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Effects of elevated carbon dioxide on contraction force and proteome composition of sea urchin tube feet

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Abstract

This study examined how contraction force and protein profiles of the tube feet of the sea urchin (Pseudocentrotus depressus) were affected when acclimated to 400 (control), 2000 and 10000 μatm CO₂ for 48 days. Acclimation to higher CO₂ conditions significantly reduced contraction force of the tube feet. Two-dimensional gel electrophoresis showed that eight spots changed in protein volume: six up-regulated and two down-regulated. Using matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight mass spectrometry, three up-regulated spots (tubulin beta chain, tropomyosin fragment, and actin N-terminal fragment) and two down-regulated spots (actin C-terminal fragment and myosin light chain) were identified. One possible interpretation of the results is that elevated CO₂ weakened contraction of the tube feet muscle through an alteration of proteome composition, mainly associated with post-translational processing/proteolysis of muscle-related proteins.

Keywords: Sea urchins, Tube feet, Muscle contraction, Proteomics
1. Introduction

There are two processes by which seawater is acidified by the addition of carbon dioxide (CO₂), ocean acidification and accidental leakage from a carbon capture and storage (CCS) site. Ocean acidification occurs by absorption of CO₂ across sea surface from the atmosphere, and now widely recognized as a serious threat to the structure and function of marine ecosystems in the coming decades. Reductions in surface ocean pH by ocean acidification have been confirmed at a number of observation sites in the world oceans (Orr, 2011). It is estimated that approximately 30% of anthropogenic CO₂ emission has been absorbed by the ocean since 1960s (Le-Quéré et al., 2009). Dissolved CO₂ reacts with seawater to shift carbonate equilibria and thereby reduce seawater pH (Zeebe and Wolf-Gladrow, 2001). Average surface seawater pH is considered to have decreased by 0.1 units since the beginning of industrial revolution (Sabine et al., 2004) and is expected to decrease by further 0.8 units by 2300, when the atmospheric CO₂ concentration is projected to reach 1900 ppm (Caldeira and Wicket, 2003). The CCS technology has been developed to mitigate the effect of CO₂ related to the continued reliance on fossil fuels as prevalent energy source (IPCC, 2005). With CCS technology, CO₂ is separated from flue gas at large industrial and energy-related sources, transported to a storage site and injected into a geological formation, and hence isolated from the atmosphere for a long period of time (Schrag, 2009). Globally, there are 15 large-scale projects in operation, with a further seven under construction (Global CCS Institute, 2015). Accidental leakage of stored CO₂ through the seabed, which might impact benthic ecosystems near a leakage site, has been one of the major concerns with CCS technology (Taylor et al., 2014). The spatial extent of an acidification event will depend on the position of the CCS infrastructure and the nature of the leak. At the present time, we have little to no experience with CCS leakage, and therefore it is difficult to predict how and
to what extent marine faunas and floras near the leakage site would be affected. If CO₂ leakage occurs, CO₂ concentrations in the surrounding seawater can reach much higher levels than those predicted from ocean acidification. Therefore, the CO₂ levels used in the recent risk assessment studies of leakage from CCS sites are as high as 18325 µatm (Murray et al., 2013), 20000 µatm (Restelli et al., 2015), 29000 µatm (Ishida et al., 2013), or even up to 313862 µatm (De Orte et al., 2014), one or two orders of magnitude higher than in ocean acidification studies (typically up to 1000 or 2000 µatm).

Effects of CO₂ on marine organisms have become a focus of marine biological research during the past 20 years, mainly because of the increasing concern with the impacts of ocean acidification (Gattuso and Hansson, 2011) and potential CO₂ leakage from a CCS site (Noble et al., 2012) on marine ecosystem structures and ecological services. It is now generally accepted that calcification (i.e., the formation of CaCO₃ structures from Ca²⁺ absorbed from seawater and HCO₃⁻ generated by metabolism and originated from seawater carbon pool, Furla et al. 2000) is one of the most sensitive biological processes negatively affected by seawater acidification (Kroeker et al. 2013). For example, Li et al. (2016) recently demonstrated that the tubes of serpulid worm *Hydroides elegans* had lower hardness and a smaller radius when subjected to the projected acidic conditions for 2100 (pH 7.8), causing the worm to be mechanically weaker, although different organisms may show different sensitivities and response patterns (Ries et al. 2009). In addition, an increasing amount of data have become available on CO₂ effects on early development, growth, metabolism, photosynthesis, and survival of marine organisms (Kroeker et al. 2013). By comparison, very little is known on how CO₂ affects the integrity and functionality of muscular systems among marine animals. Wood et al. (2008) reported that the muscle mass in the arms of the brittlestar, *Amphiura filiformis*, decreased in lowered pH (7.7, 7.3 and 6.8) but
without structural changes in muscles, while in another species *Ophiura ophiura* muscle density remained unaffected in lowered pH (7.7 and 7.3; PCO$_2$ 1300–1400 and 2300–2500 μatm, Wood et al. 2010). Schalkhausser et al. (2013) found significant declines in the force generated by the adductor muscle in the king scallop, *Pecten maximus*, collected in Norway and reared under PCO$_2$ of 1120 μatm at 10°C. However, the effect was not detected in the same species from France under the same experimental conditions (Schalkhausser et al. 2014).

