ICES Journal of Marine Science Advance Access published July 22, 2016

ICES Journal of Marine Science

ICES International Council for the Exploration of the Sea
CIEM Conseil International pour l'Exploration de la Mer

ICES Journal of Marine Science (2016), doi:10.1093/icesjms/fsw124

Effects of potential future CO_2 levels in seawater on emerging behaviour and respiration of Manila clams, *Venerupis philippinarum*

Jung-Ah Lee[‡] and Tae Won Kim[*,‡]

Marine Ecosystem and Biological Research Centre, Marine Life and Ecosystem Division, Korea Institute of Ocean Science and Technology, Ansan 15627, Republic of Korea

*Corresponding author: tel: +82 31 400 7796; fax: +82 31 408 5934; e-mail: ktwon@kiost.ac.kr
[‡]These authors contributed equally to this work.

Lee, J. A., and Kim, T. W. Effects of potential future CO_2 levels in seawater on emerging behaviour and respiration of Manila clams, *Venerupis philippinarum*. – ICES Journal of Marine Science, doi:10.1093/icesjms/fsw124.

Received 31 March 2016; revised 8 June 2016; accepted 13 June 2016.

High atmospheric CO_2 dissolves into the surface of the ocean and lowers the pH of seawater and is thus expected to pose a potential threat to various marine organisms. We investigated the physiological and behavioural responses of adult Manila clams, *Venerupis philippinarum* ($n = 96$, shell length 25.32 ± 1.66mm and total wet weight 3.10 ± 0.54 g), to three levels (400, 700, and 900 µatm) of CO_2 partial pressure (pCO_2) for 48 days. There were no significant differences in mortality, growth, respiration rate, or emergence from the sediment between the three levels, indicating that near future atmospheric levels of CO_2 do not seem to have a serious effect on the physiology and behaviour of adult Manila clams. However, Manila clams could be exposed to notably higher pCO_2 and lower pH levels at local conditions due to the other issues, including eutrophication. Thus, the younger clams ($n = 240$, shell length 16.71 ± 0.96mm and total wet weight 0.70 ± 0.13 g) were exposed to pCO_2 levels of 900 µatm (pH 7.8) and higher, such as 1300 and 2300 µatm (pH 7.7 and 7.5, respectively), for 39 days. Although mortality and growth were not significantly different between treatments, the emergence rates at the two higher pCO_2 levels were higher than that at the lowest level during the last 10 days of the experiment. The oxygen consumption rate (OCR) was reduced after 39 days of exposure to 2300 µatm of pCO_2. The increase in emerging behaviour and the decrease in the rate of oxygen consumption indicated worse physiological conditions of the clams; the population may be negatively influenced due to worse conditions or increased probability of predation.

Keywords: behaviour, growth, Manila clam, ocean acidification, oxygen consumption rate, *Venerupis philippinarum*.

Introduction

According to the Representative Concentration Pathways (RCP) scenario presented by the 5th Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), atmospheric CO_2 levels are expected to increase to between 421 µatm (RCP 2.6) and 936 µatm (RCP 8.5) in the year 2100 as CO_2 emissions from anthropogenic activities increase. Accordingly, surface ocean water continues to absorb the rising levels of CO_2, resulting in a decrease in the availability of carbonate ions and a drop in the pH of seawater. This phenomenon of ocean acidification (OA) is expected to pose a potential threat to marine organisms (Orr *et al.*, 2005; Fabry *et al.*, 2008).

Recognizing the potential impact of OA on the marine ecosystem, ecologically relevant CO_2 perturbation experiments have been conducted worldwide, revealing substantial adverse (e.g., Sung *et al.*, 2014; Kim *et al.*, 2016) or no effects (e.g. Heinrich *et al.*, 2016; Sundin and Jutfelt, 2016; Zhang *et al.*, 2016) on a wide range of marine organisms. Many studies of the biological effects of OA have focused on the predicted change of mean pH of ocean surface waters derived from IPCC climate scenarios (e.g. Orr *et al.*, 2005; Heinrich *et al.*, 2016; Sundin and Jutfelt, 2016; Zhang *et al.*, 2016). However, nearshore environments are also influenced by low pH waters (Hofmann *et al.*, 2011) due to various factors such as eutrophication through increased microbial

respiration (Cai *et al.*, 2011; Kim *et al.*, 2013a; Wallace *et al.*, 2014), freshwater input by river discharge (Kim *et al.*, 2014), and upwelling from below the continental shelf and advected to shallow nearshore environments (Feely *et al.*, 2008). Future pH of nearshore water could be much lower than the expected pH of the open ocean.

The biological responses and effects of OA could vary depending on species specificity and life cycles (Langer *et al.*, 2006; Kroeker *et al.*, 2010; Sung *et al.*, 2014). Decreases in the pH of seawater may increase mortality and inhibit growth of certain species (Langer *et al.*, 2006; Lischka *et al.*, 2011; Range *et al.*, 2011; Kim *et al.*, 2013b). Although not directly detrimental, a decreased pH can change the behaviour and physiology of animals (Kim *et al.*, 2016; Munday *et al.*, 2016). The olfactory function of animals could be impaired, and thus, their prey detection or predator avoidance can be retarded (Kim *et al.*, 2016). Metabolism could be also influenced, thus changing oxygen consumption rates (Lannig *et al.*, 2010; Kim *et al.*, 2016).

This study aims to observe the effects of potential future CO_2 levels in seawater on the behaviour and physiology of adult and juvenile Manila clams, *Venerupis philippinarum*. The native range of *V. philippinarum* stretches from Siberia to China, and this species has been introduced to habitats ranging from Japan to the West Coast of North America. Manila clams are now cultured worldwide, including in North and Central America, Europe, and Asian countries, where it is one of the most popular shellfish resources (Becker *et al.*, 2008).

