

Effects of Long-Term Ocean Acidification Exposure on the Structural, Mineralogical, and Mechanical Properties of Sea Urchin (*Echinometra* spp.) Skeletons at a Natural Volcanic CO₂ Seep

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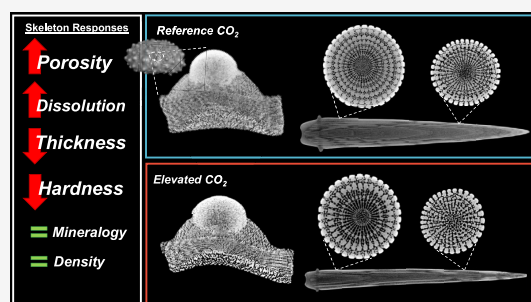
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ABSTRACT: Two decades of mesocosm studies document generally negative effects of ocean acidification (OA) on adult sea urchin growth, feeding performance, skeletal structure, and strength. Whether experimental observations hold true in natural systems will determine whether they can be extrapolated to predict responses under ecologically relevant contexts. Here, we employ a suite of imaging, chemical, and mechanical techniques to examine the skeletal properties of two closely related sea urchin species (genus *Echinometra*) living at a natural carbon dioxide (CO₂) seep in Japan. Test plates and spines from urchins living under elevated CO₂ conditions were thinner, more porous, and had less biomineral than those at reference sites; however, tooth structure was resilient to elevated CO₂. The magnesium content of the test and spines did not differ between sites; however, they exhibited reduced nanohardness and became more brittle under elevated CO₂. Together, altered structural and mechanical properties may compromise the protective function of urchin skeletons at the CO₂ seep. These responses have implications for ecosystem structure if urchin function is suppressed at the population level. Future work might explore the repeatability of these findings across successive species and localities to recognize generality, its limits, and the conditions that mediate the influence of OA.

KEYWORDS: ocean acidification, calcification, climate change, sea urchins



INTRODUCTION

The continued rise in atmospheric carbon dioxide (CO₂) is modifying seawater–carbonate chemistry, simultaneously reducing ocean pH and carbonate (CO₃²⁻) mineral saturation, processes known jointly as ocean acidification (OA).¹ Organisms that form carbonate shells and skeletons are particularly sensitive to OA,² with lowered saturation states (Ω)³ and elevated [H⁺] levels increasing the energetic demands required to maintain calcification.^{4–6} Sea urchins are a calcifying group of benthic macroherbivores that play a critical role in preserving ecosystem balance by exerting top-down control over algal growth via grazing. Urchins help to maintain heterogeneous benthic communities^{7,8} and prevent the spread of opportunistic, fast-growing algal turfs: degraded, less-productive, and increasingly prevalent ecosystem states.^{9,10} Exposure to elevated pCO₂ can negatively impact both adult and larval sea urchin calcification, as evidenced by the production of weaker, more porous, and deformed skeletal structures under low pH conditions.^{2,5,11–18} If OA compromises the integrity of their skeletons,^{17,19} ecologically important urchin functions may be suppressed at the population level in future oceans.²⁰

To complement laboratory-based observations, a growing body of research has emerged utilizing naturally extreme environments to examine species' responses to long-term

acidification under ecologically relevant conditions.^{21–27} CO₂ seeps have the potential to serve as analogues for OA^{27–31} due to the natural pH gradients they generate. At these sites, low pH levels representative of future climatic conditions under IPCC Shared Socioeconomic Pathways (SSPs) SSP1-2.6 (low CO₂ emissions) and SSP5-8.5 (high CO₂ emissions) have been observed.³² CO₂ seeps are not perfect proxies, however, and their caveats are well-documented. Temporal (i.e., tidal or seasonal) variability in seawater pH is common at CO₂ seeps and increases with decreasing pH means,^{24,33–35} limiting the ability to disentangle the effects of mean pH from pH variability. Potential heavy-metal contamination may also influence organismal responses^{36,37} and can be exacerbated by elevated CO₂.³⁸ Nevertheless, remarkable reproducibility in species responses across laboratory, mesocosm, and field-based CO₂ seep approaches has been observed in both tropical and temperate systems,³⁹ highlighting the rare and valuable

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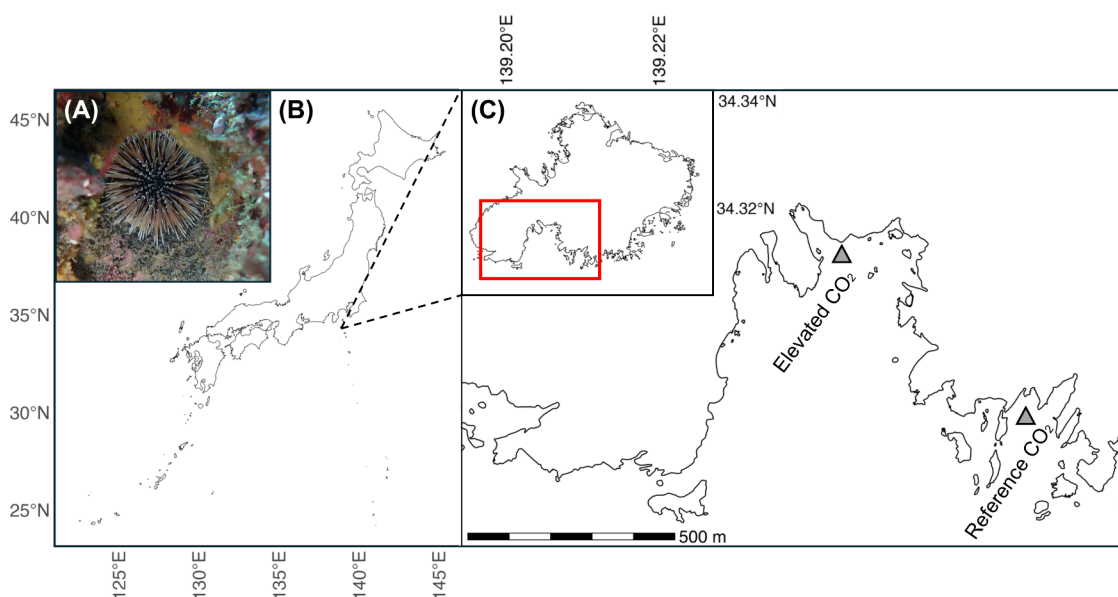


Figure 1. (A) Rock-boring urchin (*Echinometra* sp.). Photo credit: Ben P. Harvey. (B) Location of Shikine Island, Japan. (C) Map of Shikine Island showing the reference and elevated CO₂ sampling locations, created using data (C23-06_01_GML to C23-06_47_GML) from the National Land Information Division, National Spatial Planning and Regional Policy Bureau, MLIT of Japan.

opportunity CO₂ seeps and their resident populations provide to examine the mechanisms and traits underpinning species persistence under future ocean conditions.

In this study, we tested for baseline differences in the structure, mineralogy, and mechanical properties of skeletons from two closely related sea urchin species (*Echinometra* sp. A and *Echinometra* cf. sp. E) either living under reference *p*CO₂ conditions (~350 μatm, pH_T ~ 8.14) or acclimatized to elevated *p*CO₂ (~900 μatm, pH_T ~ 7.79) throughout their postsettlement lifetime at a natural volcanic CO₂ seep in Japan. The porosity, thickness, and relative density of the ambital test plates, primary spines, and teeth were quantified using X-ray microcomputed tomography (μCT), while scanning electron microscopy (SEM) was used to identify dissolution on the skeleton surface. X-ray diffraction (XRD) crystallography and energy-dispersive X-ray spectroscopy (EDX) were employed to identify mineralogical changes in skeletal calcite (MgCO₃ and Mg/Ca content, respectively). Finally, the influence of structural and mineralogical changes on skeleton mechanical resilience (nanohardness and elasticity) was assessed by using nanoindentation.

