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Adult acclimation to combined temperature and pH stressors significantly enhances reproductive outcomes compared to short-term exposures

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Summary

1. This study examined the effects of long-term culture under altered conditions on the Antarctic sea urchin, *Sterechinus neumayeri*.

2. *Sterechinus neumayeri* was cultured under the combined environmental stressors of lowered pH (−0·3 and −0·5 pH units) and increased temperature (+2 °C) for 2 years. This time-scale covered two full reproductive cycles in this species and analyses included studies on both adult metabolism and larval development.

3. Adults took at least 6–8 months to acclimate to the altered conditions, but beyond this, there was no detectable effect of temperature or pH.

4. Animals were spawned after 6 and 17 months exposure to altered conditions, with markedly different outcomes. At 6 months, the percentage hatching and larval survival rates were greatest in the animals kept at 0 °C under current pH conditions, whilst those under lowered pH and +2 °C performed significantly less well. After 17 months, performance was not significantly different across treatments, including controls. However, under the altered conditions urchins produced larger eggs compared with control animals.

5. These data show that under long-term culture adult *S. neumayeri* appear to acclimate their metabolic and reproductive physiology to the combined stressors of altered pH and increased temperature, with relatively little measureable effect. They also emphasize the importance of long-term studies in evaluating effects of altered pH, particularly in slow developing marine species with long gonad maturation times, as the effects of altered conditions cannot be accurately evaluated unless gonads have fully matured under the new conditions.

Key-words: CO_2, echinoderm, gonad maturation, larval development, oxygen consumption, vitellogenesis

Introduction

To provide more accurate predictions of the consequences of climate change on biodiversity, it is essential to understand the complex synergistic effects of multiple environmental stressors across the different life-history stages of species, and the resulting ecosystem interactions (Crain, Kroeker & Halpern 2008; Kroeker *et al.* 2010; Kroeker *et al.* 2011; Russell *et al.* 2012). Despite considerable research in this area, largely fuelled by concern about the increasing acidification of the World's oceans, there are still critical gaps in our knowledge, such as species' abilities for long-term acclimation or adaptation to altered conditions and their phenotypic plasticity (Munday *et al.* 2009; Sunday *et al.* 2014), as most experiments to date have been short-term. Data arising from recent long-term exposure (in excess of a year) and transgenerational studies are revealing contrasting results to the short-term data,

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with adult acclimation to altered conditions an important factor (Donelson *et al*. 2012; Miller *et al*. 2012; Form & Riebesell 2012; Dupont *et al*. 2013; Calosi *et al*. 2013). Extended studies are therefore imperative for accurate future predictions.

High-latitude seas are regarded as particularly vulnerable to climate change effects, with regions of the Southern Ocean experiencing rapid rates of warming (IPCC 2013) and predictions that seasonal winter carbonate ion undersaturation will begin by 2030 (McNeil & Matear 2008). In tandem, there is currently very limited knowledge on capacities of this region's fauna to acclimate to altered conditions (Peck *et al*. 2014). A keystone Southern Ocean species is the sea urchin *Sterechinus neumayeri*. It is a circum-polar endemic, and in shallow water, it can be the most abundant macrobenthic invertebrate, playing a crucial role in the benthic ecosystem (Brey & Gutt 1991). This species is a major transformer of energy from detrital and depositing sources. It modifies seabed communities by intense grazing pressure, especially at the high densities that *S. neumayeri* naturally occurs at, limiting the density and diversity of encrusting and attached forms, including macroalage (Bowden *et al*. 2006; Campana *et al*. 2009). As a marine calcifier, it also contributes significantly towards global $CaCO_3$ sequestration within a depth range of 0–800 m (Lebrato *et al*. 2010; Sewell & Hofmann 2011). Therefore, any negative impacts from climate change on this species will have major impacts on the benthic food web, and also potentially impact global $CaCO_3$ cycling (Sewell & Hofmann 2011). In common with many Antarctic marine invertebrates, *S. neumayeri* is stenothermal, with low metabolic rates, slow annual growth and extended longevity, living up to 40 years and requiring up to 6 months to acclimate to elevated temperatures (Brey *et al*. 1995; Brockington 2001; Peck *et al*. 2009, 2014; Peck, Morley & Clark 2010). Consequently, adaptation to new environmental conditions will be strongly dependant on phenotypic plasticity and acclimatization (Somero 2010).

To date, all acidification studies on *S. neumayeri* have been short-term, and mostly focussed on pH (Clark, Lamare & Barker 2009; Ericson *et al*. 2010, 2012; Byrne *et al*. 2013; Yu *et al*. 2013), with only two studies on the combined effects of pH and temperature (Ericson *et al*. 2012; Byrne *et al*. 2013). Furthermore, all were restricted to early developmental life stages and did not cover the full course of larval development. In each case, the offspring were produced from parents held under ambient pH conditions with the offspring directly introduced into sea water at low pH, which may produce misleading responses for long-term predictions (Suckling *et al*. 2014a, b). Only temperate and tropical examples exist for adult pre-conditioning of echinoderms prior to spawning over variable time-scales, from 6 weeks in *Echinometra mathaei* (Uthicke *et al*. 2013) through to 10 weeks (*Psammechinus miliaris,* Suckling *et al*. 2014b), 9 months (*Hemicentrotus pulcherrimus,* Kurihara *et al*. 2013) and up to 16 months for *Strongylocentrotus droebachiensis* (Dupont *et al*. 2013). In the latter example, spawning after 4 months and 16 months (encompassing part and full gametogenesis, respectively) showed marked differences. There was improved fecundity and larval survival at the longer acclimation period, albeit with potentially deleterious carry over effects reported when the larvae and juveniles were cultured under the altered conditions.

In *S. neumayeri*, vitellogenesis takes between 18 and 24 months and spermatogenesis 12 months. Female gonads contain two cohorts of eggs; one in its second year maturing until spawned with another cohort <12 months old and in an early vitellogenic state (Pearse & Cameron 1991; Brockington, Peck & Tyler 2007). In this study, we determined the physiological impacts of altered sea water pH and temperature on adult *S. neumayeri* over a 2 year period to encompass the full development of both egg cohorts. We aimed to determine whether adult urchins had the physiologically flexibility to cope with the altered culture conditions and to identify the effects of short and long-term adult exposures on the performance of the early life stages of the next generation.

