EFFECTS OF ELEVATED SEAWATER CO2 ON FEED INTAKE, OXYGEN CONSUMPTION AND MORPHOLOGY OF ARISTOTLE'S LANTERN IN THE SEA URCHIN ANTHOCIDARIS CRASSISPINA

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EFFECTS OF ELEVATED SEAWATER CO_2 ON FEED INTAKE, OXYGEN CONSUMPTION AND MORPHOLOGY OF ARISTOTLE'S LANTERN IN THE SEA URCHIN *Anthocidaris crassispina*

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ABSTRACT

Adult sea urchins, *Anthocidaris crassispina*, were reared individually in running seawater equilibrated with gas mixtures containing 380 ppm (control), 1,000 ppm or 3,000 ppm CO_2, at ambient natural temperature for 140 days to study effects of elevated CO_2 on feed intake, fecal production, oxygen consumption, and the morphology of Aristotle's lantern. Feed intake became significantly lower in the two high CO_2 groups than in the control after 90 (1,000 ppm) or 110 (3,000 ppm) days and remained suppressed until the end of the experiment (control, 0.16 ± 0.01 (mean $\pm$ SE) g dry wt ind^{-1} day^{-1}; 1,000 ppm, 0.10 ± 0.01; 3,000 ppm, 0.11 ± 0.01, as determined on day 140). Fecal production showed similar responses. Oxygen consumption was 35% lower in the two high CO_2 groups when measured on day 50, but subsequently became similar between the groups except a significant difference between control and 3,000 ppm urchins on day 140. When determined on day 140, magnesium concentration of the coelomic fluid was significantly elevated, but calcium concentration was unaffected in the high CO_2 urchins. Exposure to 3,000 ppm CO_2 resulted in undulating lateral plates and ridges of the teeth, and the distal edges of tooth ridges appeared worn out. These results suggest that energy available for growth and reproduction was compromised under the elevated CO_2 conditions, which might lead to reduced growth and reproductive output when exposure prolonged.

I. INTRODUCTION

It is estimated that approximately 25% of anthropogenic carbon dioxide emission has been absorbed by the oceans during the last 200 years since the beginning of the industrial revolution [34]. Elevations of dissolved CO_2 shift carbonate equilibria of seawater, thereby increasing concentrations of H^+ (lowering pH) and HCO_3^- but decreasing concentration of CO_3^{2-} and therefore saturation state of calcium carbonate ($CaCO_3$) in seawater. The process is usually termed ocean acidification. It is considered that the global mean pH of surface seawater has decreased by 0.1 units until today, and is projected to further decrease by 0.3 to 0.4 units by the end of this century [7], with potentially adverse consequences for marine ecosystem structures and functions [2, 13, 14, 21].

Echinoderms are one of the animal groups that are thought to be most severely impacted by predicted oceanic environmental changes due to increasing CO_2 concentrations [15]. This is probably because echinoderms build skeletons with high-magnesium calcite (a polymorph of $CaCO_3$ containing >4% mol Mg/mol Ca) [10], which is more soluble than aragonite (another polymorph seen in e.g., coral and pteropods) [33]. In addition, although data are not available for all five classes of echinoderms (Crinoidea, Asteroidea, Ophiuroidea, Echinoidea, and Holothuroidea), sea urchins have a poor capacity for acid-base regulation under hypercapnic (elevated CO_2) conditions so that body fluid pH shows only limited, if any, recovery towards normal values during hypercapnic exposure ([30, 37], but see [39]), and one species of sea star *Asterias rubens* was reported to show no pH compensation in high CO_2 seawater [3]. In addition to effects on acid-base status, exposure to high CO_2 seawater is known to affect fertilization [6, 19], early development [29, 46], gene expression [38, 31], and calcification [11] in sea urchins. In comparison, only a few studies examined how high CO_2 would affect feed intake in sea urchins; Our previous study demonstrated that CO_2 suppressed feed intake to <30% of the control levels after 16 days

Table 1. Carbonate chemistry and saturation states with respect to calcite (Ω_C) and aragonate (Ω_A) of the experimental seawater (mean ± SE).

	CO₂ condition		
	380 ppm	1,000 ppm	3,000 ppm
pH (NBS)	8.15 ± 0.003	7.83 ± 0.003	7.33 ± 0.004
Salinity	33.9 ± 0.04	33.9 ± 0.05	33.8 ± 0.1
TA (μmol kg SW^{-1})	2205.0 ± 1.52	2205.0 ± 1.52	2205.0 ± 1.52
pCO_2 (μatm)	401.5 ± 3.45	907.9 ± 7.31	3126.1 ± 29.03
[HCO$_3^-$] (μmol kg SW^{-1})	1837.7 ± 3.95	2010.3 ± 3.15	2141.1 ± 1.92
[CO$_3^{2-}$] (μmol kg SW^{-1})	147.7 ± 1.30	78.3 ± 0.90	25.7 ± 0.31
Ω_C	3.6 ± 0.03	1.9 ± 0.02	0.6 ± 0.01
Ω_A	2.3 ± 0.02	1.2 ± 0.01	0.4 ± 0.01