The purpose of this study was to examine the effect of CO$_2$ on contraction force and protein composition of the tube feet of the sea urchin, *Pseudocentrotus depressus*. Tube feet are unique hydraulic mechano-sensory adhesive organs found in echinoderms. Tube feet have a variety of functions including light sensitivity, respiration, chemoreception and locomotion (Lesser et al., 2011). They consist of a basal extensible cylinder, the stem, which bears an apical flattened disc that makes contact with and adheres to the substratum. The stem wall of a tube foot consists of an outer epidermis, a basiepidermal nerve plexus, a connective tissue layer (mutable collagenous tissue), a myomesothelium (retractor muscle) and an inner epithelium that surrounds the water-vascular lumen (Santos, 2005). Recent proteomic characterization of tube feet from the sea urchin *Paracentrotus lividus* identified 328 non-redundant proteins, including 44 phospho-proteins and 18 glycoproteins, and revealed that the organs are composed of sensory-perception-related proteins, nerve-related proteins, muscle-related proteins, development/regeneration-related proteins, immunological-response-related proteins, and temporary-adhesion-related proteins (Santos et al., 2013). While in-depth proteomics has been reported in sea urchin tube feet, a comparative analysis of the tube feet proteomes in response to an environmental change, such as ocean acidification, remains to be elucidated. We examined the contraction force and proteome composition of tube feet under three CO$_2$ concentrations based on the present-day level (400
µatm), the prediction by IPCC in the year 2300 (2000 µatm) and extreme conditions (10000 µatm) predicted as the model of CCS leakage. Adult sea urchins (P. depressus) were acclimated to these CO2 concentrations for 48 days. The contraction force was measured using isolated tube foot preparations. Proteomic analysis was applied to elucidate the response of muscle contraction to elevated CO2 at the molecular level.

2. Materials and methods

2.1. Animal collection and maintenance

Wild adult sea urchins were collected by a local fisherman in Saga Prefecture, Japan (33°32'55.82" N; 129°50'56.74" E). Their shell diameter and wet body weight were 54.26 ± 2.26 mm and 56.12 ± 6.24 g (Mean ± SD, n = 42) respectively. The sea urchins were transferred to the Institute for East China Sea Research of Nagasaki University, Japan and stocked for 10 days in a 100 L tank supplied with a continuous flow of filtered seawater at 2 L/min at ambient pH (8.17) and temperature (23.3–24.0 °C). The sea urchins were fed with artificial food pellets prepared according to the recipe by Hiratsuka and Uehara (2007) every second day.

2.2. CO2 exposure

The sea urchins were acclimated for 48 days (from 13 July to 27 August 2014) in seawater equilibrated with ambient air (400 ppm CO2, seawater pH 8.2, control), or CO2-enriched air at a concentration of 2000 ppm (pH 7.6, ocean acidification) or 10000 ppm (pH 7.0, CCS leakage). The sea urchins were reared individually in seven replicate containers (14 × 22 × 14 (depth) cm) per treatment placed in a water bath (120 × 75 × 20 (depth) cm) with a water depth of 12 cm.
Seawater was double filtered (150 µm and with string-wound cartridges) before being supplied to
the header tanks. The filtered seawater was gravity-fed from the header tank to each of the 7
rearing containers at a flow rate of 50 mL/min. Seawater in the header tanks was bubbled
continuously with an outside air at flow rate of 10 L/min for the control condition, or with CO2-
enriched air (2000 or 10000 ppm), which was prepared with a gas blender (Kofloc, GB-2C,
Japan) by mixing dried air and pure CO₂ for the two higher CO₂ conditions. In addition, seawater
in the rearing containers was gently bubbled with the same gases. The seawater temperature was
kept stable at 25°C. The water baths were covered with plastic sheet to avoid CO₂ exchange with
room air. Seawater pH (NBS) and dissolved oxygen concentration were monitored every second
day with a digital multiparameter meter (WTW multi 3420, Germany) calibrated with a standard
buffer solution of pH 4.01, 6.86 and 9.18. Salinity and temperature were monitored daily
throughout the experiment using a salinity refractometer (S/Mill-E, Atago, Japan) and a solar
digital thermometer (SN-1200, Netsuken, Japan). Alkalinity was measured weekly with a total
alkalinity titrator (Kimoto, ATT-05, Japan). Partial pressure of CO₂ (PCO₂) was calculated from
the data of seawater pH, temperature, salinity and alkalinity, using the CO2SYS program (E.
Lewis, Brookhaven National laboratory).

2.3. Measurement of tube feet contraction

Seven sea urchins, one from each rearing container, were used per treatment for the
measurement of the contraction force of isolated tube foot. A small piece (0.5 cm²) of the lateral
side of test was snipped off with the tube feet, submerged in seawater at room temperature with
the same PCO₂ level as during acclimation, and continuously superfused with the seawater using
a gravity-feeding apparatus. The cut end of the test and the tip of a tube foot were each pinched
using a pair of micro bulldog clamps. The clamp at the tip of the tube foot was connected to the probe of an isometric transducer (SB-1T, Nihon Kohden, Tokyo, Japan). The tube foot was gradually extended to a length of about 20-30 mm from its base, using a micromanipulator, until spontaneous rhythmic contractions occurred as seen in intact animals. To induce maximal contraction, the perfusate was switched from seawater to seawater containing \(10^{-4}\) M acetylcholine (Ach) (Florey and Cahill, 1980), and the response was recorded until contraction force began to decrease. ACh is the natural excitatory transmitter substance in sea urchin tube feet (Florey et al., 1975). The maximal value was used as a contraction force in each measurement. The number of successful measurements in each animal varied from 1 to 3 (i.e., 1 to 3 tube feet because each tube foot was used only once), depending on the animal's physiological conditions. We used average values of each sea urchin for statistical comparison (Table S1).

