p\text{CO}_2$  conditions (i.e., high  $p\text{CO}_2$  and greenhouse;  $p < 0.05$ ).

We also found a relatively higher ratio of abnormal nauplii without symmetrical appendages, and abnormal eggs with abnormal embryonic development in the

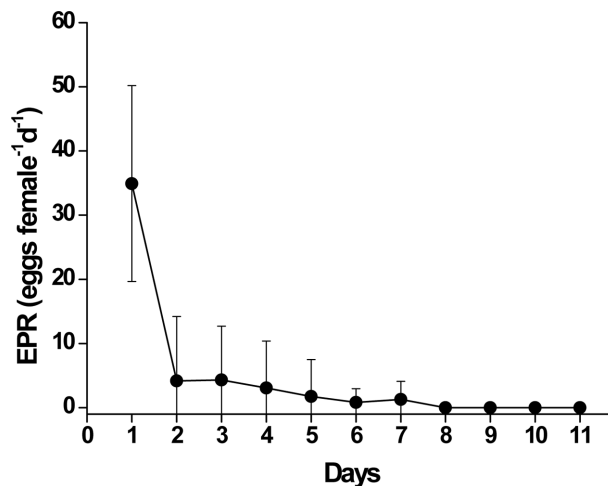


Fig. 7. Variation of *in situ* egg production rate (EPR) of adult female *Calanus sinicus*

greenhouse treatment compared with those in the ambient treatment (Fig. 6), indicating that combined higher  $p\text{CO}_2$  and temperature negatively affected egg development in copepods.

#### *In situ* EPR of *C. sinicus*

The *in situ* EPR was highest on day 1, and then decreased sharply, showing the same EPR pattern as observed in Experiment-II (Fig. 7). Therefore, it is likely that the EPR pattern in Experiment-II was not biased under laboratory conditions. The mean EPR was  $0\text{--}34.9$  eggs female<sup>-1</sup> d<sup>-1</sup> (mean,  $4.7$  eggs female<sup>-1</sup> d<sup>-1</sup>). The *in situ* mean EPR on day 1 was not different from that on day 2 for the ambient treatment in Experiment-II (Mann-Whitney *U*-test,  $p > 0.05$ ), indicating that the adult female copepods showed strong spawning activity by day 2.

## 4. Discussion

We tested the combined effects of enhanced  $p\text{CO}_2$  and

Table 3. Summary of the Tukey's post hoc comparison for all pair-wise treatments [ambient ( $n = 6$ ), high  $p\text{CO}_2$  ( $n = 10$ ), high temperature ( $n = 10$ ), and greenhouse ( $n = 10$ )] for egg hatching success of *Calanus sinicus* in Experiment-II

Treatment	<i>p</i> -Value	Egg hatching success
Greenhouse vs. High $p\text{CO}_2$	0.0441*	Lower in greenhouse
Greenhouse vs. High temperature	0.2162	No difference
Greenhouse vs. Ambient	0.0033**	Lower in greenhouse
High $p\text{CO}_2$ vs. High temperature	0.8611	No difference
High $p\text{CO}_2$ vs. Ambient	0.5051	No difference
High temperature vs. Ambient	0.1798	No difference

\* $p < 0.05$ , \*\* $p < 0.01$

temperature on reproduction and survival rate of the copepod *C. sinicus* from the Yellow Sea for the first time, using a rather lower  $p\text{CO}_2$  treatment compared to that used in previous studies (Kurihara et al. 2004; Mayor et al. 2007; Kurihara and Ishimatsu 2008; McConville et al. 2013). In this study, we found that EHS of *C. sinicus* decreased significantly at 753 ppm  $p\text{CO}_2$  and 12°C temperature for 10 d compared to that in the control.