The pH, salinity and total alkalinity (TA) were measured, but the other parameters were calculated from pH, salinity and temperature using CO2sys (see text).

of exposure to CO₂ partial pressure (pCO_2) of 1,000 μatm (for a gas phase, 1 μatm = 1 ppm under the barometric pressure of 1 atm) in the sea urchin *Hemicentrotus pulcherrimus* [25]. Under a higher pCO_2 condition (284 Pa ≈ 2800 μatm), feed intake was significantly depressed in adult green sea urchin *Strongylocentrotus droebachiensis* as determined at 45 days of exposure [39], which agrees with earlier results reported for the same species reared under an even higher pCO_2 of 8,000 μatm for 8 weeks [36]. In contrast, feed intake and growth in the sea star *Pisaster ochraceus* were shown to increase when reared under 780 ppm for 21 days [18]. Thus, feeding responses to elevated ambient CO₂ seem to depend upon levels of CO₂ stress, and possibly vary between different echinoderms.

The aim of this research was to investigate feed intake and associated anatomical and physiological changes of adult *Anthocidaris crassispina*, one of the most common and commercially important sea urchins in the Pacific coasts of Japan, Taiwan, and southeastern China [1], under elevated seawater CO₂ conditions. The sea urchins were reared individually for 140 days in seawater equilibrated with CO₂-enriched air containing 1,000 or 3,000 ppm CO₂. To explore possible mechanisms for reduced feed intake in sea urchins under elevated CO₂ conditions, we investigated morphology of the masticatory apparatus (Aristotle's lantern) of the sea urchin. The Aristotle's lantern is pentaramous, composed of five pyramids (= jaws), to each of which a tooth is firmly attached [8, 10]. The tooth is a ceramic-fiber-reinforced ceramic-matrix composite with the matrix being composed of fine calcite crystals with a very high magnesium content [5, 28, 42]. The working tip of the tooth is composed of 4.5-13% Mg calcite plates and needles embedded in a matrix of polycrystalline calcite that contains 40-45% Mg [26]. The skeletal elements are mobilized by a complex set of myocytes and ligaments [45]. The effect of CO₂-acidified seawater on the function and morphology of Aristotle's lantern has not been addressed yet.

II. MATERIALS AND METHODS

1. Experimental Animals

Two hundred adult *A. crassispina* were collected at a rocky shore near Saga Fisheries Research Institute (33°29'01"N, 129°56'27"E) on October 17th 2011, and transported to the Institute for East China Sea Research, Nagasaki University (ECSER, 32°48'39"N, 129°46'20"E), Nagasaki, Japan. The sea urchins were kept in five 100 L flow-through tanks with sand-filtered running seawater (10 L min^{-1}) at ambient pH (8.12) and fed a sufficient amount of the green alga *Undaria pinnatifida* prior to use.

2. CO₂ Exposure

The experimental system consisted of three sets of header tanks (capacity 50 L) and aquaria (120 cm × 75 cm × 20 cm (depth)). Twenty-one containers (22 cm × 14 cm × 14 cm (depth)) were placed in each aquarium for individual rearing of test animals. Each set was used for one of three CO₂ treatments (control, 380 ppm, seawater pH 8.16; the year 2100 prediction by A1FI scenario, 1,000 ppm, pH 7.84 [22]; the year 2300 prediction, 3,000 ppm, pH 7.35 [7]). Natural seawater was filtered (1 μm), pumped into the header tanks, and equilibrated with either outdoor air (control group) or CO₂-enriched air (1,000 and 3,000 ppm groups), which was prepared with a gas blender (Kofloc, GB-2C, Japan) by mixing dried air and pure CO₂. Seawater was gravity-fed from a header tank to each of the 21 containers (water depth 13 cm) at a flow rate of 80 mL min^{-1}, without recirculation of the outflow from the animal containers. Seawater pH (NBS) in each container was checked daily with a pH meter (Mettler Toledo, MP 125, USA), calibrated with standard buffer solutions (pH 4.01, 6.86, and 9.18) every day. The salinity was measured using a refractometer (Atago, 100-S, Japan). Alkalinity was measured with a total alkalinity titrator (Kimoto, ATT-05, Japan) every month. Partial pressure of CO₂ (pCO_2), bicarbonate (HCO$_3^-$) and carbonate (CO$_3^{2-}$) concentrations, and the saturation states for calcite (Ω_C) and aragonite (Ω_A) in seawater were calculated from the measured seawater pH, alkalinity, temperature and salinity, using the program CO2SYS (E. Lewis, Brookhaven National Laboratory, Table 1). Dissolved oxygen concentration during the experiment was measured
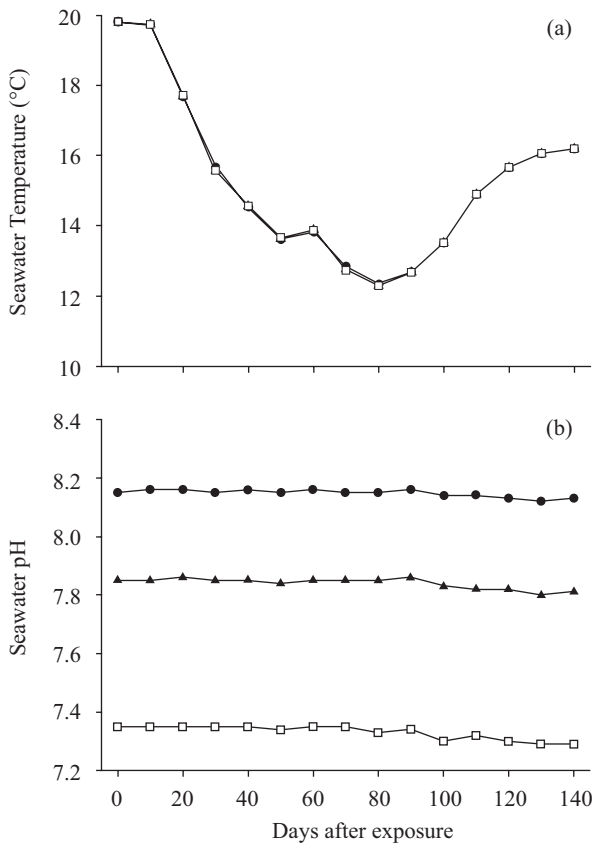
Fig. 1. **Temporal changes in seawater temperature (a) and pH (b) during 140-day exposure experiment. Filled circles represent control, filled triangels 1,000 ppm, and open squares 3,000 ppm urchins. Note that the data points in (a) are nearly overlapped.**