Materials and methods

ANIMAL COLLECTION AND CULTURING

Sterechinus neumayeri were collected by scuba divers in the austral summer of 2008–2009 from 3–10 m depth at Back Bay Lagoon, Ryder Bay, Antarctic Peninsula (67°34′ S, 68°08′ W). Specimens were transported to the UK and held in the British Antarctic Survey recirculating aquarium at 0 °C (±0·1 °C) in Cambridge for approximately 2 months before being introduced to the recirculating CO_2 microcosm system [adapted from Widdicombe & Needham (2007) and fully described in Suckling *et al*. (2014b)]. In the microcosm (200 L), UV sterilized and 50 μm filtered sea water was delivered to 80-L closed cylindrical mixing tanks. CO_2 gas (BOC) was introduced via a ceramic diffuser using an Aquamedic pH controlled computer and electrode system and mixed with sea water by an Aquamedic ocean runner power head 2000. Treated sea water was gravity fed to each experimental tank at a rate of 0·56 ± 0·03 L min^{-1}. The control mesocosm had no pH control system, but did include the Aquamedic ocean runner power head 2000. Sea water quality was assessed every 2–3 days using Nutrafin Aquarium test kits. Ammonia, nitrite and nitrates were maintained well below 0·4, 0·2 and 5 mg L^{-1}, respectively, by a combination of biological filtration, protein skimming and partial sea water exchanges (approximately 5–15% every 2–3 days) to prevent toxicity from metabolic by-products.

Sea water temperature in the experimental microcosms was approximately +2 °C (Table 1), with pH initially set at ambient levels in all tanks. Urchins were held in these conditions for 14 days before gradually decreasing the pH in selected tanks in equal, twice daily increments, over a period of 3 days until the desired pH was achieved. Adult urchins (total $n = 288$; 26·18 ± 0·21 mm mean test diameter) were selected at random and distributed between 36 three litre aquaria ($n = 8$ urchins per aquarium = 164 cm^2 $urchin^{-1}$). In addition, large animals were

Table 1. Mean (±SE) water parameters in the adult *Sterechinus neumayeri* microcosm over the course of the experiment following the format of Barry *et al*. (2010). Values for pCO_2, Ω calcite, Ω aragonite and total alkalinity were modelled from CO2SYS (Lewis & Wallace 1998) with refitted constants (Mehrbach *et al*. 1973; Dickson & Millero 1987). Values represent sea water values before and after sea water exchanges

Sea water parameter	Low-temperature control*	High-temperature control	−0·3 pH	−0·5 pH
pCO_2 (μatm)	392 ± 4	437 ± 8	928 ± 39	1405 ± 33
pH_{NIST}	7·98 ± 0·01	7·99 ± 0·01	7·70 ± 0·01	7·53 ± 0·01
Ω calcite	1·10 ± 0·01	1·50 ± 0·03	0·75 ± 0·03	0·54 ± 0·02
Ω aragonite	0·69 ± 0·01	0·94 ± 0·02	0·47 ± 0·02	0·34 ± 0·01
Temperature (°C)	−0·3 ± 0·1	1·8 ± 0·1	2·1 ± 0·1	2·4 ± 0·1
Salinity (psu)	34 ± 1	35 ± 1	35 ± 1	35 ± 1
Total alkalinity (mmol L^{-1})	1609 ± 14	1871 ± 12	1838 ± 11	1877 ± 11

*Calculated data from Cross, Peck & Harper (2015). Sea water temperature and pH_{NIST} values for each experimental year have been presented in Fig. S7 (Supporting information).

designated broodstock (31·18 ± 0·37 mm mean test diameter; total $n = 72$) and kept separately within the microcosm (Fig. S1, Supporting information). All animals were fed *Pollachius virens* at approximately 2% mean body mass every 14 days.

Treatments used in this study were based on the IPCC 'business-as-usual' scenario with the forecasted reduction of 0·3–0·5 pH units in oceanic surface waters by 2100 (IPCC 2013). The treatments used for the adult physiology were control at present day conditions, ~ pH 8·0; moderate acidification with a reduction of 0·3 pH units at ~ pH 7·7 and high acidification with a reduction of 0·5 pH units at ~ pH 7·5, with all water temperatures elevated to values between 1·8 and 2·4 °C ('high temperature'; Table 1). A low-temperature control was not used for the adult physiology assessments because the long-term effects of 2 °C warming are already known for adults of this species (Peck *et al*. 2014).

WATER CHEMISTRY

Temperature was recorded daily for all treatments (Digital Testo 106, Alton, UK). Salinity (Tropical Marine Centre V2 Handheld refractometer, Chorleywood, UK), pH_{NIST} (temperature compensated; HANNA bench top meter pH/ORP 115 v pH21-01) and TCO_2 (mmol L^{-1}; Ciba Corning TCO_2 Analyzer 965, Olympic Analytical, Malvern, UK) were recorded weekly (Table 1). The TCO_2 analyzer was calibrated with 2 g L^{-1} CO_2 standard prior to measurements. Aquamedic pH probes were calibrated with NIST certified pH buffer solutions at least two times per week. Sea water samples were analysed for phosphate and silicate levels following Nickell *et al*. (2003).

ADULT METABOLIC MEASUREMENTS: OXYGEN CONSUMPTION

Within each treatment, the animals were divided into two groups. One group (24 adults) was used to measure oxygen consumption across the 2 year experimental period. Oxygen consumption was measured every 4 months for 10 animals randomly selected from this group of 24 in each of the four treatments using closed bottle techniques (Obermüller *et al*. 2010). In summary, urchins were placed individually into open chambers, without aerial exposure, and left overnight to adjust to conditions prior to closing chambers. Chamber habituation and respiratory measurements were carried out at approximately the same time of day on all measurement days to minimize circadian effects. Oxygen consumption was measured using a Fibox-3 fibre optic oxygen sensor (PreSens Precision Sensing GmbH, Regensburg, Germany) until there was a 15–25% drop in oxygen saturation. Saturation was not allowed to drop below 70% to minimize stress effects (Peck & Prothero-Thomas 2002). Control chambers (without urchins) were run simultaneously, and all calculated rates of oxygen consumption were corrected for controls. Chamber volumes were corrected for animal volumes prior to calculating oxygen consumed. Animal volume, whole animal wet mass (g ± 0·01) and test diameter (mm ± 0·1) were recorded for each urchin. The second group was allocated for destructive sampling to obtain ash free dry masses (AFDM) for each treatment at each sampling time point ($n = 8$). Within each treatment, live wet volumes vs. urchin diameter regressions were used to relate oxygen consumption measurements to animal AFDM.