We conducted two experiments to determine if *V. philippinarum* is affected by OA. In Experiment I, we investigated how emerging behaviour from the sediment and the physiological responses of adult clams change under the predicted atmospheric pCO_2 levels of the year 2100 based on the RCP scenario presented by the 5th Assessment Report of the IPCC. Clams usually burrow into sandy or muddy sediment to escape predation (Lee *et al.*, 2012). However, they may emerge from the sediment into an environment that is not favourable to them. We used this emerging behaviour as an indicator of environmental stress. To compare the physiological responses, we measured the oxygen consumption rates of individuals exposed to each treatment. Because Manila clams live in the intertidal mudflat which experiences low pH (less than pH 7.5) which is induced by various factors such as eutrophication and other issues for up to 1–2 months (Kim *et al.*, 2013a; Kozuki *et al.*, 2013; Kim *et al.*, 2014; Wallace *et al.*, 2014), there needs an exposure experiment to lower pH. Therefore, in Experiment II, we tested how the younger clams responded to lower pH levels induced by notably higher levels of atmospheric CO_2.

Methods

Collection and maintenance of *V. philippinarum*

For Experiment I, approximately 200 individuals of adult Manila clams (*V. philippinarum*) were collected in a sealed Styrofoam box of low temperature without seawater on 16 May, 2014 and brought within 2.5 hr from the intertidal mudflats around Namdang-ri, Seobu-myeon, Hongseong-gun, Chungcheongnam-do, Republic of Korea (ROK), to the laboratory (Korea Institute of Ocean Science & Technology, Ansan). For Experiment II, about 700 much younger Manila clams were collected from Jugyo-ri, Jugyo-myeon, Boryeong-si, Chungcheongnam-do, ROK and brought to the laboratory in the same manner on 27 June, 2014. All clams were acclimatised in well-aerated seawater in a closed, recirculating aquaculture system (500 l, 150 cm × 70 cm × 80 cm, $W \times L \times H$) for a period of time before the experiments. The conditions were gradually changed from the *in situ* environmental conditions to the experimental temperature of 23 °C and the salinity of 34 ppt. The experimental temperature was regulated by a chiller and/or heater connected to a thermostat. The clams were sufficiently fed once per day with Shellfish Diet 1800® (Reed Mariculture Inc., USA). The photoperiod followed the natural light:dark (L:D) cycle of the outside laboratory.

Experimental setup

Experiment I

A total of 96 healthy clams (shell length (SL) 25.32 ± 1.66mm and total wet weight (TWW) 3.12 ± 0.54 g: mean ± SD) were selected from the individuals acclimatised to the common experimental condition (23 °C, 34 ppt, and over 85% DO). Generally, Manila clams live in mudflat sediments at a constant depth, acquiring nutrients from seawater by inhalant opening and eliminating waste matter by exhalent opening. Eight clams were allocated randomly to each of twelve 500 ml transparent glass jars containing 4 cm deep sediment. Every clam in each jar was marked from 1 to 8 on both sides of the shell with a meteor pen for individual identification. Four randomly selected jars were assigned to each of three nominal pCO_2 levels (i.e. 400, 700, and 900 µatm) for 48 days from 27 May to 14 July 2014. Feeding of each experimental clam was stopped 48 hr prior to the beginning and the end of the experiment to ensure a post-absorptive digestive state for the measurements of size and oxygen consumption rate. Experimental clams were fully fed once per day with Shellfish Diet 1800® during the entire period except for the beginning and end of the experiment.

The water of exposure tank was primarily filtered by ϕ 10µm filter, and then ϕ 1µm filter. Experimental seawater for each treatment group was delivered to each jar at a 75 ml min^{-1} flow rate by a gas-controlled, flow-through automatic circulatory system. In total, 12 jars (each housing eight clams) divided into three treatment groups were placed in an aquarium (91 l, 75 cm × 55 cm × 22 cm) containing one large platform with grooves to hold the jars. Each jar was overflowed with the water corresponding to the specific treatments.

Experiment II

A total of 240 healthy clams (SL 16.71 ± 0.96 mm and TWW 0.70 ± 0.13 g: mean±SD) were selected from individuals acclimatised to the experimental temperature and salinity. Ten clams were allocated randomly to each of twenty-four 500 ml transparent glass jars containing 4 cm deep sediment. Every clam in each jar was marked from 1 to 10 with a method used in Experiment I. Eight randomly selected jars were assigned to each of three nominal pCO_2 levels (i.e. 900, 1300, and 2300 µatm) for 39 days from 8 August to 16 September 2014. The feeding was done following the same method used in Experiment I. We used clams exposed to 900 µatm of pCO_2 (pH 7.8) as a control group for the experiment because 900 µatm of pCO_2 did not show any adverse effect on Manila clams in Experiment I and many Manila clams generally live in the mudflat of local estuaries which experience low pH water (e.g. pH 7.2–7.6 in Gwangyang Bay and pH 6.9–8.0 in Jinhae Bay, Korea during the summer season) frequently (Kim *et al.*, 2013a, 2014)

The experimental exposure tank consisted of three separate sets of the recirculation system, which included a filtration pump (EHEIM, 250/300 l h^{-1}) and the aquarium (22 l, 32 cm × 48 cm × 14.5 cm) containing a grooved platform to hold eight jars that were overflowed with the treatment water. The experimental seawater corresponding to each treatment group was delivered by a gas-controlled circulatory system to each jar at 77 ml min^{-1}.

Sediment

The sediment for Experiment I was collected from mudflats where Manila clams for the experiment were collected. Fine mud and sand were mixed at the same ratio and provided as experimental sediment. Although the sediment composition is close to that of the habitat, it was hard to maintain small particle sediment (mud) in the experimental system and standardize the composition. Therefore, we used normal sand by cleaning several times with freshwater, drying in the sun and then passing through a sieve of diameter (ϕ) 1 mm. The compositions (%) of the sediments used in the experiments were analysed using a Gradex 2000 particle size analyser (Rotex Inc., USA) and the principle of X-ray monitored gravity sedimentation; the values obtained were represented as percentages by weight for each grade (Table 1). The major textural class of sediment (Table 1) was determined from the statistical variables, such as average size, degree of sorting, skewness value, and kurtosis of percentages by weight for each grade using the Inclusive Graphic Method (Folk and Ward, 1957). The statistical processing program SdiGraph3 V1.04 was used for the particle size analysis.