2.4. Collection of tube feet for proteomic analysis

Four sea urchins were used per treatment for this purpose. Approximately 10 to 60 mg of tube feet were isolated on ice under a microscope from each sea urchin. To prevent protein digestion, the tube feet were soaked in 1 mL protease inhibitor solution (one tablet of cOmplete\textsuperscript{TM} Mini/10 mL seawater, Roche, Indianapolis, IN, USA) during collection, and then placed into a 2-mL screw-cap centrifuge tube (Sarstedt, Nümbrecht, Germany). The samples were centrifuged at 12000 \(\times\) g for 1 min to remove the seawater. Subsequently, the samples were weighed and kept at \(-80^\circ\)C until analysis.

2.5. Protein extraction
To accomplish extraction, separation and identification of proteins from a small amount of tube feet, we applied a small-scale proteomic approach (Yamaguchi, 2011; Khandakar et al., 2013)

One milliliter of Trizol reagent (Life Technologies, Carlsbad, CA, USA), 100 µg of zirconia beads (0.6 mm in diameter, BMS, Tokyo, Japan), and a stainless-steel bead (5 mm in diameter, BMS, Tokyo, Japan) were added to the frozen tube feet in a 2 mL screw-cap centrifuge tube (Sarstedt, Nümbrecht, Germany). The tubes were mounted in a Master Rack aluminium block (BMS, Tokyo, Japan) and agitated for 2 min at 25ºC in a ShakeMaster Auto ver 1.5 (BMS, Tokyo, Japan). The homogenates were incubated at 25ºC for 5 min and 0.2 mL of chloroform was added to each tube. The tubes were then shaken vigorously by hand for 15 s and incubated at 25ºC for 3 min. The mixture was centrifuged (12000 × g, 15 min, at 4ºC) to separate it into a lower organic phase, an interphase, and an upper aqueous phase. After removing the aqueous phase, 300 µL of ethanol was added to the tube. The sample was mixed by inversion 3–5 times, incubated at 25ºC for 3 min, and then centrifuged (12000 × g, 1 min, at 4ºC) to remove the DNA pellets.

The resulting supernatant was transferred to dialysis tubing with a 3500 Da molecular weight cut-off (Spectra/Por RC dialysis membrane 3, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), and dialyzed against 400 volumes of Milli-Q water (once renewed) for 72 h at 4ºC. The dialysate was dehydrated with solvent-absorbent powder (Spectra/Gel Absorbent, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), suspended in 300 µL of IEF solution A without carrier ampholite (8 M urea, 50 mM DTT, 2% w/v CHAPS, 0.001% w/v bromophenol blue), incubated at 25ºC for 16 h, and centrifuged (15000 × g, 15 min, 25ºC). The supernatant was transferred to a new tube and the remaining pellet was re-suspended with 100 µL of IEF solution A, centrifuged (15000 × g, 15 min, 25ºC) and the supernatant was combined with the
first supernatant (total 400 µL). The combined supernatant (4 aliquots of 100 µL in a 0.5 mL Safe-Lock Protein LoBind Tube, Eppendorf, Hamburg, Germany) was stored at –70ºC. Protein concentration was determined using RC DC™ Protein Assay Kit (Bio-Rad, Hercules, CA, USA), with bovine serum albumin (BSA) as a standard.

2.6. Two-Dimensional gel electrophoresis (2-DE)

The first dimensional IEF separation was carried out using a 7-cm ReadyStrip® IPG Strips (Linear pH gradient, pH 3–10, Bio-Rad). The IPG strips were passively rehydrated for 12 h at room temperature in 125 µL of resuspension solution (8 M urea, 50 mM DTT, 2% w/v CHAPS, 0.2% carrier ampholytes, 0.0001% bromophenol blue), containing 30 µg protein/strip. IEF was carried out at 20°C, for a total of 20000 Vh (15 min with a 0–250 V linear gradient; 2 h with a 250–4000 V linear gradient; and finally 4000V held constant until 20000 Vh had been reached). After IEF, the IPG strips were incubated in 2.5 mL of 2% DDT-containing equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol) for 20 min and then incubated with 2.5% iodoacetamide-containing equilibration buffer for 20 min at room temperature. The second dimensional electrophoresis was performed with a Mini-PROTEAN Tetra electrophoresis cell (Bio-Rad, Hercules, CA, USA). The equilibrated IPG strips were placed on the top of a Mini-PROTEAN TGX® precast gels, sealed with ReadyPrep® overlay agarose (Bio-Rad) and electrophoresed at 200 V in running buffer (25 mM Tris base, 192 mM glycine and 0.1% w/v SDS), for 30 min at room temperature. Gels were fixed with a fixing solution (40% ethanol, 10% acetic acid) for 2 h. Following this, the gels were stained with Flamingo® fluorescent stain (Bio-Rad) and the gel images for figure presentation were captured using a GELSCAN® laser scanner (iMeasure, Nagano, Japan). For quantitative analysis of 2-DE patterns and spot volumes, a total
of 24 gel images, consisting of four biological replicates (i.e., proteins individually prepared from
four specimens per treatment) and two technical replicates for each protein sample, were
compared using the Prodigy SameSpots® software package (Non-linear Dynamics, Newcastle,
UK). Reproducible gel spots were counted using the same software during spot alignment and
pre-filtering stages.