METHODS

Study Site and Environmental Context. Shikine (34°19'32.9"N 139°12'38.5"E) is a volcanic island located within the Izu archipelago off the coast of southern Shizuoka, Japan (Figure 1). Reference rocky reef habitats around the island are typical of the subtropical-temperate region,²⁵ comprising a mixture of canopy-forming fleshy macroalgae and zooxanthellate scleractinian corals. At "venting" sites, roughly 600 m from the reference reef, volcanic activity causes almost pure (98 ± 3%) CO₂ to bubble from the seabed, creating localized gradients of CO₂.²⁹ Long-term continuous monitoring has revealed that the reference and elevated CO₂ sites used in this study have mean pH_T values of 8.137 ± 0.056 (SD) and 7.781 ± 0.105 (SD), respectively.^{10,35} The elevated CO₂ site close to the venting area offers the potential for space-for-time analyses of OA, with mean pH analogous to future

(year 2100) conditions expected under projections of changing ocean pH (IPCC SSP5-8.5).³² Despite following natural diurnal, oceanographic, and seasonal variation, the CO₂ conditions are temporally stable over the long-term (mean carbonate chemistries presented in Table S1) and are not confounded by differences in temperature, salinity, dissolved oxygen, total alkalinity, nutrients, or depth relative to reference sites.^{10,29,35}

Ethics Statement. All sea urchins used in this study were collected and euthanized in accordance with institutional guidelines, with approval from the University of Tsukuba and Shikine Island Fisherman's Union. No animal ethics approval was required for this study under the Japanese regulations and legislation.

Specimen Collection and Sample Preparation. Adult sea urchins (*Echinometra* spp.; Figure 1A) were collected by SCUBA at depths of 3–5 m from two near-shore locations at Shikine Island, Japan, on July 25–27, 2023. The sites comprised a bay outside the influence of the CO₂ seep (reference CO₂) and a nearby area within the adjacent Mikawa Bay, close to the CO₂ vent (elevated CO₂). A total of 33 specimens (test diameter 13.31–43.43 mm) were collected at random from the reference (*n* = 16) and elevated (*n* = 17) CO₂ sites to test for baseline, OA-driven differences in the skeletal properties. Urchins were placed in aerated buckets filled with seawater from their respective collection sites and transported back to the University of Tsukuba Shikine Island field research laboratory. After collection, urchins were weighed (wet weight) and photographed beside a scale. The test diameter was later calculated as an average of three measurements using *ImageJ* v2.16.0.⁴⁰ Urchins were then split into halves along their transverse plane, the soft tissue was removed, and their skeletons were frozen until processing. An additional six specimens were collected from the reference site on October 8, 2024, solely for DNA barcoding to verify the species identification.

The skeletons were cleaned of remaining soft tissue following the methods of Collard et al.⁴¹ by soaking in 2.5%

Table 1. Structural and Mineralogical Properties of Urchin Tests at Reference (350 μatm) and Elevated (900 μatm) CO_2 Sites^a

	reference CO_2	elevated CO_2	test diameter			site		species	
			F	p value		F	p value	F	p value
Urchin wet weight (g)	16.72 $\pm$ 2.11	5.57 $\pm$ 0.973				22.52	<0.001	0.0376	0.848
test diameter (mm)	31.96 $\pm$ 1.61	21.35 $\pm$ 1.34				22.56	<0.001	0.0685	0.796
test thickness (μm)	779 $\pm$ 33.5	486 $\pm$ 25.4	54.6	<0.001	***	13.8	<0.01	0.804	0.378
porosity (%)	31.2 $\pm$ 0.646	40.3 $\pm$ 0.517	0.065	0.8007		61.2	<0.001	0.149	0.703
density (GSV)	36143 $\pm$ 640	34251 $\pm$ 843	0.575	0.455		3.20	0.085	0.240	0.628
MgCO_3 (mol %)	13.72 $\pm$ 0.098	13.70 $\pm$ 0.142	0.049	0.827		0.0001	0.991	1.55	0.232
Mg/Ca in Calcite	0.063 $\pm$ 0.003	0.068 $\pm$ 0.004	1.43	0.242		3.54	0.071	0.904	0.350

^aReported are the group mean values ($\pm\text{SE}$) and ANOVA results for the urchin wet weight and test diameter and the ANCOVA results for the test plate thickness, porosity, density, MgCO_3 , and Mg/Ca. ANCOVA models include the test diameter as a covariate. Only the main effects were tested where the site $\times$ species interaction term was nonsignificant ($p > 0.2$). The level of significance is indicated with asterisks, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

NaOCl for 1.5 h, followed by 5.25% NaOCl for a further 2.5 h. Samples were placed on an orbital shaker throughout tissue digestion to prevent lactic acid formation.¹⁷ After digestion, the samples were triple-rinsed with ultrapure water. Interambulacral test plates were collected from the ambitus rather than the younger apical plates to assess the impacts (if any) of chronic exposure to elevated CO_2 on skeletal properties. Primary spines were also collected from each urchin, avoiding those with broken or regenerating tips. The length and diameter of the largest spines ($n = 8$ per individual) were measured with electronic calipers (± 0.1 mm accuracy).

DNA Barcoding. The species-level taxonomy within the *Echinometra* genus remains uncertain,⁴² despite being the focus of many ecological^{43–46} and reproductive studies.^{47–49} Morphological markers alone cannot reliably distinguish members of this species complex,⁵⁰ and further genomic data are required to clearly delineate species boundaries. To determine the species identity of the collected urchins, a small portion of the gonads was subjected to DNA barcoding. DNA was extracted from the tissue using Promega Maxwell RSC Tissue DNA Kits (Promega, USA) and the associated Maxwell RSC Instrument according to the manufacturer's protocols. The extracted DNA concentration was measured by fluorometry with a Qubit 4.0 Fluorometer Assay Kit (Thermo Fisher Scientific, USA) and, if required, diluted to ~ 20 ng μL^{-1} with double distilled water (ddH_2O). Polymerase chain reaction (PCR) was performed using the primers COI-f (5'-CCTGCAGGAGGAGGAGAYCC-3') and COI-d (5'-GAA-CATGATGAAGAAGTGCACCTTCCC-3') to target a partial sequence (~ 600 bp) of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene.⁵¹ PCR reactions were performed in a 20 μL total volume: 10 μL of GoTaq Flexi DNA Polymerase (Promega), 8.6 μL of ddH_2O , 0.2 μL (100 mM) of each primer, and 1 μL of DNA template. Cycling parameters were an initial denaturation at 94 $^\circ\text{C}$ for 4 min, followed by 35 cycles of 94 $^\circ\text{C}$ for 30 s, 55 $^\circ\text{C}$ for 30 s, and 72 $^\circ\text{C}$ for 30 s, with a final elongation at 72 $^\circ\text{C}$ for 3 min and a hold at 4 $^\circ\text{C}$. PCR products were purified using an ECO-CIP Rapid PCR Cleanup Kit (New England Biolabs, USA) and sequenced by Eurofins Genomics K.K. (Tokyo, Japan) using the forward and reverse primers on an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific, USA).

Sequence data were analyzed in Geneious Prime v2024.0.7. Chromatograms were manually curated and deposited in GenBank under the Accession Numbers PX411491–PX411529. Processed sequences were pooled with additional *Echinometra* spp. sequences obtained from GenBank (Table

S2). *Heliocidaris crassispina* (Echinoidea: Camarodonta: Echinometridae; GenBank Accession Number JN716400) was used as the outgroup. Sequences were aligned using MUSCLE⁵² with default settings, and a neighbor-joining phylogenetic tree was constructed with branch support assessed from 1000 bootstrap replicates. Species identity was inferred based on tree topology relative to known species.