pCO_2 treatments and seawater chemistry

Experiment I

To regulate the experimental pCO_2 levels and to maintain the normal dissolved oxygen (DO) level of seawater (85–100% saturated DO), we used a high-precision pCO_2 control system, AICAL, which includes CO_2 dissolution and measurement towers of 1.78 m in height (Fujita *et al.*, 2011; Suwa *et al.*, 2013). Gaseous CO_2 was well dissolved in the filtered (pore size, 10 and 1μm seriatim) seawater by being bubbled from the bottom of the towers. The pCO_2 levels of seawater were continuously monitored and recorded every minute in the equilibrated air flowing out from the measurement tower surface using a pCO_2 measuring/monitoring system (CO2-07; Kimoto Electric) equipped with a non-dispersive infrared (NDIR) analyser (LI-840; LI-COR, Lincoln, NE, USA). The pCO_2 levels were adjusted as needed in a pCO_2-regulation system (CGM-07 and DGG-07; Kimoto Electric, Osaka, Japan) by controlling the CO_2 concentration in the air by mixing CO_2 and dilution-air (dried air with a low CO_2 concentration into the filtered seawater). The pCO_2-equilibrated seawater was pumped from the tower into four replicate experimental jars for each treatment using a peristaltic pump.

The physical and carbonate chemistry variables for the experimental seawater of each treatment are shown in Table 2. Temperature (*T*) and salinity (*S*) were measured with a model YSI 30 electronic conductivity/temperature meter (Yellow Spring Instruments Inc., USA) two times a day (am 10:00 and pm 4:00). DO was only measured in treatments A and C with a YSI 5010 BOD Probe connected to a YSI 5000 dissolved oxygen meter (Yellow Spring Instruments Inc., USA) three times during the

Table 1. Composition and type of sediment used in Experiments I and II

	Composition (%)				Statistical Parameters				
	Gravel	Sand	Silt	Clay	Mean (ϕ)	Sorting	Skewness	Kurtosis	Sediment Type
Experiment I	0.00	73.07	19.13	7.80	2.66	2.75	0.70	1.04	zS
Experiment II	0.00	99.75	0.25	0.00	1.19	0.76	0.10	0.98	S

Table 2. Seawater physical and carbonate chemistry variables (mean ± *SD*) of three pCO_2 treatments in Experiment I: temperature (*T*) and salinity (*S*) were measured every day; dissolved oxygen (DO) was measured in treatments A and C on the 16th, 30th, and 48th day of the experiment; pH and total alkalinity (TA) were measured four times and twice during the experiment, respectively; the partial pressure of CO_2 in seawater (pCO_2) was monitored continuously using an NDIR analyser during the experiment; dissolved inorganic carbon (DIC), the calcite and aragonite saturation states, and the remaining parameters were calculated with the CO2SYS Calc XLS program (v2.1) using TA and pCO_2

	Nominal pCO_2 (μatm)		
Parameter	400 (A)	700 (B)	900 (C)
T (°C)	23.1 ± 0.7 (23.9 ± 0.3)	23.1 ± 0.7 (23.9 ± 0.2)	23.1 ± 0.7 (24.0 ± 0.1)
S (ppt)	33.9 ± 0.8 (34.3 ± 0.5)	33.9 ± 0.8 (34.3 ± 0.5)	33.9 ± 0.8 (34.3 ± 0.5)
DO (mgl^{-1})	6.79 ± 0.21	–	6.72 ± 0.26
DO (%)	97.9 ± 4.0	–	96.7 ± 4.6
TA ($\mu molkg^{-1}$)	2398.31 ±54.86	2397.11 ± 56.05	2393.56 ± 55.06
pCO_2 (μatm)	438.6 ± 13.1 (442.1 ± 5.1)	738.3 ± 12.5 (740.4 ± 8.9)	932.9 ± 90.9 (955.1 ± 13.9)
$[CO_2]$ ($\mu molkg^{-1}$)	12.93 ± 0.14	21.63 ± 0.19	27.83 ± 0.40
$[HCO_3^-]$ ($\mu molkg^{-1}$)	1897.55 ± 32.18	2048.32 ± 39.24	2106.40 ± 39.46
$[CO_3^{2-}]$ ($\mu molkg^{-1}$)	205.88 ± 9.88	143.56 ± 7.25	118.22 ± 6.71
DIC ($\mu molkg^{-1}$)	2116.37 ± 41.80	2213.51 ± 46.29	2252.46 ± 45.77
pH (NBS scale)	8.04 ± 0.04 (8.05 ± 0.03)	7.86 ± 0.03 (7.86 ± 0.01)	7.77 ± 0.03 (7.77 ± 0.01)
Calcite saturation	4.98 ± 0.22	3.47 ± 0.16	2.86 ± 0.15
Aragonite saturation	3.26 ± 0.14	2.28 ± 0.11	1.88 ± 0.10

The values in parentheses were measured using the samples prepared for TA measurements.

entire experimental period. pH_{NBS} was measured with a Seven Multi pH meter (Mettler-Toledo International Inc., Germany) four times during the experiment. The pH meter was regularly calibrated with the pH values of YSI Buffer Solution (pH 4.00, pH 7.00, and pH 10.00 at 25 °C). To measure the total alkalinity (TA), the samples were titrated automatically with HCl (~0.25 M HCl in a solution of 0.45 M NaCl) past the endpoint of pH 4.5 with an accuracy of ± 2 μmol kg^{-1} (Dickson *et al.*, 2005). The accuracy of the TA measurements was controlled using certified reference material supplied by A.G. Dickson (Scripps Institution of Oceanography, San Diego, USA). TA analysis was performed two times during the experiment. The remaining parameters except those mentioned previously and pCO_2 were calculated with the CO2SYS Calc XLS program (v2.1) (Pierrot *et al.*, 2006) using the values of T, S, TA, pCO_2, and pH_{NBS} with the dissociation constants for carbonic acid and the dissociation constants for the reaction (KSO_4) (Dickson, 1990). The values in parentheses given in Table 2 were measured from the samples prepared for TA measurements.

