Effects of high CO_2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations

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Key words. CO_2, decreased pH, ocean acidification, copepod, all life stages, reproduction

Abstract

We studied the effects of exposure to seawater equilibrated with CO_2-enriched air (CO_2 2,380 ppm) from eggs to maturity and over 2 subsequent generations on the copepod *Acartia tsuensis*. Compared to the control (CO_2 380 ppm), high CO_2 exposure through all life stages of the 1st generation copepods did not significantly affect survival, body size or developmental speed. Egg production and hatching rates were also not significantly different between the initial generation of females exposed to high CO_2 and the 1st and 2nd generation females developed from eggs to maturity in high CO_2. Thus, the copepods appear more tolerant to increased CO_2 than other marine organisms previously investigated for CO_2 tolerance (i.e. sea urchins and bivalves). However, the crucial importance of copepods in marine ecosystems requires thorough evaluation of the overall impacts of marine environmental changes predicted to occur with increased CO_2 concentrations, i.e., increased temperature, enhanced UV irradiation, and changes in the community structure and nutritional value of phytoplankton.

1. Introduction

The recent report from the Intergovernmental Panel on Climate Change (IPCC) pointed out that warming of the climate system is unequivocal, and that most of the warming is very likely due to the increase in anthropogenic green-house gas concentrations, of which carbon dioxide (CO_2) is the most important (IPCC, 2007). The present atmospheric CO_2 concentration (380 ppm) has already risen by 100 ppm since the pre-industrial level, and exceeds by far the natural range over the past 650,000 years (IPCC, 2007). Increased atmospheric CO_2 diffuses into the ocean surface waters and lowers the pH; the present surface ocean pH (8.2) is already lower by 0.1 pH unit than pre-industrial values, and is predicted to decrease by 0.77 units along with the elevation of atmospheric CO_2 to ca. 2,000 ppm within the next 300 years (Caldeira and Wickett, 2003).

Ocean acidification due to increased CO_2 may have profound impacts on marine biota. Although pertinent information is only recently emerging, the most well-understood biological impact of high seawater CO_2 is the reduction of the calcification rate in calcifying organisms due to acidification-driven lowering of the

calcium carbonate ($CaCO_3$) saturation state (Feely et al., 2004; Langdon and Atkinson, 2005; Raven et al., 2005; Kleypas et al., 2006). Recent studies, however, have revealed that long-term hypercapnia could disrupt the physiology of exposed marine organisms. For example, three-month exposure to hypercapnic seawater (pH 7.3) reduced both growth and oxygen consumption in marine mussels *Mytilus galloprovincialis* (Michaelidis et al., 2005). Changes in metabolic enzyme activities and a transient acid-base disturbance were reported for the teleost *Sparus aurata* exposed to seawater acidified to pH 7.3 by adding CO_2 for 10 days (Michaelidis et al. 2007). These two studies used CO_2 to acidify test seawater, but did not report CO_2 partial pressure (pCO_2) of the seawater. In addition, early development was shown to be disturbed by exposure to high ambient CO_2. Kurihara and Shirayama (2004) demonstrated that CO_2 reduces fertilization, cleavage, developmental speed and pluteus larval size of the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* in a concentration-dependent manner. More recently, Kurihara et al. (2007) reported that exposure to 2,268 ppm CO_2 conditions (pH 7.4) leads to significant disruption of shell formation and considerable tetrogenesis in the oyster *Crassostrea gigas*. Reduced metabolic rates and growth could

be fatal for organisms if such conditions occur over a prolonged period. Effects on early development and reproduction would lead to a reduction of community size if they continue over generations. On the other hand, there is a possibility that marine organisms will adapt to persistent high CO_2 environment such that they resume environmental fitness to proliferate. However, to our knowledge, there has been no study that investigated the effects of increased CO_2 conditions over a full life cycle and subsequent generations for metazoan organisms.

In the present study, we first examined the effects of exposure to seawater equilibrated with CO_2-enriched air (2,000 ppm above ambient air (CO_2 380 ppm), hereafter as +2,000 ppm) through all life stages, i.e., from eggs to maturity, on the copepod *Acartia tsuensis* to compare survival, body size and developmental speed. We then compared egg production and hatching rate between the initial generation of females exposed to high CO_2 and the 1^{st} and 2^{nd} generation females that had developed from eggs to maturity in high CO_2 conditions. Planktonic copepods constitute the bulk of the biomass in most pelagic zooplanktonic communities and are important food source for higher trophic organisms including krill and fishes (Nybakken, 2001).

