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Experimental influence of pH on the early life-stages of sea urchins II: Increasing parental exposure times gives rise to different responses

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Abstract

Many studies into the responses of early life-stages to ocean acidification utilise offspring obtained from parents reared under present-day conditions. Their offspring are directly introduced to altered-pH. This study determined whether this approach is suitable by pre-exposing parent sea urchins (*Psammechinus miliaris*) to altered seawater-pH (~1000µatm) for several durations, spawning them and rearing their offspring to settlement. Parents acclimated when exposed to low seawater-pH for extended periods (>42d). Longer adult pre-exposures reduced larval survival and less competent offspring were removed from populations earlier than in controls. Control offspring were larger during earlier development-stages (2-7d) but

smaller during later development-stages (14+d) than offspring reared under low pH. Juvenile settlement levels were similar across all treatments. After 17d, offspring sourced from parents pre-exposed to low pH for 42 and 70d were larger than those pre-exposed for 28d and ambient sourced offspring directly transferred to low pH. These different responses show that the use of ambient derived offspring utilised in many studies are likely not an ideal approach when assessing larval development responses via morphometric measurements and survivorship prior to settlement. This study also suggests that calcifying organisms have capacities to acclimate and possibly adapt towards conditions beyond natural rates of ocean acidification.

Keywords: CO_2; echinoderm; larvae; ocean acidification; *Psammechinus miliaris*; settlement.

Introduction

Predicting the responses of marine organisms to rapid climate change has been a focus in interdisciplinary science during the last decade. One area in particular is that of ocean acidification. The oceans have absorbed up to a third of anthropogenic atmospheric carbon dioxide (CO_2) since the onset of the industrial revolution. As a consequence oceanic surface waters have reduced by 0.1 pH units during the last 250 years and are forecasted to reduce by a further 0.3-0.5 units by the year 2100 (Houghton et al., 2001; Caldeira and Wickett, 2003; 2005; Royal Society, 2005; Canadell et al., 2007). This in turn, will likely cause the undersaturation of calcium carbonate which may have important consequences for marine organisms, particularly those which biomineralise (Doney et al., 2009).

Controlled laboratory experiments are a widely utilised proxy to assess the responses of these marine organisms with undersaturated seawater conditions generally achieved by the mixing of seawater and carbon dioxide gas (Barry et al., 2010). A large number of studies that use this approach have demonstrated a range of impacts on adult marine organisms and that these impacts are species specific (e.g. Ries et al., 2009). Additionally early life stages are often identified as the most sensitive part of an organism's life cycle and have been portrayed

as the bottleneck for the success of populations and therefore the continuation of a species under a changing climate (Martin et al., 2011). Information on this section of the life cycle is therefore of great importance in making predictions on organismal responses.

An increasing number of laboratory based studies focussing on early life stages of marine organisms have emerged in recent years (e.g. Dupont et al., 2010; Byrne, 2011). Although these studies, like those on adults, show a range of responses, the majority report negative impacts (e.g. abnormality and mortality; Dupont et al., 2008; Kurihara, 2008; Doo et al., 2011). However, such studies largely utilise offspring derived from ambient sources; the adults were generally collected from present day coastal areas and then spawned under present day conditions in the laboratory, after which the subsequent gametes or offspring were then directly introduced into low seawater pH. The responses of offspring using this approach may reflect scenarios such as a CO_2 sequestration leak that causes reduced seawater pH in a localised area. However, ocean acidification occurs over many years and in a wider global context and at this pace, these organisms could acclimate, produce and deposit gametes, reproduce and perhaps even adapt to change (Suckling, 2012). These approaches utilise direct introduction of ambient sourced gametes/offspring into low pH conditions may therefore report responses that are likely to be acute and chronic demonstrating only the plasticity of present-day organisms.

The responses of organisms have been shown to differ when introduced at different rates to a range of environmental parameters (e.g. salinity (Anger, 1996), temperature (Peck et al. (2009) and seawater pH (Suckling et al. submitted)). Suckling et al. (submitted) showed that sea urchin (*Psammechinus miliaris* (P.L.S Müller, 1771) larvae directly introduced to reduced seawater pH had lower survival and were smaller compared to controls while those introduced at slower rates showed less severe negative responses. These studies highlight that direct transfer approaches may not be ideal for making predictions on organismal responses under future environmental change, such as ocean acidification, especially when focussing on early life stages.