ADULT DATA COLLECTION – GROWTH, CALCIFICATION AND GONAD INDEX

Animal volume, whole animal wet and dry mass (±0·01 g), test diameter and thickness (measured at mid-test height from interambulacral plates) (±0·01 mm), gonad wet and dry tissue mass (±0·01 g), AFDM (±0·01 g), sex and gonad index were recorded for each animal used in the destructive sampling analyses. Sex was determined from wet-squash preparations of fresh gonad tissue in sea water using phase-contrast light microscopy (Kelly 2001). Gonad index (GI%) was calculated as gonad dry mass/whole animal dry mass. Gonad and remaining tissues plus tests were oven dried at 60 °C to constant mass. Skeleton mass was measured as ash remaining after ignition in a muffle furnace at 470 °C for 24 h. For the broodstock, maximum test diameters were recorded before spawning.

SPAWNING AND LARVAL REARING

Broodstock urchins were spawned after they had been in the microcosms for 6 months and 17 months. A low-temperature control (0 °C; Table S1, Supporting information) was included because *S. neumayeri* development is hypothesized to have an optimal temperature window of +0·2 to +1·7 °C (Stanwell-Smith & Peck 1998), just under our proposed elevated temperature treatments, and the effects on larval from adults pre-exposed to elevated temperatures are currently unknown. Low temperature adults were reared under similar husbandry conditions but held instead in the British Antarctic Survey recirculating aquarium at 0 °C (±0·1 °C). Six females and two males were used in each

spawning trial following Suckling *et al.* (2014a,b), with three replicates per treatment. In summary, *S. neumayeri* were spawned using intracoelomic injections of 1–2 mL of 0·5 M KCl through the peristomal membrane. After injection, urchins were stored in ~300-mL glass jars filled with 5 μm filtered treatment sea water until gametes were shed. Eggs were distributed across three replicate treatment buckets until they formed a monolayer at the bottom of each bucket. Buckets were sealed (with lids) and contained 12 L of filtered treatment sea water. Sperm from two males were combined, diluted and added, 1–2 mL per bucket following Catarino *et al.* (2012). Sperm concentration was not calculated but was subject to the same dilution series from concentrated sperm across all treatments (1 : 5; Kelly *et al.* 2000; Suckling *et al.* 2014a,b). Cultures were gently mixed to encourage fertilization. Spawning, fertilization and larval rearing were all carried out at 12L : 12D and approximately −1 °C (air temperature), irrespective of the parental culture temperature, as it was not technically possible to maintain appropriate numbers of replicates for all treatments at all temperatures. Complete sea water exchanges were carried out every 4 days, with larvae retained in a 50-μm mesh sieve in a water bath while buckets were cleaned and filled. Once the stomach had fully formed (~day 21), larvae were fed *Dunaliella tertiolecta* (Culture Collection of Algae and Protozoa, code 19/6B) at 1500 cells mL^{-1} every 2nd day. Sea water pH in development trials was maintained at parental culture levels using specialized mixed gases (1000 and 1500 ppm CO_2 blended with air, BOC) or air bubbled through the culture medium (Table S1, Supporting information). Sea water chemistry measurements followed the regime as described above but with twice weekly measurements and daily pH and temperature readings for all cultures. Twenty-four hours after fertilization hatched blastulae densities were quantified for each treatment (three pseudoreplicates) using a 1 mL Sedgewick rafter cell and compound microscope. Larval survival was assessed every 2–5 days in triplicate (per treatment pseudoreplicate, $n = 3$). Cultures were gently mixed prior to each 15 mL of culture being removed, and all larvae counted under a dissection microscope and then returned to the respective culture. The mean of these pseudoreplicates was then used to represent each treatment replicate. Embryos and larvae were fixed for morphometric measures after 7–8 and 25 days, following McEdward & Herrera (1999) and Kelly *et al.* (2000). Larval rearing was terminated at 25 days. Offspring were photographed on an inverted compound microscope with digital camera and graticules at relevant magnifications. Calibrations and measurements were then quantified using ImageJ Analysis software, NIH, Bethesda, MD, USA (Abramoff, Magelhaes & Ram 2004). Approximately, 30 larvae per replicate in each treatment were measured for various parameters including post-oral arm length in the most advanced larvae, which was used as a metric of skeletal development.

STATISTICAL ANALYSES AND DATA ACCESSIBILITY

Each treatment (temperature and pH) was analysed as an independent factor nesting tank or replicate for oxygen uptake, test diameter, whole animal wet mass (balanced from random removal of unbalanced data), egg size and larval post-oral arm data. Data were analysed in Minitab (Statistical Software version 15, Coventry, UK) using either Nested ANOVA via a general linear model (GLM) (larval data) or one-way ANOVA (adult data) after assessing normality using Anderson–Darling ($P > 0·05$) and homogeneity of variance using Levene's test. After significant ANOVA results, Tukey's or Bonferroni's pairwise comparisons were used to determine treatment differences. Nonparametric Kruskal–Wallis tests were carried out where data consisted of ranks or heterogeneous residual variability remained after transforming data following Sokal & Rohlf (1995). Proportional data were arcsin transformed prior to analysis (Kelly *et al.* 2000). Adult growth was assessed for normality (Anderson–Darling), and relationships with time were determined using correlation and regression analyses. Multiple comparisons of treatment slopes were assessed with a GLM of interacting treatments with time and using time as a covariant. Results were accepted after homogeneity of residuals was met. Growth and standard error of slopes were quantified from the GLM's resulting coefficient terms. Due to multiple testing, *P*-values were adjusted for larval morphometrics, development stages and abnormalities using the method of Benjamini & Hochberg (1995). Data are available via the Polar Data Centre (Suckling *et al.* 2014).

Results

Over the 2 years of the experiment, there were no adult mortalities and sea water parameters were stable (Fig. S2, Supporting information). Saturation states of the microcosm control tanks were slightly undersaturated with respect to aragonite ($\Omega < 1$), but calcite was supersaturated ($\Omega > 1$). Saturation states for lowered pH sea water (−0·3 and −0·5 pH) were undersaturated with respect to calcite and aragonite (Table 1), which was probably due to the cold temperatures (Guinotte & Fabry 2008). Sea water chemistry parameters for the larval experiment were very similar to those of the long-term adult treatments (Table S1, Supporting information).