2.7. Protein in-gel digestion and MALDI-QIT-TOF mass spectrometry

Protein spots were manually excised from the 2D gels using a spot image analyzer
(FluoroPhoreStar 3000®, Anatech, Tokyo, Japan) equipped with a gel picker (1.8 mm in
diameter). The methods we used for in-gel digestion and peptide extraction were slightly
modified from the previously described methods of Shevchenko et al. (2006) and Khandakar et
al. (2013). To reduce self-digestion of trypsin and avoid contamination, we used Trypsin Singles
(proteomics grade, Sigma-Aldrich, MO, USA) at a lower concentration (10 ng/µL trypsin in 25
mM ammonium bicarbonate containing 10% acetonitrile) instead of Trypsin (sequencing grade,
modified, Promega Corp. 13 ng/µL trypsin in 10 mM ammonium bicarbonate containing 10%
acetonitrile) used by Shevchenko et al. (2006). Expecting faster peptide dryness, we used an
extraction solution of 0.05% TFA in 50% acetonitrile, instead of 5% formic acid in 50%
acetonitrile (Yamaguchi, 2011; Khandakar et al., 2013). In brief, the samples were dehydrated in
100 µL of acetonitrile and agitated for 10 min. The supernatant was withdrawn. The residues
were added with 8 µL of the Trypsin Singles and left on ice for 20 min. After removal of an
excess amount of the solution, the gels were incubated at 37°C overnight. Twenty µL of the
extraction solution was added to the tube, incubated at 37°C in a shaker (100 r/min) for 15 min,
and sonicated in a cup horn sonicator (Astrason Ultrasoinic Processor XL2020, Misoinix, at
output level 4) for 1 min. The solutions were transferred to a new 0.5-mL centrifuge tube and then centrifuged with a Speed Vac (SPD 131 DDA, Thermo Scientific) at 200 rpm and at 45°C for 15 min. The dried samples were dissolved in 5 µL of 0.5 mg/mL of 2,5-dihydroxybenzoic acid (DHBA, Shimadzu, Japan) in 33% acetonitrile, 0.1% trifluoroacetic acid. The 1 µL samples of the solution were spotted onto a µFocus MALDI target plate (Hudson Surface Technology, NJ, USA) and dried at room temperature. MS and MS/MS spectra were obtained using a MALDI-QIT-TOF mass spectrometer (AXIMA Resonance, Shimadzu, Kyoto, Japan). MS/MS ion search was performed using MASCOT® version 2.3 (Matrix Science, London, UK) against SwissProt 2014_07 (5456000 sequences; 194259968 residues) and EST Echinidae 2015_04 (851028 sequences; 212054698 residues) in our own MASCOT server. Search parameters used were: enzyme, trypsin; maximum missed cleavages, 2; fixed modification; carbamidomethyl (C); variable modifications, oxidation (HW and M); peptide mass tolerance, ± 0.3 Da; fragment mass tolerance, ± 0.2 Da; mass values, monoisotopic. Mascot score was assigned to identify protein with significance threshold at $p < 0.05$ as shown in figure S1.

2.8. Determination of $pI$ and MW

Theoretical isoelectric point ($pI$) and sequence mass of precursor protein were calculated using ProtParam (http://www.expasy.ch/tools/protparam.html). Observed $pI$ was calculated from the horizontal migration of the spot. Observed mass was estimated from the vertical migration of the spot by the method of Weber and Osborn (1969).

2.9. Statistical analysis
One sample t-test was applied to determine difference between measured and nominal PCO$_2$ values in each treatment. One-way ANOVA was applied to determine difference between group means and Tukey’s HSD run to confirm group differences using PASW statistics 18. For 2-DE pattern analyses, four biological replicates for each condition and two technical replicates for each protein sample were compared between different CO$_2$ levels. Differences in spot volumes were statistically evaluated using ANOVA at $p < 0.05$, using Prodigy SameSpots® software package (Non-linear Dynamics, Newcastle, UK). Differentially accumulated proteins were defined as significant at fold ratios $\geq 1.4$.

3. Results

3.1 Seawater chemistry

Daily seawater temperature fluctuated slightly as air temperature varied between 23.4–25.2°C. Throughout the experiment, salinity remained at 34–35 PSU and the dissolved oxygen saturation was always above 90% (Table 1). Seawater pH varied slightly but remained relatively stable throughout the experiment (Fig. 1). The seawater pH, PCO$_2$, and HCO$_3^-$ and CO$_3^{2-}$ concentrations all differed significantly between treatments (one-way ANOVA, Table 1). Measured PCO$_2$ values were not significantly different from corresponding nominal values at 400 or 2000 µatm, whereas it was not the case at 10000 µatm (one-sample t-test, $t_{400\mu atm} = 0.035$, $P = 0.975$; $t_{2000\mu atm} = 2.039$, $P = 0.111$; $t_{10000\mu atm} = 3.548$, $P = 0.024$).