Previous studies on echinoid early life stage responses have largely used ambient derived offspring, without considering parental pre-exposure or pre-conditioning to reduced pH conditions (e.g. Havenhand et al., 2008; Kurihara, 2008; Clark et al., 2009; Dupont et al., 2010a; Byrne et al., 2011), thus not addressing wider parts of the life-cycle. Species such as *Arbacia dufresnei* (Blainville, 1825), *A. punctulata* and *Paracentrotus lividus* (Lamarck, 1816) appear to not be affected by near-future ocean acidification (Carr et al., 2006; Moulin et al., 2011; Martin et al., 2011; Catarino et al., 2012). However, negative impacts such as smaller larval sizes, have been reported for several species due to physiological stress under lower carbonate saturation states (e.g. *Evichinus chloroticus* (Havenhand et al., 2008); *Heliodaris erythrogramma* (Valenciennes, 1846): Kurihara & Shiryama, 2004; Kurihara et al., 2004); *Strongylocentrotus droebachiensis* (Müller, 1776): Stumpp et al., 2011)). These studies generally concluded that marine calcifiers will respond negatively when exposed to altered seawater pH at forecasted year 2100 conditions. However, organisms will develop, mature, deposit gametes and reproduce along with the gradual process of ocean acidification. Many studies have demonstrated the influence that external factors can have on gamete development and reproduction, especially in the area of aquaculture (e.g. Shpigel et al., 2004; Hammer et al., 2006). Therefore these studies, which conclude negative responses, likely demonstrate instead the physiological flexibilities of present day echinoids to altered seawater pH conditions.

Few studies have considered maternal and paternal effects with respect to ocean acidification laboratory studies. Parker et al. (2012) exposed oysters (*Saccostrea glomerata* Gould, 1850) for 5 weeks to forecasted seawater pH conditions prior to assessment of offspring success. Although the study's focus was limited to adult and pre-metamorphosis development stages of offspring, it suggested that *S. glomerata* have the capacity to acclimate and adapt to future conditions. Additionally, Dupont et al. (2012) carried out a 16-month trial that exposed adults of the urchin *Strongylocentrotus droebachiensis* to forecasted seawater pH, and reported effects over most life history stages. Adults in their experiments were spawned after 4 and 16 months to assess the reproductive and offspring responses. The adults

exhibited evidence of acclimation but subsequent juvenile survival was low. The conclusion presented was that there was a negative carry-over effect from adults to larvae and then to juveniles. However, the results for the juveniles were unclear due to the occurrence of high mortality within one of the replicates. In contrast, Uthicke et al. (2012) found that the responses of the offspring of the sea urchin *Echinometra mathaei* were not more resilient to ocean acidification conditions after pre-exposing the parents for 6-weeks, but only focussed on the pre-settlement stages of offspring. Great care should be employed when interpreting results from these studies in an ecological context due to the restrictions of laboratory based studies, but they are a marked improvement into assessing the impacts of climate change on reproduction. They also provide examples of how responses can be species specific and therefore need to be carried out on a wider range of organisms.

In this study, we assessed the responses of early life stages of sea urchins (*P. miliaris*) when derived from parents previously exposed to either control or altered seawater pH for increasing periods of time (up to 70 days). This species was utilised as it has well-established reproductive and larval rearing methodology (McEdward and Herrera, 1999; Kelly et al. 2000; Liu et al, 2007). We follow established methods undertaken by earlier studies and also take into consideration the effects of parental exposure of different durations on offspring. In contrast to some studies, we also assessed the full cycle of larval development and survival to include settling juvenile stages.

Materials and Methods

Sea urchin source and maintenance

Subtidal *Psammechinus miliaris* (total n = 192) were hand collected by scuba divers in March 2009 from a site at 3-10m depth at Rubha Garbh, Loch Creran (56º30’36.5088” N 5º22’57.1791” W), Scotland described by Symonds et al. (2009). Animals were transferred to aquaria at the Scottish Association for Marine Science, Oban. The urchins (30.74 ± 0.14 mm test diameter (TD ± SE)) were selected at random and distributed between 24 three-litre aquaria (n = 8 urchins per aquaria = 164 cm^2 $urchin^{-1}$). Each aquarium had an independent

supply of UV sterilized, 250 µm filtered seawater of ambient temperature and salinity and a photoperiod of 16 h light and 8 h dark (Kelly et al., 2000). The urchins were acclimated to these conditions for 14 days prior to the experiment. They were fed an artificial diet (see section 'Artificial diet preparation') previously shown to be suitable for this species (Suckling, 2012), satiating individuals at approximately 5 % mean body weight every 2 to 3 days. Uneaten food and faeces were removed by siphoning three times weekly and before new feed was introduced.

Artificial diet preparation

Laminaria digitata (Hudson, J.V. Lamouroux, 1813) free from epifauna was collected by hand during spring low tides from Connel Bridge, West Coast of Scotland, dried at 60 ºC for 24 h then homogenised into a fine powder and stored in an air tight container at -20 ºC until required. *Mytilus edulis* (Linnaeus, 1758) were hand collected during low spring tides from Loch Creran Head, West Coast of Scotland, dissected and homogenised to a paste. These homogenised components were mixed with molten agar and alginic acid according to the volumes in Table 1. After setting, the mixture was cut into approximately 1cm cubes and stored at -20 ºC until use. Diets were defrosted prior to introduction to adult *P. miliaris*.