daily in all containers with a DO meter (Eyela, NCB-1200, Japan). The DO meter was daily calibrated with humidified N_2 gas and air. Sixty-three adult *A. crassispina* with mean wet weight of 58.1 ± 1.4 (SE) g and mean test diameter 50.6 ± 3.4 mm were transferred into the 63 containers (21 containers per treatment × 3 treatments) individually after an acclimation period of 40 days. Prior to experimentation, the sea urchins were treated with a 300 ppm solution of H_2O_2 as a preemptive measure against the spotting disease [40].

The exposure experiment started on November 26th 2011, and ended on April 14th 2012, lasting for 140 days. Water temperature and salinity ranged between 12.3°C and 19.8°C (Fig. 1(a)), and between 33.4 and 35.1 (not shown), respectively, during the experiment. Seawater pH showed slight daily fluctuations, but overall remained stable throughout the experiment (Fig. 1(b)). Dissolved oxygen saturation was always above 95%. Light regime was adjusted to replicate the natural daylight cycle over both the acclimation and experimental periods. When found, dead individuals were removed immediately and excluded from any further measurements.

3. Growth

Test diameter and wet body weight were measured at the beginning and the end of the experiment (14 individuals per treatment). The wet body weight was measured to the nearest 0.01 g with an electric balance after briefly blotting excess seawater with a paper towel. The test diameter was measured with a caliper to the nearest 0.01 mm. At the end of the experiment, the urchins were dissected open, and the wet weights of the gonads and the gut were determined. The gut was rinsed in seawater to remove any contents, placed on a paper towel to remove excess water, and then weighed. The gonad index (GoI) was calculated as wet gonad weight (g)/wet body weight (g) × 100. Similarly, the gut index (GuI) was calculated as wet gut weight (g)/wet body weight (g) × 100.

4. Feed Intake and Fecal Production

Artificial food pellets (1.5 cm × 1.5 cm × 1 cm, 5-7 g) were prepared every 4 days by mixing 5 g dried algae (*U. pinnatifida*) powder, 3 g agar, and 100 mL seawater according to the method described by Hiratsuka and Uehara [20]. The pellets were stored in a refrigerator until feeding. Preliminary study demonstrated that percentage weight loss of the pellets after 24-h immersion in running seawater (80 mL min⁻¹) was 0.11 ± 0.17% (n = 10), and this was taken into account in the calculation of feed intake. The sea urchins were fed every second day. Seven individuals of the 21 sea urchins in each treatment received pre-weighed pellets (one pellet per sea urchin) and feed intake and fecal production were obtained for them. Sixteen h after feeding, residual pellets were removed by large tweezers, placed on a paper towel to remove excess water, and weighed. Feed intake was calculated as dry weight per individual by incorporating a conversion factor from wet to day weight, which was determined by drying pre-weighed 10 moist food pellets at 50°C for 48 h to a constant weight.

Feces of the 7 sea urchins were collected in bottles twice per day by suctioning, filtered and dried to constant weight in an oven at 50°C for 48 h. Fecal production was calculated as dry weight per individual per day. Fecal matter was daily removed by siphoning for the other 14 sea urchins. Fecal production was determined every 10 days after day 40.