Therefore, evaluating the impacts of increased CO_2 on copepods is essential for projecting possible alterations of marine ecosystems in future acidified oceans. We selected *A. tsuensis* as our experimental material because it has a short life cycle (they develop from eggs to adults in 9 days at 25 °C) and is readily reared over several generations under laboratory conditions.

2. Material and methods

2.1. Test animals

Adults of *Acartia tsuensis* used in this study were obtained from stock cultures that have been reared for ca. 1 year in our laboratory. The copepods were kept in 5-L plastic beakers filled with filtered (GF/C 1.2 μm) and sterilized (121°C, 15 min) seawater (FSW) at 25°C and under 24 h light conditions, and fed on a mixture of three phytoplankton species, *Isochrysis galbana, Chaetoceros gracilis* and *Pavlova lutheri*. The seawater pH of the stock beakers ranged between 8.0 and 8.2.

2.2. Seawater preparation

Control seawater was prepared by aerating FSW enriched with 5×10^5 cells mL^{-1} of *I. galbana*, with air (CO_2 concentration: 380 ppm). Seawater of higher CO_2 concentration was bubbled with CO_2-enriched air of which the CO_2 concentration was adjusted to 2,000 ppm above the control concentration (+2,000 ppm, hereafter referred to as CO_2 seawater). The CO_2-enriched air was prepared by mixing air and pure CO_2 at flow rates of 500 mL min^{-1} and 1.0 mL min^{-1}, respectively, using a flow meter (Kofloc 250, Japan). The seawater salinity (35) was measured with a refractometer (Atago, 100-S, Japan).

2.3. Survival and growth rate

Approximately 200 adult individuals of both sexes were divided into 2 groups and reared for 24 h in two beakers (capacity 1 L), each containing 1 L of control (380

ppm) or CO_2 seawater. Cultures were continuously aerated in an incubator at 25 ± 1°C and under 24 h dark conditions. The seawater pH was checked at 0 h (control: pH 8.23 ± 0.01 (SD), CO_2: pH 7.31 ± 0.02) and at 24 h of rearing (control: pH 8.18 ± 0.02, CO_2: pH 7.31 ± 0.01). After 24 h, produced eggs that had been dispersed directly into the seawater were collected from each beaker by filtering (mesh size 300 μm) the seawater to separate females, and 100 eggs were transferred into each of eight 500 mL Erlenmeyer flasks filled with the control or CO_2 seawater. All flasks were continuously bubbled with air or CO_2-enriched air at a flow rate of 10 mL min^{-1} in the incubator. The seawater in the flasks was fully renewed once a day to restore the *I. galbana* concentration to the predetermined level (5×10^5 cells mL^{-1}). The seawater pH was measured daily before and after renewing the seawater with a pH meter (Mettler Toledo, MP125, USA). The pH values of the control and CO_2 seawater were 8.17 ± 0.04 and 7.32 ± 0.01, respectively, and remained stable throughout the experiments. On days 3, 5, 7 and 9, one flask from each experimental group was randomly selected to count the number of individuals therein and classify the developmental stages after fixation (see

Takahashi and Ohno, 1996 for criteria used for staging). Four replicate experiments were conducted.

2.4. Egg production and hatching rate of generation 0, 1 and 2 females reared in high CO_2 seawater

Ten adult females were collected from the stock cultures, and transferred individually into 50 mL vials. The vials were filled with the control or CO_2 seawater and stoppered leaving no air space, and kept in dark conditions at 25°C (generation 0). To prevent egg consumption by the females, the seawater was enriched with enough food (5×10^5 cells mL^{-1} *I. galbana*). After 24 h, the number of eggs produced from each female was counted. Ten eggs were transferred into another set of 50 mL vials filled with the control or CO_2 seawater. After a further 24 h in the same conditions, hatching rate was estimated by counting the number of egg's shells in the 10 eggs. The seawater pH remained stable during the 24 h incubation. To study the egg production ability and egg hatchability of females developed from eggs in the high CO_2 conditions (generation