LONG-TERM ADULT CULTURE

Adult metabolic measurements: oxygen consumption

Rates of oxygen consumption varied significantly across treatments during the experimental period as well as across time (treatment: $F_{(2,162)} = 10·31$, $P < 0·001$; time: $F_{(8,162)} = 26·73$, $P < 0·001$; treat*time: $F_{(16,162)} = 1·80$, $P = 0·036$; tank: $F_{(2,162)} = 2·72$, $P = 0·069$) (Fig. 1). There was a clear pattern to the data. Oxygen consumption was high during the first 2 weeks of the study, but then decreased to a relatively constant level for 8 months and then decreased again, remaining relatively constant for the remainder of the experimental period (Fig. 1). Oxygen consumption measured at the start of the experiment (0 months) was higher under the low pH conditions (−0·3 and −0·5 pH) than in controls ($F_{(2,24)} = 5·90$, $P = 0·048$), but no other treatment differences were observed at other times including the end of the experimental period of 2 years (0·5 months: $F_{(2,24)} = 1·35$, $P = 0·448$; 1 month: $F_{(2,24)} = 3·58$, $P = 0·129$; 4 months: $F_{(2,24)} = 0·51$, $P = 0·664$; 8 months: $F_{(2,24)} = 0·53$, $P = 0·664$; 12 months: $F_{(2,24)} = 0·60$, $P = 0·664$; 16 months: $F_{(2,24)} = 0·56$, $P = 0·664$; 20 months: $F_{(2,24)} = 3·92$, $P = 0·117$; 24 months: $F_{(2,24)} = 0·07$, $P = 0·909$).

Somatic growth

There was no somatic growth in the adults, as test diameters; whole animal wet and dry mass ad AFDM showed no significant change over the 2 year experiment (Figs S3–6, Supporting information).

Calcification

Skeleton ash mass did not differ significantly between treatments at any given time point over the 2 year study (treatment: $F_{(2,237)} = 0{\cdot}06$, $P = 0{\cdot}938$; time: $F_{(8,237)} = 2{\cdot}91$, $P = 0{\cdot}004$; interaction: $F_{(16,237)} = 0{\cdot}76$, $P = 0{\cdot}727$; time across each treatment: control: $F_{(8,79)} = 1{\cdot}18$, $P = 0{\cdot}453$; −0·3 pH: $F_{(8,79)} = 1{\cdot}53$, $P = 0{\cdot}299$; −0·5 pH: $F_{(8,79)} = 1{\cdot}77$, $P = 0{\cdot}230$; Fig. S7, Supporting information). Test thickness measured after 2 years exposure did not differ significantly across treatments (control = 0·98 ± 0·16, −0·3 pH = 1·00 ± 0·20, −0·5 pH = 1·01 ± 0·17 mm; $F = 0{\cdot}33$, $P = 0{\cdot}721$).

Gonad index (GI%)

There were no significant differences in GI% during the course of the experiment in animals held in either control or in the lowest pH conditions (treatment: $F_{(2,189)} = 0{\cdot}81$, $P = 0{\cdot}448$; time: $F_{(8,189)} = 4{\cdot}47$, $P < 0{\cdot}001$; interaction: $F_{(16,189)} = 0{\cdot}95$, $P = 0{\cdot}511$; time across each treatment: control: $F_{(8,63)} = 2{\cdot}10$, $P = 0{\cdot}131$; −0·3 pH: $F_{(8,63)} = 2{\cdot}78$, $P = 0{\cdot}053$; −0·5 pH: $F_{(8,63)} = 1{\cdot}33$, $P = 0{\cdot}417$; Fig. 2).

EGG SIZE, FERTILIZATION AND DEVELOPMENT

Egg size

At 6 months, eggs produced under low-temperature control conditions (0 °C) were significantly larger (~12%) than all other treatments and −0·5 pH eggs were larger than −0·3 pH (treat: $F_{(3,1184)} = 157{\cdot}78$, $P < 0{\cdot}001$; replicate: $F_{(8,1184)} = 18{\cdot}32$, $P < 0{\cdot}001$) (Fig 3). This situation was different at 17 months as females in the low temperature produced significantly smaller eggs (154·86 ± 1·93 μm) compared to all other treatments, and −0·5 pH eggs were larger than high-temperature controls (166·92 ± 1·19, 169·62 ± 1·14 μm, respectively) (+1·8 °C: $H_{(3)} = 58{\cdot}12$, $P < 0{\cdot}001$) (Fig 3).

Fertilization success

Fertilization success exceeded 50% in all treatments. After 6 months exposure, high-temperature controls (+1·8 °C) produced 17% more fertilized eggs compared to all other treatments ($F_{(3,8)} = 51{\cdot}21$, $P < 0{\cdot}001$). After 17 months exposure, high-temperature controls (0 °C) had a fertilization rate 30% higher than animals under lowest pH conditions (−0·5 pH; $F_{(3,8)} = 9{\cdot}30$, $P = 0{\cdot}006$).

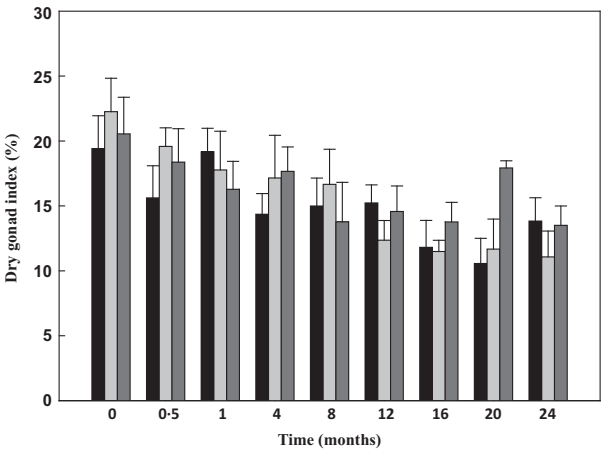

Fig. 2. Mean dry gonad index (%; ±SE) over 2 years of adult *S. neumayeri* exposed to control and reduced pH conditions. ■ = control, ▒ = −0·3 pH, ▒ = −0·5 pH.