Experiment II

To regulate the experimental pCO_2 levels and to maintain the normal DO level of the seawater (85–100% saturated DO), we used two sets of the gas concentration control system with mass flow controllers (MFCs) and an air-blower. The principle of this system is to continuously supply experimental seawater to each treatment group with mixed gas via the MFCs using the standard gas (a mixture of 30% CO_2 and 70% N_2) and the dilution gas (100% air). The concentration of each treatment group reached equilibrium states that were close to the target pCO_2 levels. Thereafter, we measured the pH and TA of the experimental seawater to calculate the pCO_2 reached. For the targeted level of pCO_2, we regulated the mass and the flow of the MFC. This whole procedure was continued until the targeted nominal pCO_2 levels were attained.

The physical and carbonate chemistry variables for experimental seawater of each treatment are shown in Table 3. T and S were measured with a YSI 30 electronic conductivity/temperature meter once or twice a day. DO and pH_{NBS} were measured with the same methods used in Experiment I once a day. TA analysis was done with the same methods as Experiment I only once during the entire experiment. The rest of the parameters, such as pCO_2, dissolved inorganic carbon (DIC), and the other carbonate chemistry parameters, were calculated with the CO2SYS Calc XLS program (v2.1) (Pierrot *et al.*, 2006) using the values of T, S, TA, and pH_{NBS} with the dissociation constants for carbonic acid and the dissociation constants for the reaction (KSO_4) (Dickson, 1990). The values in parentheses given in Table 3 were also measured from the samples prepared for TA measurements.

Survival rate and emergence

The mortality of clams in each jar was checked daily during the entire experimental period (48 days in Experiment I and 39 days in Experiment II). To determine the live/dead status of the clams, the pores of the siphons shown in the surface of sediment and the status of gaping were checked with the naked eye. When visual checking was not possible, the power of shell closure or heaviness of the clam was checked using pincers. Individuals that did not close the shell in response to touching or were too lightweight were considered dead. Dead individuals were immediately removed from the jar after checking. The survival rate (%) was calculated by dividing the number of surviving individuals by the number of initial individuals.

The location of each clam in each jar (in/out of the sediment) was also checked between 10:00 and 11:00 am daily during each entire experiment period. The emergence rate (%) was calculated by dividing the number of clams that emerged from the sediment by the number of live individuals and was thereafter presented as a percentage.

Growth

The SL (mm) and TWW (g) of each clam were measured at the beginning and end of the experiment to observe the effects of pCO_2 on the growth of living clams during the exposure (on day 48 in Experiment I and on day 39 in Experiment II). The growth rates (%) of the clams were calculated individually over the whole period of exposure as follows:

$$\text{Growth rate during the whole period (\%)} = \frac{\text{SL(or TWW)}_{\text{final}} - \text{SL(or TWW)}_{\text{initial}}}{\text{SL(or TWW)}_{\text{initial}}} \times 100.$$

Table 3. Seawater physical and carbonate chemistry variables (mean ± *SD*) of three pCO_2 treatments in Experiment II: temperature (*T*), salinity (*S*), dissolved oxygen (DO), and pH were measured every day; total alkalinity (TA) was measured once during the experiment; partial pressure of CO_2 in seawater (pCO_2), Dissolved inorganic carbon (DIC), and calcite and aragonite saturation states were calculated with the CO2SYS Calc XLS program (v2.1) using TA and pH_{NBS}

	Nominal pCO_2 (μatm)		
Parameter	**900 (D)**	**1300 (E)**	**2300 (F)**
T (°C)	22.6 ± 0.3 (22.2 ± 0.0)	22.6 ± 0.3 (22.2 ± 0.1)	22.6 ± 0.3 (22.3 ± 0.0)
S (ppt)	33.9 ± 0.4 (33.9 ± 0.0)	33.9 ± 0.4 (33.8 ± 0.0)	34.0 ± 0.4 (34.2 ± 0.0)
DO (mg l^{-1})	6.64 ± 0.35	6.10 ± 0.34	6.24 ± 0.34
DO (%)	93.6 ± 4.9	85.9 ± 4.7	87.8 ± 4.8
TA (μmol kg^{-1})	2486.17 ± 1.38	2554.21 ± 2.31	2726.10 ± 4.72
pCO_2 (μatm)	927.5 ± 0.5	1339.3 ± 0.5	2342.7 ± 261.8
$[CO_2]$ (μmol kg^{-1})	28.39 ± 0.02	41.01 ± 0.14	71.40 ± 7.98
$[HCO_3^-]$ (μmol kg^{-1})	2193.43 ± 1.24	2327.58 ± 0.90	2567.19 ± 11.58
$[CO_3^{2-}]$ (μmol kg^{-1})	121.28 ± 0.07	94.39 ± 0.61	66.84 ± 6.87
DIC (μmol kg^{-1})	2343.10 ± 1.33	2462.98 ± 1.37	2705.43 ± 12.69
pH (NBS scale)	7.80 ± 0.10 (7.90 ± 0.00)	7.69 ± 0.08 (7.76 ± 0.00)	7.47 ± 0.10 (7.56 ± 0.05)
Calcite saturation	2.93 ± 0.00	2.28 ± 0.02	1.61 ± 0.17
Aragonite saturation	1.91 ± 0.00	1.49 ± 0.01	1.05 ± 0.11

The values in parentheses were measured using the samples prepared for TA measurements.

The experimental clams were fasted for 2 days before the size measurements to ascertain the post-digestion growth rates.

Respirometry

Experiment I

The oxygen consumption rates (OCRs) were measured using the experimental treatment seawater for each of the four subsets (three clams per subset), which included the Control and the highest pCO_2 experimental treatment, at four points: (i) at the initial conditions before exposure to the Control and the highest pCO_2 experimental treatment; (ii) at the 16th day into exposure; (iii) at the 30th day into exposure; (iv) ∼ at the 48th day into exposure. For the first respirometry point, three clams were selected randomly from each jar of the group; the same clams were continuously used for each subsequent respirometry point. Before measurement, each measured individual was freed from the sediment and its shell surface was cleaned using the experimental treatment seawater.