5. Oxygen Consumption

Oxygen consumption rates were determined 48 h after feeding on day 50, 80, 110, and 140 at natural seawater temperature. Eight sea urchins of each group, different from those used for feed intake determinations, were placed individually in a respiration chamber (approximately 1 L capacity), which was continuously supplied with seawater, 4 h prior to determinations for acclimation. A magnetic stirrer bar at the bottom of the chamber was used to gently mix seawater in the chamber. At the start of a measurement, a water sample was withdrawn by a glass, gas-tight syringe, and the partial pressure of oxygen (pO_2) was determined with a Strathkelvin electrode (Model 1302 Strathkelvin, USA) thermostatted at experimental temperatures and a meter (Model 782, Strathkelvin). Then, the chamber was closed for 1 h, and another water sample was taken. Oxygen consumption rate was

Table 2. Comparison of test diameter (TD), wet body weight (WBW), and gonad (GoI) and gut (GuI) indices of *Anthocidaris crassispina* reared for 140 days at 380, 1,000 and 3,000 ppm CO₂.

CO_2 (ppm)	TD (mm)		% ΔTD	WBW (g)		% ΔWBW	GoI (%)	GuI (%)
	Day 0	Day 140		Day 0	Day 140			
380	46.9 ± 0.1	51.4 ± 0.1	9.9 ± 1.3[a]	57.9 ± 2.5	53.9 ± 2.6	− 6.9 ± 2.7[a]	7.8 ± 0.4	2.3 ± 0.1
1,000	48.8 ± 0.1	52.0 ± 0.1	6.7 ± 1.4[ab]	56.4 ± 3.6	51.5 ± 3.6	− 8.7 ± 2.3[ab]	6.2 ± 0.8	2.2 ± 0.1
3,000	48.1 ± 0.1	50.5 ± 0.1	5.1 ± 1.1[b]	59.4 ± 3.6	50.7 ± 3.7	− 15.2 ± 1.3[b]	5.4 ± 0.4	2.3 ± 0.1

Values represent means ± SE (n = 12-14). Values with different alphabets are significantly different from each other ($p < 0.05$, see text for more details of statistical results).

calculated from the water volume of the chambers, difference between the initial and final pO_2, oxygen solubility at the experimental temperature and salinity [12], and sea urchin's body weight.

6. Ca and Mg Concentrations in Coelomic Fluid

Seven individuals from each group were used to measure the calcium and magnesium concentrations in coelomic fluid. Approximately 0.5 mL of coelomic fluid was withdrawn from each sea urchin at the end of the experiment with a hypodermic needle inserted into the perivisceral coelomic space through the peristomial membrane. The coelomic fluid was immediately centrifuged at 12,000 rpm. The supernatant was diluted 200 times with Milli-Q water to achieve determination ranges for Ca and Mg of an ion chromatograph (Dionex ICS-1000, ICS-1100, RFC-30) with an eluent of 30 mmol L^{-1} methanesulfonic acid (cations) or 10-40 mmol L^{-1} KOH (anions) at a flow rate of 1 mL min^{-1}.

7. Morphology of Aristotle's Lantern (AL)

Four individual in each group were used to determine the effect of high pCO_2 on AL anatomy. AL's were treated with 1 M NaOH for 5 days to digest organic matters, and dried in an oven at 37°C for 2 days. Teeth were isolated from AL, coated with platinum with an ion sputter (E-1010, HITACHI, Japan), and observed under a scanning electron microscope (S-3400N, HITACHI, Japan) at 20 kV.

8. Statistical Analysis

Data were analyzed using SigmaStat 3.5 (Systat Software Inc., USA). Effects on feed intake, growth, and oxygen consumption were analyzed by two-way repeated measures analysis of variance (ANOVA) followed by Holm-Sidak multiple comparison test. One-way ANOVA was used to examine effects on body size, calcium and magnesium concentrations of the coelomic fluid, and morphometries of the Aristotle's lantern. Significance level was set at $p < 0.05$.

III. RESULTS

1. Survival, External Morphology and Gonad Development

Survival over the experimental period were 100% at 380
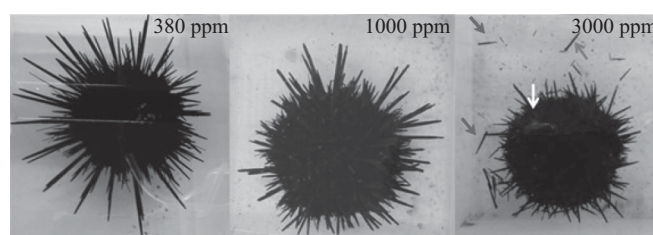
Fig. 2. External morphology of *Anthocidaris crassispina* reared for 140 days at 380, 1,000 and 3,000 ppm CO₂. White arrow in 3,000 ppm indicates the black scabs. Gray arrows indicate the broken spines.

ppm, 95.2% at both 1,000 ppm (one died on day 130) and 3,000 ppm (died on day 81) conditions. Though survival was not largely reduced under the elevated CO_2 conditions, the high-CO_2 urchins appeared unhealthy after the 140-day exposure; most of those reared at elevated CO_2 levels had black lesions and spines broken, which was not observed in any of the control urchins (Fig. 2).