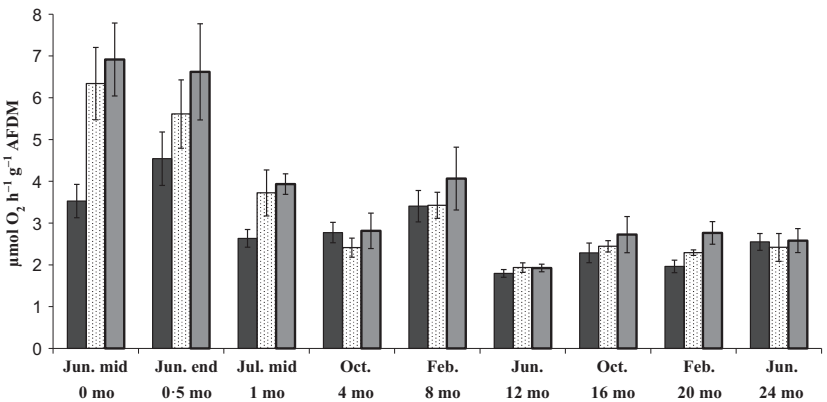

Fig. 1. Mean metabolic responses (μmol O_2 h^{-1} g^{-1} AFDM ± SE) over 2 years of adult *S. neumayeri* exposed to control and reduced pH conditions. ■ = Control, ▒ = −0·3 pH, ▒ = −0·5 pH. Statistics for the time points with significant differences only at 0 and 20 months (0 months: $F_{(2,27)} = 5{\cdot}66$, $P = 0{\cdot}042$; 0·5 months $F_{(2,26)} = 2{\cdot}10$, $P = 0{\cdot}250$; 1 month: $F_{(2,27)} = 2{\cdot}72$, $P = 0{\cdot}196$; 4 months: $F_{(2,27)} = 0{\cdot}51$, $P = 0{\cdot}705$; 8 months: $F_{(2,25)} = 0{\cdot}38$, $P = 0{\cdot}729$; 12 months: $F_{(2,27)} = 0{\cdot}37$, $P = 0{\cdot}729$; 16 months: $F_{(2,27)} = 0{\cdot}60$, $P = 0{\cdot}686$; 20 months: $F_{(2,27)} = 5{\cdot}22$, $P = 0{\cdot}042$; 24 months: $F_{(2,27)} = 0{\cdot}07$, $P = 0{\cdot}937$.

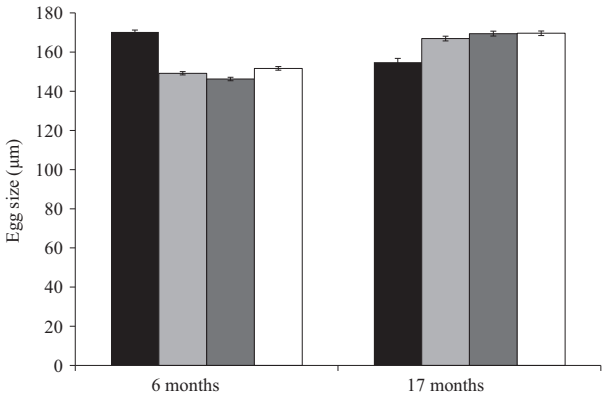

Fig. 3. Egg sizes of *S. neumayeri* exposed to low-temperature (■) and high-temperature (▩) controls and reduced pH conditions −0·3 pH (■) and −0·5 pH (□) after 6 and 17 months.

Hatching success

In the 6-month spawning, hatching success was variable across treatments, with the highest success in the low-temperature controls (0 °C) (63% ±4) and lowest in the lowest pH conditions (−0·5 pH; 18% ±16), with high-temperature controls (+1·8 °C) and moderately low pH conditions (−0·3 pH) intermediate at 39% ±12 and 46% ±11, respectively ($F_{(3,8)} = 4{\cdot}99$, $P = 0{\cdot}031$). At 17 months, there was no significant difference between hatching success in any of the four treatments (low-temperature control (0 °C) = 46% ±9; high-temperature control (+1·8 °C) = 36% ±3; −0·3 pH = 38% ±3; −0·5 pH = 47% ±5) ($F_{(3,8)} = 3{\cdot}09$, $P = 0{\cdot}089$).

Larval survival

In the 6-month spawning, survival rates across treatments were not significantly different until day 17, after which the offspring derived from parents kept at both low- and high-temperature control conditions (0 and +1·8 °C, respectively) had higher survival rates compared to those in the lowest pH conditions ($F_{(3,8)} = 10{\cdot}12$, $P = 0{\cdot}043$; Fig. 4a). At 17 months, larval survival across all treatments was not significantly different until day 15 ($F_{(3,8)} = 5{\cdot}89$, $P = 0{\cdot}048$; Fig. 4b). After this, survival in high-temperature controls (+1·8 °C) was higher than both lowest sea water pH (−0·5 pH) and low-temperature controls (0 °C) ($F_{(3,8)} = 7{\cdot}89$, $P = 0{\cdot}048$; Fig. 4b).

DEVELOPMENTAL STAGE AND ABNORMAL DEVELOPMENT

The cultures were evaluated both for developmental stage and morphometric measurements with data presented here for the final 25 day time point only.

Six-month trial

Larvae in the high-temperature controls (+1·8 °C) were the most developmentally advanced compared of all treatments with the highest percentages of larvae at the gastrula and prismatic stages (Fig. 5a). Proportions of offspring at the earlier blastula stage were low but similar across all treatments ($F_{(3,8)} = 4{\cdot}00$, $P = 0{\cdot}097$), and there was no significant difference in either the later pluteus stage ($F_{(3,8)} = 0{\cdot}93$, $P = 0{\cdot}612$) or levels of abnormal development ($F_{(3,8)} = 0{\cdot}80$, $P = 0{\cdot}622$), both of which averaged 5% across all treatments (Fig. 5a). Morphometric assessments of post-oral arm lengths in pluteus stage larvae were only possible in the low-temperature controls (0 °C) and moderate pH conditions (−0·3 pH), and these were not significantly different, averaging 43·17 µm (treatment: $F_{(1,14)} = 0{\cdot}26$, $P = 0{\cdot}615$; replicate: $F_{(1,14)} = 0{\cdot}27$, $P = 0{\cdot}608$).

17-month trial

At 25 days, these larvae were more highly developed than those in the 6-month trial, with higher proportions at the later prismatic and pluteus stages in each culture (Fig. 5b). Larvae from low-temperature controls (0 °C) were generally the least advanced with the highest proportion of gastrulae when compared with other treatments ($F_{(3,8)} = 11{\cdot}17$, $P = 0{\cdot}007$). The high-temperature controls (+1·8 °C) were the most advanced (Fig. 5b). The proportion of abnormal development was 19% significantly higher under lowest pH conditions (−0·5 pH) compared with both the low- and high-temperature controls ($F_{(3,8)} = 6{\cdot}43$, $P = 0{\cdot}029$; Fig. 5b). At 17 months, there were pluteus stage larvae with measureable post-oral arms in all cultures, which were not significantly different in length at approximately 53 µm in all treatments (treatment: $F_{(3,54)} = 0{\cdot}42$, $P = 0{\cdot}741$; replicate: $F_{(4,54)} = 4{\cdot}47$, $P = 0{\cdot}003$).