In order to estimate the microbial oxygen consumption in the unfiltered experimental treatment seawater, measurements were also simultaneously performed using the same treatment seawater without clams. The estimated rates of oxygen decline due to microbial respiration were subsequently subtracted from the measured rates of oxygen decline when the clams were present.

The clams were fasted for at least 48 hr before being placed into the tailor-made DO bottle with a volume of ca. 487 ml for measurement. Oxygen consumption (OC) was calculated from the difference between the initial and final DO concentrations induced by experimental individuals and the other factors involved in the calculation of OC, including the bottle volume and measuring time (hr). The DO concentration was measured with a DO meter (YSI 5000 and YSI 5010 BOD Probe) using the polarographic method. The initial DO concentration was steady at 92–100%, and the final DO was steady at 71–81%. The OCRs were calculated as ml of O_2 consumed per hour and were normalised to the flesh dry weight (FDW) of the clams to remove the variability in metabolic rates associated with changes in biomass. During the measurement, each DO bottle was sealed with tinfoil to provide dark conditions and then immersed ca. 2/3 in height in a large aquarium filled with temperature-regulated seawater together with the experimental jars to maintain the experimental temperature. After 1 hr, the clams were removed from the DO bottle and returned to their treatment jars. At the end of the experiment, a measured clam was sacrificed and immediately the flesh was extracted and dried in a drying oven (VS-1202D3, Vision Scientific Co., Ltd, Korea) at 80 °C for 24 hr. Finally, the FDW was determined for use in the calculation of OCR.

Experiment II

OCRs were measured using the experimental treatment seawater for each of the three subsets (five clams per subset) of the three experimental treatments at three points: (i) at initial conditions before exposure to the three experimental treatments; (ii) at the 20th day into exposure; and (iii) at the 39th day into exposure. For the first respirometry point, five clams were selected randomly from each jar of the group; the same clams were used for the remaining respirometry points. In cases in which a clam died during the course of the experiment, it was replaced with another clam within the same subset. The initial DO concentration was steady at a saturation level of 89–99%, and the final DO was steady at a saturation level of 86–96%. All other measurement procedures followed the same method as Experiment I. The dried flesh was weighed for use in the calculation of OCR on the 39th day of exposure. In cases in which the FDW could not be measured from the clam used in the metabolic weight experiment (when the clam died during the exposure period or was used in the initial and middle period for measurement), the relationship between SL(mm) and FDW (g) was derived from independent individual samples to estimate the FDW of the measured clam. The relation formula used was $FDW = 0.0038 \times SL - 0.0414$ ($r^2 = 0.7223$, $n = 105$, the range of SL 11.71–20.64 mm).

Statistical analysis

All proportional data (cumulative mortality and the emergence rate of clams) were arcsine root square transformed. Then, the repeated measures ANOVA test was applied. In all repeated-measures analyses, the assumption of equal between-group correlations and group variances ("sphericity") was not violated (Mauchly's test, all $p > 0.05\%$). If there was a significant difference, Tukey's post-hoc test was applied. The growth rates of SL and TWW were compared between treatments using a one-way ANOVA test. Because OCRs may be variable depending on the time of measurement and by an endogenous respiration rhythm such as circadian and(or) circatidal rhythm (Lee *et al.*, 2007), we compared respiration rates between treatment groups separately at each measurement time (Kim *et al.*, 2016). Because OCRs are also non-normally distributed, the Mann–Whitney *U* test was used to compare OCRs between two treatment groups and the Kruskal–Wallis test was used for three treatment groups.

Results

Experiment I

Mortality, growth and emergence

During 48 days of exposure, the survival rates of clams did not differ between the different pCO_2 treatments (repeated-measures ANOVA: $F_{2,\ 9} = 1.963$, $p = 0.196$). Although SL growth rate was negative in 700 and 900 µatm of pCO_2, neither the SL growth rate (one-way ANOVA: $F_{2,87} = 1.456$, $p = 0.239$) nor the TWW growth rate (one-way ANOVA: $F_{\ 2,\ 87} = 0.838$, $p = 0.436$) were significantly different between treatments (Table 4). The emergence rate of clams was not significantly different between treatments ($F_{\ 2,\ 9} = 0.361$, $p = 0.706$, Figure 1a). Neither exposure time ($F_{\ 47,\ 423} = 1.120$, $p = 0.279$) nor the interaction between exposure time and treatments ($F_{\ 94,\ 423} = 0.981$, $p = 0.534$) influenced emergence rate.

Oxygen consumption rate

Before and after exposure, the OCR of clams was not significantly different between the treatments of 400 and 900 µatm pCO_2 throughout the entire experiment (Mann–Whitney *U*-test: before exposure, $Z_{\ 4,4} = -0.726$, $p = 0.468$; 16 days after exposure, $Z_{\ 4,\ 4} = -0.289$; $p = 0.773$, 30 days after exposure, $Z_{\ 4,\ 4} = 0.000$, $p = 1.000$; and 48 days after exposure, $Z_{\ 4,\ 4} = 0.000$, $p = 1.000$, Figure 1b).

Experiment II

Mortality, growth, and emergence

Over 39 days, the survival rate of clams did not differ significantly between different pCO_2 treatments (repeated measures ANOVA: $F_{\ 2,\ 21} = 1.379$, $p = 0.274$). Although SL growth rate was negative in 1300 and 1700 µatm of pCO_2, neither the SL growth rate ($F_{\ 2,}$