CO_2 microcosm

A flow-through CO_2 microcosm (132 L; Fig. 1) was developed and adapted from a seawater acidification tank system similar to that of Widdicombe and Needham (2007). UV sterilized and 250 µm filtered seawater was delivered to 60 L closed cylindrical mixing tanks. This was mixed with CO_2 gas via a ceramic diffuser using an Aquamedic pH controlled computer and electrode system circulated by a 2000 L h^{-1} pump. The computer was set to a designated pH level ± 0.02 pH units hysteresis. Seawater of set pH was fed by gravity to 12 experimental tanks in parallel, at a rate of 0.92 ± 0.6 L min^{-1} per tank. A control system used an identical set up but excluded the pH control system.

Twice weekly, temperature (ºC; YSI Model 63), salinity (YSI Model 63), pH_{NIST} and TCO_2 (mmol L^{-1}; Ciba Corning TCO_2 Analyzer 965, Olympic Analytical, UK) were measured and recorded for three replicate experimental tanks per treatment. The TCO_2 analyzer was calibrated with 2 g L^{-1} CO_2 standard prior to measurements. Aquamedic pH probes were calibrated every second day with NIST certified pH buffer solutions. Sixty mL were also extracted from each treatment mixing tanks, filtered through GFF filter paper, and stored at -20 ºC in a light proof container until defrosted for nutrient analysis (Phosphate and silicate; duplicate samples; for methods see Nickell et al. (2003)).

Adult exposure treatments

The treatments established in this study were derived from the ambient surface seawater pH measured in March 2009 (pH 7.93) prior to the start of the experiment as described in Dupont et al. (2008). Based on the ambient level in the Scottish Association for Marine Science seawater system and the IPCC 'business-as-usual' scenario (year 2100, ~1000 ppm, ~ pH 7.7; Houghton et al., 2001; Caldeira & Wickett, 2005; IPCC, 2007; Guinotte and Fabry, 2008) the reduced pH treatment utilised in this study was pH 7.73. Seawater parameters under which *P. miliaris* were exposed are presented in Table 2. Values for control pCO_2 may appear high but are within the normally experienced range for shallow coastal seawater (Barry et al., 2010; Gazeau et al., 2010). Control seawater was supersaturated ($\Omega > 1$) and the reduced pH treatment undersaturated ($\Omega < 1$) with respect to calcite and aragonite (Table 2).

Adult *P. miliaris* were exposed for various periods of times reduced pH conditions: 0 days, 28 days, 42 days and 70 days. Control and 0 d offspring were sourced from ambient reared parents. Control offspring were reared under ambient conditions whilst 0 d offspring were placed directly into reduced pH conditions. For the other exposure durations, offspring treatments were reared under reduced pH conditions derived from parents exposed to reduced pH for 28 d, 42 d or 70 d (Table 3).

Prior to introduction to reduced pH conditions, the adult *P. miliaris* were held under ambient control conditions. Hence, to avoid stress, control seawater was gradually displaced by reduced pH seawater. Trials during establishment of the pH controlled system showed no abnormal animal behaviour or indication of stress (e.g. spawning, loss of appetite) during this period. Treatments (lengths of time) were allocated randomly to the aquaria using Minitab's (Statistical Software™ Version 15) random block design. Each treatment had three replicates of eight animals per time period (28, 42 and 70 d) in the reduced seawater pH treatment. The control treatment comprised of three replicates of eight animals. Animals were randomly selected for spawning from across these replicates until a ratio of five females and 2 males were achieved for gamete introduction (see section 'spawning and larval rearing'). Remaining animals which were not selected for spawning in each replicate per treatment and time period were dissected to assess somatic growth and gonad index (see section 'adult data collection').

Adult data collection

Various somatic parameters were measured for adult *P. miliaris* following Suckling et al. (2011). Whole wet weight (± 0.01 g) and maximum horizontal test diameter (TD, vernier callipers ± 0.1 mm) were recorded before dissection. Gonad wet weight (± 0.01 g) was also recorded. Sex was determined from a wet-squash preparation of fresh gonad tissue in seawater using phase-contrast light microscopy (Kelly, 2000). The gonad index (GI %) was calculated by dividing the gonad wet weight by the whole wet weight, expressed as a proportion.

