Key biological responses over two generations of the sea urchin *Echinometra* sp. A under future ocean conditions

S. Uthicke^{1,*}, F. Patel¹, S. Karelitz², H. M. Luter¹, N. S. Webster^{1,3}, M. Lamare²

¹Australian Institute of Marine Science, PMB No 3, Townsville, Queensland 4810, Australia

²Department of Marine Science, University of Otago, 9016 Dunedin, New Zealand

³Australian Centre for Ecogenomics, University of Queensland, Brisbane, Queensland 4072, Australia

ABSTRACT: Few studies have investigated the effects of ocean warming and acidification on marine benthic organisms over ecologically relevant time scales. We used an environmentally controlled coral reef mesocosm system to assess growth and physiological responses of the sea urchin species *Echinometra* sp. A over 2 generations. Each mesocosm was controlled for temperature and $p\text{CO}_2$ over 29 mo under 3 climate change scenarios (present day and predicted states in 2050 and 2100 under RCP 8.5). The system maintained treatment conditions including annual temperature cycles and a daily variation in $p\text{CO}_2$. Over 20 mo, adult *Echinometra* exhibited no significant difference in size and weight among the treatments. Growth rates and respiration rates did not differ significantly among treatments. Urchins from the 2100 treatment had elevated ammonium excretion rates and reduced $\text{O}_2\text{:N}$ ratios, suggesting a change in catabolism. We detected no difference in spawning index scores or oocyte size after 20 mo in the treatments, suggesting that gonad development was not impaired by variations in $p\text{CO}_2$ and temperature reflecting anticipated climate change scenarios. Larvae produced from experimentally exposed adults were successfully settled from all treatments and raised for 5 mo inside the mesocosm. The final size of these juveniles exhibited no significant difference among treatments. Overall, we demonstrated that the mesocosm system provided a near natural environment for this urchin species. Climate change and ocean acidification did not affect the benthic life stages investigated here. Importantly, in previous short-term (weeks to months) experiments, this species exhibited reductions in growth and gonad development, highlighting the potential for short-term experiments with non-acclimated animals to yield contrasting, possibly erroneous results.

KEY WORDS: Ocean acidification · Ocean warming · Climate scenarios · Calcifying invertebrates

1. INTRODUCTION

Many calcifying marine organisms exhibit reduced calcification rates or survival due to increased sea surface temperature (SST), ocean acidification (OA) or a combination of these (Kroeker et al. 2010, 2013, Poloczanska et al. 2016). Both stressors are a direct result of anthropogenically increased atmospheric carbon dioxide (CO_2). SSTs in coral reef areas have

already risen by nearly 1°C compared to pre-industrial levels (Lough et al. 2018), and a further increase by $1\text{--}3^\circ\text{C}$ is predicted by the end of the 21st century (Collins et al. 2013). CO_2 absorbed into the oceans decreases the saturation state of the carbonate ion (CO_3^{2-}) and lowers pH. Without mitigation, by the year 2100, ocean pH will have decreased by 0.2–0.4 units, and the partial pressure of CO_2 ($p\text{CO}_2$) will have increased to 750–900 μatm (Orr et al. 2005, Meins-

*Corresponding author: s.uthicke@aims.gov.au

hausen et al. 2011). Coral reefs are particularly vulnerable to both warming and OA because their ecosystem engineers (scleractinian corals) (Hoegh-Guldberg et al. 2007), like many other tropical marine animals (Stuart-Smith et al. 2015, Negri et al. 2020), already live close to their thermal limit (Hoegh-Guldberg 1999), and calcification (aragonite accretion) is inhibited in seawater with lower aragonite saturation state. In addition, a large variety of other functionally important organisms on coral reefs, such as calcifying algae (Koch et al. 2013), foraminifera (Doo et al. 2014) and echinoderms (Byrne 2011, Byrne et al. 2018), are also threatened by increased SST and/or OA.

Sea urchins of the genus *Echinometra* are abundant and ecologically important members of most coral reefs, as they play a primary role in algal grazing and bioerosion (McClanahan & Muthiga 2007). In the Indo-Pacific region, this genus has now been divided into 5 species (Arakaki et al. 1998, Bronstein & Loya 2013), 3 of which have not been assigned full species names (sp. A, C and EE). Several short-term, aquarium-based studies investigating the effects of OA on *Echinometra* sp. A showed subtle negative effects of OA on growth and physiology (Uthicke et al. 2013). Similarly, in the Caribbean *E. viridis*, growth of adults was reduced under elevated $p\text{CO}_2$ (Courtney et al. 2013), and juveniles of *Echinometra* sp. also grew slower after 12 wk of exposure to elevated $p\text{CO}_2$ (Shirayama & Thornton 2005). By contrast, Indian Ocean *E. mathaei* and *Echinometra* sp. EE exhibited no measurable physiological responses to elevated $p\text{CO}_2$ with exposure times between 45 d and 12 mo (Hazan et al. 2014, Moulin et al. 2014, 2015). While very few studies have investigated the effect of temperature increase on *Echinometra* species, a limited immunological response to ocean warming was evident in *E. lactuner* (Branco et al. 2013), and effects on growth and other physiological parameters were more pronounced under simultaneous temperature and $p\text{CO}_2$ stress in *Echinometra* sp. A (Uthicke et al. 2014b).