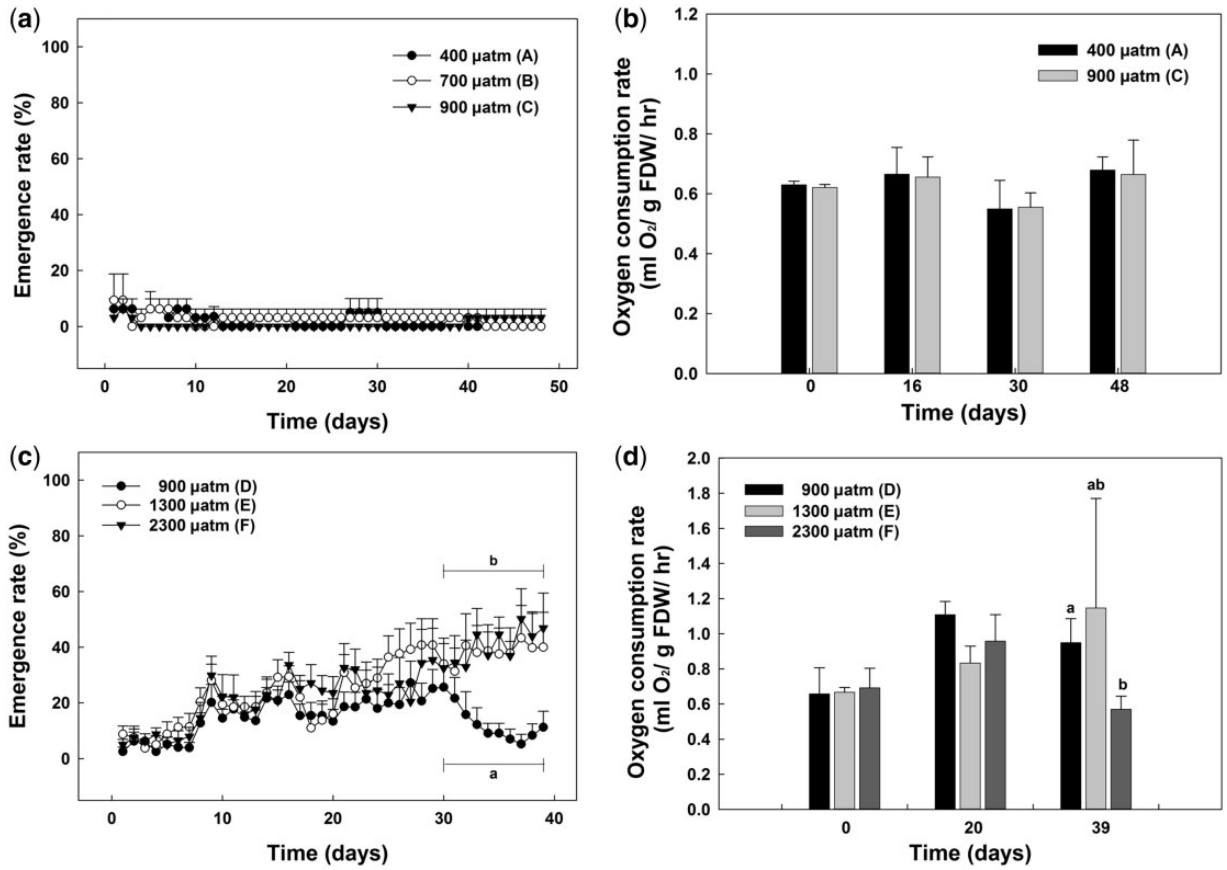

Figure 1. (a) Percentage of clams emerging from sediment out of the total number of clams at three pCO_2 concentrations in Experiment I. (b) Oxygen consumption rate of clams at two pCO_2 concentrations in Experiment I. (c) Percentage of clams emerging from sediment out of the total number of clams at three pCO_2 concentrations in Experiment II. Different letters indicates the periods when the two groups showed significant differences. (d) Oxygen consumption rate of clams at three different pCO_2 concentrations in Experiment II. Different letters indicate significant differences between the groups.

Table 4. Percent change in shell length and total wet weight during the experiment

Percent change of	Nominal pCO_2 (µatm)					
	Experiment I (48 days)			Experiment II (39 days)		
	400 (A)	700 (B)	900 (C)	900 (D)	1300 (E)	2300 (F)
Shell length	0.38 ± 0.49 ($n = 27$)	−0.15 ± 0.05 ($n = 31$)	−0.19 ± 0.05 ($n = 32$)	0.13 ± 0.24 ($n = 33$)	−0.08 ± 0.03 ($n = 36$)	−0.23 ± 0.03 ($n = 26$)
Total wet weight	0.03 ± 0.94 ($n = 27$)	0.86 ± 0.23 ($n = 31$)	0.97 ± 0.33 ($n = 32$)	3.04 ± 0.67 ($n = 33$)	3.21 ± 0.35 ($n = 36$)	4.29 ± 0.54 ($n = 26$)

$_{92} = 1.507$, $p = 0.227$) nor the TWW growth rate of clams ($F_{2,\ 92} = 1.438$, $p = 0.243$) significantly differed between treatments (Table 4). The emergence rate of clams was not significantly different between the treatments (Repeated measures ANOVA: $F_{2,\ 21} = 2.836$, $p = 0.081$, Figure 1c). However, there was a significant effect of exposure time ($F_{47,\ 423} = 6.787$, $p < 0.0001$) and interaction between time and treatment ($F_{76,\ 798} = 1.625$, $p = 0.001$) on the emergence rate (Figure 1c) which means that treatment influenced the emergence rate as time went by. Especially, the emergence rate became significantly different between the treatments between 30 and 39 days after exposure ($F_{2,\ 21} = 5.085$, $p = 0.016$, Figure 1c).The emergence rate of the clams treated with high pCO_2 (1300 and 2300 µatm) was greater than that of clams treated with 900 µatm of pCO_2 (Tukey's post-hoc test: 900 µatm vs. other treatments, all $p < 0.05$).

Oxygen consumption rate

Before and 20 days after exposure, the OCR was not significantly different between the treatments (Kruskal–Wallis test: before exposure, $\chi^2 = 0.622$, $df = 1$, $p = 0.733$; 20 days after exposure, $\chi^2 = 2.756$, $df = 1$, $p = 0.252$). However, 39 days after exposure, the OCR of clams under the highest pCO_2 (2300 µatm) became lower than that of the clams under 900 µatm of pCO_2, and the differences between treatments were marginally significant (Mann–Whitney U-test: $Z_{3,\ 3} = -1.964$, $p = 0.05$, Figure 1d).