Spawning and larval rearing

Larvae were reared in static systems adapted from Kelly et al. (2000) supplied with premixed CO_2 (~ pH 7.7; Table 1; Fig. 2) or compressed air at low flow rate to maintain larval cultures in suspension until settlement. Control offspring were reared under control conditions and 0 mo gametes were placed directly into reduced pH conditions. Remaining

219 treatment offspring (derived from parents exposed to low pH for 28 d, 42 d or 70 d) were held
220 under reduced pH conditions.

221 Randomly selected adult *P. miliaris* were spawned using injections of 1-2 mL of 0.5
222 M KCl through the persitomal membrane into the coelom until 5 females were successfully
223 spawned for each treatment exposure. After injection urchins were stored in ~ 300 mL glass
224 jars filled with 5-µm filtered treatment seawater until gametes were shed and then they were
225 returned to flow-through aquaria. Jars were kept closed during spawning and prior to
226 fertilisation to prevent crossing of gametes/fertilisation prior to targeted fertilisation. Clean
227 gloves and pipettes were utilised for handling of each *P. miliaris* and transfer of gametes as a
228 further measure to prevent non-target fertilisation/gamete transfer from occurring. This
229 ensured that samples for fertilisation success were within a precise time. Eggs from the 5
230 females for each treatment were combined and divided equally into three replicate volumes,
231 rinsed with filtered seawater and placed as a monolayer on the bottom of sealed buckets
232 containing 12 L of filtered treatment seawater. Sperm concentration was not calculated but
233 was subject to the same dilution series from concentrated sperm across all treatments (1:5;
234 Kelly et al., 2000). Eggs were fertilised by adding 1-2 mL of diluted sperm from the 2 males
235 from each treatment. Forty-five minutes later the seawater in each bucket was gently well
236 mixed and five 15 ml sub-samples were fixed to determine egg density and fertilisation rate.
237 To ensure that the pH of the seawater remained relatively uniform over the initial
238 development period when no aeration can occur, premixed CO_2 gas was introduced at a rate
239 of 0.2 L min^{-1} into the header space between the seawater surface and lid for 24 hours.

240 Twenty four hours after fertilisation hatched blastulae densities were quantified for
241 each treatment (three pseudoreplicates) using a 1 mL sedgewick rafter cell and compound
242 microscope. Blastulae were decanted into their respective treatment buckets at a density of ~ 1
243 individual mL^{-1}. Glass tubes were used to deliver relevant treatment gases (at ~ 200 mL min^{-1})
244 to the bottom of the buckets. Complete seawater exchanges were carried out every two days,
245 with larvae retained in a 40 µm mesh sieve in a water bath while buckets were cleaned and
246 filled.

Once the stomach had fully formed (gastrula stage, 48 hours after fertilisation) feeding of larvae was initiated. *Dunaliella tertiolecta* (Culture Collection of Algae and Protozoa, code 19/6B) was cultured following methods described in Liu et al. (2007). For larvae with two, three and four pairs of arms, food was provided daily at 1500, 4500 and 7500 algal cells mL^{-1}, respectively (Kelly et al., 2000).

Larval settlement success was assessed following the methods described by Suckling et al. (submitted). In summary, larvae were considered competent for settlement when the rudiment was similar in size to the stomach and spines and tube feet were visible in the rudiment. Capacity to settle was quantified by placing larvae into Petri-dishes conditioned with a natural biofilm of marine bacteria, algae including diatoms (Hinegardner, 1969). Thirty echinoplutei were decanted into pre-fouled 90 mm Petri-dishes in triplicate and assessed for settlement every 24 h for 3 days. Larvae that metamorphosed into juveniles were classed as successful settlers. Settlement Petri-dishes for the control were placed on a small tray and left exposed to the ambient air in the CT room at ~ 16 ºC. To maintain a constant high CO_2 environment for larvae reared in reduced pH, Petri dishes were housed in propagators. Pre-mixed CO_2 gas was supplied to the propagator ~ 0.1 L min^{-1}. A 0.5 m HDPE hose vent was installed to inhibit back diffusion of ambient air. Blank reduced pH seawater samples were maintained in this environment simultaneously and were measured for pH each day and were found to remain constant. Numbers of successfully settled juveniles were counted directly and calculated as a percentage utilising the last larval survival density count. Absolute numbers settled per 1000 eggs were calculated by utilising an artificial count of egg numbers (base of bucket surface area divided by measured egg area) because true egg numbers were not recorded.

Temperature and pH_{NIST} were recorded daily for the static larval-systems. Seawater parameters (see 'CO_2 microcosm') were measured for fresh seawater and existing seawater prior to water exchange. Seawater samples in the static cultures were filtered through a 40 µm mesh to prevent the uptake of larvae into the sampling devices.