### Comparison of EPR, EHS, and survival rate among copepods

The EHS of most copepods, including *Acartia tsuensis*, *A. erythraea*, *A. steueri*, *Calanus finmarchicus*, *Centropages typicus*, and *Temora longicornis* was affected at > 2000 ppm  $p\text{CO}_2$  (Table 4). However, the EPR of copepods was less sensitive than the EHS using only  $p\text{CO}_2$ . For example, the EPR of *C. sinicus* from the Yellow Sea was not affected at 800, 2000, and 5000 ppm  $p\text{CO}_2$  at 16°C, except at 10000 ppm at which the EPR decreased (Zhang et al. 2011). The EHS of *C. finmarchicus* from the northern Atlantic Ocean was affected at 8000 ppm  $p\text{CO}_2$  at ambient temperature (e.g., 9°C), but not the EPR (Mayor et al. 2007).

The EPR and EHS of *Acartia clausi* decreased at enhanced  $p\text{CO}_2$  and/or temperature. However, the *A. clausi* EPR and EHS were not affected at 450 ppm  $p\text{CO}_2$  and either temperature, indicating that enhanced  $p\text{CO}_2$  was more effective than increasing temperature for *A. clausi* EPR and EHS (Zervoudaki et al. 2013).

In this study, the *C. sinicus* EHS decreased significantly at both 753 ppm  $p\text{CO}_2$  (i.e., future  $p\text{CO}_2$  concentration in 2100) and a 4°C increase in temperature (Table 4). Therefore, the combined effects of acidification and increased temperature were more effective at decreasing EHS than the single effect of acidification. However, the EPR and survival rate of adult females were not affected by the experimental treatments. Survival rates of *A. erythraea*, *A. steueri*, and *C. sinicus* were not affected by 10000 ppm  $p\text{CO}_2$  for 10 d (Table 4). Larval mortality and growth rate of the copepod *C. finmarchicus* decreased at 7300 ppm and 9700 ppm  $p\text{CO}_2$  during a medium-term exposure (e.g., 28 d) (Pedersen et al. 2013).

### Potential mechanism of the hypercapnia effects

In general, hypercapnia affects the metabolic processes of copepods by disturbing the extracellular acid-base balance, as energy demand increases to maintain the acid-base status (e.g., increased respiration). As a result, the acquired energy will be used to maintain cell homeostasis

rather than for growth and reproduction. Therefore, growth and reproduction, which require more energy, may be inhibited temporarily (Langenbuch and Pörtner 2002; Langenbuch et al. 2006; Pörtner 2008). However, the capacity to regulate acid-base status varies depending on the species, so the species sensitivity to acidification will change.

The impact of the climate change is influenced additively or synergistically by various factors (e.g., temperature elevation, anoxia, and pollutants), rather than a single factor (Invidia et al. 2004; Kurihara and Ishimatsu 2008; Feng et al. 2009; Vehmaa et al. 2012). In general, the impact of hypercapnia on an organism is greater as temperature and anoxia increase (Pörtner et al. 2005). Increased  $\text{CO}_2$  and anoxia narrow the thermal window of an organism, as exposure to the thermal extreme increases sensitivity to high  $\text{CO}_2$  or anoxia (Pörtner 2008; Vehmaa et al. 2012). Increased temperature could benefit the copepod EPR (Jang et al. 2013). However, when the optimum temperature of copepods is narrowed by interacting with acidification, the EPR could be affected depending on the capacity of acid-base regulation (Vehmaa et al. 2012).

In this study, EHS was reduced most by both temperature and  $p\text{CO}_2$  increase (e.g., greenhouse treatment) compared with either the normal condition (e.g., ambient treatment) or enhanced  $p\text{CO}_2$  alone (e.g., high  $p\text{CO}_2$  treatment). In fact, EHS decreased ~35% in the greenhouse treatment compared to that in the ambient treatment as a control (i.e., relatively lower temperature and  $p\text{CO}_2$ ). Increased  $p\text{CO}_2$  alone (e.g., high  $p\text{CO}_2$  treatment) had relatively little effect on *C. sinicus* EHS. However, when temperature increased to a particular level (e.g., +4°C elevation), the enhanced  $p\text{CO}_2$  negatively affected EHS (e.g., greenhouse treatment). Temperature elevation alone had little effect on *C. sinicus* EHS when  $p\text{CO}_2$  was normal (i.e., below the present level). However, when  $p\text{CO}_2$  increased to a particular level (e.g., 753 ppm  $p\text{CO}_2$ ), EHS of the copepods decreased as temperature increased (e.g., greenhouse treatment).