Discussion

ADULT PERFORMANCE UNDER LONG-TERM CULTURE

Adults were kept for 2 years in these experimental conditions, without mortality or any apparent effects on growth, feeding, behaviour. There was no significant change in size or mass of the urchins during the 2-year culture (Figs S3–7, Supporting information). This result correlates with previous calcein marking mark-recapture experiments, which showed that *S. neumayeri* grows very slowly, particularly in the large size range used here, with test diameters above 20 mm (Brey *et al.* 1995; Brockington 2001). Importantly, the tests did not lose mass throughout the 2 year exposure, which suggests that there was no effect on calcification despite aragonite and calcite being undersaturated, which means that the animals were able to protect the test from dissolution.

Over the 2-year period, oxygen consumption showed a very clear pattern. Short exposure periods (0 month) had significantly higher values than the rest of the experimental period, with urchins in the lowest pH having the highest

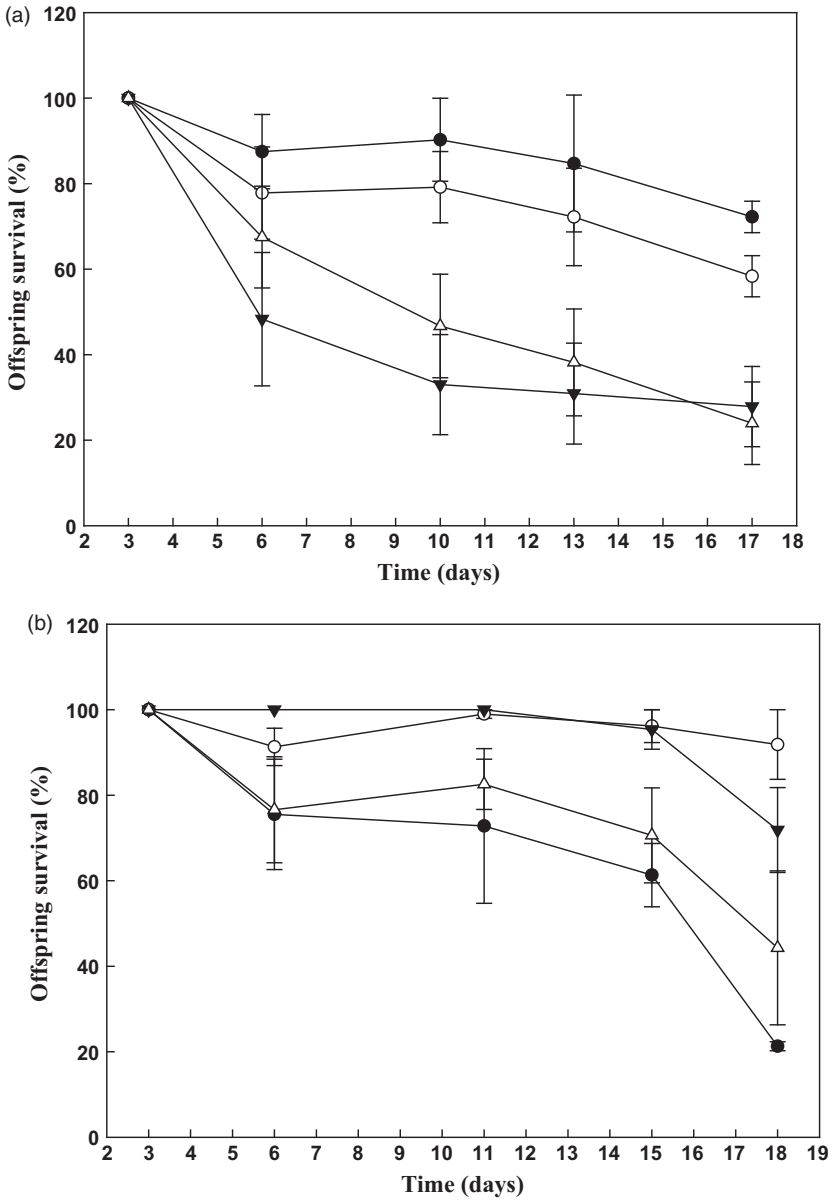

Fig. 4. Survival of *S. neumayeri* offspring (%; ±SE) derived from adults exposed for (a) 6 months and (b) 17 months to control and forecasted pH conditions. –●– = Low-temperature control, –○– = high-temperature control, –△– = −0·3 pH and –▼– = −0·5 pH.

respiration rates (Fig. 1). It is likely that the decrease in oxygen consumption after month 8 was due to the successful acclimation to the new conditions. The metabolic rates of the animals investigated here exhibited the classic biphasic response to increase in temperature (Bouchard & Guderley 2003). There was an initial 'stress' response as the animals' physiology responded to the increase in temperature, which was followed by a gradual lowering of metabolism to a new steady state as the animals adjusted their physiology to the new conditions, demonstrating physiological flexibility with respect to the altered environments (Piersma & Drent 2003). A similar pattern was shown in resting metabolic rates in the Antarctic fish *Pagothenia borchgrevinki* when acclimated to 5 °C, albeit at much faster rates (Robinson & Davison 2008). However, our experiments included exposure to low pH and it is likely that physiological processes were also adjusting to the undersaturated carbonate conditions. Diffusion of CO_2 into internal body fluids (hypercapnia) leading to a reduction in internal pH (acidosis) has been reported to affect physiological processes (e.g. metabolism; Pörtner 2008; Melzner *et al.* 2009; Collard *et al.* 2013). Homeostasis of inner fluids incurs enhanced costs and elevated respiration plus the diversion of energy away from other processes such as reproduction (Pörtner, Bock & Reipschläger 2000; Melzner *et al.* 2009; Collard *et al.* 2013). Reports for *S. droebachiensis* showed a negative impact on acid-base homeostasis after 5 days (Spicer *et al.* 2011), which disappeared after 10–45 days exposure (Stumpp *et al.* 2012). The rates of oxygen consumption after 8 months were still higher than the average data reported for *S. neumayeri* in the field (Brockington & Peck 2001), but the animals in our experiment were kept at around 2 °C and oxygen consumption rates here were similar to those reported from measurements conducted during the austral summer when temperatures usually increase above 0 °C (Brockington & Peck 2001; Clarke *et al.* 2008).