3.2. Tube feet contraction force
There was no difference in test diameters or body weights of the sea urchins between treatments (one-way ANOVA, $F_{2,18} = 1.127; P = 0.346$ and $F_{2,18} = 0.475, P = 0.63$, respectively).

Tube feet contraction force was significantly lowered with increasing $CO_2$ levels in a $PCO_2$-dependent manner (one-way ANOVA, $F_{2,18} = 13.89, P = 0.000$; Fig. 2). Multiple comparison analysis revealed that contraction force differed significantly between all pH treatments (Tukey’s HSD, $P < 0.05$; Table S1).

### 3.3. Protein profile under changing in $CO_2$ concentration

The total protein amounts soluble to CHAPS-based IEF solution obtained from the tube feet in 400 µatm, 2000 µatm and 10000 µatm treatments were $0.65 \pm 0.27$, $1.60 \pm 0.48$ and $1.40 \pm 0.33$ mg g$^{-1}$ fresh weight, respectively. The number of protein spots that were resolved from protein extracts from 400 µatm, 2000 µatm and 10000 µatm treatments were 120 ± 2, 120 ± 2 and 119 ± 1 respectively (Fig. 3. Comparative quantitative image analysis of 2-DE patterns showed that a total of eight protein spots changed significantly in spot volume (i.e. spot intensity); two spots were down-regulated and six spots were up-regulated under increasing $CO_2$ conditions (Figs. 3 and S1). Eight differentially accumulated protein spots (spots 1–8) and four constantly expressed protein spots (spots a-d, prominent spots selected for easier protein extraction/identification) were excised from the 2-DE gels (Figs. 3 and S1), digested in-gel with trypsin, and the extracted tryptic peptides were subjected to MALDI-QIT-TOF mass spectrometry. Although nucleotide/protein sequences reported from *Pseudocentrotus depressus* number less than one thousand (56 proteins and 941 ESTs), 9 of the 12 protein spots were positively identified by cross-species searches (Tables 2 and S2). Four constantly expressed proteins were identified as actin-1 (spot a), tropomyosin (spot b), voltage dependent anion channel 2 (spot c), and
calmodulin-A (spot d). Of the 6 up-regulated proteins, three were identified as tubulin beta chain (spot 2), tropomyosin (spot 3) and actin (spot 4). The other three (spots 1, 5, and 6) were not identified. Two down-regulated proteins were identified as myosin light chain (spot 7) and actin (spot 8). The observed masses of actins, spots 4 (25.4 kDa) and 8 (17.4 kDa), were significantly lower than the theoretical masses of actin (41.8 kDa) (Table 2). In contrast, the observed mass of constantly expressed actin (spot a, 39.0 kDa) is close to the theoretical mass. These observations and the peptides assigned to the actin sequence by MS/MS ions searches indicated that spots 4 and 8 were N-terminal and C-terminal fragments of actin, respectively (Tables 2 and S2). In a similar way, spot b (34.7 kDa) was considered to be intact tropomyosin, and spot 3 (28.3 kDa) a fragment of tropomyosin (Table 2).

4. Discussion

This study demonstrated that the contraction force of the tube feet was significantly reduced by 48 day exposure to 2000 and 10000 µatm CO₂ in the sea urchin, *P. depressus*. The mechanisms underlying this functional impairment are yet to be elucidated, but our proteomic analysis demonstrated alterations of proteome composition, mainly a consequence of post-translational processing and/or proteolysis of muscle-related proteins, which could give a clue for a mechanistic understanding of the physiological disorder. Presently, very little is known about the effects of ocean acidification and CO₂ leakage from CCS sites on muscular systems of marine invertebrates (see Introduction). We recently showed that CO₂ significantly reduced the locomotion speed of the sea urchin, *Hemicentrotus pulcherrimus*, reared under 1000 µatm PCO₂ both at ambient and at an elevated temperature (+ 2°C) for seven months (Yin, Lee, Kurihara and Ishimatsu, in preparation). Similarly, some studies showed negative impacts on muscle systems
in fish. Chambers et al. (2014) and Frommel et al. (2016) showed subtle histological alterations in skeletal muscle of the fish larvae (Paralichthys dentatus and Thunnus albacares) under elevated CO$_2$ conditions (1800 and 4700 μatm, and 2000–9600 μatm, respectively). Bignami et al. (2014) reported that maximum swimming velocity was reduced in pelagic larvae of Coryphaena hippurus, reared under 1460 μatm PCO$_2$ for 21 days, but this effect was absent in larvae of another pelagic fish, Rachycentron canadum (Bignami et al. 2013) reared under 2100 μatm for 22 days. On the other hand, there are studies demonstrating that fish were relatively insensitive to elevated CO$_2$. Maneja et al. (2013) found swimming activity of larval Atlantic cod, Gadus morhua, was robust to high CO$_2$ exposure, and Melzner et al. (2009) also found that critical swimming speed of adult G. morhua was unaffected by exposure to 5800 μatm PCO$_2$ for 12 months or 3100 μatm PCO$_2$ for 4 months. Furthermore, we have recently published the data on the effect of CO$_2$ and temperature on the escape response of the Japanese anchovy, Engraulis japonicus to mechanical stimuli, and found no significant difference in various parameters of the response except turning rate which was elevated at a higher temperature (Nasuchon et al. 2016).