The test diameter and wet body weight of the sea urchins did not vary among different CO_2 groups at the beginning of the rearing period (Table 2). By 140 days, the percent increment of test diameter showed a small difference between groups (one-way ANOVA:F (2,39) = 3.636, $p < 0.05$), with a significant difference between 380 and 3,000 ppm groups (Tukey's test), but not between 380 and 1,000 ppm groups or between 1,000 and 3,000ppm groups. The reductions of body weight were most likely due to loss of spines, which was more pronounced at 3,000 ppm (Fig. 2). The gonad or gut indices showed no significant differences between groups (Table 2). Our experimental period (November to April) corresponded to the non-reproductive period for the species in the southern part of Japan, and therefore the gonads remained immature (spawning in June to July in Saga Prefecture, Japan, personal communication).

2. Feed Intake and Fecal Production

Two-way repeated measures ANOVA demonstrated that feed intake was significantly affected by CO_2, exposure period and their interaction (Table 3). Fecal production showed nearly the same response. Significant differences of feed intake between treatments were not observed during the first 40 days of exposure. Thereafter feed intake began to fall in high CO_2 urchins, and became significantly lower after day 90

Table 3. ANOVA table for analyses of feed intake, fecal production and oxygen consumption

Feed intake			
Source of variation	df	F	P
CO_2	2	4.420	0.027
Day	14	53.979	<0.001
$CO_2 \times Day$	28	2.414	<0.001
Residual	252		
Total	314		
Fecal production			
Source of variation	df	F	P
CO_2	2	4.345	0.029
Day	10	43.243	<0.001
$CO_2 \times Day$	20	1.619	0.052
Residual	180		
Total	230		
Oxygen consumption			
Source of variation	df	F	
CO_2	2	2.429	0.112
Day	3	45.729	<0.001
$CO_2 \times Day$	6	4.961	<0.001
Residual	61		
Total	93		

Two-way repeated measures ANOVA was used for the analyses.

(1,000) or day 110 (3,000) than in control (Holm-Sidak multiple comparison test, Fig. 3(a)). Similarly, a reduction was observed in fecal production of high CO_2 sea urchins with time, and the difference between control and high CO_2 groups were significant after day 100 (Holm-Sidak multiple comparison test, Fig. 3(b)).

3. Oxygen Consumption

Oxygen consumption was affected by exposure period and its interaction with CO_2 (two-way repeated measures ANOVA, Table 3). The CO_2 exposure significantly reduced oxygen consumption on day 50 at both CO_2 levels, but the difference became insignificant subsequently. On day 140, only oxygen consumption at 3,000 ppm was significantly lower than control (Holm-Sidak multiple comparison test, Fig. 4).

4. Ca and Mg Concentrations of Coelomic Fluid

Table 4 shows that the magnesium concentration of coelomic fluid in 1,000 and 3,000 ppm animals were significantly higher (one-way ANOVA: $F(2,12) = 11.25$, $p < 0.01$) than in 380 ppm urchins after 140 days of CO_2 exposure. In contrast, there was no significant difference in the calcium concentrations of coelomic fluid between the 3 groups (one-way ANOVA: $F(2,12) = 1.269$, $p > 0.05$).

