**Question**: How are T.gratilla affected by climate change, specifically increased temperatures and decreased pH?

**Hypothesis**:

- Increased T = decreased body size

- Decreased pH = decreased calcification, alterations in composition

- Both = smaller and less calcified, alterations in composition

**Treatments**: T/OA, T, OA

**Controls**: Ambient

**Responses**: Growth (Diameter, mm), Calcification and/or dissolution.

**Experimental Units**: Technically header tanks… n=2

**Sample Units**: Individual urchins… n=24

**Materials and Methods Sections:**

[1. **Experimental System**](#ExpSystem)

**-** Describe the actual system -> seawater from bay to header buckets to tanks

- Flow rates and turnover

- Keishas 2 papers ( (Bahr & Jokiel, 2016; Jokiel, Bahr, & Rodgers, 2014))

• **FIGUREs**:

[**2. Study Animal Collection and Acclimation**](#Animal)

- describes how got urchins

- acclimation period? Amp up period?

**• FIGUREs**: urchin diagram?

- Growth plot here??

[**3. Experimental Treatments**](#ExpTreatments) **and** [**Data Collection**](#DataCollection)

- Describe what and how treatments were done

- What was measured

- How data was collected

• **FIGURES**

- Table of treatment means

[5. **SEM**](#SEM)

**-** Sample collection and mounting prep as well as actual use of machine

[6.](#Stats) **[Statistical Analysis](#Stats)**

- Describe what tests done and how and significance.

- Include residuals shown for normality and variances

- Image Analysis?

[**- Possible Figures**](#Figures)

**[-References](#Refs)**

**1. Experimental Design**

The experiment was conducted in a flow-through system at the University of Hawaiʻi at Manoa’s Hawai’i Institute of Marine Biology (HIMB) on Moku o Loʻe in Kāne’ohe Bay Hawai’i and followed a similar low-cost, high-flow system outlined in Jokiel et. al. 2014. Four treatment groups represented future projected climate change conditions (IPCC Ref.): control (ambient temperature and pCO2); high temperature (+2°C); high pCO2 (to -0.3 pH units); and combined high temperature and CO2 (+2°C, -0.3 pH units) (Przeslawski, Ahyong, Byrne, Wörheide, & Hutchings, 2008). Six replicate urchins were assigned to each treatment.

Unfiltered seawater was pumped directly from the bay from a 3m depth into eight 5-gallon header buckets where seawater conditions were manipulated. Each header flowed into 3 randomly assigned 8L tanks where individual urchins were housed, for a total of 24 microcosms with 6 replicates per treatment. Water flowed into tanks at a rate of 0.9 - 1.2 L min-1, resulting in a complete volume exchange every 6 - 8 minutes, providing over 95% dissolved oxygen. The experiment ran for 138 days, not including a two-week acclimation period. By the end of the experiment, urchins had reached mature adult size of at least 60mm (mean 72.12 ± 0.99mm) (Byrne et al., 2014; Dworjanyn & Byrne, 2018).

**2. Study animal collection and acclimation**

Urchins were collected from the Hawaiʻi Department of Land and Natural Resources (DLNR) Division of Aquatic Resources (DAR) sea urchin hatchery, transported to HIMB, and acclimated to Kaneohe Bay ambient conditions for two weeks in flow-through seawater tables. Test diameters were not significantly different upon initial collection (7.53 ± 0.15 mm) or after acclimation (16.11 ± 0.35 mm). Urchins were fed *Gracilaria salicornia* collected from the shores of HIMB in excessto ensure consistent food availability. Throughout the experiment, there was only one mortality on day 116 of an urchin in a heated treatment.

**3. Experimental Treatments and Data Collection**

Temperature was controlled using ﻿submersible titanium heaters (Finnex©, TH-800, 800 W). Seawater pH was manipulated by direct CO2 bubbling through a hydro pump to maximize diffusion (Jokiel et al., 2014). All ambient and heated conditions were bubbled with air to ensure consistency in dissolved oxygen concentrations.

Because of the open-system, all treatments tracked natural daily and seasonal fluctuations. No treatment was applied for the first two weeks during acclimation. After which, treatments were ramped up for two more weeks at a rate of -0.06 pH units day-1. Temperature (°C), salinity ﻿(‰), and dissolved oxygen (% saturation) were measured at mid-day (11:00 – 14:00 h) to ensure consistent desired environmental conditions. Water samples were collected once a week for use in titrations and photometric pH determinations.