Skeletal Structure. X-ray Microcomputed Tomography (μCT). 3D features of the ambital test plates ($n = 3$) and primary spines (base, $n = 3$; tip, $n = 1$) from each specimen were reconstructed by μCT using Zeiss Xradia 510 Versa and VersaXRM 730 X-ray microscopes (Carl Zeiss, USA). Scans were performed with a $4\times$ objective at a voltage of 80 kV and a current of 87 μA without system recalibration. Images were acquired with an exposure time of 1 s per projection and a total of 401 projections over 180° . Features of the teeth ($n = 1$ per individual) were reconstructed using a Zeiss VersaXRM 730 microscope with a $40\times$ objective at a voltage of 60 kV and a current of 108 μA , without system recalibration. Scans of the teeth were acquired with an exposure time of 3 s per projection and a total of 401 projections over 180° . For all scans, an LE1 filter was used to attenuate low-energy X-rays and reduce error associated with beam-hardening artifacts such as cupping, shadowing, and partial-volume effects⁵³ during threshold segmentation. Voxel sizes ranged from 1.45 to 4.44 μm for test plates, from 1.54 to 4.44 μm for spines, and from 1.31 to 1.60 μm for teeth, and images were reconstructed in 16-bit gray-scale format (intensity value range 0–65535). Structural properties (i.e., thickness and porosity) of the scanned samples were determined in 3D Slicer (v5.4.0)⁵⁴ using the Segment Editor module.⁵⁵ To determine the skeletal porosity, solid calcareous material was differentiated from void space (pores) by thresholding intensity values within the subvolume. The subvolume porosity (%) was calculated using the following formula:

$$\text{porosity} = \frac{\text{void space volume}}{(\text{biomineralized volume} + \text{void space volume})} \times 100$$

Fast-growing peripheral sutures and ambulacral pores at the test-plate margins (from which the tube feet extend) and high-density spine bosses and test-plate tubercles were avoided to prevent values from being skewed, respectively, toward higher or lower porosity.¹⁷ Following the protocols of Chatzinikolaou et al.,⁵⁶ mean gray-scale values (GSVs) of the segmented biomineralized volume were used as a proxy for skeletal

density.⁵⁷ These values represent relative solid-phase X-ray attenuation without calibration to absolute density values.

Chemical Analysis. *X-ray Diffraction (XRD) and Scanning Electron Microscopy with Energy-Dispersive X-ray Spectroscopy (SEM–EDX).* XRD analysis was used to determine the magnesium (Mg) content of the crystalline phase of the skeletal Mg-calcite. Dried interambulacral test plates and primary spines were ground into fine powders by using an agate mortar cleaned with ethyl alcohol. Approximately 1 g of powder was then transferred to a sample holder and loaded into the diffractometer. Measurements were carried out using a D8 DISCOVER X-ray diffractometer (Bruker, USA) with Cu K α radiation. Operating conditions were maintained at 40 kV and 40 mA for all measurements. An angular range of 5–80° 2 θ was measured with a step size of 0.0150° and 0.5 s counting time per step. The skeletal Mg-calcite content (mol %; Table 1) was calculated by measuring the d_{104} peak position (Å) in the DIFFRAC^{plus} EVA program (Bruker AXS) software, assuming a linear interpolation between calcite (CaCO₃) and magnesite (MgCO₃). The linear relationship between the d_{104} spacing and Mg content has been validated within the range of 0–17.4 mol % MgCO₃,^{58,59} values typical for high Mg-calcite in sea urchins.^{13,60}

SEM–EDX was used to assess the nanostructural, elemental, and compositional changes associated with sea urchin skeletons under acidified conditions. A total of six ambital interambulacral test plates were collected from each specimen. Test plates were gold-sputter-coated for 2.5 min (20 nm thickness) and examined with a JEOL JSM-7900F Benchtop scanning electron microscope (JEOL, Japan). The inner surface of three plates and the outer surface of another three plates were first examined for signs of skeletal dissolution (e.g., surface pitting or deformation). The elemental composition of each surface was then determined by elemental mapping using an Oxford Instruments X-Max Extreme Windowless EDS Detector (Oxford Instruments, U.K.). Three maps (~6500 μm^2) were collected for each test plate with a normalized acquisition time of 40 s. Quantitative elemental analysis was performed in Aztec (Oxford Instruments, U.K.) to determine the elemental composition in weight percent (wt %). The energy spectrum was background- and baseline-corrected, adjusting for the presence of gold coating. No reference material was used; therefore, a relative molar ratio of magnesium to calcium (Mg/Ca [mol/mol]) was calculated for each test plate surface using elemental weight percent data, averaged across measurements for each specimen. Mg/Ca on inner and outer test-plate surfaces were compared between sites by ANCOVA.

Mechanical Properties. *Nanoindentation.* Nanoindentation was performed on ambital test plates and primary spines to examine the mechanical properties of the skeleton (one plate and one spine from each urchin: reference CO₂, $n = 11$; elevated CO₂, $n = 12$). Plates and spines were first photographed beside a scale and later measured in ImageJ (3 s.f.). Spines were cross-sectioned by transverse fracturing at the base and tip for separate analyses. Samples were mounted with cyanoacrylate glue: test plates were placed with the outer surface of the skeleton facing up, while spine bases and tips were mounted vertically. The samples were then ultrapolished with whetstones of decreasing particle size (from 17.3 to 2 μm), followed by a final polishing with diamond paste (particle size ~ 0.5 μm) to ensure a flat surface upon which indents could be made. A nanoconical Hysitron TS 77 Select

TriboIndenter (Bruker, USA) with a tip radius of 150 nm was used to perform load-controlled indents at a loading rate of 40 $\mu\text{N s}^{-1}$ with a maximum load of 200 μN and a 5 s hold. The low load value (and corresponding low indentation depth) was chosen to avoid sample fracture and tip slippage during the loading process. For spine bases and tips, indents were performed on the outer rings of the radially arranged septa, avoiding the porous meshwork stereom at the spine center (Figure 4). For test plates, care was taken to ensure that indents were performed on the center of stereom trabeculae rather than the resin within the stereom pores. Five indents were performed for each test plate, and three indents were performed for each spine base and tip. A minimum distance of 20 μm between indents was maintained to avoid interference from residual deformation (cracks and faults) caused by previous impressions. For each sample type, the calcite elastic modulus (Young's modulus; E) and hardness (H) were determined from the unloading curve of the indentation⁶¹ and averaged across measurements for each individual.

Statistical Analyses. Statistical analyses were performed in R (version 4.2.0⁶²), with the rstatix,⁶³ stats,⁶² and car⁶⁴ packages. The ggplot2⁶⁵ and ggpubr⁶⁶ packages were used for figure production. Body-size metrics (test diameter and wet weight) were treated as descriptors of the site-level size structure and compared with two-way type II ANOVAs (car:Anova) including site and species with an interaction term. Skeletal end points that could coexist with the body size (e.g., test thickness/porosity, spine dimensions, tooth metrics, and mechanical properties) were analyzed with two-way ANCOVAs (car:Anova) of site, species, and their interaction, using the test diameter as a covariate to account for the influence of differences in urchin size between sites. The data normality was checked (car:qqPlot), and where multiple measurements existed per individual, responses were pooled before analysis. If the site $\times$ species interaction was nonsignificant ($p > 0.2$), models were rerun with the main effects only; if significant ($p < 0.2$), species-specific ANCOVAs were fitted. For visualization, when the urchin test diameter was a significant covariate, size-standardized values (covariate-adjusted to the pooled mean diameter; stats:predict) were plotted. Otherwise, raw values were shown. Full stats are available in Tables S3–S31.

RESULTS

DNA Barcoding. The results of the neighbor-joining phylogenetic tree showed that all individuals clustered with one of the main clades of *Echinometra* (Figure S1). A total of 16 of 32 sequences were identified as *Echinometra* sp. A (bootstrap support = 99.9), and a further two individuals were identified as *Echinometra mathaei* (bootstrap support = 99.9), subspecies well documented throughout southern Japan.⁴⁵ Surprisingly, the remaining specimen ($n = 15$) formed a distinct monophyletic clade with *Echinometra* sp. E (a subspecies not previously documented in Japan) from the Red Sea, Persian Gulf, Zanzibar, and Indo-Malay Peninsula.^{50,67,68} Low bootstrap support (84.5) limits our ability to confidently identify these individuals with a single mitochondrial barcode; thereby, the specimens are hereby collectively referred to as *Echinometra* cf. sp. E. The two *Echinometra mathaei* individuals were omitted from subsequent analyses due to low sample size. The remainder of our analyses considered the responses of *Echinometra* sp. A and *Echinometra* cf. sp. E.