Discussion

When the adult *V. philippinarum* clams were exposed to 400, 700, and 900 µatm of pCO_2 for 48 days, the mortality, oxygen consumption rate, and emergence from sediment were not significantly different among the three pCO_2 levels. Although 48 days are the short experimental period to determine the effect of OA on long-term scale, this indicates that near future atmospheric levels of pCO_2 may not influence the physiology and behaviour of adult Manila clams. In contrast, when younger Manila clams were exposed to pCO_2 levels of 900, 1300, and 2300 µatm (i.e. pH 7.8, 7.7, and 7.5, respectively) for 39 days, although mortality was not significantly different between treatments, emerging behaviour increased at the two higher pCO_2 level during the late period of the experiment. Furthermore, the oxygen consumption rate was significantly lower under a pCO_2 level of 2300 µatm at 39 days than any other treatment after exposure. This suggests that the population of *V. philippinarum* may be negatively influenced by higher CO_2 levels than expected in the near future or within this century. Although atmospheric pCO_2 levels of 1300 and 2300 µatm are difficult to expect in the near future, pH 7.7 and 7.5 could be attained in the water depending on local conditions, such as eutrophication and hypoxia (Kim *et al.*, 2013a, 2014; Wallace *et al.*, 2014).

Emerging from sediment is not a normal clam behaviour. Clams usually dig burrows in the sediment to escape predation, exposing the siphon to the surface of the sediment in order to feed. However, when conditions are unfavourable, they may emerge from their burrows (Lee *et al.*, 2012). Although we did not confirm the correlation between emerging behaviour and eventual death of individual Manila clams, we frequently observed that emerging clams did not dig burrows again and were eventually found dead on the surface of the sediment. Manila clams might express this emerging behaviour under conditions of hypercarpenia because they cannot maintain the acid/base balance in their body fluids (Lannig *et al.*, 2010). Emergence from burrows of calms can also significantly increase predation risk by other predators such as crabs and birds (Seitz *et al.*, 2003; Saloom and Duncan, 2005).

The decrease in the OCR indicated that the clams reduced their metabolism in response to increased CO_2 levels. Though the DO of seawater was maintained close to a saturation point, increased CO_2 may be quite stressful to the clams, potentially leading them to reduce their respiration. Underlying diverse mechanisms can lead to change in oxygen consumption (Pörtner *et al.*, 2004). Acid–base regulation and $GABA_A$ receptor functioning related to respiration may be impaired by OA (Clements and Hunt, 2015). The respiratory responses to high CO_2 and/or low pH are quite variable and species dependent. Some species increase respiration (Kim *et al.*, 2016), other species do not change respiration (Carey *et al.*, 2014), while still other species decrease respiration (Edmunds, 2012). Life history stages, CO_2 level, and exposure duration as well as species characteristics may complexly influence OCR.

In conclusion, near future levels of OA by atmospheric CO_2 may not severely influence the population of *V. philippinarum*. However, much lower seawater pH induced by eutrophication and other factors which local estuaries can experience now and in near future may severely affect the stability of the population by changing their physiology and behaviour. Acidification in estuaries or bays is usually accompanied by hypoxia and other local stressors (Cai *et al.*, 2011; Kim *et al.*, 2013b). However, in contrast to enormous amount of studies on the effects of acidification *per se*, not many studies were conducted on the effects of multiple stressors including acidification and hypoxia (Gobler and Baumann, 2016). If high pCO_2 levels are combined with other local conditions, such as ocean eutrophication and hypoxia, the sustainability of the Manila clam population may be questionable. Additional studies are needed to answer whether younger individuals are more vulnerable to future OA; to determine how much variation exists depending on local populations; and whether other factors, such as temperature and DO, can synergistically influence the population of Manila clams.

Acknowledgements

The authors thank Ok Hwan Yu, Dongseon Kim, Yu Jeong Choi, and Chae Young Yoo for the experimental support.

Funding

This research was supported by the KIOST internal research fund PE99358 and PE9944F, and the Ministry of Oceans and Fisheries fund PG49260.

References

Becker, P., Barringer, C., and Marelli, D. C. 2008. Thirty years of sea ranching Manila clams (*Venerupis philippinarum*): successful techniques and lessons learned. Reviews in Fisheries Science, 16: 44–50.

Cai, W. J., Hu, X. P., Huang, W. J., Murrell, M. C., Lehrter, J. C., Lohrenz, S. E., Chou, W. C. *et al.* 2011. Acidification of subsurface coastal waters enhanced by eutrophication. Nature Geoscience, 4: 766–770.

Carey, N., Dupont, S., Lundve, B., and Sigwart, J. D. 2014. One size fits all: stability of metabolic scaling under warming and ocean acidification in echinoderms. Marine Biology, 161: 2131–2142.

Clements, J. C., and Hunt, H. L. 2015. Marine animal behaviour in a high CO_2 ocean. Marine Ecology Progress Series, 536: 259–279.

Dickson, A. G. 1990. Thermodynamics of the dissociation of boric-acid in synthetic seawater from 273.15-K to 318.15-K. Deep-Sea Research Part a-Oceanographic Research Papers, 37: 755–766.

Dickson, A. G., Sabine, C. L., and Christian, J. R. 2005. Guide to best practices for ocean CO_2 measurements. In: PICES Special Publication No. 3 IOCCP Report 8: 191 pp.

Edmunds, P. J. 2012. Effect of pCO_2 on the growth, respiration, and photophysiology of massive *Porites spp.* in Moorea, French Polynesia. Marine Biology, 159: 2149–2160.

Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. Ices Journal of Marine Science, 65: 414–432.

Feely, R. A., Sabine, C. L., Hernandez-Ayon, J. M., Ianson, D., and Hales, B. 2008. Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science, 320: 1490–1492.

Folk, R. L., and Ward, W. C. 1957. Brazos River bar: study in the significance of grain size parameters. Journal of Sedimentary Petrology, 27: 3–27.

Fujita, K., Hikami, M., Suzuki, A., Kuroyanagi, A., Sakai, K., Kawahata, H., and Nojiri, Y. 2011. Effects of ocean acidification on calcification of symbiont-bearing reef foraminifers. Biogeosciences, 8: 2089–2098.

Gobler, C. J., and Baumann, H. 2016. Hypoxia and acidification in ocean ecosystems: coupled dynamics and effects on marine life. Biology Letters, 12: 20150976.