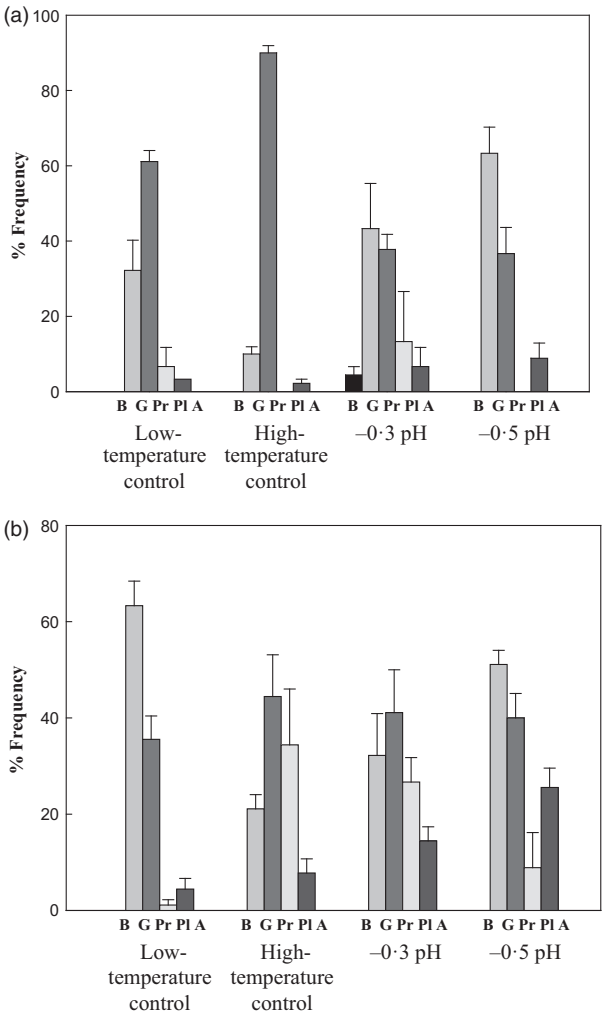

Fig. 5. Mean frequencies of larval development stages (%; ±SE) of 25 day old *S. neumayeri* offspring derived from adults exposed for (a) 6 months and (b) 17 months to low- and high-temperature controls and forecasted pH conditions. B = blastula, G = gastrula, Pr = prismatic larva, Pl = pluteus and A = abnormal development.

Thermal acclimation trials previously conducted on *S. neumayeri* showed that it takes up to 6 months to acclimate to 3 °C (Peck *et al.* 2014). The animals in this experiment may have taken slightly longer to acclimate to the altered conditions (circa 8 months from the oxygen consumption data) due to the interacting effects of temperature and pH (c.f Clark *et al.* 2013), especially since oxygen consumption levels in high-temperature controls were always lower than those of urchins in lowered pH. Metabolic rates have been measured in other invertebrate ocean acidification studies, with mixed results. Increased metabolic rates were observed in the urchin *Paracentrotus lividus,* the brittle star *Amphiura filiformis* and selectively bred oysters *Saccostrea glomerata* (Wood, Spicer & Widdicombe 2008; Parker *et al.* 2011; Catarino, Bauwens & Dubois 2012). However, no increase in metabolic rate was observed in wild populations of *S. glomerata* or the temperate urchin, *S. droebachiensis* when subjected to altered pH (Parker *et al.* 2011; Stumpp *et al.* 2011). These conflicting data may be species/population dependent, but the time of exposure to altered conditions might also be important; sufficient time may not have elapsed for acclimation processes to be completed, as was demonstrated here and the 16-month study of Dupont *et al.* (2013). In the latter, metabolic rates were not measured, but the lack of impact on fecundity after 16 months of culture, suggested that the urchins were able to replenish energy supplies for successful reproduction, which was taken to indicate full acclimation (Dupont *et al.* 2013).

Overall, there appeared to be no major long-term effects of altered conditions on adult *S. neumayeri* beyond 8 months exposure. The lack of major effects may be due to inherent variability in the natural pH of the coastal Southern Ocean. Significant seasonal variation in pH has been recorded in two coastal sites (Prydz Bay and the Ross Sea) with threefold changes in the aragonite saturation state (McNeil, Sweeney & Gibson 2011) across the year equivalent to 0·3–0·5 pH units (Hofmann *et al.* 2011). Thus, shallow coastal populations of *S. neumayeri* may already be habituated to variable sea water pH.

LARVAL PERFORMANCE UNDER LONG-TERM CULTURE

The spawning and larval culture trials conducted after 17 months showed enhanced survival and larval development than the 6-month trial. In these experiments, it was possible to include a low-temperature control (0 °C) spawning at current pH, in addition to the high-temperature control (+1·8 °C), which was a useful extra comparison, as technical constraints required all larvae to be reared at the same temperature. At 6 months, the eggs produced by the low-temperature control (0 °C) females were larger compared with the other treatments, with a higher rate of hatching success (63%) (Fig. 3). However, after 17 months exposure hatching success (36–47%) and larval survival were similar across all treatments. This agrees with Dupont *et al.* (2013), where fecundity and larval survival 'recovered' after increased adult exposure, which was attributed to acclimation in *S. droebachiensis*. In this study, the spawned animals under lowest pH conditions produced larger eggs after extended adult exposure time. This potentially indicates that acclimation has resulted in a higher investment per egg, as a mechanism to increase developmental success and recruitment (Llodra 2002). Larger eggs produce better targets for sperm, thus increasing fertilization success (Levitan 1993). Egg size also correlates with juvenile performance (Emlet & Hoegh-Guldberg 1997).

Whilst survival of embryos and larvae improved with adult exposure time to altered sea water pH, there was also a parallel increase in abnormal larval development for larvae raised from animals kept in high temperatures. These adults were all cultured at around 2 °C and abnor-

mal development in their progeny doubled after 17 months while abnormalities in low-temperature controls (0 °C) remained at similar levels throughout the 2 years (Fig. 5). Temperature was previously shown to significantly affect *S. neumayeri* larval development, with an optimal temperature window of +0·2 to +1·7 °C proposed for embryo viability (Stanwell-Smith & Peck 1998; Tyler, Young & Clarke 2000). Therefore, it is likely that levels of abnormal development observed here were due to a cumulative temperature effect. However, a treatment effect was also observed, with most abnormal larvae produced in the lowest pH conditions (−0·5 pH) (Fig. 5). This potentially indicates an interacting effect of exposure to two different stressors. In a previous study, settlement success and metamorphosis was the same across control and reduced pH treatments in the temperate urchin *Psammechinus miliaris* and less competent larvae appeared to fail earlier under reduced pH compared to control conditions (Suckling *et al.* 2014b). The increase here in abnormally developed larvae after 17 months adult preexposure, may again be a result of adult acclimation effects, with less competent larvae surviving longer under reduced pH conditions, but this can only be verified by using longer culture periods.