The EPR and EHS of *A. clausi* from the Mediterranean Sea decrease significantly under combined increases of  $\text{CO}_2$  and temperature (e.g., near future acidification condition of pH 0.83 and ambient and a +4°C temperature of 20°C), but acidification alone does not affect the survival rate of this copepod (Zervoudaki et al. 2013).

### Caution applying laboratory results to the field

Caution is needed to apply the results obtained from

**Table 4. Summary of physiological impacts of increased  $p\text{CO}_2$  and/or temperature on copepods (modified from Halsband and Kurihara 2013)**

Species	$p\text{CO}_2$ (ppm)	Temp. (°C)	Duration (days)	Parameter	Effect	Reference
<i>Acartia tsuensis</i>	2380	25	9	Egg survival	no	Kurihara and Ishimatsu (2008)
			9–12	Egg production	no	
			(3 generation)	Hatching success	negative	
<i>Acartia erythraea</i>	2365/5360/10365	27	8	Adult survival	no	Kurihara et al. (2004)
<i>Acartia steueri</i>		24		Hatching success	negative	
				Offspring survival	negative	
<i>Calanus finmarchicus</i>	8000	9	5	Egg production	no	Mayor et al. (2007)
				Hatching success	negative	
<i>Centropages typicus</i>	9830	15	4	Egg production	no	McConville et al. (2013)
				Hatching success	negative	
<i>Temora longicornis</i>	9830	15	4	Egg production	no	McConville et al. (2013)
				Hatching success	no	
<i>Calanus sinicus</i>	800/2000/5000/10000	16	8	Adult survival	no	Zhang et al. (2011)
	800/2000/5000			Egg production	no	
	10000			Egg production	negative	
<i>Acartia grani</i>	1203	18	4	Ingestion rate	no	Isari et al. (2015)
<i>Oithona davisae</i>				Egg production	no	
				Hatching success	no	
				Respiration rate	no	
<i>Acartia clausi</i>	463–466	16	5	Egg production	no (Control)	Zervoudaki et al. (2013)
				Hatching success	no (Control)	
	463–466	20	5	Egg production	no	
				Hatching success	no	
	823–824	16	5	Egg production	negative	
				Hatching success	negative	
<i>Calanus sinicus</i>	823–824	20	5	Egg production	negative	This study
				Hatching success	negative	
	289	8	10	Adult survival	no (Control)	
				Egg production	no (Control)	
				Hatching success	no (Control)	
	289	12	10	Adult survival	no	
				Egg production	no	
				Hatching success	no	
	753	8	10	Adult survival	no	
				Egg production	no	
				Hatching success	no	
	753	12	10	Adult survival	no	
				Egg production	no	
				Hatching success	negative	
	1305	17	5	Adult survival	no	
				Fecal pellet production	no	

short-term exposure of copepods to either lower or higher  $\text{CO}_2$  concentration in the laboratory to the field because the impact of acidification on organisms may lessen through adaptation (i.e., phenotypic plasticity) (Vehmaa et al. 2012; Lewis et al. 2013; Isari et al. 2016). Isari et al. (2016) suggested that the short-term positive or negative response of an organism to elevated  $\text{CO}_2$  is only the initial response; therefore, the reaction weakens as time goes by through physiological adaptation.

For example, although an organism is affected by a particular level of  $\text{CO}_2$ , the same organism may occur at the more elevated  $\text{CO}_2$  concentration in the field, suggesting adaptation to the changing environment (Gaitán-Espitia et al. 2014). Interestingly, as the response of EPR to acidification is reversible, the EPR will recover if copepods are transferred to the control conditions (Kurihara et al. 2004). Therefore, it is likely that the EHS response of *C. sinicus* observed in this study will be reversible. However, it is questionable whether the reproductive response of *C. sinicus* to the combined effects of  $\text{CO}_2$  and temperature will be reversible.