5. Morphology of Aristotle's Lantern (AL)

The skeletal weight of AL between different groups did not
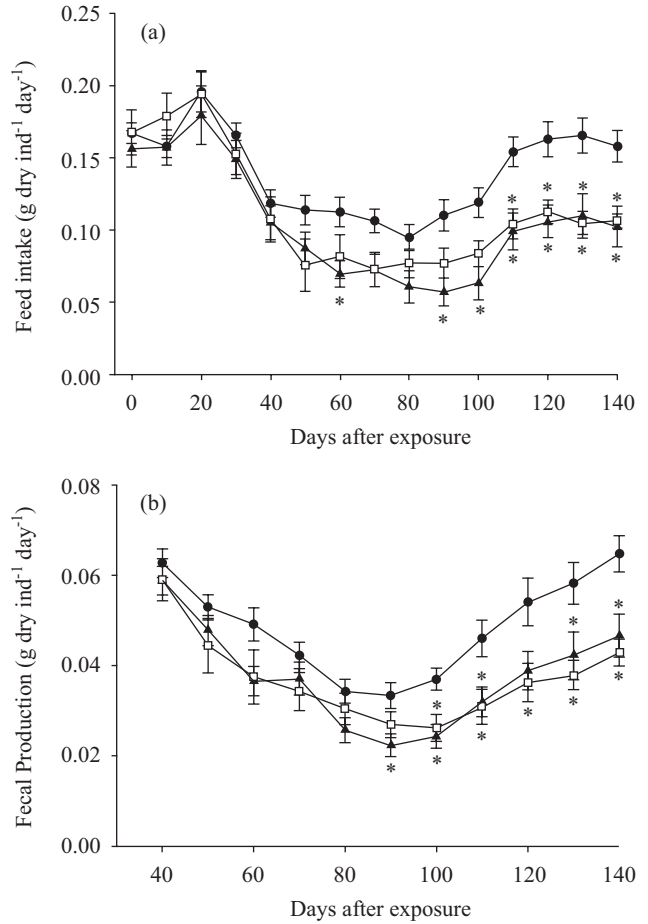
Fig. 3. Feed intake (a) and fecal production (b) of *Anthocidaris crassispina* reared for 140 days at 380, 1,000 and 3,000 ppm CO_2. Filled circles represent control, filled triangels 1,000 ppm, and open squares 3,000 ppm urchins. Asterisks indicate significant difference from the respective control values at the same sampling time. Mean ± SE, n = 7. For the details of statistical analysis, see Table 3.
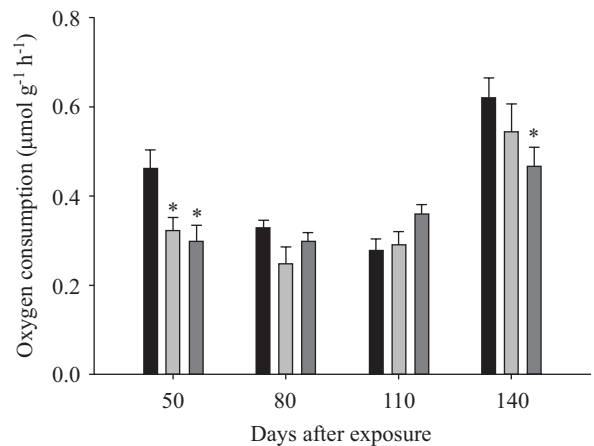
Fig. 4. Oxygen consumption of *Anthocidaris crassispina* reared for 140 days at 380, 1,000 and 3,000 ppm CO_2. Black bars represent control, light gray bars 1,000 ppm, and dark gray bars 3,000 ppm urchins. Mean ± SE, n = 7-8. Asterisks indicate significant difference from the respective control values of the same measurement day.

Table 4. Calcium and magnesium content of coelomic fluid of Anthocidaris crassispina reared for 140 days at 380, 1,000 and 3,000 ppm CO_2.

CO_2 (ppm)	$[Mg^{2+}]$ (mmol L^{-1})	$[Ca^{2+}]$ (mmol L^{-1})
380	54.6 ± 1.4^a	9.2 ± 1.1
1,000	67.6 ± 2.3^b	9.1 ± 1.1
3,000	67.5 ± 2.8^b	7.2 ± 0.8

Mean $\pm$ SE (n = 5). Values with different alphabets are significantly different from each other ($p < 0.01$, see text for more details of statistical result).

Table 5. Weight of Aristotle's lantern and morphological measurements of the tooth of Anthocidaris crassispina reared for 140 days at 380, 1,000 and 3,000 ppm CO_2.

CO_2	Weight of Aristotle's lantern (g)	Tooth width (mm)	Tooth thickness (mm)	Tooth tip angle (degree)
380 ppm	0.88 ± 0.08	1.60 ± 0.03	1.44 ± 0.04^a	57.5 ± 1.5
1,000 ppm	0.86 ± 0.10	1.56 ± 0.09	1.33 ± 0.02^b	57.4 ± 2.8
3,000 ppm	0.94 ± 0.07	1.56 ± 0.05	1.23 ± 0.02^c	62.5 ± 2.5

Mean $\pm$ SE (n = 4). Values with different alphabets are significantly different from each other ($p < 0.01$, see text for more details of statistical result).

show any significant difference when determined on day 140 (Table 5). However, the tooth of all the 3,000 ppm sea urchins, showed morphological abnormalities such as undulating lateral plates (upward arrow in Fig. 5d, enlarged view in Fig. 5f) and ridges (downward arrows in Fig. 5d). Individuals in 1,000 and 3,000 ppm group had thinner teeth than control (one-way ANOVA: F (2, 9) = 17.63, $p < 0.01$, Table 5). Neither tooth width nor tip angle was affected by CO_2 treatments.

IV. DISCUSSION

This study has unequivocally demonstrated that feed intake of the sea urchin was suppressed after 90-110 days of exposure to CO_2 higher than 1,000 ppm. We reared our urchins individually to avoid pseudoreplication, which has been one of major drawbacks in the experimental design employed in a number of climate change experiments on marine organisms [43]. Since oxygen consumption was much less affected, the reductions in feed intake seen in this study implies decreased scope for growth [44] in our urchins. It was previously reported that scope for growth was decreased by 20% when *Strongylocentrotus droebachiensis* was reared for 45 days at 284 Pa (2,800 μatm) but unaffected at 102 Pa (1,000 μatm) pCO₂ [39].