Heinrich, D. D. U., Watson, S. A., Rummer, J. L., Brandl, S. J., Simpfendorfer, C. A., Heupel, M. R., and Munday, P. L. 2016. Foraging behaviour of the epaulette shark *Hemiscyllium ocellatum*

is not affected by elevated CO_2. ICES Journal of Marine Science, 73: 633–640.

Hofmann, G. E., Smith, J. E., Johnson, K. S., Send, U., Levin, L. A., Micheli, F., Paytan, A. *et al.* 2011. High-frequency dynamics of ocean pH: a multi-ecosystem comparison. PLoS One, 6: e28983.

Kim, D., Choi, S. H., Yang, E. J., Kim, K. H., Jeong, J. H., and Kim, Y. O. 2013a. Biologically mediated seasonality of Aragonite saturation states in Jinhae Bay, Korea. Journal of Coastal Research, 29: 1420–1426.

Kim, D., Yang, E. J., Baek, S. H., Kim, K. H., Jeong, J. H., and Kim, Y. O. 2014. Aragonite undersaturation in Gwangyang Bay, South Korea: effects of fresh water input. Ocean Science Journal, 49: 223–230.

Kim, T. W., Barry, J. P., and Micheli, F. 2013b. The effects of intermittent exposure to low-pH and low-oxygen conditions on survival and growth of juvenile red abalone. Biogeosciences, 10: 7255–7262.

Kim, T. W., Taylor, J., Lovera, C., and Barry, J. P. 2016. CO_2-driven decrease in pH disrupts olfactory behaviour and increases individual variation in deep-sea hermit crabs. ICES Journal of Marine Science, 73: 613–619.

Kozuki, Y., Yamanaka, R., Matsushige, M., Saitoh, A., Otani, S., and Ishida, T. 2013. The after-effects of hypoxia exposure on the clam *Ruditapes philippinarum* in Omaehama beach, Japan. Estuarine Coastal and Shelf Science, 116: 50–56.

Kroeker, K. J., Kordas, R. L., Crim, R. N., and Singh, G. G. 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecology Letters, 13: 1419–1434.

Langer, G., Geisen, M., Baumann, K. H., Klas, J., Riebesell, U., Thoms, S., and Young, J. R. 2006. Species-specific responses of calcifying algae to changing seawater carbonate chemistry. Geochemistry Geophysics Geosystems, 7: Q09006 ,

Lannig, G., Eilers, S., Portner, H. O., Sokolova, I. M., and Bock, C. 2010. Impact of ocean acidification on energy metabolism of oyster, *Crassostrea gigas* – changes in metabolic pathways and thermal response. Marine Drugs, 8: 2318–2339.

Lee, A. C., Lee, Y. C., and Chin, T. S. 2012. Effects of low dissolved oxygen on the digging behaviour and metabolism of the hard clam (*Meretrix lusoria*). Aquaculture Research, 43: 1–13.

Lee, J. A., Kim, J. W., and Kim, W. S. 2007. Effect of tremata closures on the oxygen consumption rhythm of ezo abalone *Haliotis discus hannai*. Aquaculture, 270: 312–320.

Lischka, S., Budenbender, J., Boxhammer, T., and Riebesell, U. 2011. Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth. Biogeosciences, 8: 14.

Munday, P. L., Watson, S. A., Parsons, D. M., King, A., Barr, N. G., Mcleod, I. M., Allan, B. J. M. *et al.* 2016. Effects of elevated CO_2 on early life history development of the yellowtail kingfish, *Seriola lalandi*, a large pelagic fish. ICES Journal of Marine Science, 73: 641–649.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A. *et al.* 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature, 437: 681–686.

Pörtner, H. O., Langenbuch, M., and Reipschlager, A. 2004. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. Journal of Oceanography, 60: 705–718.

Pierrot, D., Lewis, E., and Wallace, D. W. R. 2006. MS Excel program developed for CO2 system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, TN.

Range, P., Chicharo, M. A., Ben-Hamadou, R., Pilo, D., Matias, D., Joaquim, S., Oliveira, A. P. *et al.* 2011. Calcification, growth and mortality of juvenile clams *Ruditapes decussatus* under increased pCO_2 and reduced pH: variable responses to ocean acidification at local scales? Journal of Experimental Marine Biology and Ecology, 396: 177–184.

Saloom, M. E., and Duncan, D. S. 2005. Low dissolved oxygen levels reduce anti-predation behaviours of the freshwater clam *Corbicula fluminea*. Freshwater Biology, 50: 1233–1238.

Seitz, R. D., Marshall, L. S., Hines, A. H., and Clark, K. L. 2003. Effects of hypoxia on predator–prey dynamics of the blue crab *Callinectes sapidus* and the Baltic clam *Macoma balthica* in Chesapeake Bay. Marine Ecology Progress Series, 257: 179–188.

Sundin, J., and Jutfelt, F. 2016. 9-28 d of exposure to elevated pCO_2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (*Ctenolabrus rupestris*). Ices Journal of Marine Science, 73: 620–632.

Sung, C. G., Kim, T. W., Park, Y. G., Kang, S. G., Inaba, K., Shiba, K., Choi, T. S. *et al.* 2014. Species and gamete-specific fertilization success of two sea urchins under near future levels of pCO_2. Journal of Marine Systems, 137: 67–73.

Suwa, R., Nojiri, Y., Ono, T., and Shirayama, Y. 2013. Effects of low pCO_2 conditions on sea urchin larval size. Marine Ecology-an Evolutionary Perspective, 34: 443–450.

Wallace, R. B., Baumann, H., Grear, J. S., Aller, R. C., and Gobler, C. J. 2014. Coastal ocean acidification: the other eutrophication problem. Estuarine Coastal and Shelf Science, 148: 1–13.

Zhang, H. Y., Shin, P. K. S., and Cheung, S. G. 2016. Physiological responses and scope for growth in a marine scavenging gastropod, *Nassarius festivus* (Powys, 1835), are affected by salinity and temperature but not by ocean acidification. ICES Journal of Marine Science, 73: 814–824.

Handling editor: David M. Fields