Importantly, most previous studies assessing the effects of climate change on reef organisms were conducted over relatively short terms (weeks to months) using small aquaria, with experimental treatments often not replicated or only pseudo-replicated (Cornwall & Hurd 2016). Short-term studies do not address the potential for adaptation or acclimation of individuals (Kroeker et al. 2013), and studies over several generations are required to investigate trans-generational plasticity and selection/adaptation (Donelson et al. 2018). An alter-

native to short-term experiments for studying the effects of OA is to measure responses of long-term residents at carbon dioxide vents, which are subjected to higher $p\text{CO}_2$ than adjacent control reefs (Hall-Spencer et al. 2008, Fabricius et al. 2011). However, the small spatial extent of vents means that the developmental stages (typically free living and planktonic) and resulting offspring of vent-acclimated adults are not likely to recruit within the same vent environment, hindering multi-generational acclimation studies of vent taxa (Lamare et al. 2016, Uthicke et al. 2016). Selection and adaptation to climate change can also be studied on taxa inhabiting vent systems or natural upwelling areas using population genomic approaches (Pespeni et al. 2012, 2013).

An alternative approach to overcome limitations of short-term experiments is the use of mesocosm systems housing multiple species in large manipulated water volumes over several generations. This approach is relatively common in marine plankton communities (e.g. Riebesell et al. 2007, Allgaier et al. 2008). However, in benthic coral reef science, few examples of these exist, possibly due to the large expense and difficulties in maintaining water quality and treatment conditions over the long term. A large-scale mesocosm system at a Great Barrier Reef research station was used to study bioeroding sponges in a 12 wk experiment under different climate scenarios (Fang et al. 2013, 2014). Sea urchin growth (Leblud et al. 2014, Moulin et al. 2015) and OA effects on crustose coralline algae and corals (Langdon et al. 2003, Jokiel et al. 2008) have also been investigated in reef mesocosms.

In the present study, we introduce a large-scale mesocosm system for climate change related research at the Australian Institute of Marine Science's National Sea Simulator (SeaSim). Temperature and $p\text{CO}_2$ were controlled in combination independently for each replicate to simulate present-day conditions and 2 future (2050 and 2100) climate scenarios assumed under RCP 8.5 (Meinshausen et al. 2011). We present data on 2 generations of the tropical rock-boring sea urchin *Echinometra* sp. A, measuring growth, physiology and reproductive performance of the parent generation over 20 mo, and presenting growth of their post-larval offspring up to 5 mo post settlement. Given that this species has been previously studied in shorter-term experiments both under elevated temperature and increased $p\text{CO}_2$ (Uthicke et al. 2013, 2014b), we contrast performance and response in this long-term experiment to previous findings.

2. MATERIALS AND METHODS

2.1. Species and study sites

Approximately 230 specimens of *Echinometra* sp. A (sensu Arakaki et al. 1998) (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m637p087_supp.pdf) were collected on the Great Barrier Reef, from depths <5 m at Trunk Reef (18.3188° S, 146.8662° E) on 18 and 19 February 2016. Animals were collected by SCUBA divers and returned to the research vessel, where they were maintained in 60 l aquaria supplied with flow-through ambient reef seawater, and later transferred to the SeaSim until the start of the experiment.

2.2. Mesocosm systems and treatment levels

A detailed description of the mesocosm outdoor system is given in Text S1 in the Supplement. Experimental conditions aimed to mimic temperature and $p\text{CO}_2$ increases in the years 2050 and 2100 following an RCP 8.5 scenario (Meinshausen et al. 2011, Collins et al. 2013). Thus, present day $p\text{CO}_2$ was assumed to be 410 μatm , in accordance with present-day measurements in GBR inshore and offshore areas (Uthicke et al. 2014a). Projected 2050 conditions were assumed to be 670 μatm and 2100 conditions were 900 μatm . For temperature, the ambient treatment tracked temperatures recorded on a nearby mid-shelf reef (Davies Reef, 18.8313° S, 147.6355° E) by the AIMS data centre from 1991 to 2012 (available at: <https://apps.aims.gov.au/metadata/view/38f2c8ae-bdab-47fe-99fe-2b4513938fa5>). A 1°C temperature increase was applied to the 2050 treatment, and a 2°C increase applied to the 2100 treatment. Given that temperatures have already risen by approximately 0.9°C, these treatments represent an increase of 1.9°C and 2.9°C above pre-industrial levels. We prepared a total of 9 mesocosms, with 3 of these randomly assigned to each treatment.

2.3. Nutrient seawater carbonate chemistry analysis

In addition to continuous logger measurements of $p\text{CO}_2$, temperature and light (see Text S1), weekly water quality samples were collected from each system. For carbonate chemistry, 250 ml of water were collected from Tank A (see Fig. S2) of each system and fixed with 125 μl of a saturated mercury chloride solution. These samples were analysed for dissolved

inorganic carbon (DIC) and total alkalinity (TA) on a Vindta 3c, using certified reference seawaters (A. G. Dickson, Scripps Institute of Oceanography, Dixon, Batch 141, 159 and 164) for calibration. CO_2 system parameters were calculated using CO2sys.xls (Pelletier et al. 2007). Samples for dissolved inorganic nutrients (NO_2 , NO_3 , NH_4 , PO_4 and SiO_4) were collected at the same time (2 per system), syringe filtered through 0.45 μm filters (Sartorius Minsart N) into acid-washed (10% HCl) plastic 10 ml tubes and stored frozen (-20°C) until analysis. Dissolved inorganic nutrient concentrations were determined by standard methods (Ryle et al. 1981) on a segmented flow analyser. Here, NH_4 , NO_2 and NO_3 were combined and are reported as total dissolved inorganic nitrogen (DIN).