Larval morphometrics

Survival was assessed every 2-5 days in triplicate (per treatment replicate, n = 3). Cultures were gently mixed prior to each 15 mL pseudoreplicate being removed and counted under a dissection microscope and then returned to respective cultures. Early life stages were fixed for morphometric measures after 2, 7, 11, 14 and 17 days, based on the protocol of McEdward and Herrera (1999) and Kelly et al. (2000). Blastulae, gastrulae and echinopluteus larvae were photographed on an inverted compound microscope with digital camera and 1 mm graticules at relevant magnifications. Calibrations and measurements were then quantified using ImageJ Analysis software (Abramoff et al., 2004). Approximately 30 larvae per replicate in each treatment were measured for the parameters described in Fig. 3, dependent upon developmental stage. Development stages were recorded under the main categories of 2-armed, 4-armed, 6-armed and 8-armed plutei as described in McEdward and Herrera (1999). Larvae were classified as abnormal developers when development diverged strongly away from the norm described in McEdward and Herrera (1999) and Kurihara (2008). Examples of abnormal development included missing arms, abnormal tissue growth and the presence of two rudiments (Suckling et al. submitted).

Statistical analysis

Data were analysed by using either Minitab (Statistical Software™ Version 15) Nested ANOVA via a General Linear Model or One Way ANOVA after assessing normality and homogeneity of variance ($P > 0.05$). After significant ANOVA results, Tukey's or Bonferronni's Pairwise Comparisons were used to determine which treatments differed. Non-parametric Kruskal-Wallis tests were performed where heterogenous residual variability remained after transforming data following procedures presented by Sokal and Rohlf (1995). Proportional data were arcsine transformed prior to analysis (Kelly et al., 2000). Where Type I errors occurred the means and 95 % confidence intervals were graphically analysed to illustrate the data under normal assumptions. Graphical and tabulated representations of data were presented as the mean and standard errors of treatments unless otherwise indicated. Due

to multiple testing, P values were adjusted for larval morphometrics, development stages and abnormalities following Benjamini and Hochberg (1995).

Results

Adults

One hundred percent survival was observed for adult *P. miliaris* held under the two treatment conditions (Control and low pH) from April until July 2009. There were no significant differences between treatment exposures for test diameter (TD, One-way ANOVA, $F_{(3,51)} = 0.49$, $P = 0.693$), total wet weight (One-way ANOVA, $F_{(3,51)} = 0.93$, $P = 0.435$) or gonad index (GI, One-way ANOVA, $F_{(3,51)} = 0.44$, $P = 0.727$) amongst adult *P. miliaris*. Adult data separated by sex showed no significant differences between treatment exposures (TD, ♂, Kruskal-Wallis, $H_{(3)} = 1.94$, $P = 0.584$, ♀, One-way ANOVA, $F_{(3,27)} = 0.49$, $P = 0.690$; total wet weight, ♂, One-way ANOVA, $F_{(3,20)} = 1.95$, $P = 0.154$, ♀, One-way ANOVA, $F_{(3,27)} = 0.69$, $P = 0.568$; GI, ♂, One-way ANOVA, $F_{(3,20)} = 1.04$, $P = 0.395$, ♀, One-way ANOVA, $F_{(3,27)} = 0.46$, $P = 0.712$).

Females exposed to reduced pH for 70 d released the smallest eggs (One-way ANOVA, $F_{(4,2504)} = 26.77$, $P < 0.001$; Fig. 4). Eggs fertilization was least successful for gametes derived from adults exposed to reduced pH for 28 d compared to gametes derived from shorter and longer exposure times (One-way ANOVA, $F_{(4,23)} = 14.95$, $P < 0.001$; Fig. 4).

Early life stages

Survival decreased in larval cultures with time (Fig. 5), with significant treatment effects observed from day 11 onwards. On day 11 survival was highest in control larvae compared to larvae derived from parents reared in reduced pH for 28 and 70 d (One-way ANOVA, $F_{(4,10)} = 9.79$, $P = 0.002$). Survival of larvae in controls remained highest by day 16 compared to echinoplutei derived from adults exposed for 42 and 70 d in reduced pH (One-way ANOVA, $F_{(4,10)} = 5.69$, $P = 0.012$).

Stage and abnormal development

Significantly less larvae from controls developed preoral arms compared to larvae reared under reduced pH on day 17 (One-way ANOVA, $F_{(4,10)}$ =27.42, P = 0.002). All other development stages were not significantly different across samples days (One-way ANOVAs, P > 0.05; Table 4). On day 11 Control larvae had significantly fewer rods protruding from tissues compared to larvae reared under reduced pH (One-way ANOVA, $F_{(4,10)}$ = 77.35, P = 0.047). Although no other significant abnormalities were found, there was a notable increase in abnormal development with exposure time across all treatments (Table 5).