#### Food limitation and a decrease in the EPR

In this study, the *C. sinicus* EPR in the control and Experiment-II treatments decreased with time (Fig. 4). The *in situ* EPR with an ambient diet in surface waters also tended to decrease with time (Fig. 5). The reason why the EPR of *C. sinicus* decreased drastically after 2 d in this study is unclear. We supplied excess cultured phytoplankton and adult females produced a lot of fecal pellets during the experiments. However, considering the food quality, the phytoplankton diet may have been insufficient to maintain the EPR in the laboratory. Also, the quantity of a natural diet from surface waters in the field may not be enough due to rather low chlorophyll-*a* concentrations (e.g., 1.2–2.2  $\mu\text{g L}^{-1}$ ). Therefore, it is likely that a potential food limitation is responsible for the decreased EPR of *C. sinicus* in the laboratory. Park and Lee (1995) reported that the EPR of *C. sinicus* collected in Asan Bay, the Yellow Sea, decreased drastically with time and then the copepod stopped spawning in the laboratory. Mayor et al. (2007) also suggested that a food limitation during laboratory incubation was responsible for a sharp decrease in the EPR of *C. finmarchicus*.

We have no available data on whether *C. sinicus* is spawning continuously in the field. However, it is apparent that the sharp decrease of the EPR in the laboratory did not affect our test of the combined impact of  $\text{CO}_2$  and temperature elevation on reproduction and

survival rate of adult females.

#### Re-mating necessary for continuous spawning

We do not know whether *C. sinicus* must re-mate to spawn continuously. In this study, *C. sinicus* spawned a few eggs over 10 d and most hatched successfully. *Calanus helgolandicus* from the northern Atlantic Ocean produces fertilized eggs without re-mating in the laboratory eating a dinoflagellate diet and the eggs hatched successfully (Kang and Poulet 2000). So, apparently *C. sinicus* in the Yellow Sea do not need to re-mate over 10 d.

#### Was 289 ppm $p\text{CO}_2$ suitable as a control in Experiment-II?

$p\text{CO}_2$  in the control seawater in Experiment-II (e.g., ambient treatment) was 289 ppm and similar to  $p\text{CO}_2$  in a pre-industrial environment (e.g., 280 ppm; Rose et al. 2009), but was lower than that of the current  $p\text{CO}_2$  (e.g., ~400 ppm in 2011; IPCC 2013). However, no difference in mean EHS was detected between the *in situ* mean EHS over 3 d with surface seawater and the mean EHS over 10 d in the ambient treatment of Experiment-II (Mann-Whitney *U*-test,  $p > 0.05$ ;  $n = 16$  for *in situ* EHS,  $n = 6$  for mean EHS over 10 days at ambient treatment). Therefore, it is likely that the EHS results in the ambient treatment (i.e., 289 ppm) were not biased by the lower  $p\text{CO}_2$  (i.e., 289 ppm) compared to the current  $p\text{CO}_2$ .

#### 5. Conclusion

We evaluated the impact of enhanced  $p\text{CO}_2$  and temperature on copepods under laboratory conditions. The reproductive response (e.g., EPR and EHS) and survival rate of adult *C. sinicus* females from Asan Bay, the Yellow Sea were measured. *Calanus sinicus* EHS decreased significantly in the greenhouse treatment, indicating the combined effect of  $p\text{CO}_2$  and temperature on the copepods was more effective than that of the single effect of increased  $p\text{CO}_2$ . According to previous studies, acidification disturbs the intra and extracellular acid-base status, which can affect the reproduction, particularly egg hatching rate, by changing energy allocation. This effect may vary depending on the acid-base regulatory capacity. Laboratory results should be carefully applied to the field. To overcome this problem, experiments must be designed to test the long-term impacts of increased  $p\text{CO}_2$  and temperature on multi-generations as well as a single generation in the future. This kind of long-term incubation experiment may help to better understand a potential

adaptation of the copepods.

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