2.4. Growth measurements

The initial size (maximum diameter, measured by callipers) and wet weight (after blotting) of each urchin was determined on 24 February 2016, after which the urchins were divided haphazardly into 3 groups of 70–71 individuals. Weight and size measurements of all urchins were repeated after 6 mo and at the end of the experiment. On 4 March 2016, ~0.5 ml of the fluorochrome tetracycline (5 g l⁻¹ dissolved in seawater) was injected into the coelom of each individual, a standard method to fluorescently tag sea urchin skeletons as tetracycline becomes incorporated and marks the actively calcifying regions of skeletal elements. Previous laboratory studies on other urchin species have confirmed that tetracycline tagged and untagged animals exhibited no difference in growth or survival 6 mo after administration of the polyfluorochrome (Ellers & Johnson 2009). Following tagging, urchins ($n = 22\text{--}24$) were released into the main tank of each of the 9 mesocosm systems. All systems were run at ambient sea temperature and $p\text{CO}_2$ conditions at the start of the experiment. Over the following 4 wk, temperature and $p\text{CO}_2$ of the 2050 and 2100 treatments were adjusted linearly until target values were reached on 27 March (2050 treatment) and 5 April (2100). The adult growth experiment ended after about 20 mo, with spawning experiments between 26 October and 15 November 2017 (see Section 2.7).

2.5. Growth estimates

Urchins were starved for 24 h by moving them to Tank C (see Fig. S2) of their respective mesocosm

and thus separating them from their food source prior to all measurements, to reduce influence of gut contents on weight and metabolism. At the end of the 20 mo experiment, animals were dissected and the feeding structure of the urchins (Aristotle's Lantern) was cleaned in 5% commercial bleach. From the lantern, a single demi-pyramid (a large calcified skeletal element) was examined and digitally photographed under UV light using a Leica inverted microscope fitted with a DAPI filter cube. Animals that had been tagged successfully yielded demi-pyramids that contained an internal fluorescent mark representing the size of the structure at the time of tagging, with growth represented by an increase in the size of the demi-pyramid beyond the fluorescent mark. Demi-pyramid (jaw) length (J_t) at the time of tagging was measured as the distance between the oral tip and the upper edge of the tetracycline tag. The growth of the demi-pyramid (ΔJ) was measured as the distance from the tetracycline tag to the new epiphysis junction (Ebert 1982, Lamare & Mladenov 2000). Growth was determined as an increase in the demi-pyramid length over 20 mo.

Differences in growth among the treatments were investigated by comparing the slope of the log-transformed demi-pyramid growth ($\ln(\Delta J)$) vs. the initial size of the demi-pyramid (J_t) (Uthicke et al. 2016). For comparison with field growth data based on mark and recapture studies (McClanahan & Muthiga 2007), we also modelled growth parameters using the von Bertalanffy growth function, based on changes in test diameter. To convert demi-pyramid size to test size, we used a linear model of maximum test diameter (TD_{t+1}) on demi-pyramid length (J_{t+1}) at the end of the experiment to calculate respective maximum test diameter at the start (TD_t) from initial demi-pyramid length (J_t). Using Walford plots of changes in TD, we estimated von Bertalanffy growth parameters (k and L_∞) for urchins in all treatment groups combined, using methods described by Lamare & Mladenov (2000).

2.6. Respiration and ammonium excretion

Respiration rates of 10 individual urchins from each mesocosm were measured over a period of approximately 60 min in enclosed 495 ml Perspex jars between 15 and 24 August 2017 (~18 mo of experimental exposure). Water temperatures in that week ranged between 23.3 and 23.5°C for the control, 24.3 and 24.5°C for the 2050 treatment and 25.3 and 26.3°C for the 2100 treatment. Incubations were con-

ducted in the dark and monitored to ensure that oxygen levels did not drop below 80%. Oxygen concentrations were measured in the initial water, after incubations and in blanks using a handheld optode-based oxygen meter (HQ30d, equipped with LDO101 IntelliCAL oxygen probe, Hach).

Ammonia excretion was determined concurrently during respiration incubations. Ammonia concentrations were measured in duplicate initial water samples, after incubations and blanks. Samples were immediately filtered through 0.45 μm syringe filters (Sartorius), placed on ice and frozen until analysis. Ammonia concentrations were determined on an auto analyser following methods described by Ryle et al. (1981). Incubations for respiration and excretion measurements for each group of animals took place in the mesocosm of their origin, thus under the exact treatment conditions. After incubations, animals were released back into their habitat.

Respiration and ammonia excretion rates were calculated considering incubation water volume (minus urchin volume, calculated from wet weight following a regression established by Uthicke et al. 2013), time of incubation and removal of background (from blank incubations), and data were expressed as standard metabolic rates (SMR, $\mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$) by dividing the respiration or excretion rate by the wet weight of the individual.

2.7. Spawning index, oocyte diameter and juvenile growth

After approximately 20 mo of exposure to treatments, adult urchins were spawned at 2 discrete time periods for the production of larvae and resulting juveniles. The first spawning period was conducted in 3 'Blocks' (24/26/30 October 2017), each of these containing urchins from 1 mesocosm of each treatment level. For the present study, we used only data for oocyte diameter from these spawnings. Oocyte diameter ($N = 100$ per female) from 4–5 females per mesocosm was measured directly without preservation after spawning. For this, photos were taken under a compound microscope and later measured with Image-J (Schneider et al. 2012). A second spawning induction was made on 15 November 2017, where individuals from all mesocosms (with the exception of mesocosm outdoor system No. 7 where too few animals were available) were spawned.