Actual numbers of offspring surviving to 18 days postfertilization did show differences between treatments. At the 6-month spawning, the numbers of surviving larvae derived from the parents maintained in reduced pH were significantly lower compared with the other treatments. Conversely, at the 17-month spawning, survival was lowest for larvae derived from the low-temperature control (0 °C) and lowest pH (−0·5 pH) cultured parents, indicating there was little difference between treatments. In each spawning, the larvae were cultured for 25 days. In a temperate species, we previously demonstrated the importance of culturing larvae until settlement (Suckling *et al.* 2014b), but the slow development of the Antarctic *S. neumayeri* larvae precluded this, as at least 115 days are required to reach metamorphosis (Bosch *et al.* 1987). Predicting the final effect of the culture conditions on recruitment and juvenile development can be complex, as demonstrated by Dupont *et al.* (2013). When they analysed juvenile survival from 4-month acclimated adults, they identified a potential carry over effect with reduced juvenile survival 3 months post-settlement in some treatments. However, these results may have been confounded by high mortality rates a result of potential technical problems in their system, which averaged 33–54% with 95% in juveniles raised at high pCO_2.

The beneficial effects of allowing adults to acclimate to altered sea water pH, prior to spawning, as demonstrated here, have also been reported for marine invertebrates from lower latitudes (Parker *et al.* 2011; Dupont *et al.* 2013; Suckling *et al.* (2014b). Studies which included the maturation of gonads under altered conditions are rare and show variable results (Parker *et al.* 2011; Uthicke *et al.* 2013) and focussed largely on offspring success (Dupont *et al.* 2013). The experimental time-frame is critical (Suckling *et al.* 2014b). Even if adults acclimate quickly, gametogenic cycles can take much longer to complete, for example the tropical sea urchin, *E. mathaei* has a gametogenic cycle encompassing 5–6 months (Pearse 1969), which is much longer than the timing of adult metabolic acclimation (Uthicke *et al.* 2013).

The fact that embryos and larvae were able to develop in lowered pH with elevated temperatures agrees with previous studies on *S. neumayeri*. Ericson *et al.* (2012) observed reduced frequencies of normally developing cleaving embryos at pH 7·5 with an 11% reduction in both fertilization success and normally developed blastulae. Whilst Byrne *et al.* (2013) showed that larval growth was reduced at lower pH, but that size was partially compensated for by temperature. They also observed more abnormal larvae and smaller echinoplutei with decreased arm lengths at low pH and elevated temperature. These previous studies are not directly comparable with our data as they used offspring derived from adults held in ambient conditions and without significant gametogenesis under the altered conditions. However, our data confirm these previous findings with the demonstration of a significant temperature effect, further emphasizing the requirement to include both temperature and pH in marine acidification experiments.

One further aspect relevant to these studies is that of source populations and population genetic variability. All of the experiments listed to date on *S. neumayeri* (with the exception of this study) were performed with Ross Sea populations. Populations of circumpolar species from the Antarctic Peninsula differ significantly in their physiology compared with the same species from the Ross Sea. Acclimation capacities of notothenioid fish (Bilyk & deVries 2011) or the thermal tolerances of marine invertebrates (Morley *et al.* 2012) differ, even though the ambient temperature regimes differ by only 1–2 °C. The basis for this remains unknown, but there is probably a genetic component. Such an effect was demonstrated in an F1 selection experiment on *Strongylocentrotus purpuratus* urchin populations from along a 1200 km stretch of the United States coastline. Offspring were raised under a variety of altered pH conditions and showed allelic selection on genes for several biochemical functions, including membrane composition and ion transport (Pespeni *et al.* 2012). In the Southern Ocean, the main area with significant warming is the western Antarctic Peninsula and this is where biodiversity is potentially most at threat. Hence, in these populations, there is a need to understand not only how much phenotypic plasticity exists, but also the fine detail of circumpolar population structure and the consequences for functional biodiversity. These data are needed in order to better predict the effects of climate change on Antarctic marine species (Somero 2010; Peck 2011).

Conclusions

Sterechinus neumayeri adults successfully acclimate in long-term culture to the combined stressors of reduced pH and elevated temperature, with relatively little measurable effect on metabolism and reproduction after 2 years. Since these animals were exposed to altered conditions at rates much faster than is occurring naturally, they seem highly resilient to such changes. At the end of 2 years the animals under lowest pH conditions (−0·5 pH) were producing fewer, but larger eggs. But losses during development and metamorphosis are high and selection may operate at different rates under the different treatments. Thus, it is only with culture through to settlement and raising of juveniles that accurate predictions can be made for future recruitment rates. These data highlight the importance of experiments using long-term culture in altered conditions for marine species, especially those with poor acclimation abilities. Data here also emphasize that sampling following rapid introduction to reduced sea water pH conditions (e.g. up to 8 months for polar species and likely many weeks for temperate taxa) may be evaluating animals in physiological transition and not acclimated. Exposing adults long term, so that the full gametogenic cycle is undertaken in altered conditions, significantly affects reproductive success and larval outcomes and therefore this practise should be implemented in future studies to increase the accuracy of predictions with regard to species' response to climate change.

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Data accessibility

Data available from: http://doi.org/wrh (Suckling *et al.* 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Mean (±SE) seawater parameters of the *Sterechinus neumayeri* larval cultures derived from parents pre-exposed to low temperature and high temperature seawater controls and lowered carbonate conditions.

Fig. S1. Experimental set-up for each of the treatments with two tray tanks for each treatment, fed by a single mixer tank.

Fig. S2. Mean (±SE) seawater parameters of the *Sterechinus neumayeri* adults exposed to low temperature and high temperature (A) seawater controls and lowered carbonate conditions (B) across each year of the experimental period.

Fig. S3. Mean test diameter (mm; ±SE) of adult *S. neumayeri* exposed to control and reduced pH conditions.

Fig. S4. Mean whole animal wet mass (g; ±SE) of adult *S. neumayeri* exposed to control and reduced pH conditions.

Fig. S5. Mean whole animal dry mass (g; ±SE) of adult *S. neumayeri* exposed to control and reduced pH conditions.

Fig. S6. Mean whole animal ash free dry mass (g; ±SE) of adult *S. neumayeri* exposed to control and reduced pH conditions.

Fig. S7. Skeleton mass (measured as ash remaining after ignition) (g; ±SE) of adult *S. neumayeri* exposed to control and reduced pH conditions.