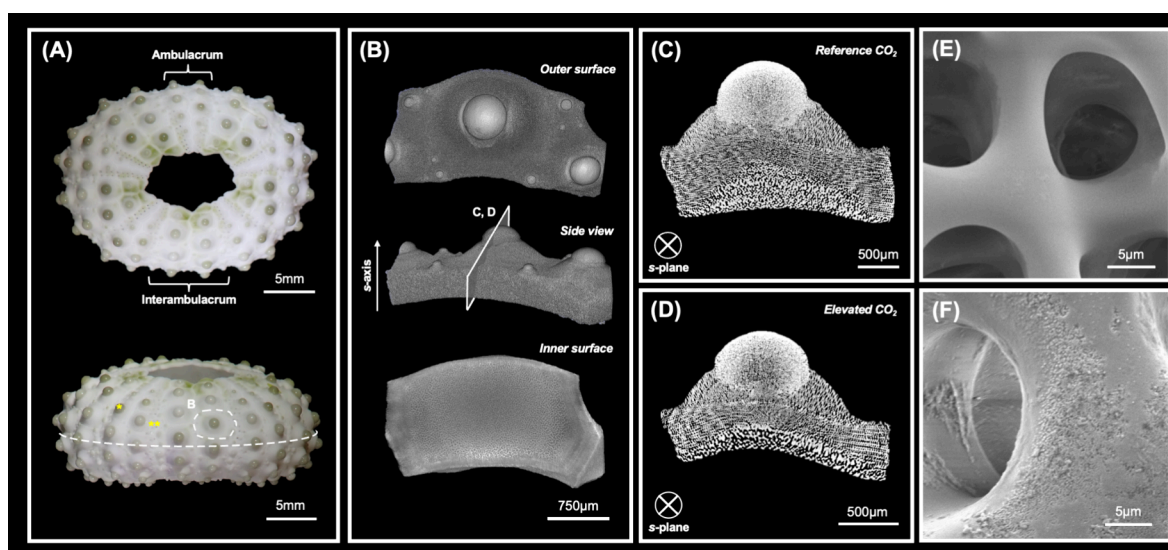


Figure 2. Comparison of the *Echinometra* spp. test-plate morphology between reference and elevated CO₂ sites. (A) Top: Aboral view of the sea urchin test showing the ambulacral and interambulacral regions. Bottom: Lateral view of the sea urchin test with the ambitus indicated by the dashed line (*tubercle; **tube feet pores). (B) Dorsal, lateral, and ventral views of an interambulacral test plate from the ambitus, indicated in the bottom (A), reconstructed with μ CT. Representative μ CT images of test-plate cross sections through the sagittal *s*-plane (indicated by a crossed circle) of urchins living under reference (C) and elevated (D) CO₂ conditions. SEM micrographs of the inner surface of test plates from living urchins (E) and elevated CO₂ (F) conditions. Signs of varying degrees of dissolution, as indicated by the pitted structure, were visible for elevated CO₂ urchins, compared to the typically smooth surface of reference CO₂ urchin test plates. The outer surface of the test plates showed little to no sign of dissolution.

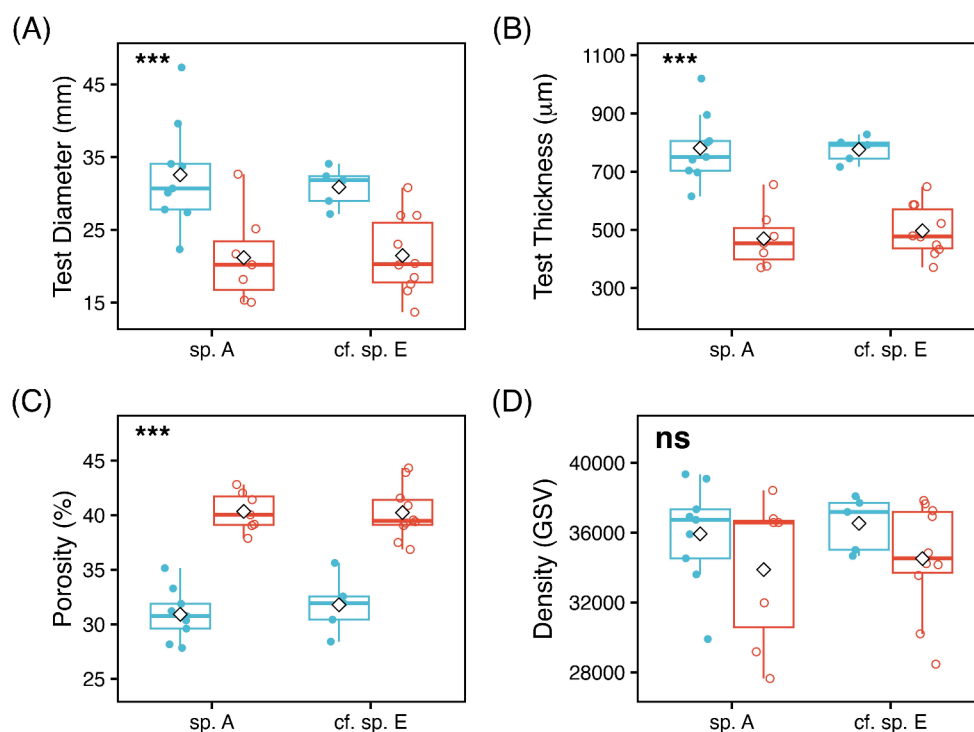


Figure 3. Comparisons of the test size and structure between reference (filled/blue points; $n = 14$) and elevated (nonfilled/red points; $n = 17$) CO₂ sites for *Echinometra* sp. A ($n = 16$) and *Echinometra* cf. sp. E ($n = 15$). These are plotted for the test diameter (A), ambital test plate thickness (B), and porosity (C) determined from μ CT reconstructions ($n = 3$ per individual). Test thickness values are corrected for the urchin test diameter. Diamonds indicate the mean values. Significant site effects across species are indicated (top left). The level of significance is indicated with asterisks, where ns is $p > 0.05$, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

Skeletal Structure. Sea urchin size structure differed between sites, with the mean test diameter (ANOVA site: $F_{1,27} = 22.23$, $p < 0.001$) and wet weight (ANOVA site: $F_{1,27} = 22.52$, $p < 0.001$) lower at the elevated CO₂ site (Figure 3A

and Table 1). However, the size distributions overlapped between sites (Figures S2–S4). The thickness, but not porosity, of skeletal test plates correlated with individual size: larger urchins produced thicker (ANCOVA test diameter: $F_{1,27}$