1), a sufficiently large number of both adult males and females were reared in 1 L flasks bubbled with air or CO_2-enriched air at a flow rate of 20 mL min^{-1}, and produced eggs were collected 24 h afterwards. These eggs were transferred into other 1 L flasks aerated in the same manner, and reared for ca. 10 days until they developed into the adult stage. Ten females were randomly selected and reared individually to study egg production ability and egg hatchability as for the generation 0 females. The same procedure was repeated to obtain data for the generation 2 females. When no eggs from a female hatched in 24 h, the female was considered not to have copulated, and the data not included in the analysis. Seawater pH was measured at the beginning and termination of each experiment to verify that pH had not changed during the experiments. The pH values of the control and CO_2 seawater were 8.14 ± 0.06 and 7.32 ± 0.04, respectively.

2.5. Statistical analysis

Treatment effects on survival, egg production and hatching rate were tested for significance at the 5% level using two-way ANOVA. When there was a significant difference between the groups using two-way ANOVA, Student t-test was applied.

Student t-test was used to analyze effects on promosome length of different stages. Values are presented as the mean ± SD throughout.

3. Results

Survival was not significantly different between the copepods reared in the control and CO_2 seawater from eggs to the adult stage (Fig. 1, two-way ANOVA). The overall averages of survival rates were 53% and 43% for the control and the CO_2 copepods, respectively, and there was no significant difference in survival with time (Fig. 1, two-way ANOVA). The sex ratio did not also differ between the groups (female 40 ± 4.7% (control) and 45 ± 8.3% (CO_2)).

Figure 2 compares developmental stages of the control and CO_2 group on 3, 5, 7 and 9 days after culture. By day 3, all eggs had developed into nauplii with nauplius stage 4 (N4) comprising ca. 60% of the total copepods in both groups. By day 5, half of the copepods had developed into the copepodite stage, and by day 7, most copepods had developed into the copepodid stage 3 or 4 (C3/C4). Adult copepods (C6) first appeared

on day 9, and egg production began on day 11 in both groups. There was no difference in the promosome length in all stages between the control and CO_2 copepods (Fig. 3, t-test).

The overall egg production rates did not differ between the control and CO_2 copepods (Fig. 4, two-way ANOVA), although there was a significant difference between generations (Fig. 4, $P < 0.05$, two-way ANOVA). The overall hatching rate of CO_2 eggs was significantly lower than that of the control eggs (Fig. 5, $P < 0.05$, two-way ANOVA) however there was no significant difference in the hatching rate between the control and CO_2 eggs when compared separately for each generation (Fig. 5, t-test).

4. Discussion

The present study revealed that exposure to seawater equilibrated with high CO_2 (+2,000 ppm) air did not significantly affect the survival rate of the copepod *A. tsuensis* (Fig. 1). The initial reductions of survival seen in both the control and CO_2

groups by day 3 (Fig. 1) agree with the previous findings of 40% mortality during the nauplius stages 1 to 3 (Takahashi and Ohno, 1996). Takahashi and Ohno (1996) suggested that the high mortality rate during early stages of the nauplii was due to the difficulty in shifting energy source from yolk-related endogenous food to an exogenous one. Our previous study also demonstrated that the survival of adult *A. erythraea* and *A. steueri* was unaffected even in seawater equilibrated with +10,000 ppm CO_2 in air (pH 6.8; Kurihara et al., 2004). Survival of *A. erythraea* nauplii was unaffected when reared in seawater equilibrated with +2,000 ppm CO_2, but depressed above +5,000 ppm (Kurihara et al., 2004). Yamada and Ikeda (1999) found a progressive increase in LC_{50} and LC_0 with increasing exposure time to acidified (pH 7.2-4.5) seawater by mineral acids for six copepod species. Thus, short-term exposure to CO_2 concentration lower than ca. 2,000 ppm, the maximum atmospheric CO_2 concentration predicted to occur by the year 2300 (Caldeira and Wickett, 2003), may not reduce, but exposure to higher CO_2 concentrations does reduce survival of copepods.