Spawning was induced by intracoelomic injection of 0.5 ml KCl (0.5 M). Spawning females were inverted on beakers with 250 ml filtered seawater, and

oocytes were spawned directly into the water. Males were kept outside the water and sperm was collected dry from the aboral surface and stored in 1.5 ml Eppendorf tubes. Spawning for both sexes was scored into 3 broad categories (little or no spawn, poor, good) following Uthicke et al. (2013). Eggs from 4 females per mesocosm were pooled in 1000 ml beakers. Sperm (1 μ l) was pooled from each male within each mesocosm into 20 ml filtered seawater, and 1 ml of this solution was used for fertilisation under respective treatment pH and temperature conditions. Fertilisation was checked under a microscope (presence of the fertilisation envelope) after 10 min, when we generally observed 90–100% fertilisation. After fertilisation rate was deemed adequate, excess sperm was flushed out by several washes with fresh filtered seawater. Approximately 2500 embryos were added to each of 3 Schott bottles (500 ml) filled with 0.5 μ m filtered seawater per mesocosm, resulting in densities of $\sim$ 5 embryos ml^{-1} . Schott bottles were filled with seawater at respective treatment temperatures and $p\text{CO}_2$. Bottles were placed on an under-water roller, submerged in water to keep temperatures constant at the desired treatment level. Bottles were cleaned and filled with fresh treatment water every second day. pH was measured during the first spawning (Karelitz et al. 2020), revealing that pH remained constant at treatment levels over the incubation period and thus $p\text{CO}_2$ was expected to remain near treatment levels. After 48 h, the developing pluteus larvae were fed twice daily with *Chaetoceros muelleri* (final concentration of 5000 cells ml^{-1} , increased to 8000 cells ml^{-1} after 10 d). Larvae from the 2050 and 2100 treatments were competent (as judged by development of the rudiment and appearance of tube feet and pedicellaria on the larval body) to settlement at 19 d post fertilisation (dpf) (4 December), whereas larvae in the ambient treatment were competent at 26 dpf (11 December). To induce settlement, larvae were placed in 500 ml seawater in square aquaria (10 $\times$ 10 $\times$ 10 cm), containing aragonite plugs conditioned with a mixed crustose coralline algae (CCA) consortium grown at SeaSim. For logistical reasons, all larvae had to be settled under control conditions. Four to 6 d after settlement, plugs containing metamorphosed sea urchins were transferred into meshed (150 μ m) 50 ml polypropylene centrifuge tubes (Uthicke & Altenrath 2010), with approximately 40 juveniles in each tube (see Section 3.6). Tubes containing juvenile urchins were returned to the same mesocosm the parents originated from. CCA plugs were checked twice per week, and new plugs were added in cases where the original ones became CCA

depleted. Juveniles were kept until 31 May, and digital images of the aboral view of all juveniles were taken to determine size using a scale bar. Two perpendicular diameter measurements (diameter presented as average of these) was analysed using Image-J (Schneider et al. 2012).

2.8. Statistical analyses

Generalized mixed-effects models were used to test for differences among treatments for most parameters tested, using log as a link function. Each individual mesocosm represents an independently controlled unit and was used as a random factor in each model. Thus, we used 'glmer($y \sim \text{Treatment} + (1|\text{Mesocosm})$ family = Gamma(link = 'log'))' as a model for most parameters (respiration, ammonium release, jaw growth), using the lme4 (Bates et al. 2015) library in R. Changes in average size of the urchins included an interactive effect of time ('Period', initial, 6 mo and 20 mo measurements) in the model. Spawning of the urchins for egg size measurements were conducted over 3 different experimental days (24/26/30 October 2017). The model used to test for differences in egg size included 'Female' and 'Mesocosm' as random factors.

Data for the spawning index were scored as categories, and thus comprised ordinal data which were analysed with cumulative link models using the 'Ordinal' library in R.

For juvenile grow out, we aimed to place a similar number of newly metamorphosed urchins ($\sim$ 20–40) per tube. However, due to their small size it was not possible to achieve this with high accuracy, and subsequent counts confirmed greater variability in stocking density. Initial data analysis suggested that densities had an influence on size of the urchins (see Section 3.6). To account for this, we averaged the size in each replicated tube (2–4 per mesocosm) and removed 4 tubes which had extreme (<16 or >50) numbers of urchins. To further account for differences in densities, we used 'number' as a covariate in an ANCOVA. Initial data analysis did not indicate deviation from the homogeneity of slopes assumption for this analysis. Linear mixed-effects models (with a log link function) with mesocosm as a random factor were then used for analysis. Assumptions for all analyses were tested by inspecting residual plots and boxplots of data within all treatment groups. All statistical analyses were conducted in R version 3.5.2 (R Core Team 2018).

3. RESULTS

3.1. System performance

Both temperature and $p\text{CO}_2$ tracked the desired range for the 3 treatments throughout the experimental duration (Fig. 1). Temperatures reached target levels as planned in the initial ramp-up period, but a system error kept temperatures constant (but different between treatments) for ~12 wk. Thereafter, temperatures tracked the variation in annual winter and summer cycles, maintaining the temperature difference between treatments as programmed. The $p\text{CO}_2$, as read from the logging system, reached target levels after the ramp-up period and remained at constant levels over time. Average temperature and carbon chemistry varied little among individual

replicated mesocosm systems (Table 1A). As expected, $p\text{CO}_2$ was higher during night time and reduced during the day (Table 1B), with a daily range of up to 100 μatm $p\text{CO}_2$ for all treatments. Daily light integral also varied with the annual cycle, with lowest values in winter, and no apparent difference between mesocosms. Dissolved inorganic nitrogen (DIN) and phosphate values were in the low range typical for coral reef ecosystems and showed no obvious difference between treatments (Table S1).