Larval morphometrics and settlement

Morphometric parameters measured in echinoplutei are summarised in the 'larval morphometrics' section. Generally a similar trend in response to pH treatment conditions was observed across these parameters (Fig. 6-9; Supplementary Tables S1-S4). Therefore only seven key larval morphometrics will be reported in these results (larval length (LL), larval width (LW), body length (BL) and rod (BR), postoral arm length (PO) and rod (POR) and rudiment length (RUD)).

During earlier development stages (days 2-7) offspring in controls were larger (larval length (LL) and width (LW)) than larvae reared in reduced pH treatments (day 2: length One-way ANOVA, $F_{(4,605)}$ = 18.92, P < 0.001; width, One-way ANOVA, $F_{(4,605)}$ = 41.86, P < 0.001; day 7: length, Kruskal-Wallis, $H_{(4)}$ = 109.87, P = 0.002, width, One-way ANOVA, $F_{(4,336)}$ = 19.89, P = 0.002; Fig. 6a and b). However, from day 14 onwards the trend in the size of offspring appeared to reverse, with controls being smaller than those larvae in reduced pH, and persisted until metamorphosis and settlement (day 14: length, Kruskal-Wallis, $H_{(4)}$ = 92.10, P = 0.002, width , Kruskal-Wallis, $H_{(4)}$ = 13.70, P = 0.015; day 17: length, Kruskal-Wallis, $H_{(4)}$ = 58.61, P = 0.002, width, One-way ANOVA, $F_{(4,385)}$ = 5.18, P = 0.002; Fig. 6a and b). On day 17 the differing parental pre-exposure times demonstrated varying impacts on offspring morphometrics. The offspring derived from parents exposed longest to low seawater pH (42 and 70 d) were significantly larger than those pre-exposed for 28 days (28 d) and

ambient sourced offspring directly transferred to low seawater pH (0 d; length, Kruskal-Wallis, $H_{(4)}$ = 58.61, P = 0.002, width, One-way ANOVA, $F_{(4,385)}$ = 5.18, P = 0.002; Fig. 6a and b). Similar responses to all those described above were observed for postoral arm (PO) and rod (POR) lengths (day 7: arm, Kruskal-Wallis, $H_{(4)}$ = 135.83, P = 0.002, rod, Kruskal-Wallis, $H_{(4)}$ = 131.43, P = 0.002; day 11: arm, One-way ANOVA, $F_{(4,380)}$ = 11.01, P = 0.064, rod, Kruskal-Wallis, $H_{(4)}$ = 6.81, P = 0.189; day 14: arm, One-way ANOVA, $F_{(4,393)}$ = 24.72, P = 0.002, rod, One-way ANOVA, $F_{(4,392)}$ = 16.83, P = 0.002; day 17: arm, Kruskal-Wallis, $H_{(4)}$ = 34.25, P = 0.002, rod, Kruskal-Wallis, $H_{(4)}$ = 45.38, P = 0.002; Fig. 7a and b).

Body lengths (BL) were longer in controls compared to reduced pH treatments during early-stages of development (day 7: One-way ANOVA, $F_{(4,339)}$ = 12.48, P = 0.002; day 11: Kruskal-Wallis, $H_{(4)}$ = 23.46, P = 0.003; Fig. 8a). Towards metamorphosis body lengths (BL) were similar across all treatments (day 14: Kruskal-Wallis, $H_{(4)}$ = 4.45, P = 0.460; day 17: One-way ANOVA, $F_{(4,387)}$ = 2.79, P = 0.040; Fig. 8a). The skeletal body rods (BR) demonstrated a contrasting response to seawater treatments. During earlier development stages, 28 d and 42 d offspring generally had longer body rod lengths (BR) than controls (day 7: Kruskal-Wallis, $H_{(4)}$ = 43.05, P = 0.002; day 11: One-way ANOVA, $F_{(4,370)}$ = 8.47, P = 0.004; Fig. 8b). During later development, controls comprised of longer body rod lengths (BR) than echinoplutei reared under reduced pH conditions (day 14: One-way ANOVA, $F_{(4,388)}$ = 17.64, P = 0.002; day 17: One-way ANOVA, $F_{(4,384)}$ = 7.40, P = 0.002; Fig. 8b).

Echinoplutei growing rudiments (RUD) towards metamorphosis were largest under reduced pH conditions by 36 % and 62 % on days 14 and 17 respectively compared to 'present-day' controls (day 14: Kruskal-Wallis, $H_{(4)}$ = 65.53, P = 0.002; day 17: One-way ANOVA, $F_{(4,350)}$ = 16.15, P = 0.002; Fig. 9).

Analyses of the percentage of successfully settled echinpoplutei show that 28 d, 42 d and 70 d had significantly higher settlement compared to controls (One-way ANOVA, $F_{(4,10)}$ = 5.81, P = 0.002; Table 6). Further analyses of total numbers of larvae settled in the context of Day 16 densities and absolute numbers settled per 1000 fertilized eggs revealed no significant

differences between treatments (One-way ANOVA, $F_{(4,10)} = 0.48$, $P = 0.752$ and $F_{(4,10)} = 1.50$, $P = 0.275$ respectively; Table 6).