Development of copepods was unaffected by the CO_2 treatment at all stages (Figs. 2 and 3). Egg production and hatching rates were also unaffected even when adult

females were reared for 2 generations in the CO_2 seawater (Figs. 4 and 5). Meanwhile, egg production and hatching rates of the two species of copepods, *A. steueri* and *A. erythraea*, were decreased in a concentration-dependent manner, though effect was significant only above +5,000 ppm CO_2 (Kurihara et al., 2004). Mayor et al. (2007) demonstrated that exposure to seawater acidified by equilibrating with 8,000 ppm CO_2 in air did not affect both growth and egg production of the copepod *Calanus finmarchicus*, but significantly reduced egg hatching rate. Again, these results indicate that CO_2 concentrations higher than ca. 2,000 ppm potentially have negative impacts on reproduction of copepods, while lower concentrations appear not to have significant impact.

In contrast to the present results for copepods, early development was severely impacted in the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra mathaei* (CO_2 concentration +500 - +2000 ppm, Kurihara and Shirayama, 2004) and in the oyster *Crassostrea gigas* (2,268 ppm, Kurihara et al., 2007). The two studies demonstrated that synthesis of $CaCO_3$ exoskeleton was disrupted and that most larvae became morphologically abnormal to the extent that they would not probably survive.

These findings suggest that the projected high CO_2 conditions in neritic oceans may alter marine ecosystem structure through different vulnerability of marine organisms to CO_2. In addition, fishes are in general far more tolerant to high CO_2 than invertebrates, which may lead to restructuring of marine ecosystems through increased predation pressure (Ishimatsu et al., 2005).

Despite seemingly higher CO_2 tolerance of copepods than other marine invertebrates, the ecological importance of copepods in the marine ecosystem requires more careful and extensive evaluation of overall impacts of marine environmental changes that are predicted to occur with increased CO_2 concentrations. Of particular importance are potential synergistic effects of ocean acidification and other abiotic changes associated with climate change. These include increases in seawater temperature, increased irradiance of ultraviolet light, and changes in salinity (IPCC, 2007). Moreover, some anthropogenically induced factors such as anoxia and high heavy metal concentrations may compound impacts of ocean acidification (Harley et al., 2006). Indeed, Invidia et al. (2004) reported that egg production of *A. tonsa* was significantly depressed by anoxia at low pH (pH 6.5). Furthermore, indirect effects

through altered food-web structures are also likely. Several studies showed that the C : N : P ratio of phytoplankton, which determines the food quality for herbivores (Anderson and Hessen, 1995), changes with increased seawater CO_2 (Burkhardt and Riebesell 1997; Burkhardt et al., 1999; Wolf-Gladrow et al., 1999). Wolf-Gladrow et al. (1999) demonstrated that the C : P ratio of the marine diatoms, *Skeletonema costatum*, *Asterionella gracilis* and *Coscinodiscus wailesii* increased with increasing seawater CO_2 concentration. Urabe et al. (2003) demonstrated that the C : P ratio of freshwater algae increased when cultured under elevated CO_2 levels, which led to suppressed growth of *Daphnia* feeding on them. In addition, species compositions of phytoplankton may also be altered by reduced pH (Hinga, 2002; Tortell et al., 2002). The above are only a few examples that need to be scrutinized, and much more must be understood to accurately predict the fate of marine copepod community in future oceans.

Acknowledgments

We thank Dr. Atsushi Hagiwara, Nagasaki University and Dr. Takashi Ishimaru, Tokyo University of Marine Science and Technology for providing the copepods, and Dr.

Mitsunori Iwataki, Nagasaki University for providing the phytoplankton. This study was partially supported by a Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (JSPS, 18510011).