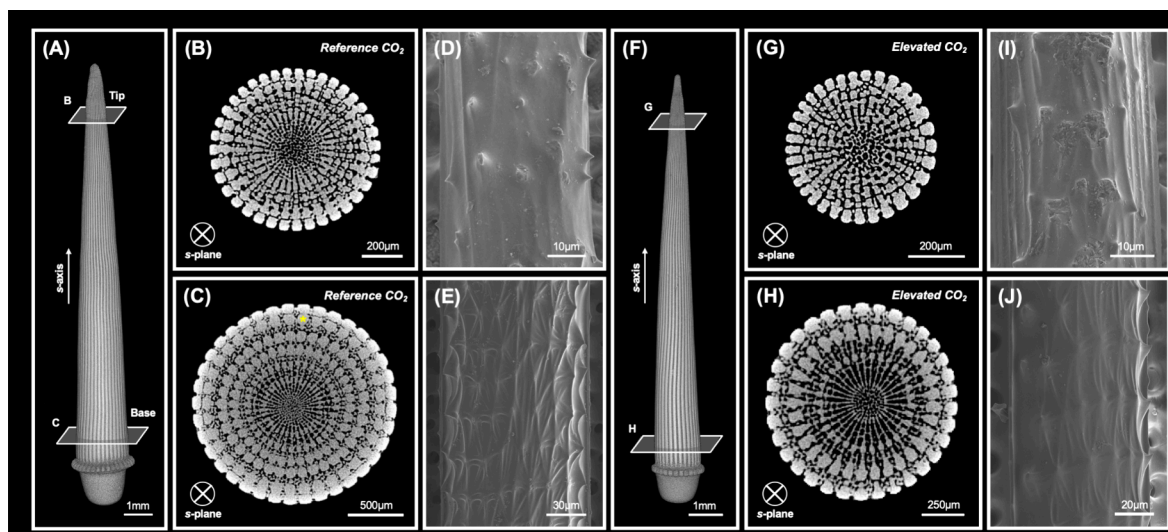


Figure 4. Comparison of the primary spine morphology between reference and elevated CO₂ sites. μ CT reconstructions of primary spines from urchins living under reference (A) and elevated (F) CO₂ conditions, respectively (*septa). Representative μ CT images of spine tip (B and G) and base (C and H) cross sections through the sagittal *s*-plane (indicated by a crossed circle) of urchins living under reference (B and C) and elevated (G and H) CO₂ conditions. SEM micrographs of spine tip septa from urchins living under elevated CO₂ (I) showed signs of dissolution greater than those from urchins at the reference CO₂ site (D). Septa at the base of spines showed little to no sign of dissolution at either site (E and J).

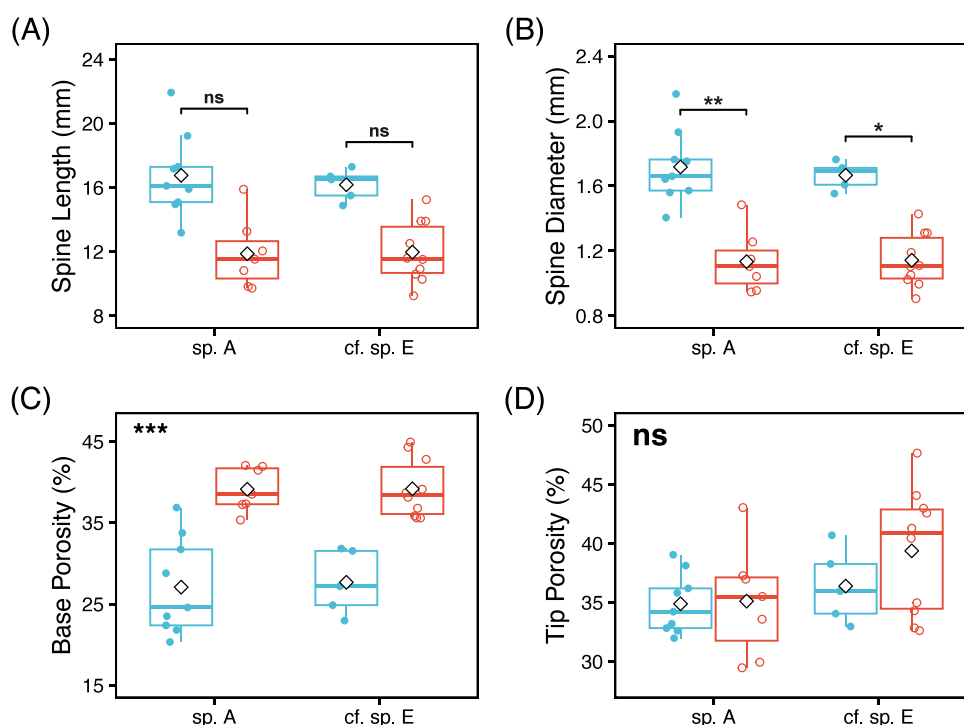


Figure 5. Comparison of the spine size and structure between reference (filled/blue points; $n = 14$) and elevated (nonfilled/red points; $n = 17$) CO₂ sites for *Echinometra* sp. A ($n = 16$) and *Echinometra* cf. sp. E ($n = 15$). These are plotted for the primary spine length (A), diameter (B), and porosity at the base (C) and tip (D) determined from μ CT reconstructions. Diamonds indicate mean values. The spine length and diameter values are corrected for differences in the test diameter. Significant site effects across species are indicated (top left). Where the site $\times$ species interaction term was significant ($p < 0.2$), significant site effects for within species ANCOVAs are indicated above the box and whisker plots. The level of significance is indicated with asterisks, where ns is $p > 0.05$, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

= 54.6 , $p < 0.001$) but not significantly more porous (ANCOVA test diameter: $F_{1,27} = 0.065$, $p = 0.801$) skeletons (Table 1). After size standardization, test plates were thinner (ANCOVA site: $F_{1,27} = 13.8$, $p < 0.001$; Figure 3B) and more porous (ANCOVA site: $F_{1,27} = 61.2$, $p < 0.001$; Figure 3C) for urchins from sites with elevated CO₂. On average, the ambient plates were $68.8 \pm 0.65\%$ biomineral by volume for the

reference urchins and $59.7 \pm 0.52\%$ biomineral for those from the elevated CO₂ site, representing a 29% increase in porosity and a 13% reduction in biomineral (relative to the test-plate size). At high magnification (1000–4000 $\times$), dissolution ranging from acute to severe was observed on the surface of test plates from individuals living under elevated CO₂

Table 2. Structural and Mineralogical Properties of Urchin Spines at Reference (350 μatm) and Elevated (900 μatm) CO_2 Sites^a

				test diameter			site		species	
		reference CO_2	elevated CO_2	<i>F</i>	<i>p</i> value		<i>F</i>	<i>p</i> value	<i>F</i>	<i>p</i> value
spine length (mm)	sp. A	15.1 $\pm$ 0.4	12.5 $\pm$ 1.0	5.33	0.038	*	0.615	0.447		
	cf. sp. E	16.2 $\pm$ 0.8	12.0 $\pm$ 0.7	17.8	<0.01	**	0.672	0.428		
spine diameter (mm)	sp. A	1.6 $\pm$ 0.1	1.1 $\pm$ 0.1	131	<0.001	***	9.52	<0.01	**	
	cf. sp. E	1.5 $\pm$ 0.1	1.1 $\pm$ 0.1	17.9	<0.01	**	6.08	0.030	*	
base porosity (%)		27.3 $\pm$ 1.37	39.2 $\pm$ 0.767	1.05	0.315		23.7	<0.001	***	0.019
tip porosity (%)		31.5 $\pm$ 1.18	37.6 $\pm$ 1.30	0.449	0.509		1.33	0.258		0.893
base density (GSV)	sp. A	39895 $\pm$ 1079	30894 $\pm$ 3097	0.706	0.416		2.89	0.113		
	cf. sp. E	34128 $\pm$ 2650	35277 $\pm$ 3448	3.38	0.091		1.26	0.167		
tip density (GSV)		39218 $\pm$ 1279	33208 $\pm$ 2253	2.73	0.110		0.186	0.670	0.054	0.819
MgCO_3 (mol %)		7.88 $\pm$ 0.123	7.76 $\pm$ 0.109	0.612	0.446	*	0.003	0.957	5.42	0.033

^aReported are the group mean values ($\pm$ SE) and ANCOVA results for the spine length, diameter, base and tip porosity, density, and spine MgCO_3 content. ANCOVA models include the test diameter as a covariate. Only the main effects were tested where the site $\times$ species interaction term was nonsignificant ($p > 0.2$). Where the interaction term was significant, separate ANCOVAs were run for each species. The level of significance is indicated with asterisks, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