**pH Spectrophotometer:** (Bahr & Jokiel, 2016)**:**

Seawater pH was determined spectrophotometrically (pHTotal) each week using m-cresol purple dye (Sigma-Aldrich© #857890). All procedures were followed according to SOP 7 (Dickson, Sabine, & Christian, 2007). In addition to the above procedure, pH was checked frequently using an Accumet© AP72pH/mV/temperature meter (pHNBS) to ensure stability in between sampling.

**Titrations:**

**﻿**Total alkalinity was measured weekly using an automatic titrator (Titrino© Plus 877, Metrohm) with pH glass electrode (9101 Herisau, Metrohm©). The automatic titrator was checked and confirmed for precision with certified reference materials (CRM) (Batch 127 from A. Dickson Laboratory, Scripps Institution of Oceanography). Additional seawater parameters were calculated using the program CO2SYS (Pierrot, D., 2012) with stoichiometric dissociation constants (K1, K2) as redefined (original by Mehrbach et. al., 1973) by Dickson & Millero, 1987.

**4. SEM and Image J**

At the end of the 138-day experimental period, spines were plucked from each individual urchin (n=23) for use in scanning electron microscopy (SEM) at the Biological Electron Microscope Facility (BEMF) at the University of Hawaiʻi at Mānoa. Approximately 10–20 spines were haphazardly selected from the skirt of the urchin test (*possible diagram from (Collard et al., 2016)*). Spines were soaked in 10mL of 10% bleach solution for 20 minutes to remove organic material, rinsed in distilled water to remove bleach residue, and dried at 60°F for 24 hours. All spines selected were measured for length using Neiko digital calipers (Resolution: 0.01mm, Accuracy: 0.02mm) and three were randomly selected to be prepped for SEM imaging. Each selected spine was cut 2 mm from the tip and base using a fresh razor blade and air blasted to remove dust particles. In the event that a spine broke during the mounting process, another spine was randomly selected. The mid-section was saved for potential future compositional analysis. The base and tip selections were mounted cut-side upward on aluminum stubs using carbon conductive adhesive paint and coated with gold/palladium for 45 seconds in a Hummer 6.2 sputter coater. Specimens were viewed with a Hitachi S-4800 Field Emission Scanning Electron Microscope at an accelerating voltage of 5.0 kV. Because the SEM allows for maneuverability on the X, Y, and Z planes, stubs could be rotated to ensure a direct overhead image could be captured.

Images were analyzed using ImageJ (NIH, Bethesda, MD, USA). A manual selection of each spine cross-section was made to include only the inner portion of the spine. A custom macro script was run to convert each image to binary (black for voids; white for CaCO3) and adjust for outlying bright spots. The image was then scanned to divide it in half with equal area on each side. Although great care was taken to remove dust before SEM imaging, some spines still had small specks of dust that could interfere with results. If dust was visible, images were rotated so the dust occupied only one side. If there was too much dust across the entire cross-section, that image was not used in analysis (n=1). Three ratios of black to white (left, right, and full) were calculated to represent calcified to non-calcified material. There was no difference between left and right non-dusty sides, so analysis was conducted on only one side per image.

**6. Statistical Analyses**

Data analysis was conducted using JMP and R programs. This study followed a blocked factorial design in which the headers were randomly assigned to tanks. Residuals were checked for normality and variances determined for equality. Linear mixed models with nested…