3.2. Survival

A total of 211 *Echinometra* sp. A were tagged and exposed to the 3 climate treatments in the mesocosm

Fig. 1. Daily averages of temperature and $p\text{CO}_2$, and daily light integral (DLI) in experimental mesocosms, colored by treatment, between March 2016 and July 2018

Table 1. Temperature and carbon chemistry during the 26 mo experimental period. (A) Temperature and carbon chemistry based on weekly measurements of dissolved inorganic carbon (DIC) and total alkalinity (TA). Values are taken during day time and thus partial pressure of CO₂ ($p\text{CO}_2$) values represent minima. For each mesocosm, temperature and carbonate chemistry parameters (mean $\pm$ SD of range) including $p\text{CO}_2$, DIC, TA and calcite saturation state (Ω_{ca}), are reported. N = range of sample sizes for each parameter across all mesocosms. (B) $p\text{CO}_2$ values and daily light integrals (DLI) taken by Telair and Licor loggers, respectively, averaged over all available days and mesocosms per treatment. $p\text{CO}_2$ – Min: minimum $p\text{CO}_2$ values averaged for the period between 10:00 and 14:00 h; $p\text{CO}_2$ – Average: mean value of daily averages $p\text{CO}_2$ – Max: maximum $p\text{CO}_2$ values averaged for the period between 22:00 and 02:00 h. SD of the averages are given in parentheses. MOS: mesocosm outdoor system

(A) Treatment	MOS no.	Temperature (°C)	pH _T	$p\text{CO}_2$ (µatm)	DIC (µmol kg ⁻¹)	TA (µmol kg ⁻¹)	Ω_{ca}
Ambient	3	26.42 (1.64)	8.06 (0.04)	374.0 (39.4)	1933.8 (37.4)	2259.3 (46.6)	5.41 (0.56)
	6	26.42 (1.64)	8.06 (0.02)	368.5 (24.6)	1924.4 (42.4)	2249.6 (41.2)	5.38 (0.27)
	8	26.41 (1.64)	8.06 (0.03)	375.9 (35.6)	1942.9 (35.9)	2267.3 (34.4)	5.39 (0.36)
2050	1	27.38 (1.63)	7.89 (0.03)	606.6 (50.1)	2047.3 (42.6)	2286.5 (38.8)	4.15 (0.35)
	4	27.37 (1.62)	7.90 (0.03)	588.8 (48.8)	2022.5 (47.2)	2263.5 (45.7)	4.15 (0.32)
	7	27.37 (1.63)	7.89 (0.03)	612.8 (53.5)	2038.6 (43.7)	2273.7 (36.2)	4.07 (0.33)
2100	2	28.34 (1.61)	7.79 (0.04)	808.3 (77.00)	2103.3 (42.5)	2297.3 (35.9)	3.51 (0.33)
	5	28.33 (1.61)	7.79 (0.04)	803.1 (73.6)	2085.5 (41.6)	2277.2 (34.4)	3.46 (0.33)
	9	28.34 (1.61)	7.79 (0.06)	792.2 (96.4)	2094.0 (52.2)	2290.8 (47.9)	3.55 (0.50)
N	844	117–124	117–124	117–124	117–124	117–124	
(B) Treatment	$p\text{CO}_2$ – Min (µatm)	$p\text{CO}_2$ – Average (µatm)	$p\text{CO}_2$ – Max (µatm)	DLI (mol photons m ⁻² d ⁻¹)			
Ambient	373.87 (25.43)	444.51 (24.05)	487.34 (31.79)	4.12 (1.83)			
2050	621.72 (34.69)	670.69 (31.95)	716.77 (34.70)	4.06 (1.73)			
2100	853.20 (73.46)	904.91 (69.23)	950.44 (71.51)	4.57 (1.88)			
N	2531–2532	2534–2535	2531–2532	2374–2526			

systems. Overall, survival in all treatments was high (>90% in 7 of 9 mesocosms) after 6 and 20 mo of the experiment (Table S2). An exception was mesocosm outdoor system No. 7 (2050 treatment), where only 4 out of 24 specimens survived. Further investigation revealed the presence of a large predatory hermit crab in this system, which was only discovered and removed after 12 mo, by which time a substantial proportion of the urchins had been preyed upon.

3.3. Size and growth

At the beginning of the experiment, *Echinometra* sp. A had a broad uni-modal size frequency distribution both of its maximal diameter and weight (Fig. S3), with very similar average weights and maximum diameter in each mesocosm (Table S3). Over the course of the experiment, the mode for both measures of size shifted right and distributions narrowed. Neither the diameter nor the weight of the urchins was significantly different among the treatments in any of the 3 measuring periods (Fig. 2, Table 2, non-significant factors Treatment and Treatment $\times$ Period interaction). As suggested by the shift in mode of the

size frequency distributions, the average diameter and weight of the urchins significantly increased over the course of the experiment (Table 2, factor Period).

At the end of the experiment, all lanterns of the urchins were dissected and bleached. Under UV-light, we detected a clear fluorescent mark in 155 (88.6%) of the lanterns, and these were used to perform individual based growth analysis. The percentage of tagged animals at the end of the experiment ranged from 81.2% in the ambient treatments to 89.2 and 89.7% in the 2100 and 2050 treatments, respectively.

The change in jaw size ($\ln(\Delta J)$) was negatively correlated with the initial jaw size (J_i) (Fig. 3), indicating slower growth for larger individuals. However, we found no significant difference in growth among treatments (Table 3). Further analysis showed there was also no difference in growth within males and females separately across the experimental treatments, or between males and females (Table 3).

For comparison with previously reported measurements of field growth rates of *Echinometra* spp., we fitted a von Bertalanffy growth function. This was done for all data combined since previous analyses (see above) indicated no difference among treatments or sexes. Based on intercept (25.51, 1 SE = 1.26) and

Fig. 2. Changes in maximum diameter and weight of *Echinometra* sp. A adults after 20 mo in the 3 climate scenarios. Boxes outline the inter-quartile range, whiskers denote 1.5× the inter-quartile range, and the line in each box indicates the median

slope (0.5019, 1 SE = 0.0297) of the Ford-Walford analysis (Figs. S4 & S5), we calculated a maximum test diameter (L_{∞}) of 51.21 mm and a growth constant (k) of 0.376.