Discussion

Many ocean acidification studies reproduce adults collected from the natural world, and then introduce their offspring directly into reduced pH conditions. In this study, we tested whether this direct transfer is a suitable technical design. This is especially important because predictions of organismal responses to forecasted climate change are largely derived from reports that utilised such approaches (Suckling et al., submitted).

In this study offspring derived from adults pre-exposed to altered seawater did respond differently to offspring directly transferred from ambient conditions to low seawater pH. This is evident from our results for survival and morphometrics during pre-settlement stages. Controls had highest survival from day 11 compared to offspring derived from parents previously exposed to low seawater pH (28, 42 and 70 d). However, offspring that were sourced from ambient reared parents and directly transferred to low seawater pH conditions (0 d) were not significantly different from controls (C) nor from offspring from pre-exposed parents (28, 42 and 70 d). As offspring reached later stages of development (day 17+) different responses in morphometrics were observed across experimental treatments. Offspring sourced from parents pre-exposed to low seawater pH for longest periods of time (42 and 70 d) were larger than those derived from parents pre-exposed for only 28 days (28 d) and offspring from ambient sourced parents directly transferred to low pH (0 d).

Twenty-eight days of parental pre-exposure appears to be an insufficient amount of time to demonstrate any observable impact on offspring morphometrics but extending this up to 42 days gives rise to different responses. Therefore, a minimum period of time of parental pre-exposure may be required to observe effects on subsequent offspring that would therefore indicate that these sea urchins have acclimated. Other studies have also shown that parents have capacities to acclimate when pre-exposed to other environmental changes. Gezelius (1962) demonstrated that *P. miliaris* has physiological plasticity with regards to salinity

environments and reproduction. Deep-water specimens were transferred to lower salinity surface water conditions and *vice versa*. After 50 days exposure (approximately close to 42 days) to different treatments, *P. miliaris* demonstrated acclimation to salinity, as fertilisation and development was best at the salinity at which parents were maintained. Similarly for the asteroid *Luidia clathrata*, Hintz and Lawrence (1994) showed that parents exposed for 1 month during gametogenesis to low or high salinities demonstrated acclimation of the gametes.

Acclimation of parents in the longest exposure treatments was also demonstrated by improved fertilisation success. After 28 days (28 d) exposure to reduced pH fertilization success was lowest. Increasing the exposure time further (up to 70 days), however, showed fertilization success levels increased back towards control levels. Low fertilization success after 28 days could be a result of reduced sperm swimming speed or due to a delay as eggs guard themselves against polyspermy (becoming fertilised by more than one sperm; Levitan, 2006; Havenhand et al., 2008). However, Caldwell et al. (2011) have shown that sperm swimming speeds increase for *P. miliaris* under low pH conditions. These data therefore need to be approached carefully since in these experiments sperm were diluted and not quantified which may have affected the results, despite relatively high levels of fertilisation observed for most of the treatments. It is still possible that acclimation underpins the increased fertilisation success observed with increased exposure time, similarly seen with this species when exposed to altered salinities (Gezelius, 1962) as discussed above. In conjunction eggs were smallest from urchins exposed for the longest time period (70 d) to reduced pH.

In comparison to previous research on early life-stage responses to reduced pH conditions, this study is one of the first few to pre-expose parents and expands on studies of larval success to include settlement. The pre-metamorphosis results of our study were similar to those reported in other studies which did not pre-expose parents. During earlier development stages (up to day 7), control *P. miliaris* were generally larger than counterparts reared under reduced pH treatments which is in agreement with other studies of echinoderms (e.g. Kurihara & Shirayama 2004; Dupont et al., 2008; O'Donnell et al., 2010; Stumpp et al.,

2011). Two studies also support our results of reduced larval survival (from day 11; Dupont et al., 2008; Doo et al., 2011). Similar findings have been reported for other calcifying groups in terms of size and survival development (i.e. molluscs: Watson et al., 2009; Gazeau et al., 2010; Talmage & Gobler, 2010; Crim et al., 2011). These studies generally concluded that marine calcifiers will respond negatively when exposed to altered seawater pH at forecasted year 2100 conditions.