**POSSIBLE FIGURES?**



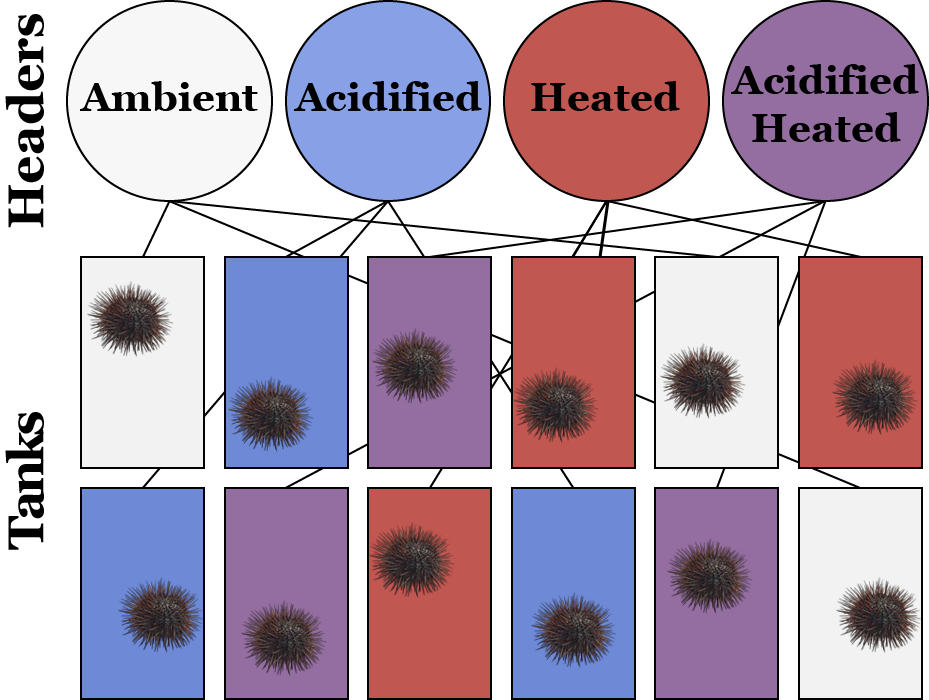


Fig. 1. Experimental set-up (top) on Moku oʻ Loʻe and schematic diagram representing the design and treatments in one side of the system.

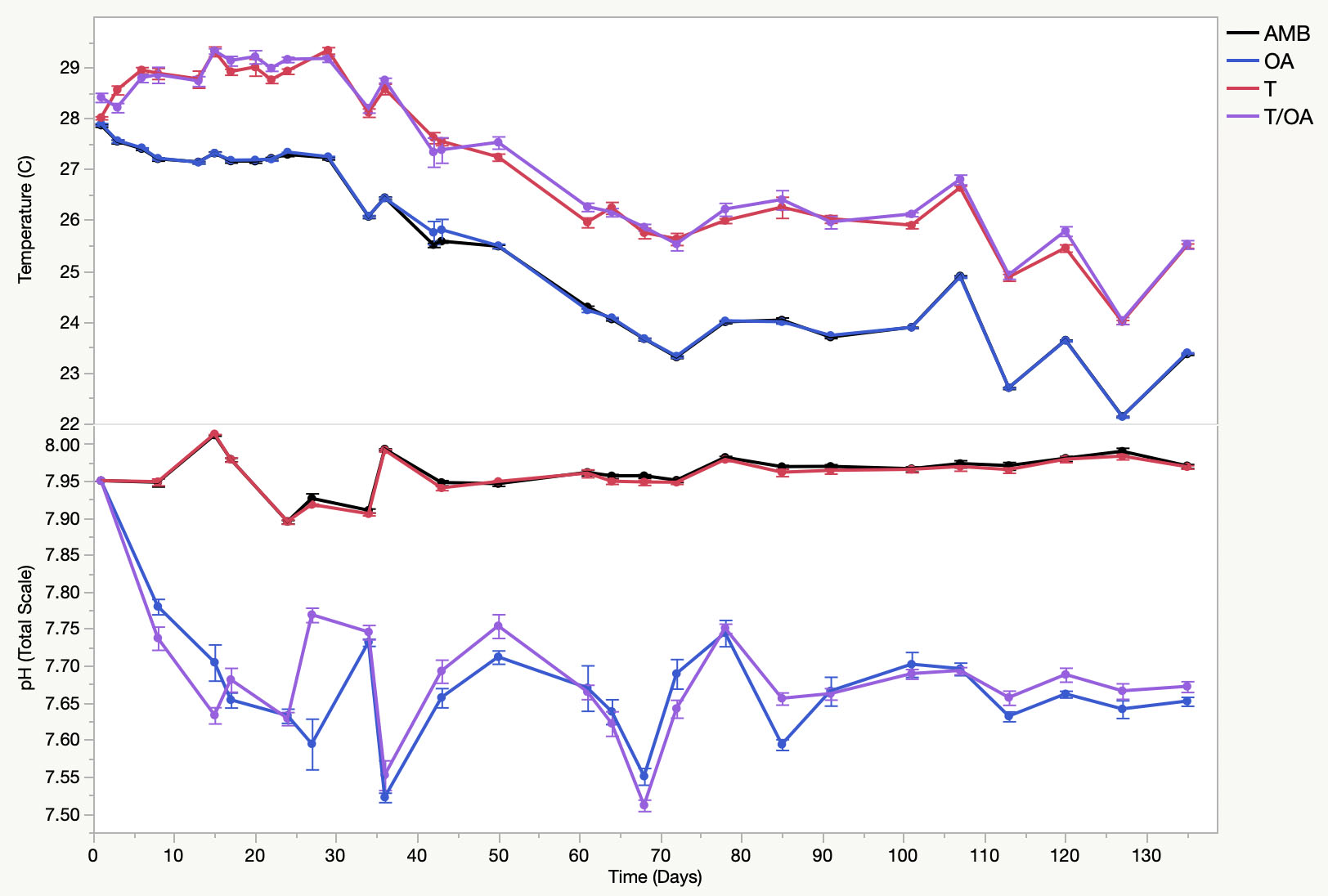
Fig 2. Temperature conditions (top) and pH conditions (bottom) over the 138-day experiment period. Desired treatment conditions of +2°C and -0.3 pH units were achieved by day 15 after a two week ramp up period.