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Figure Caption

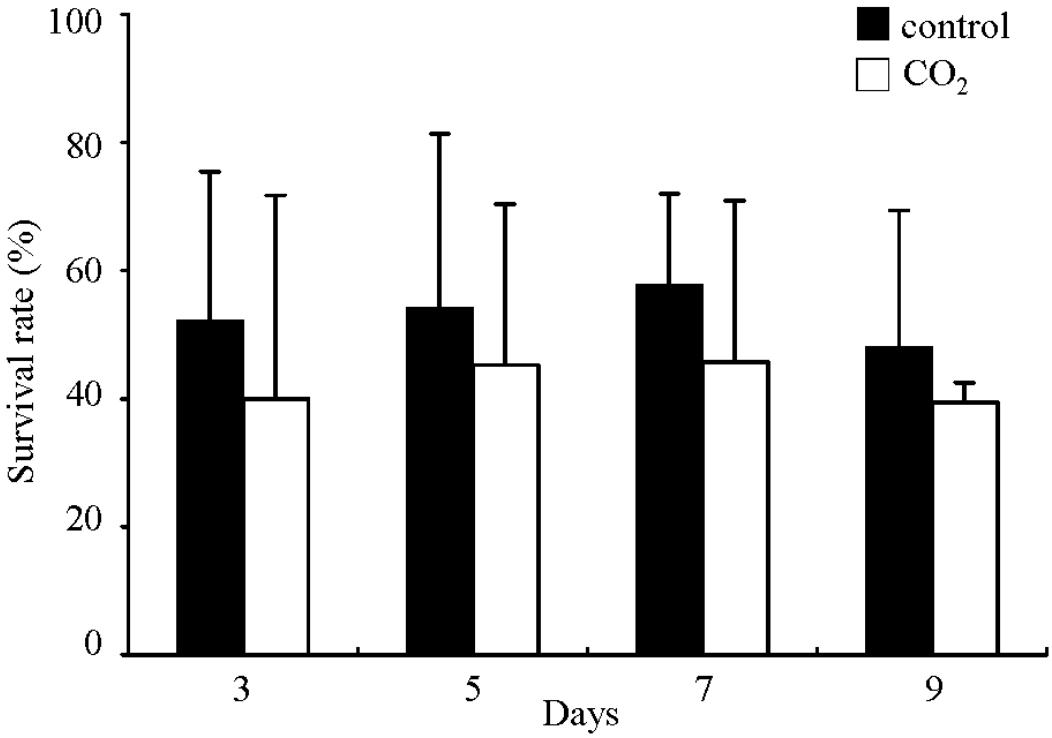

Fig. 1. Survival rate of the Acartia tsuensis eggs reared under control (CO2 380 ppm) and high CO2 (+2,000 ppm) conditions until they developed into adults over a period of 9 days. There was no significant difference ($P > 0.05$) in the survival rate between the control and CO2 group (two-way ANOVA, $P > 0,05$). Error bar = SD. n = 4.