3.4. Respiration and excretion

No significant difference was evident in the SMR among treatments measured in winter ($\chi^2 = 0.194$, df = 2, $p = 0.9076$), with an average ($\pm$ SE) rate of $11.62 \pm 0.95 \mu\text{g O}_2 \text{ g}^{-1} \text{ h}^{-1}$. Mass-specific ammonium release rates were higher in the 2100 treatment (Fig. 4), and there was a marginally significant treatment effect ($\chi^2 = 4.616$, df = 2, $p = 0.0995$). Average ammonium excretion at 2100 was 26–31% higher than in other treatments. Due to elevated ammonium release rates under constant respiration rates, O:N ratios decreased in the 2100 treatment (Fig. 4), with the overall treatment effect being marginally significant ($\chi^2 = 5.774$, df = 2, $p = 0.0557$). The average O:N ratio at 2100 was approximately 20–28% below that of other treatments.

3.5. Oocyte diameter and spawning performance

Dissection of the urchins at the end of the experiment revealed 94 females and 84 males, and 1 individual contained immature gonads which could not be sexed. The sex ratio did not deviate significantly from unity ($\chi^2 = 0.5618$, df = 1, $p = 0.4535$). A subset of animals were induced to spawn, with the majority of both sexes from each treatment producing progeny (Table 4), with no difference in the average spawning category for females (cumulative link test, $\chi^2 = 0.8927$, df = 2, $p = 0.6400$) or males ($\chi^2 = 4.1593$, df = 2, $p = 0.1250$).

A subset of eggs was collected from females ($N = 4\text{--}5$) in each treatment and oocyte diameter measured. There was no significant difference in oocyte diameter between females from the 3 treatments ($\chi^2 = 2.2126$, df = 2, $p = 0.3308$; Fig. 5).

3.6. Juveniles

Juveniles were raised for 5 mo in their respective parental treatments. Although juveniles in all treatments had peak numbers in size classes between 2 and 3 mm, there was approximately a 10-fold difference in diameter in each treatment, ranging from ~0.5 to 11.5 mm test diameter (Fig. 6).

Because size at this age was strongly density-dependent (Fig. S6), differences in average size at 5 mo were tested using ANCOVA. After accounting for differences in numbers per tube, there were no significant differences in mean diameter of the sea

Table 2. Results of general mixed-effects models for weight and length of *Echinometra* sp. A in the mesocosm experiment. Individual mesocosms ($N = 3$) were used as random factors in the model

	χ^2	df	p
Length			
Treatment (T)	0.5	2	0.7788
Period (P)	168.4887	2	<0.0001
T × P	2.3233	4	0.6765
Weight			
Treatment	0.7141	2	0.6997
Period	109.2147	2	<0.0001
T × P	0.9684	4	0.9146

Fig. 3. Final jaw length ($\ln\Delta J$) at 20 mo as a function of initial jaw length (J_t). Black line represents the common equation model ($\ln\Delta J = -0.45775 \times J_t + 5.44009$). Blue: ambient, green: 2050, red: 2100. Inset shows an example of a jaw element, with fluorescent stain at the start of the experiment clearly visible

urchins among the 3 treatments (Table 5). The average size ($\pm$ SE) modelled for average densities in the tubes was 2.54 ± 0.164 mm for the ambient treatment, and 2.77 ± 0.169 and 2.65 ± 0.163 mm for the 2050 and 2100 treatments, respectively.

4. DISCUSSION

Here we introduced and tested a large-scale mesocosm system for investigating combined climate change and OA impacts on reef organisms and assessed long-term, cross-generational effects of ocean warming and acidification on the sea urchin *Echinometra* sp. A. The mesocosm system provided a useful setting to examine the potential outcomes of climate change and OA in the laboratory under near-natural settings. The independent design and manipulation of temperature and $p\text{CO}_2$ also alleviate concerns about pseudo-replication in climate change experiments which often manipulate seawater in common sumps (Cornwall & Hurd 2016). *Echinome-*

tra sp. A grew at rates seen in its natural environment, and over 99 % of animals spawned after 20 mo, a period that would encompass 2 gametogenic cycles. Projected climate change conditions did not affect adult growth, physiology or gonad development, and there were no size differences among the offspring across treatments, contrasting with previous short-term laboratory experiments that reported deleterious responses to environmental change.

A high proportion of tagged urchins facilitated detailed growth analysis. The von Bertalanffy growth constant (k) and maximum test diameter (L_∞) calculated for mesocosm-held *Echinometra* sp. A were within the range reported for *Echinometra* spp. in the field based on mark-recapture experiments (previous reported ranges for k : 0.19–0.40, $L_\infty = 31$ –85 mm) (McClanahan & Muthiga 2007). These findings suggest that the mesocosm system provided conditions within the ranges of natural reefs with respect to nutrition and other ecological demands of the urchins that would influence growth (i.e. water quality, space).

In contrast to several previous laboratory experiments using *Echinometra* species (assessing OA alone or OA in conjunction with ocean warming) (Courtney et al. 2013, Uthicke et al. 2013, 2014b), we found no

Table 3. Modelling growth of *Echinometra* sp. A, considering the total dataset (all tagged animals), males and females, and comparing males vs. females, as change in demi-pyramid length vs. initial length and treatment (or sex). The interaction was excluded in each case after the initial analysis demonstrated homogeneity of slopes (no significant interaction). Individual mesocosms ($N = 3$) were used as random factors in the model

	χ^2	df	p
Total			
Initial demi-pyramid length	197.1297	1	<0.0001
Treatment	0.0357	2	0.9823
Males			
Initial demi-pyramid length	153.8264	1	<0.0001
Treatment	0.2342	2	0.8895
Females			
Initial demi-pyramid length	92.1016	1	<0.0001
Treatment	2.6818	2	0.2616
Males vs. Females			
Initial demi-pyramid length	199.1631	1	<0.0001
Sex	4.2901	2	0.1171