One possible interpretation of the observed negative effects of CO$_2$ on locomotive behaviors in marine invertebrates (and fish) is that elevated CO$_2$ or resultant acidification of body fluids had direct negative impacts on the integrity and/or functionality of muscular system in these animals, as suggested by our proteome analysis. However, different hypotheses are also possible. Recent papers on the effect of ocean acidification on fish have demonstrated that increasing CO$_2$ can disrupt sensory and brain functions of fish (Nagelkerken and Munday, 2016). There is some evidence for the involvement of a neurotransmitter, gamma-aminobutyric acid (GABA) in these phenomena in fish under elevated CO$_2$ (Nilsson et al., 2012; Chivers et al., 2014; Hamilton et al., 2014). The current hypothesis for the involvement of GABA assumes decreased chloride ion...
concentrations in extracellular fluid, as a result of acid-base restoration through ionic exchange with bicarbonate ions, to be responsible for the reversed (from inhibitory to excitatory) response to GABA released from a nerve terminal, as known under some pathological conditions (Nilsson et al., 2012). GABA was detected in tube foot extract of sea urchins, and known to cause excitation of cholinergic motoneurones but have no direct effect on muscle fibers (Florey et al., 1975). However, since ion concentrations of perfusate were held constant during the measurement, the reduced contraction force of the tube feet seen in this study was more likely attributable to the effect of pH/CO₂ per se, rather than through some GABA-related process triggered by lowered chloride concentrations. Sea urchins have a lower capacity for acid-base regulation of both extracellular (Spicer et al., 2011; Stumpp et al., 2012; Kurihara et al., 2013) and intracellular fluids (Stumpp et al., 2012) than fish (Ishimatsu et al., 2005), it is unlikely that elevated CO₂ levels, at least those predicted in the context of ocean acidification, will cause substantial decreases of chloride ion concentrations in the body fluids of intact sea urchins. Another possibility is that 48-day exposure to CO₂ resulted in some irreversible alterations in the functions of GABA receptors. Further, low pH per se could have reduced neuromuscular transmission (Landau and Nachchen, 1975; Takahashi and Copenhagen, 1996). Clearly, further studies are needed for mechanistic understanding of the observed reduction in the contraction force of tube feet under elevated CO₂ conditions.

Our comparative quantitative analysis of 2-DE profiles detected eight protein spots that had changed in spot volume. Of six up-regulated spots, three were identified as tubulin beta chain, tropomyosin fragment and actin N-terminal fragment, while two down-regulated ones were identified as myosin light chain and actin C-terminal fragment (see Results). These proteins are all involved in muscle contraction. An interaction between actin and myosin generates movement
relative to each other. Troponin and tropomyosin are involved in regulating actin site (Szent-
Györgyi, 1975). Myosin light chain of 17 kDa is an essential light chain (ELC), which has the
role of stabilizing the lever arm. The interaction between the C-terminal of ELC of the domain
and N-terminal sub-domain of the heavy chain of the myosin may be involved in coupling ATP
hydrolysis and rotation of the lever arm (Ushakov, 2009). Our data showed that the majority of
actin was constantly expressed as the intact protein under all the CO₂ conditions tested, whereas
minor fragments of actin were differentially expressed: the actin C-terminal fragment decreased,
while the actin N-terminal fragment increased at higher CO₂ levels. Actin has been shown to be
cleaved by caspases into N-terminal 32-kDa (Fractin) and 15-kDa (tActin) fragments. The site of
this proteolytic cleavage at the C-terminal side of ²⁴⁰\text{YELPD}²⁴⁴ is conserved from yeast to
humans, implicating it as an important regulatory sequence (Gourley and Ayscough, 2005).
However, N- and C-terminal fragments of actin identified in this study contained the uncleaved
sequence SYELPDGQVITIGNER (Figs S1 and S2), indicating the possibility that the
accumulation of the actin N-terminal fragment in this study may have been a product of caspase-
independent proteolysis of intact actin under elevated CO₂. A comparative proteomic analysis of
the oyster \textit{Crassostrea gigas} found up-regulated two isoform gene products of tropomyosin
(33.06 kDa, \textit{pI} = 4.57, and 26.91 kDa, \textit{pI} = 4.49) in mantle tissue at 2000 \textmu atm CO₂ (Wei et al
2015). In contrast, our study showed that the majority of tropomyosin was constantly expressed,
while up-regulation was found in a short form (Table 2), derived from the same tropomyosin
gene. Thus, the increased short form, most likely a fragment, of tropomyosin may also have
affected muscle contraction. Voltage dependent anion channel 2 (VDAC2, spot c) and
calmodulin-A (spot d) were identified as unchanged abundant proteins (Fig. 3 and Table 2).
Unlike actin or tropomyosin, neither fragment of VDAC2 nor calmodulin-A was detected.
Therefore, these proteins were probably not responsible for the reductions in muscle contraction force at high CO₂ conditions in our sea urchins.