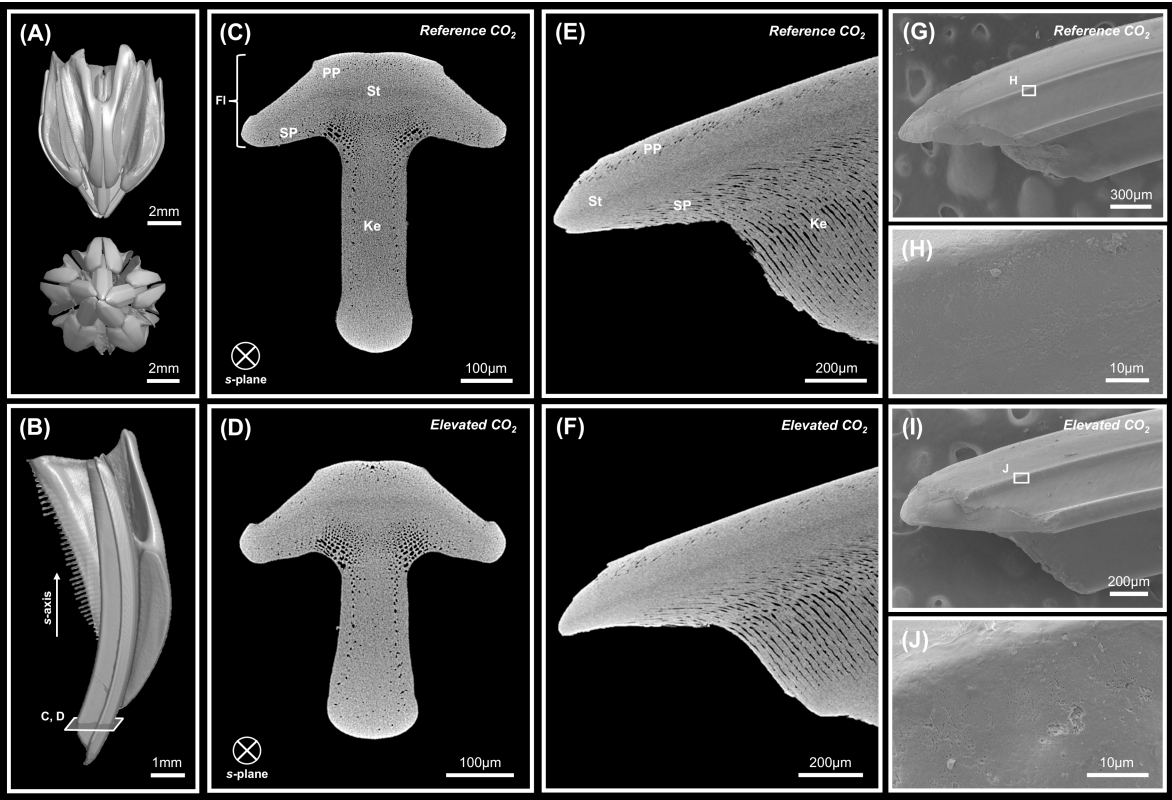


Figure 6. Comparison of the tooth morphology between reference and elevated CO_2 sites. μCT reconstructions of the Aristotle's lantern (A) and a tooth within the jaw (B). Representative μCT images of cross sections through the sagittal (C and D) and longitudinal (E and F) planes of urchin teeth from reference (C and E) and elevated (D and F) CO_2 conditions. Labeled in panels C and E are components of the tooth: the keel (Ke) and the flange (FI), the latter of which is comprised of the primary plates (PP), dense central stone (St), and secondary plates (SP). SEM micrographs of the tooth surface for urchins living under elevated CO_2 (I and J) exhibited more pronounced dissolution than those from urchins at the reference CO_2 site (G and H).

conditions (Figure 2F), compared to the smoother surface of reference urchin skeletons (Figure 2E).

For both species, primary spines were of similar length between sites (Figure 5A) but significantly thinner for elevated CO_2 urchins (Figure 5B) after size standardization. For both species, the base (ANCOVA site: $F_{1,27} = 8.42$, $p < 0.012$) but not the tip (ANCOVA site: $F_{1,27} = 1.33$, $p = 0.258$) of the spines were more porous at the elevated CO_2 site regardless of

differences in the urchin size (Table 2 and Figure 5C,D). On average, the base and tip of primary spines were $78.6 \pm 1.0\%$ and $68.6 \pm 0.8\%$ biomineral by volume for the reference urchins, compared to $66.2 \pm 1.0\%$ and $61.6 \pm 0.9\%$ for those from the elevated CO_2 site.

μCT revealed that the tooth structure was similar across the reference and elevated CO_2 sites for both species (Figure 6). The urchin size drove the differences in the tooth keel length

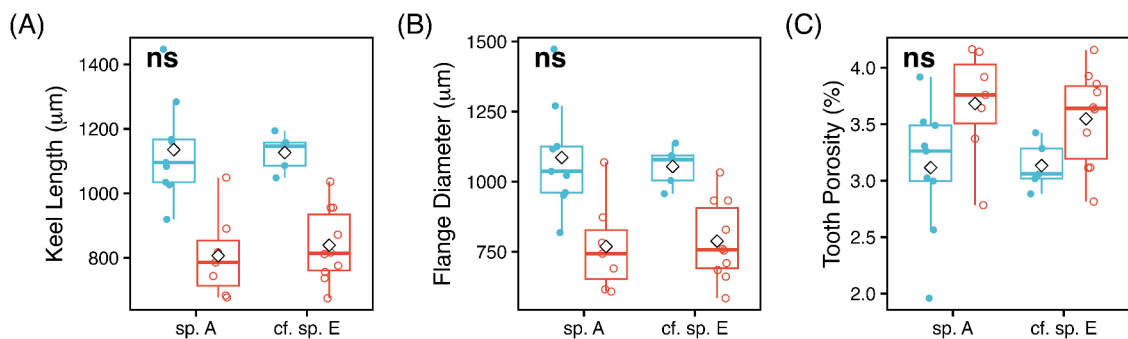


Figure 7. Comparison of the tooth size and structure between reference (filled/blue points; $n = 14$) and elevated (nonfilled/red points; $n = 17$) CO_2 sites for *Echinometra* sp. A ($n = 16$) and *Echinometra* cf. sp. E ($n = 15$). These are plotted for the keel length (A), flange diameter (B), and tooth porosity (C) determined from μCT reconstructions. Diamonds indicate mean values. For each measure, the values are corrected for differences in the test diameter. Significant site effects across species are indicated (top left). The level of significance is indicated with asterisks, where ns is $p > 0.05$, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

Table 3. Structural Properties of Urchin Teeth at Reference (350 μatm) and Elevated (900 μatm) CO_2 Sites^a

		reference CO_2	elevated CO_2	test diameter		site		species	
				<i>F</i>	<i>p</i> value	<i>F</i>	<i>p</i> value	<i>F</i>	<i>p</i> value
keel length (μm)		1132 ± 28.3	826 ± 45.3	42.5	<0.001	***	3.07	0.091	0.506
flange diameter (μm)		1074 ± 40.4	780 ± 41.5	170	<0.001	***	0.380	0.543	0.268
porosity (%)		3.12 ± 0.130	3.60 ± 0.222	15.1	<0.001	***	1.07	0.310	0.238
density (GSV)	sp. A	39316 ± 346	38899 ± 300	1.18	0.298		1.90	0.191	
	cf. sp. E	38390 ± 222	39438 ± 326	1.09	0.317		0.578	0.462	

^aReported are the group mean values ($\pm$ SE) and ANCOVA results for the tooth keel length, flange diameter, porosity, and relative density. ANCOVA models include the test diameter as a covariate. Only the main effects were tested where the site $\times$ species interaction term was nonsignificant ($p > 0.2$). The level of significance is indicated with asterisks, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

Table 4. Skeletal Hardness and Young's (Elastic) Modulus for *Echinometra* sp. A and *Echinometra* cf. sp. E Urchins Living under Reference and Elevated CO_2 Conditions (Mean $\pm$ SE) Determined by Nanoindentation^a

		species	reference CO_2	elevated CO_2	test diameter		site		species	
					<i>F</i>	<i>p</i> value	<i>F</i>	<i>p</i> value	<i>F</i>	<i>p</i> value
hardness (GPa)	test		4.66 ± 0.12	3.89 ± 0.12	1.92	0.182	7.25	0.014	*	3.15
	spine base	sp. A	3.61 ± 0.08	3.27 ± 0.14	1.98	0.194	0.598	0.459		
		cf. sp. E	3.89 ± 0.04	3.30 ± 0.10	0.263	0.622	11.1	0.01	*	
	spine tip		3.42 ± 0.06	3.18 ± 0.08	3.38	0.082	0.204	0.657		0.920
Young's modulus (GPa)	test		86.0 ± 1.58	69.5 ± 4.59	1.41	0.249	2.45	0.134		0.281
	spine base		81.5 ± 4.77	68.6 ± 3.47	0.233	0.635	3.52	0.076		0.022
	spine tip		77.0 ± 2.35	73.0 ± 3.23	0.173	0.682	0.150	0.702		1.885