Fig. 4. (A) Standard metabolic respiration rate per unit biomass, (B) ammonium excretion per unit biomass and (C) molar oxygen to nitrogen ratio of *Echinometra* sp. A measured after 17 mo among the 3 mesocosm treatments. Boxes outline the inter-quartile range, whiskers denote 1.5× the inter-quartile range, and the line in each box indicates the median

reduction in growth under combined temperature and OA scenarios. For example, previous subtle but significant size reductions in *Echinometra* sp. A were reported after 7 wk of exposure to OA ($\text{pH}_T < 7.9$) (Uthicke et al. 2013). In a second, 10 wk study on this species, a 3°C temperature increase, or a 0.2-unit pH decrease alone did not change growth. However, pH and temperature in combination ($\text{pH}_T 7.9$, +3°C, 10 wk exposure) reduced growth of the urchins (Uthicke et al. 2014b). Similar to our present findings, *E. mathaei* (Moulin et al. 2015) and *Echinometra* sp. EE (Hazan et al. 2014) from the Indian Ocean exhibited no reduction in growth in OA conditions ($\text{pH}_{\text{NBS}} 7.7$ in both studies) after ~1 yr of exposure. In addition, a previous study of *Echinometra* sp. C inhabiting CO_2 vents ($\text{pH}_T \sim 7.9$) showed that individuals grew faster under high pCO_2 , with average-sized urchins growing 0.13 and 0.31 cm per 1.46 yr at a control and CO_2 vent site, respectively (Uthicke et al. 2016). We suggest that this growth differential was not a direct effect on the

physiology of *Echinometra*, but a secondary effect of higher pCO_2 favouring higher algal biomass as food for the urchins (Uthicke et al. 2016).

Studies on somatic growth in adult temperate sea urchins typically show variable responses to reduced pH. For example, Stumpp et al. (2012) reported no

Table 4. Animals in each of 3 spawning categories during the induced spawning, separate for females and males

Treatment	Total spawned	Little/ No	Poor	Good	Average Index
Females					
Ambient	25	3	7	15	2.48
2050	24	2	6	16	2.58
2100	26	0	8	18	2.69
Males					
Ambient	24	4	4	16	2.50
2050	20	2	8	10	2.40
2100	18	0	3	15	2.83

Fig. 5. Average oocyte diameter measured from female *Echinometra* sp. A after spawning. Boxplots are based on individual datapoints ($n = 1200$ for ambient and 2100; $n = 1300$ for 2050). Boxes outline the inter-quartile range, whiskers denote $1.5\times$ the inter-quartile range, and the line in each box indicates the median

growth over 40 d in animals grown in reduced pH ($\text{pH}_{\text{NBS}} < 7.7$), with growth only observed under ambient conditions. The authors attributed this variation to the outcomes of altered resource allocation to acid-base regulation over somatic growth. In contrast, no difference in growth was seen in *Hemicentrotus pulcherrimus* between control and extremely reduced pH conditions ($\text{pH} \sim 7.0$) over a 9 mo experimental period (Kurihara et al. 2013), despite changes to reproduction, feeding rates and coelomic fluid ion composition, suggesting that energetic balances are altered by lower pH levels.

It is generally viewed that early life history stages (including juveniles) are more sensitive to environmental conditions, including ocean warming and acidification (Espinell-Velasco et al. 2018). Indeed, the few previous studies investigating the effects of OA on sea urchin juveniles detected strong growth inhibition. Three months after settlement, juveniles of the tropical urchin *Lytechinus variegatus* were significantly smaller under OA treatments ($\text{pH}_{\text{T}} < 8.0$) (Albright et al. 2012). Juvenile *Echinometra* sp. and *H. pulcherrimus* also grew slower under elevated pCO_2

($\text{pH}_{\text{NBS}} < 7.9$) in experiments lasting 6 mo (Shirayama & Thornton 2005). In juvenile tropical *Tripneustes gratilla*, decreasing pH ($\text{pH}_{\text{NBS}} 7.6$) reduced growth, but warmer temperatures ($+3^\circ\text{C}$) accelerated growth (Dworjanyn & Byrne 2018). Thus, it was surprising that we did not find a growth response of juveniles in our study using combined pCO_2 and temperature stress. In the temperate urchin *Heliocidaris erythrogramma*, both temperature increase and reduced pH ($\text{pH}_{\text{NBS}} 7.6\text{--}7.8$) negatively affected development of young (5 d old) juveniles (Byrne et al. 2011). However, some effects were ameliorated under simultaneous intermediate temperature and pCO_2 increase. Thus, it is possible that similar interactive effects are also influencing the response of *Echinometra* sp. A in the present study.

No significant effects of the climate treatments on *Echinometra* sp. A respiration were detected in this study. In contrast, after 7 wk under reduced pCO_2 alone, *Echinometra* sp. A showed a slight decrease in respiration levels at intermediate ($\text{pH}_{\text{T}} \sim 7.9$) pCO_2 reduction (Uthicke et al. 2013). A 3°C increase in temperature elevated respiration rates of *Echinometra* sp. A after 10 wk of exposure, with highest rates measured under simultaneous pH decrease and temperature increase ($\text{pH}_{\text{T}} 7.9$, $+3^\circ\text{C}$) (Uthicke et al. 2014b). There was no effect of increased pCO_2 on respiration of *Echinometra* sp. C at vent sites in

Fig. 6. Size frequency distribution of juvenile *Echinometra* sp. A ~5 mo post-settlement grown under 3 treatment conditions. Inset shows representative juveniles from 1 replicate tube; scale bar = 1 cm

Table 5. General linear model testing for differences between average *Echinometra* juvenile size taking into consideration differences in densities among replicates across treatments. Initial tests showed homogeneity of slopes (interaction, $p = 0.1040$). Individual mesocosms ($N = 3$) were used as random factors in the model

	χ^2	df	p
Treatment	0.9588	2	0.6192
Number in tube	43.6835	1	<0.0001

Papua New Guinea (Uthicke et al. 2016). Both temperature (+5°C) and pH ($\text{pH}_{\text{NBS}} 7.5$) stress increase metabolism in *H. erythrogramma* (Carey et al. 2016), with the largest effect under both stressors combined.