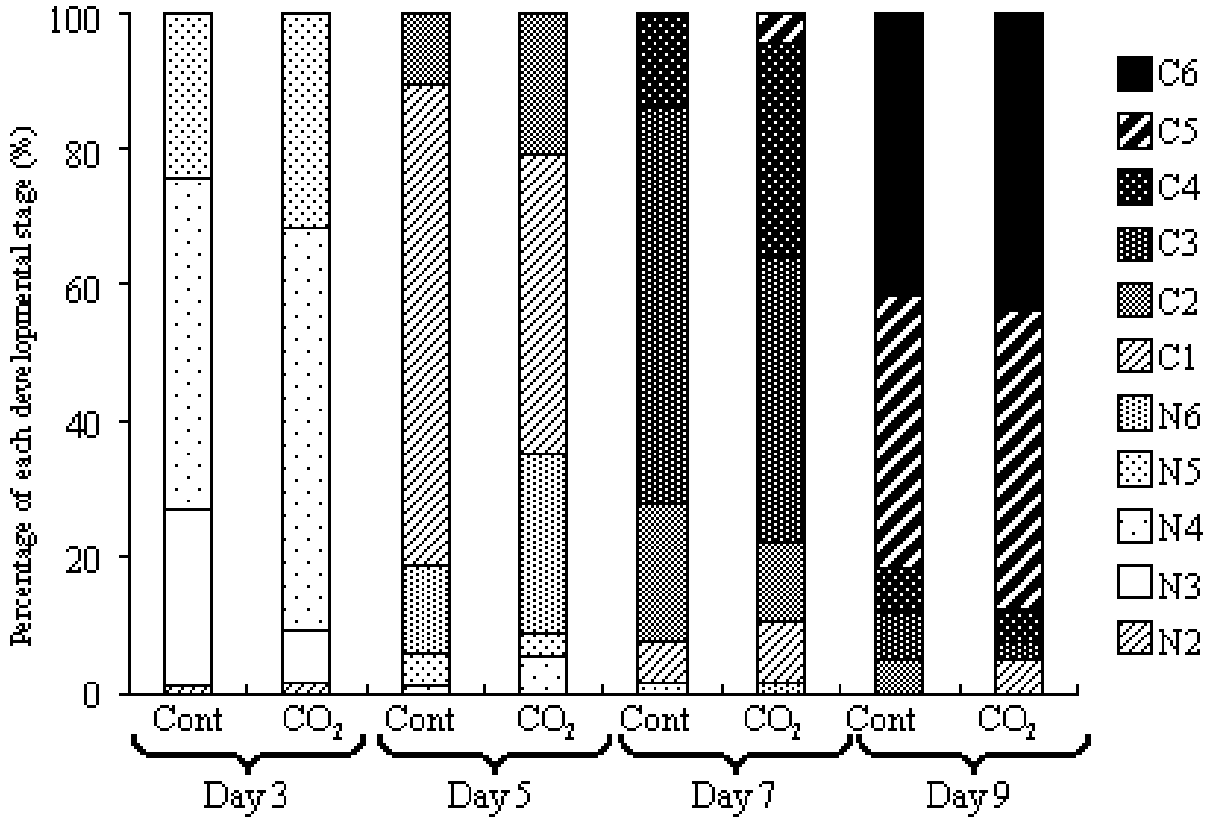

Fig. 2. Percentage of each developmental stage of Acartia tsuensis on 3, 5, 7 and 9 days after hatching from eggs reared under control (CO2 380 ppm) and high CO2 (+2,000 ppm) conditions. Error bar = SD. N2-6: Nauplius stages 2-6, C1-5: Copepodid stages 1-5. C6: Adult