^aANCOVA results are reported to 3 s.f. including the test diameter as a covariate. Only the main effects were tested where the site $\times$ species interaction term was nonsignificant ($p > 0.2$). Where the interaction term was significant, separate ANCOVAs were run for each species. The level of significance is indicated with asterisks, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

(ANCOVA test diameter: $F_{1,27} = 42.5$, $p < 0.001$) and flange diameter (ANCOVA test diameter: $F_{1,27} = 170$, $p < 0.001$) between sites (Figure 7A,B). Teeth contained the most biomineral by volume of any skeletal element, with an average porosity of $3.12 \pm 0.13\%$ by volume for reference urchins and $3.60 \pm 0.22\%$ for those from the elevated CO_2 site. Larger teeth were less porous by volume (ANCOVA test diameter: $F_{1,27} = 15.1$, $p < 0.001$); however, the overall porosity was similar between sites (ANCOVA test diameter: $F_{1,27} = 1.07$, $p = 0.310$; Figure 7C). SEM micrographs (1000–4000 $\times$) revealed that teeth from elevated CO_2 urchins had a more pitted surface, consistent with dissolution (Figure 6J), in contrast to the smoother surfaces observed in reference urchin teeth (Figure 6H).

Mineralogy. The chemical composition and density of each skeletal element was not influenced by the individual size

or chronic exposure to elevated CO_2 . For both species, the calcite comprising the test plates (Table 1), spines (Table 2), and teeth (Table 3) was of similar density between sites, as determined by μCT X-ray intensity values of the solid biomineralized material. The MgCO_3 content (mol %) was higher in the test plates (reference CO_2 , 13.72 ± 0.098 ; elevated CO_2 , 13.70 ± 0.142) than the primary spines (reference CO_2 , 7.88 ± 0.123 ; elevated CO_2 , 7.76 ± 0.109 ; Figure S6), as indicated by a shift in the d_{104} peak (Figure S8). For both skeletal elements, however, MgCO_3 was similar between sites and species (Tables 1 and 2). Similarly, test-plate Mg/Ca ratios did not differ between sites or species, were unaffected by the individual size (Table 1), but were higher on the inner surface of the test (Figure S7).

Mechanical Properties. The size of the sea urchin had no detectable influence on the hardness or elasticity (Young's

modulus) of any portion of their skeleton (Table 4 and Figure S9). Test plates produced by urchins living under reference CO_2 conditions were significantly harder (4.66 ± 0.12 GPa; $n = 11$) than those from the CO_2 seep (3.89 ± 0.12 GPa; $n = 12$) (ANCOVA site: $F_{1,19} = 7.25$, $p = 0.014$; Figure S9A). For transverse sections of spine bases, hardness was significantly reduced at the elevated CO_2 site for *Echinometra* cf. sp. E (ANCOVA_{cf.sp.E} site: $F_{1,8} = 11.14$, $p = 0.010$) but not *Echinometra* sp. A (ANCOVA_{sp.A} site: $F_{1,9} = 0.60$, $p = 0.459$; Figure S9A). At the spine tip, the hardness was similar between sites for both species (Table 4). Exposure to elevated CO_2 did not significantly alter the elasticity of any skeletal element for either species (Table 4).

DISCUSSION

Adult sea urchins, including *Echinometra* spp., are considered more resilient to OA exposure than their early life-history stages.^{12,20,69–74} However, a recent meta-analysis of primarily lab-based OA studies⁷⁵ identified consistent increases in the skeleton porosity and reductions in the weight, test thickness, and skeleton mechanical strength of adult sea urchins exposed to acidified conditions. Here, we used a natural CO_2 seep to test whether these trends hold true in an ecologically realistic setting and are consistent with the responses recorded at other CO_2 seeps.²⁴

Because of their morphological,^{50,67} ecological, and genetic similarities,⁶⁸ correctly identifying *Echinometra* subspecies is challenging, and their delineation remains uncertain. Indeed, several *Echinometra* clades identified by COI barcoding in this study were supported by low bootstrap values (Figure S1). Additional nuclear (nonmitochondrial) markers are needed to resolve the *Echinometra* species complex and clearly identify subspecies and hybrids. Such data remain sparse because most barcoding efforts to date have relied solely on COI barcodes. Despite these taxonomic ambiguities, our analyses revealed largely consistent responses to elevated CO_2 in the skeletal properties of both subspecies identified here.

Urchins exposed to chronic OA near the CO_2 seep (mean $\text{pH}_T = 7.781$) tended to be smaller and lighter than those at the reference site. Importantly, the overlap in size classes between sites suggests that differences in skeletal traits are unlikely to result solely from sampling distinct cohorts. The causes of this size–structure difference remain uncertain; it could reflect a younger age, slower growth at comparable ages, or a combination of both. If the pattern reflects younger age, it may indicate shorter lifespans at the CO_2 seep due to elevated mortality of larger/older individuals from habitat loss or predation.⁷⁶ If instead it reflects slower growth at comparable ages, it could signal a trade-off between calcification and energy balance under high CO_2 .^{77–79} Sea urchins grow continuously throughout their life, experience lower mortality rates at larger sizes, and have higher reproductive output with size.⁸⁰ Thus, whether seep urchins are smaller because they die younger or grow more slowly, a shift toward smaller body size could reduce reproductive output, survival, and recruitment.⁸⁰ Importantly, here, OA-acclimatized urchins produced more porous, thinner, and more brittle tests and spines, even after accounting for individual body size. Because *Echinometra* are largely sedentary grazers that remain near their burrows,^{43,81} individuals sampled here likely spent the entirety of their postsettlement lives under the CO_2 conditions of their collection site.²⁴ This assumption is supported by previous studies in which chemically tagged *Echinometra* were

successfully recaptured at both CO_2 seep and reference reef sites after 17 months *in situ*.²⁴ In the present study, low urchin density at the elevated CO_2 site prevented chemical tagging and therefore growth rate estimation. Where feasible, future *in situ* work should use mark–recapture, or other validated aging approaches to disentangle growth- versus age-related effects.^{24,82}

Besides individual size, the adjustment of skeletal mineralogy can also help to reduce energy consumption if the synthesis of less soluble polymorphs is favored over unstable carbonate minerals.^{83,84} Echinoderms skeletons are composed of high Mg-calcite ($\text{Mg}_x\text{Ca}_{1-x}\text{CO}_3$), an unstable polymorph of calcite prone to dissolution. For some calcifiers, the active control of skeletal magnesium incorporation (i.e., reprecipitation of less-stable high Mg-calcite as low Mg-calcite) can enhance structural stability and resistance to corrosion under acidification conditions.⁸⁵ Here, urchin skeletal calcite contained magnesium levels ($\text{MgCO}_3 \sim 7\text{--}14$ mol % and $\text{Mg}/\text{Ca} > 0.04$) typical of high Mg-calcite.⁴ However, despite mineralogical adjustment being reported in other calcifiers, here the Mg content was consistent for each skeletal component across sites. The relative density of the biomineral was also similar across sites for each skeletal element, aligning with the consistencies in the biochemical composition.