Although an increase in ammonium excretion rates under 2100 conditions and a resulting decrease in O:N ratio in our study were only marginally significant, the effect size was quite strong (~20–30%). O:N ratios measured here were slightly below those measured in a previous experiment (Uthicke et al. 2014b), but still in the high range expected for herbivorous invertebrates (Otero-Villanueva et al. 2004). Elevated temperature (+3°C) but not decreased pH ($\text{pH}_T 7.9$) was previously shown to increase ammonium release in *Echinometra* sp. A (Uthicke et al. 2014b). Similar to our findings, a temperate urchin exhibited increased ammonium release rates and reduced O:N ratios under high $p\text{CO}_2$ ($\text{pH}_{\text{NBS}} < 7.7$), which was interpreted as increased protein catabolism to maintain ion homeostasis and remove protons (Stumpp et al. 2012). Thus, it is possible that this is also occurring in *Echinometra* sp. A in the present study. However, although this may be less energy efficient, we did not detect overall changes in growth. We note that SMRs measured in the present study (global average $\sim 12 \mu\text{g g}^{-1} \text{h}^{-1}$) were distinctly lower than measured previously for the same species (25–45 $\mu\text{g g}^{-1} \text{h}^{-1}$; Uthicke et al. 2013, 2014b) or *Echinometra* sp. C (23 $\mu\text{g g}^{-1} \text{h}^{-1}$; Uthicke et al. 2016). Similar to respiration, ammonium release was also lower than previously measured (Uthicke et al. 2014b). The measurements for the present study were conducted in winter (when sea temperatures were ~ 23 – 26°C), whereas previous measurements were performed at summer temperatures (29– 30°C); therefore, it is likely that the lower rates measured reflect slower metabolism associated with cooler seawater temperatures.

In other echinoderms, respiration/metabolism usually increases with temperature, but the effect of $p\text{CO}_2$ can be variable. The brittle star *Ophionereis*

schayeri had higher respiration rates at 25°C compared to 19°C , but the effects of $p\text{CO}_2$ were variable, being reduced at intermediate $p\text{CO}_2$ levels ('metabolic depression') but increasing at higher levels (Christensen et al. 2011). In the brittlestar *Ophiura ophiura*, the effects of $p\text{CO}_2$ were dependent on temperature (Wood et al. 2010).

Most adult urchins in our experiment spawned readily upon KCl injection, and spawning index and egg size measurements exhibited no difference among treatments. Specimens successfully completed gametogenesis in all treatments and developed healthy gonads. Based on gonad morphology, a previous shorter-term study on the same species suggested that combined temperature and CO_2 stress negatively impacted gonad development in females (Uthicke et al. 2014b). A study on the effect of $p\text{CO}_2$ alone found a strong (but not statistically tested) effect of OA on male spawning performance in *Echinometra* sp. A (Uthicke et al. 2013). Gonad weight of *Echinometra* sp. C. at CO_2 vents was significantly lower than at the control sites (Uthicke et al. 2016). In *T. gratilla*, temperature and $p\text{CO}_2$ affected gonad development, with almost no gonad development at control temperatures and the highest $p\text{CO}_2$ level at each temperature (Dworjanyn & Byrne 2018). For *Heliocentrotus pulcherrimus*, gonad development occurred under reduced pH, with the gonad index similar to that of the control, although both gametogenesis and spawning were delayed by around 1 mo over a 9 mo experimental period (Kurihara et al. 2013).

In the present study, we not only examined growth and metabolic responses in long-term acclimated adults, but also in the juvenile offspring reared under the same present-day and future conditions to determine potential parental or carryover effects. Such carryover effects can be positive (Hettinger et al. 2012, 2013) or negative (Dupont et al. 2013, Donelson et al. 2018). For *Echinometra*, we saw a similar response in the juveniles as in the adults, namely, no difference in the growth across experimental treatments. These observations preclude any evidence of negative carryover effects (i.e. offspring of adults grown in future conditions did not perform more poorly than offspring from control adults). Previous studies on sea urchins have shown that negative carryover effects in response to both warming and acidification are possible. For instance, Dupont et al. (2013) found that larval exposure to high $p\text{CO}_2$ drastically reduced juvenile survival in a temperate urchin species. However, as seen here in *Echinometra*, previous studies on the same genus have indi-

cated that parental acclimation may not alleviate negative effects of OA across generations, such as abnormal development and stunted larval growth (Uthicke et al. 2013, Lamare et al. 2016). Larvae produced from adult urchins grown under 2100 conditions used in the present experiment had higher mortality than those with parents grown in controls, thus also exhibited negative carryover effects (Karelitz et al. 2020).

In conclusion, the combination of ocean warming and acidification did not affect the benthic life stages (adults and juveniles) of *Echinometra* sp. A within experimental mesocosms that closely resembled reef conditions. In contrast, *Echinometra* sp. A. exhibited reductions in growth and gonad development in previous short-term single-species experiments (Uthicke et al. 2013, 2014b). Interestingly, 2 other experiments exposing other *Echinometra* spp. to OA alone over 11–12 mo also detected no effects on growth and gonad development (Hazan et al. 2014, Moulin et al. 2015). These findings may highlight that short-term climate change experiments with non-acclimated animals can yield erroneous results.

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