1. Survival and Growth

The survival of this study is comparable to the data reported on *S. droebachiensis* exposed for 45 days to control (95%),
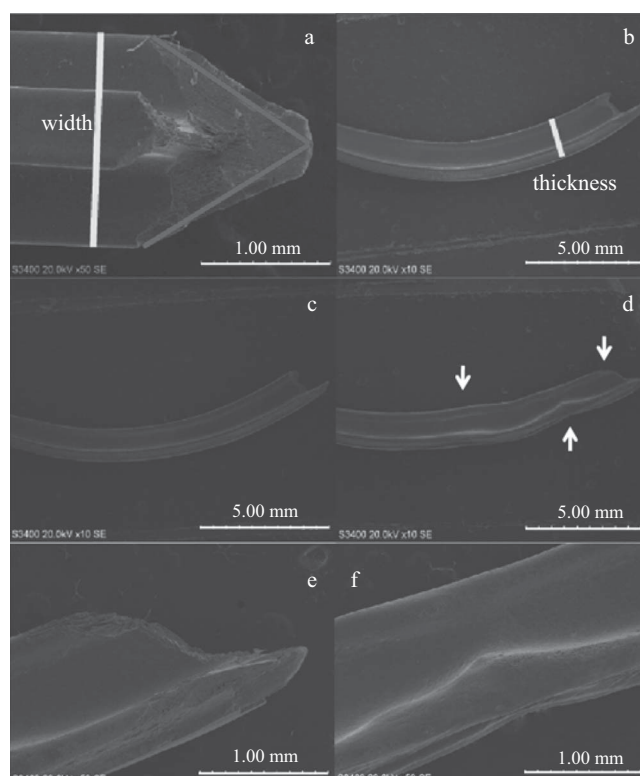
Fig. 5. Scanning electron micrographs of the grinding tips of the tooth of *Anthocidaris crassispina* reared for 140 days at 380 (a, b), 1,000 (c) and 3,000 (d, e, f) ppm CO_2. a: adaxial view of the grinding tip (control) to show measurements of width and the tip angle as given in Table 5. b: lateral view of the grinding tip (control) to show measurements of thickness. Arrows in d indicate abnormalities. e: the tip (right downward in d) of d under a higher magnification. f: the site of upward arrow in d under a higher magnification.

102 Pa (100%) and 284 Pa (95%) by Stumpp *et al.* [39]. Similarly, 100% survival was reported after up to 16 months of exposure to 400 and 1,200 μatm pCO₂ for *S. droebachiensis* [16]. Our earlier studies on *Hemicentrotus pulcherrimus* also demonstrated 100% survival after a 9-month exposure to 380 and 1,000 μatm pCO₂ [25], and 100% survival after a 10-month exposure to 1,000 μatm pCO₂ at ambient temperature, but 85% survival after a 10-month exposure to 1,000 μatm pCO₂ at an elevated temperature (+ 2°C, Yin *et al.* in preparation). Smaller individuals of sea urchins might be more vulnerable to high CO_2 conditions because *H. pulcherrimus* with a mean initial body weight of 0.84 g showed reduced survival under only 200 μatm above ambient CO_2 after 26 weeks [35]. Reduced survival under elevated CO_2 conditions was reported also on the shrimps *Palaemon pacificus* [24] and the juvenile clams *Ruditapes decussates* [32].

It has been reported that high seawater pCO₂ could exert a suppressive effect on gonad growth of sea urchins; Dupont *et al.* [16] reported a significant reduction of fecundity, estimated from the number of eggs released by intra coelomic injection of KCl, in *S. droebachiensis* acclimated for 4 months to 1,200 μatm pCO₂, whilst the effect was no longer detectable after 16

months. Stumpp *et al.* [39] demonstrated a CO_2-dependent suppression of gonad mass in *S. droebachiensis*, and attributed the effect to a lower energy allocation to gonads. Kurihara *et al.* [25] reported that a peak number of mature ova occurred one month later in *H. pulcherrimus* reared under 1,000 ppm CO_2 than control urchins, whilst the number of ova itself was unaffected.