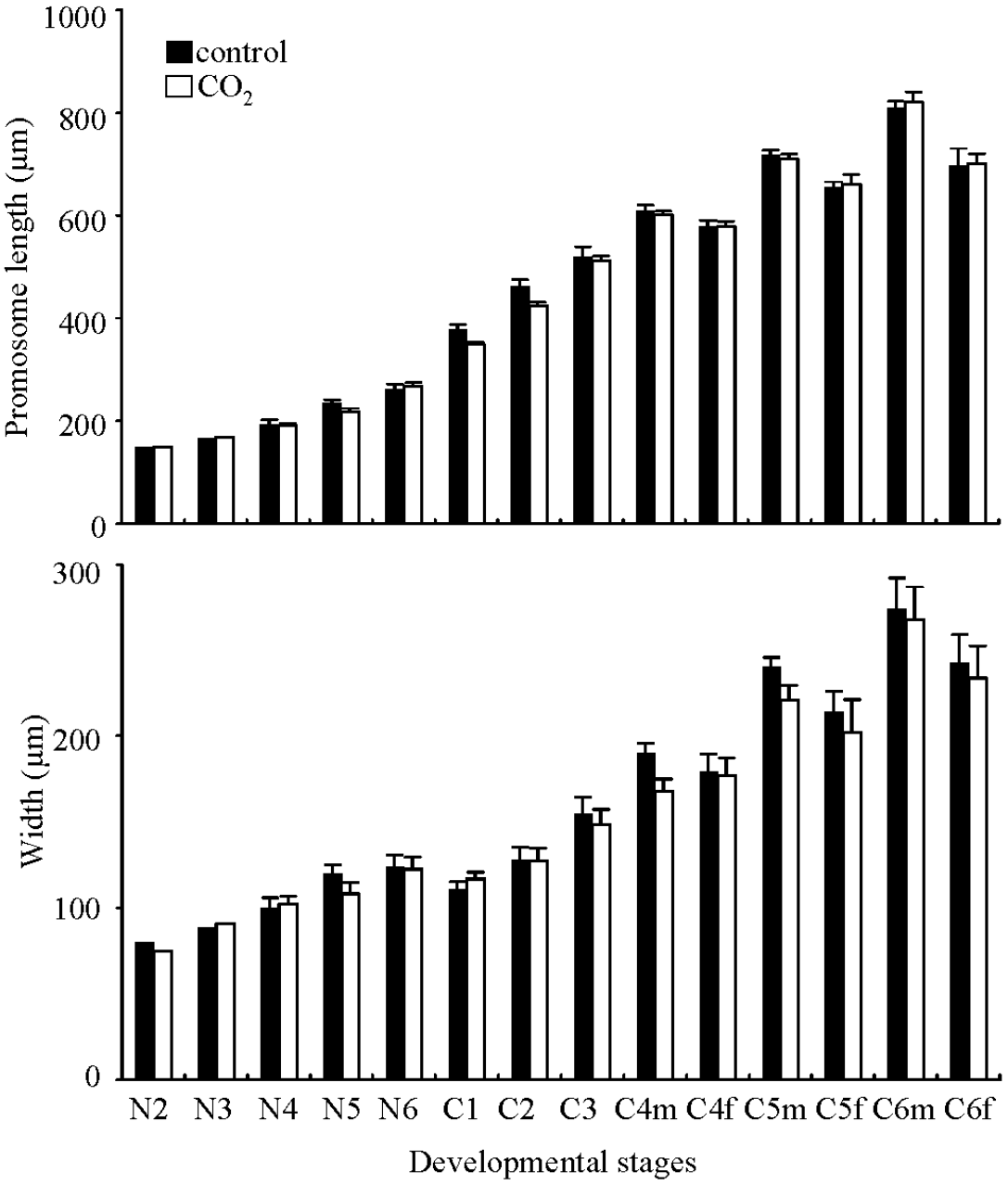

Fig. 3. Promosome length and width of each developmental stage of Acartia tsuensis copepods developed from egg to adult under control (CO2 380 ppm) and high CO2 (+2,000 ppm) conditions. There was no significant difference in both promosome length and width between the control and CO2 group (t-test, $P > 0.05$). Error bar = SD. N2-6: Nauplius stages 2-6, C1-6: Copepodid stages 1-6, f: Female m: Male. The sex of opepodids becomes identifiable from C4 by differences in the shape of the urosome and fifth legs. Numbers of individuals measured were: control; N2 = 1, N3 = 20, N4 = 39, N5 = 24, N6 = 11, C1 = 65, C2 = 25, C3 = 42, C4 = 13, C5 = 24, C6 = 60, CO2; N2 = 1, N3 = 6, N4 = 48, N5 = 26, N6 = 16, C1 = 33, C2 = 20, C3 = 29, C4 = 23, C5 = 21, C6 = 18.