Table 1. Experimental treatments and environmental conditions throughout the course of the 138-day experiment (mean ± s.e.)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Treatment** | **Salinity (ppt)** | **Temp (°C)** | **At (µmol kg-1)** | **pCO2 (ppm)** | **pH** |
| **Amb T, Amb ﻿*p*CO2** | 33.70 ± 0.03 | 25.38 ± 0.11 | 2180.6 ± 1.75 | 493.00 ± 11.41 | 7.96 ± 0.004 |
| **Amb T, High *p*CO2** | 33.70 ± 0.04 | 25.40 ± 0.11 | 2182.17 ± 1.76 | 1068.64 ± 11.41 | 7.66 ± 0.004 |
| **High T, Amb *p*CO2** | 33.70 ± 0.05 | 27.30 ± 0.11 | 2180.25 ± 1.77 | 537.34 ± 11.52 | 7.96 ± 0.004 |
| **High T, High *p*CO2** | 33.70 ± 0.06 | 27.30 ± 0.11 | 2182.53 ± 1.76 | 1120.93 ± 11.41 | 7.68 ± 0.004 |

|  |
| --- |
| macro "Sesno-ify" {  run("Clear Outside");  run("Crop");  roiManager("add");  run("Make Binary");  roiManager("Select", roiManager("count")-1);  run("Remove Outliers...", "radius=2 threshold=50 which=Bright");  run("Remove Outliers...", "radius=2 threshold=50 which=Bright");  roiManager("Rename", getTitle);  //divides current roi into left and right half  getSelectionBounds(x, y, width, height)  getRawStatistics(totalArea);  minOff = 1e9;  for (w=1; w < width; w++){  roiManager("Select", roiManager("count")-1);  setKeyDown("alt");  makeRectangle(x, y, w, height);  getRawStatistics(rightArea);  off = abs(2\*rightArea - totalArea);  if (off < minOff){  minOff = off;  xHalf = x + w;  wHalf = w;  halfArea = rightArea;  }  }  //Display result:  roiManager("Select", roiManager("count")-1);  setKeyDown("alt");  makeRectangle(x, y, wHalf, height);  run("Copy");//work around  run("Add to Manager");  roiManager("Select", roiManager("count")-1);  roiManager("Rename", getTitle + "\_Right");  //run("Measure");  wait(500);  roiManager("Select", roiManager("count")-2);  setKeyDown("alt");  makeRectangle(x+wHalf, y, width - wHalf, height);  run("Copy");//work around  run("Add to Manager");  roiManager("Select", roiManager("count")-1);  roiManager("Rename", getTitle + "\_Left");  //get histogram  roiManager("Select", roiManager("count")-3);  getStatistics(area, mean, min, max, stddev, histogram);  setResult("Skeletal", 0, histogram[255]);  setResult("Void", 0, histogram[0]);  setResult("Ratio", 0, histogram[255]/histogram[0]);  updateResults();    roiManager("Select", roiManager("count")-2);  getStatistics(area, mean, min, max, stddev, histogram);  setResult("Skeletal", 1, histogram[255]);  setResult("Void", 1, histogram[0]);  setResult("Ratio", 1, histogram[255]/histogram[0]);  updateResults();    roiManager("Select", roiManager("count")-1);  getStatistics(area, mean, min, max, stddev, histogram);  setResult("Skeletal", 2, histogram[255]);  setResult("Void", 2, histogram[0]);  setResult("Ratio", 2, histogram[255]/histogram[0]);  updateResults();  setResult("Label", 0, getTitle)  setResult("Label", 1, getTitle + "\_Right")  setResult("Label", 2, getTitle + "\_Left")  } |

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