5. Conclusion and future study

We have shown that elevated CO₂ reduced the contraction force of sea urchin tube feet in a concentration-dependent manner. Further, 2-DE based proteomics showed that proteins involved in muscle contraction changed their spot volumes under high CO₂ conditions. These results suggest that elevated CO₂ possibly affects muscular system in sea urchins through alterations in proteome composition via a post-translational proteolysis, although other interpretations are also possible. For example, disruption of neuromuscular transmission or coordination at higher neuronal levels by elevated CO₂ or lowered pH in marine animals is a possibility. On the other hand, although this study pointed out the importance of proteomic approaches to understand how sea urchin tube feet respond to elevated CO₂ in the molecular level, correlation between transcriptomic and proteomic changes in response to elevated CO₂ remain to be elucidated. In addition, the muscular systems in various organs of sea urchins and other marine invertebrates need to be examined for their CO₂ sensitivity to understand how future oceanic environmental changes will affect physiological functions driven by muscle contraction.

Acknowledgement

We thank Prof. Tatsuya Oda and his laboratory members for their helpful advices and cooperation on small-scale proteomics. We also thank Mr. Shouhei Noma, Mr. Ryosuke Ono and Mr. Mail Vǎn Hiếu for their assistance in samples preparation. Thanks are also due to Ms. Mizuri Murata and Ms. Takako Aketagawa for their help in collecting sea urchins. This study was
References

Bignami, S., Sponaugle, S., Cowen, R.K., 2013. Response to ocean acidification in larvae of a

Bignami, S., Sponaugle, S., Cowen, R.K., 2014. Effects of ocean acidification on the larvae of a
high-value pelagic fisheries species, mahi-mahi Coryphaena hippurus. Aquat. Biol. 21, 249-
260

425, 365. doi:10.1038/425365a

Chambers, R.C., Candelmo, A.C., Habeck, E.A., Poach, M.E., Wieczorek, D., Cooper, K.R.,
Greenfield, C.E., Phelan, B.A., 2014. Effects of elevated CO2 in the early life stages of
summer flounder, Paralichthys dentatus, and potential consequences of ocean acidification.

Mitchell, M.D., Corkill, K.C., Ferrari, M.C., 2014. Impaired learning of predators and lower
prey survival under elevated CO2: a consequence of neurotransmitter interference. Glob.
Change. Biol. 20, 515-522

of CO2 leakage from carbon capture and storage activities on the mobilization and speciation


**Fig. 1** Temporal changes in seawater pH during 48-day exposure experiment. Filled triangles represent 400 µatm, filled circles 2000 µatm, and filled squares 10000 µatm treatments.

**Fig. 2** Contraction force (Newton, N) of the isolated tube feet of *Pseudocentrotus depressus* induced by $10^{-4}$ M acetylcholine (Mean ± SE, number of individuals = 7/treatment). The sea urchins were acclimated to 400 µatm (control), 2000 µatm and 10000 µatm PCO$_2$ for 48 days at 25°C. *Significant difference from 400 µatm, †significant difference from 2000 µatm (ANOVA $F_{2,18} = 13.89, P = 0.000$; Tukey’s HSD, $P < 0.05$).

**Fig. 3** Two-dimensional gel electrophoretic separations. Thirty µg of tube feet proteins extracted from *Pseudocentrotus depressus* treated in **A** 400 µatm, **B** 2000 µatm and **C** 10000 µatm were separated on 7-cm IPG strips. The first dimensional isoelectric focusing of 2-DE was performed using a pH range of 3-10. The characters a-d represent the constantly expressed proteins and the numbers 1-8 represent the differentially accumulated proteins.
Fig. 1

[Graph showing the pH levels of seawater over a period of days.]
Fig. 2

The bar chart shows the effect of different CO₂ concentrations on contraction force (N). The x-axis represents CO₂ concentrations in μatm (micro-atmospheres), while the y-axis represents the contraction force in N. The chart indicates that the contraction force decreases as the CO₂ concentration increases.

- At 400 μatm, the contraction force is the highest, with a value close to 0.04 N.
- At 2000 μatm, the contraction force is significantly lower, marked with an asterisk (*).
- At 10000 μatm, the contraction force is even lower, marked with an asterisk (*) and a dagger (†) symbol.
Table 1  Seawater pH, percentage dissolved oxygen (DO) saturation, temperature (Temp.), salinity, alkalinity (TA), partial pressure of carbon dioxide (PCO₂), bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻) concentrations (mean ± SD) of each treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>400 (µatm)</th>
<th>2000 (µatm)</th>
<th>10000 (µatm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH(NBS)</td>
<td>8.16 ± 0.02</td>
<td>7.56 ± 0.03</td>
<td>6.90 ± 0.03</td>
</tr>
<tr>
<td>DO (%)</td>
<td>96 ± 2</td>
<td>96 ± 2</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Temp. (°C)</td>
<td>24.6 ± 0.6</td>
<td>24.6 ± 0.6</td>
<td>24.6 ± 0.6</td>
</tr>
<tr>
<td>Salinity (PSU)</td>
<td>34.6 ± 0.6</td>
<td>34.6 ± 0.6</td>
<td>34.6 ± 0.6</td>
</tr>
<tr>
<td>TA (µmol/Kg-SW)</td>
<td>2121.3 ± 8.4</td>
<td>2128.1 ± 6.7</td>
<td>2130.2 ± 15.0</td>
</tr>
<tr>
<td>^1PCO₂ (µatm)</td>
<td>400 ± 22</td>
<td>1912 ± 116</td>
<td>9307 ± 524</td>
</tr>
<tr>
<td>^1HCO₃⁻ (µmol/Kg-SW)</td>
<td>1677.7 ± 10.9</td>
<td>2009.0 ± 13.4*</td>
<td>2128.6 ± 12.4*†</td>
</tr>
<tr>
<td>^1CO₃²⁻ (µmol/Kg-SW)</td>
<td>196.8 ± 1.2</td>
<td>61.5 ± 3.5a</td>
<td>14.2 ± 0.7a,b</td>
</tr>
</tbody>
</table>