2. Feed Intake and Fecal Production

The feed intake determined in this study is comparable with those reported for 4 other sea urchins (*Echinometra*) from Okinawa island (26°30'N, 127°51'E), Japan, determined at 25°C [20], but higher than the rate reported for *S. droebachiensis* by Stumpp *et al.* [39] due most likely to a lower experimental temperature of the latter (10°C). Stumpp *et al.* [39] found significant reductions in feed intake and fecal production at 284 Pa pCO_2 when the data were averaged over the experimental period of 3 weeks. An important finding of our study is that the effect of CO_2 on feed intake emerged gradually as the CO_2 exposure prolonged (Fig. 3(a)). Comparison of feed intake at comparable temperatures but at different exposure periods revealed that feed intake in the two high CO_2 groups was 90% (1,000 ppm) or 92% (3,000 ppm) of the control rate (0.166 g dry ind^{-1} day^{-1}) on day 30 when seawater temperature was 15.7°C, but it decreased to 74% (1,000 ppm) or 69% (3,000 ppm) of the control (0.163 g dry ind^{-1} day^{-1}) on day 120 when seawater temperature was equal to that on day 30. These observations demonstrated that physiological responses to ocean acidification can be time-dependent, and thereby point to a risk of predicting physiological (e.g., feeding) responses to changing ocean physicochemical conditions from short-term experiments and/or from single-time sampling.

Fecal production may have incurred errors from fecal dissolution in water and admixture of unfed feed materials, though we tried to minimize these errors by frequent collection (twice a day) and careful elimination of feed materials from inclusion in our samples. The gradual divergence of fecal production in high CO_2 urchins from control ones, similar to the pattern of feed intake, seems to give some credence to our determinations. The ratios of fecal production to feed intake were similar between the groups as determined on day 140 (control 41%, 1,000 ppm 46%, 3,000 ppm 40%).

3. Oxygen Consumption

Our oxygen consumption data almost exactly match with those reported by Yamamoto and Handa [49] on the same species. Oxygen consumption was unaffected under 1,200 µatm pCO_2 both at 10 and 16°C, but significantly reduced at 2,300 µatm at 10°C (but not at 16°C) in the sea urchin *Paracentrotus lividus* after 19 days of exposure [9]. Oxygen consumption of marine invertebrates often decreases in acidified conditions [17], but no effect [41, 48] or even an increase [4, 47] has also been reported for some species. Physiological responses to changes in seawater carbonate chemistry thus

depend on animal species tested, pCO_2 levels employed, the duration of exposure, and seasonal changes in physiological states of the test animals including developmental and reproductive stages, which in turn are under the influence of environmental conditions. Again, these observations underpin the importance of long-term exposure experiment to understand integrated biological responses of marine organisms to ocean acidification, and potential risk of extrapolating impacts of ocean acidification from short and/or snap-shot determinations.

4. Ca and Mg Concentrations of Coelomic Fluid

Increased magnesium concentration of coelomic fluid was previously reported for the sea urchin *Psammechinus miliaris* by 8-day exposure to high CO_2 [30]. Stumpp *et al.* [39] reported a significant decrease in ash dry mass of the test in *S. droebachiensis*at 384 Pa CO_2. Again, responses of ion contents to high CO_2 varies between species. Kurihara *et al.* [25] reported an increase in magnesium concentration in the coelomic fluid of *H. pulcherrimus* under 1,000 µatm. Skeletal elements of the arms of the brittle star *Ophiocten sericeum* are also made of high magnesium calcite, but the magnesium and calcium contents were unaffected by up to 1,800 µatm pCO_2 exposure [41].

5. Morphology of Aristotle's Lantern

To our knowledge, this study is the first attempt to measure the effect of elevated sea water pCO_2 on the morphology of Aristotle's lantern. The distal edge of the ridge appeared worn out (Fig. 5e), which might indicate lower mechanical strength of the teeth in high CO_2 urchins. Hardness of calcite is known to be related to Mg content [23, 27]. Therefore, if exposure to high seawater CO_2 lowers Mg content of the teeth (possibly as indicated by the increase in Mg concentration in the coelomic fluid), then it will compromise grazing efficiency of the affected sea urchins. This needs to be investigated in future studies.

V. CONCLUSION

The effect of elevated seawater pCO_2 on feed intake intensified with time (Fig. 3(a)): For the first 40 days, feed intake was nearly identical among groups; the feed intake in high CO_2 groups tended to be reduced but not significantly decreased during the following 50 days; and it was only after 90-110 days where the feed intake of both high CO_2 urchins was significantly lower than the control values. A similar trend was apparent for fecal production (Fig. 3(b)). Oxygen consumption on day 100 was not significantly different between the three groups (Fig. 4) when feed intake of 3,000 ppm urchins was significantly reduced, and on day 140 oxygen consumption was significantly lower only in 3,000 ppm urchins than in the control when feed intake of both high CO_2 groups was significantly suppressed (Fig. 3(a)). Thus, it is likely that the high CO_2 urchins incurred a gradual reduction in

scope for growth [44], which would have led to a reduced reproductive output and growth if CO$_2$ exposure had prolonged. This might be responsible for a delay in gonad growth observed for *H. pulcherrimus* reared under 1,000 ppm CO$_2$ for 9 months [25].

Our examination of the skeletal elements of the Aristotle's lantern revealed little quantitative difference between the groups. Question remains as to how prolonged CO$_2$ exposure affects neuromuscular system that controls the movements of the elements of the lantern, and the mechanical durability of the teeth, both of which must be examined in future studies.

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