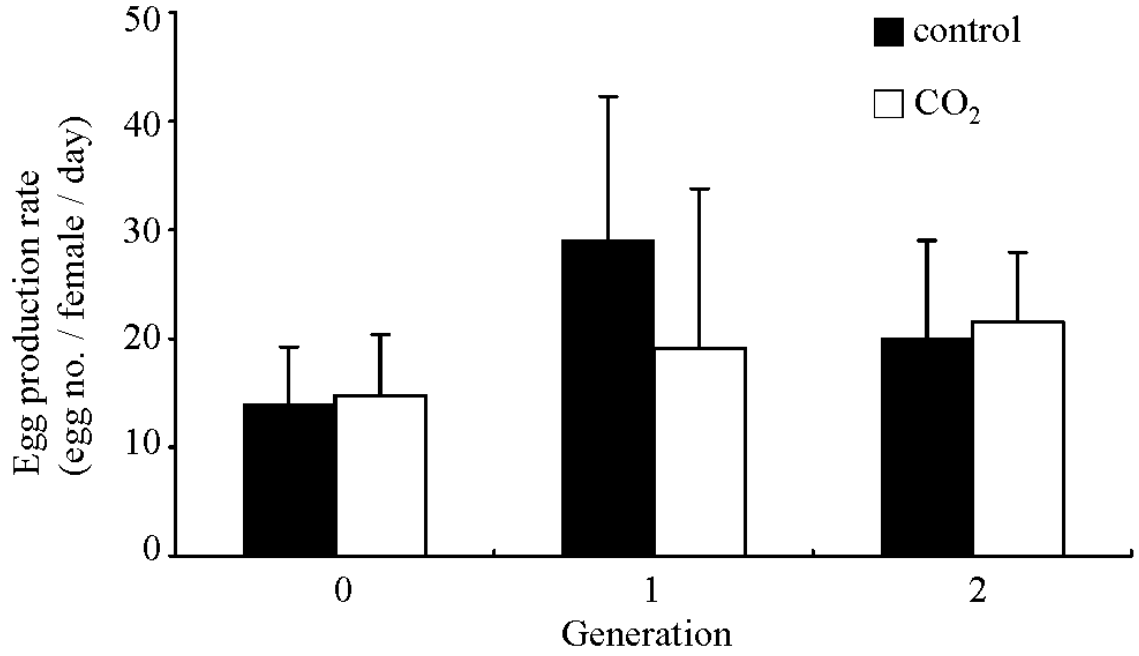

Fig. 4. Egg production rate of female Acartia tsuensis of generations 0, 1 and 2. The copepods of generation 0 were those individuals that were reared in control (CO2 380 ppm) or high CO2 (+2,000 ppm) conditions for 24 h. The copepods of generation 1 were developed in control or CO2 seawater from egg to adult, and those of generation 2 developed in control or CO2 seawater conditions into adult from the eggs obtained from the generation 1. There was no difference between control and CO2 copepods (two-way ANOVA, $P > 0.05$), although there was a significant difference between generations (two–way ANOVA, $P < 0.05$), Error bar = SD, n = 10.

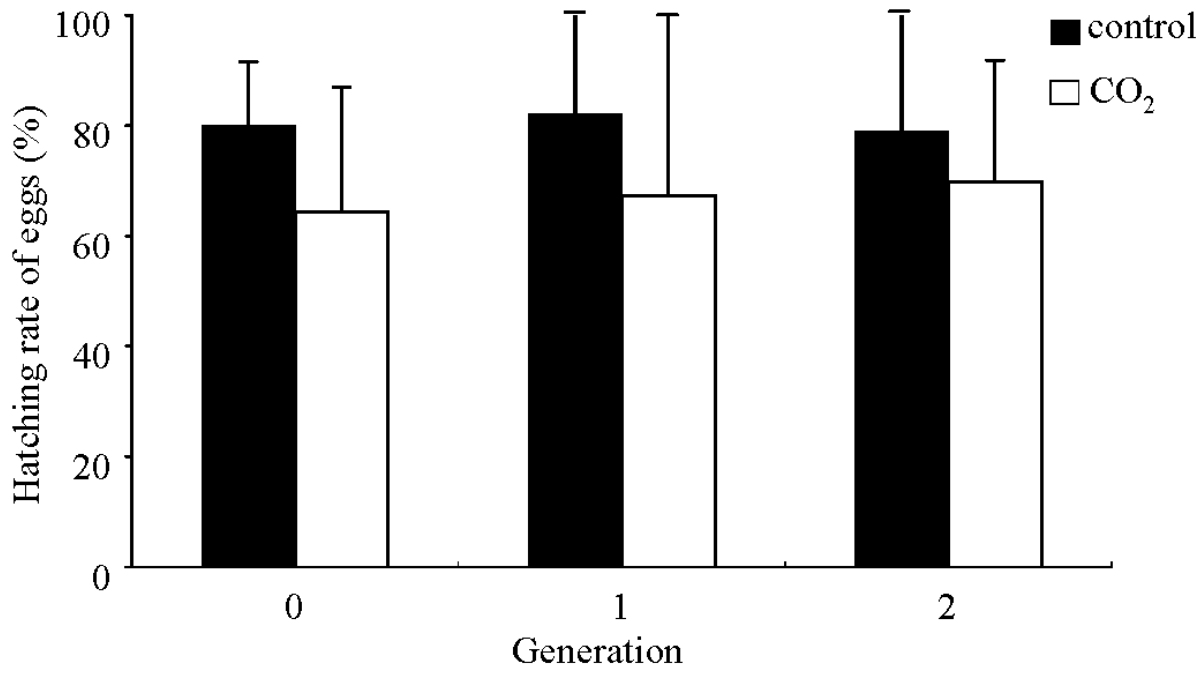

Fig. 5. Hatching rate of eggs produced by the female Acartia tsuensis of generations 0, 1 and 2. There was a significant difference in the hatching rate between control and CO2 groups ($P < 0.05$, ANOVA), although there was no difference when compared separately for each generation (t-test). Error bar = SD.