The mechanical resilience of urchin skeletons is crucial to the structural integrity of the urchins. The density and hardness of *Echinometra* teeth allow adult urchins to ingest dietary carbonate from calcareous alga and to bore into hard substrate, forming refugia from predators and wave action but also providing an important source of sediment to reefs.^{86,87} Urchin spines not only provide protection from predation⁸⁸ but also absorb and spread the impact of physical load, reducing damage to the test.⁸⁹ Importantly, investment into the test density and thickness is increasingly prioritized as urchins grow^{90,91} and the skeletal strength under impact increases with size.^{13,92} Under acidified conditions, however, dissolution, chemical shifts, and structural changes are predicted to increase the fragility of calcifier skeletons, reducing their density and increasing the skeletal porosity,^{17,56,93} rendering urchins more susceptible to predation and physical disturbance.^{13,92} If associated with increased urchin mortality,¹⁷ the reduced protective and defensive function of sea urchin skeletons would be detrimental to urchin populations and the ecological functions they perform.⁹⁴ Here, urchin test plates and the base of *Echinometra* cf. sp. E spines exhibited reduced nanohardness but maintained their elasticity, representing an overall reduction in mechanical resilience. Given the similar biochemical composition and density of the calcite produced by urchins, this mechanical weakening may suggest that other aspects of the biomineral are altered, such as the crystallographic orientation or amorphous calcium carbonate content.^{83,95} Despite some evidence of dissolution at the grinding tip, which is in direct contact with acidified seawater at the elevated CO_2 site, the tooth structure was resilient to increasing CO_2 . This may represent the prioritized maintenance of teeth over other skeletal elements to enable continued feeding and refugia formation via boring or reflect differences in construction and mineralogy between the test, spines, and teeth.

Reductions in the skeletal hardness and material elasticity are reported for other urchin species and marine calcifiers exposed to both natural^{84,96} and experimentally controlled¹⁷ low pH conditions, and urchins growing in seawater with low

Mg/Ca ratios also exhibit reduced skeletal nanohardness.⁹⁷ However, because the mechanical strength is determined by the interaction between the organic and inorganic components of calcifier skeletons (i.e., collagenous sutural ligaments),^{13,98,99} it is unclear whether the highly localized nature of nano-indentation measurements obtained here provides ecologically relevant results.^{17,100} Tests tend to mechanically fail along connective tissue suture lines rather than through plate rupturing,¹³ therefore performing mechanical tests using whole, unbleached individuals is preferred. Despite this, because the test strength correlates with their size,¹³ reductions in the urchin size at the CO₂ seep likely correspond with reduced mechanical resilience.

The sensitivity of adult *Echinometra* spp. to OA varies between studies. Uthicke et al.⁶⁹ reported slight, but not statistically significant, reductions in adult *Echinometra mathaei* growth when incubated at pH_{NIST} 7.5 for 6 weeks. Similarly, 5 weeks of continuous exposure to pH 7.7 (following a 2-week gradual acclimation) elicited no change in the growth or respiration of *E. mathaei*, which were able to maintain the pH of their coelomic fluid by increasing its bicarbonate content.¹⁰¹ Shirayama and Thornton¹⁰² reported significant reductions in growth and net calcification rates for *E. mathaei* maintained at pH 7.9, which only manifested after ~14 weeks of exposure, whereas Hazan et al.¹⁰³ reported no dissolution or structural degradation of *Echinometra* sp. EE skeletons collected following long-term incubation (~11 months) at low pH (pH_{NBS} 7.7). Acclimation of *Echinometra* to OA over multiple generations may help to ameliorate the impacts of climate change (predicted OA and warming) on benthic life stages⁷¹ observed in short-term experiments, although larval bottlenecks become more severe.^{69,72–74} For adult urchins, moderate ocean warming may also offset deleterious impacts associated with low pH by stimulating growth.^{11,104,105} For example, when incubated at typical winter (20 °C) temperatures, *Echinometra viridis* exhibited a net loss of calcified material under high pCO₂ (~830 ppm) conditions but could maintain calcification under summer (30 °C) temperatures.¹¹ As such, the location of Shikine Island toward the cold edge of tropical *Echinometra* spp.'s typically tropical/subtropical thermal range may explain the negative responses reported here.

Other indirect effects of rising CO₂, such as shifts in food identity, availability, and quality,^{24,95,106} have been proposed as factors enabling some calcifiers to “thrive” under acidified conditions. CO₂-driven dietary shifts can offset direct OA impacts by increasing energy budgets,^{107,108} enabling faster growth and the construction of stronger calcified structures.^{24,109–111} The ability of *Echinometra* to regulate its extracellular pH, and thereby maintain growth rates, may also be influenced by both food quality and quantity.^{112,113} The ingestion of calcareous food represents an important source of bicarbonate, which can be used to buffer the internal pH and maintain calcification under acidified conditions.^{106,113} As such, food represents an important factor that may contribute to the contrasting responses of *Echinometra* to OA between studies. At the tropical Upa-Upasina (Papua New Guinea) CO₂ seep, for example, *Echinometra* spp. were more abundant¹¹⁴ and grew larger and faster than their reference reef counterparts, with no difference in the urchin mortality, respiration, and test calcification between sites despite chronic exposure to elevated CO₂.²⁴ Patterns linked to increased food availability. The pH values at this site exhibited short-term variability (pH_T ~6.9–8.1)²⁴ greater than that reported at

Shikine (pH_T 7.4–7.9).³⁵ Such differences in the pH regime may explain some of the discrepancy in *Echinometra* responses between studies. As recorded for other calcifying species,^{115–117} periods of respite during which pH is elevated to ambient levels may elicit positive responses of *Echinometra* under elevated CO₂.

In other studies, CO₂ enrichment has been shown to reduce algal nutrition and palatability, alter herbivore feeding preference,¹¹⁸ and disrupt calcifier feeding rates.^{26,119} For example, at the temperate White Island (New Zealand) CO₂ seep, *Evechinus chloroticus* abundance and feeding rates were reduced despite the boosting effect of CO₂ on food (algal turf) availability.²⁶ Additional OA-driven dietary changes, such as the loss of carbonate-rich components of herbivore diets (i.e., CCA and calcifying macroalgae) due to turf proliferation and shifts in urchin gut microbiomes, may further alter energy acquisition, metabolic function,¹²⁰ and skeletal properties.^{41,93,106,110,121}

Because the sea urchin skeleton structure is influenced by the diet composition and nutritional content,^{93,122} their properties will likely be indirectly affected by OA-driven impacts on the broader benthic community. The benthic community found around the Shikine Island CO₂ seep is dominated by turf-forming diatoms,¹²³ which replace the macroalgae and calcifying algae found at reference sites.¹²⁴ Changing *Echinometra* diets, which reflect these shifts,⁴⁵ as opposed to direct CO₂ effects, may underpin the changes in the skeletal properties reported here. Understanding changes in the urchin feeding rates, metabolism, diets, nutrient content, and associated microbiome shifts will provide greater insight into how the indirect effects of elevated CO₂ influence calcifier skeletons and why responses differ across localities and between species.

The study presented here combined nanostructural (μCT and SEM), mineralogical (EDX and XRD), and mechanical (nanoindentation) analyses to investigate the long-term responses of skeletons in two closely related sea urchin species to OA at a shallow-water CO₂ seep. We show that under long-term exposure to elevated pCO₂ at Shikine Island, the chemical composition and density of *Echinometra* spp. skeletons remain unchanged, but the test and spine structures and strength are negatively impacted. The structure of the teeth, however, appears resistant to elevated CO₂. The responses of *Echinometra* spp. to OA, which differ between skeletal elements, studies, and sites, underscore the complex and multifaceted nature of OA and its impacts. Further research is needed to explore the repeatability of these findings in successive localities, urchin species, and other calcifying groups. Doing so will enable researchers to disentangle general global trends from environmental and phylogenetic noise, identify responses that show context dependency, and understand whether naturally extreme sites can serve as realistic analogues for future ocean conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.5c09757>.

Carbonate chemistry summary for reference and elevated CO₂ sites (Table S1), locality, GenBank accession numbers, and taxonomic assignments of COI sequences (Table S2), COI phylogenetic tree of

specimens (Figure S1), relationships between the test diameter and skeletal properties (Figures S2–S5), MgCO_3 content of spines and test plates (Figure S6), Mg/Ca ratios of test-plate surfaces (Figure S7), XRD spectra of spine and test-plate Mg -calcite (Figure S8), mechanical properties of test plates and spines (Figure S9), and full statistical outputs for all analyses (Tables S3–S31) (PDF)

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Notes

The authors declare no competing financial interest.

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