¹Calculated from seawater pH, temperature, salinity, alkalinity and total carbon by using CO2SYS program

*Significantly different than 400 µatm, †significantly different than 2000 µatm

aSignificantly different than 400 µatm, bsignificantly different than 2000 µatm
Table 2 MS/MS identification of the protein spots of sea urchin tube feet that showed constant (a-d) or significantly different volumes (1-8) in response to elevated CO2 conditions

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Species</th>
<th>Accession no (NCBI)</th>
<th>Theoretical mass (kDa)/pI</th>
<th>Observed mass (kDa)/pI</th>
<th>Fold 1000 μatm/400 μatm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td><strong>Constant proteins</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>Actin-1</td>
<td>Strongylocentrotus purpuratus</td>
<td>ACTA_STRPU</td>
<td>41.8/5.3</td>
<td>39.0/5.7</td>
<td>NA</td>
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<tr>
<td>b</td>
<td>Tropomyosin</td>
<td>Paracentrotus lividus</td>
<td>AM197503</td>
<td>32.7/4.7</td>
<td>34.7/4.7</td>
<td>NA</td>
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<tr>
<td>c</td>
<td>Voltage-dependent anion channel 2</td>
<td>Paracentrotus lividus</td>
<td>AM187061</td>
<td>31.1/8.8</td>
<td>29.4/7.2</td>
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<tr>
<td>d</td>
<td>Calmodulin-A</td>
<td>Strongylocentrotus intermedius</td>
<td>CALM_STRIE</td>
<td>17.6/4.1</td>
<td>18.9/3.8</td>
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<tr>
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<td>Tubulin beta chain</td>
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<td>47.9/5.2</td>
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<td>Tropomyosin fragment</td>
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<td>32.7/4.7</td>
<td>28.3/5.0</td>
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<td>-1.7</td>
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<tr>
<td>8</td>
<td>Actin C-terminal fragment</td>
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<td>ACTA_STRPU</td>
<td>41.8/5.3</td>
<td>17.4/6.8</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

NF = Not found
NA = Not available
Figure S1. Magnified images of protein spots that showed significantly different changes in sea urchin tube feet between 400 µatm, 2000 µatm and 10000 µatm treatment (Mean ±SD; P < 0.05).

Spot No. 1

Spot No. 2
Spot No.3

Spot No.4
Spot No.5

400 µatm  |  2000 µatm  |  10000 µatm

Log normalized volume

Spot No.6

400 µatm  |  2000 µatm  |  10000 µatm

Log normalized volume
Spot No. 7

400 µatm  2000 µatm  10000 µatm

Log normalized volume

Spot No. 8

400 µatm  2000 µatm  10000 µatm

Log normalized volume
Figure S2  Full peptide sequence of actin of sea urchin *Pseudocentrotus depressus*: Bold texts are peptide sequence of actin N-terminal fragment (up) and actin C-terminal (down). Highlight is amino acid sequence around the caspase-cleaved site and closed arrow is the site of cleavage by caspase.

```
MCDEEVAALVVDNGSGMCKAGFAGDDAPRAIFPSIVGRPRHQGVMVGMQKDS
YVGDEAQSKRGILTLYPIEHGIIVTNWDDMEKIWHHTFYNEVRVAPEEHPVLLT
EAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDTGDGVTH
TVPIYEGYALPHAILRLDLAGRDLTDYLMKILTERGYSFTTAEREIVRDIKEKL
CYVALDFEQEMATAASSLSLEKSYELPDGQVITIGNERFRCPEALFQPAFLGME
SPGIPvETYNSIMKCDIDIRKDLYANTVLSGGTSMYPGIADRMQKEITSLAPSTMK
IKIAPPERKYSVWIGGSILASLSTQQWMISKQHEYDESGPSIVHRKCF
```
Table S1 Contraction force of tube feet in individual sea urchin and statistics analysis

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<th>Sea urchin No.</th>
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<td>0.0550</td>
<td>0.0393</td>
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<td>0.0373</td>
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<td>0.0285</td>
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One-way ANOVA

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<th>F</th>
<th>P</th>
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<td>13.885</td>
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<td>Within groups</td>
<td>0.002</td>
<td>18</td>
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<td>Total</td>
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Multiple comparisons (Tukey HSD)

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<th>(J)Treatment</th>
<th>Mean difference (I-J)</th>
<th>Std. Error</th>
<th>P</th>
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Treatment 1 = 400 µatm, Treatment 2 = 2000 µatm, Treatment 3 = 10000 µatm
<table>
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<th>Species</th>
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<tr>
<td>a</td>
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<td>ND</td>
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<td>AM197503</td>
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Table S2: Results of MS/MS ion search and BLAST search for protein identification.