Our observations on later stages of development (from day 11 onwards) contrasted with all but one echinoderm study (Dupont et al., 2010b) that showed positive growth and size influences on lecithotrophic larvae derived from ambient sourced parents. This is in agreement with Parker et al., (2012), a study that assessed oyster (*Saccostrea glomerata*) larval responses to forecasted pH after pre-exposing parents for 5 weeks to similar conditions. Taking an overview of larval development up to settlement in this trial, an interesting phenomenon can be seen. During the earlier stages of development (days 2-7) offspring in controls were larger than larvae reared under reduced pH treatments. However, from day 14 onwards the size of reduced pH reared offspring relative to controls appeared to increase and persist until metamorphosis and settlement. This relative size increase in conjunction with the observed mortality rates suggests that less competent larvae, reared under reduced pH conditions, are removed from the population earlier than in controls with weaker larvae persisting in control populations up to settling metamorphosis. This subtle altered process of selection may have, in part, selected for larvae with specific alleles that improve performance under these conditions as discussed by Pespeni et al. (2013).

Subsequently the additional factor of adaptation requires consideration thus multigenerational responses would require investigation. This would allow for phenotypic plasticity, acclimation and adaptation to be determined (Dam, 2013; Fitzer et al., 2013). Multigenerational studies on relatively slow growing invertebrates, such as *P. miliaris* is difficult to achieve due to numerous years being required to achieve this. However organisms with rapid life-cycles, such as copepods, provide excellent models for intergenerational responses to altered seawater pH. Fitzer et al. (2012) found that *Tisbe battagliai* reallocated

resources towards maintaining reproductive output at the expense of somatic growth across three generations. This copepod therefore has the capacity for phenotypic plasticity but it is unclear when this progresses to acclimation and adaptation (Fitzer et al., 2012). Further work is therefore required to pick apart these types of responses.

On day 11, abnormal development was observed in all low seawater pH offspring in the form of broken / protruding skeletal rods. Such an abnormality is likely to disturb the streamline of larvae (McEdward and Herrera, 1999) thus increasing the effort required to obtain food particles. The reduced survival observed in this study therefore suggests that it was likely that these larvae were not able to sustain the energy required to continue to develop and grow. The direct cause of these broken / protruding skeletal rods is unknown, they are either an abnormal response to altered seawater pH or due to physical damage likely incurred during seawater exchanges. If the latter were true then this demonstrates that offspring are more susceptible to damage under low seawater pH environments.

However, expanding this pre-settlement focus to include post-settlement stages puts these larval responses into context, because, settlement numbers were similar across all treatments. These settlement results are in agreement with those described by Suckling et al. (submitted) who also found no measureable treatment effect on settlement success. These studies are among a very small number of studies (e.g. Byrne et al., 2011; Dupont et al., 2012) that take echinoderm larvae through to settlement, or at least to settlement stages. Dupont et al. (2012) reported low juvenile survival derived from parents previously exposed to low seawater pH by up to 16 months. However, the occurrence of high mortalities within one of the treatment replicates could mask any real responses. Byrne et al. (2011) and Shirayama and Thornton (2005) both reported negative impacts of laboratory induced ocean acidification on juveniles, where *Heliodaris erythrogramma, H. pulcherrimus* and *Echinometra matheri* demonstrated reduced growth, calcification and survival, however these were achieved from the direct transfer of ambient sourced offspring.

Uthicke et al. (2012) also incorporated parental exposure into their experimental design prior to assessing early life stage responses to low seawater pH. Interestingly, they

found that the responses of the offspring of the sea urchin *E. mathaei* were not more resilient to low seawater pH conditions after pre-exposing the parents for 6 weeks. These conclusions were based on the early stages of offspring pre-settlement development, which is in agreement with our study during the early development stages (days 2-7) of offspring only. In our study, however, the treatment effect on mean sizes of offspring was different during early (days 2-7) and later (days 14+) stages of offspring development. Therefore the responses of *E. mathei* offspring during later development stages in Uthicke et al's (2012) study are still unknown because the focus was limited to the earlier stages of offspring pre-settlement development only.

Despite these increased efforts to expand on life stage focuses, there is still evidence to show that responses can be species-specific (Ries et al. 2009). The investigation carried out in this study therefore needs to be performed on a wide range of organisms in order to improve our understanding of how parental pre-exposure can influence subsequent offspring success. We can conclude that incorporating parental pre-exposure in assessments of early life stage responses to low seawater pH gives rise to different outcomes. Our data show that careful consideration is needed when studies report the responses of offspring development, derived from ambient conditions, introduced directly to forecasted ocean acidification. Furthermore, this study highlights the importance of wider life-cycle approaches when forecasting organismal responses to ocean acidification (e.g. including settlement success following larval development). Therefore the organismal predictions for future ocean acidification, based only on data from studies of pre-settlement larval responses, need to be carefully considered due to their restricted focus. Moreover it is critical that settlement and early-juvenile stages are incorporated into any developmentally based studies investigating the impacts of ocean acidification, as the final effect on settlement and recruitment is critical to predicting future ecosystem level effects.

Acknowledgements

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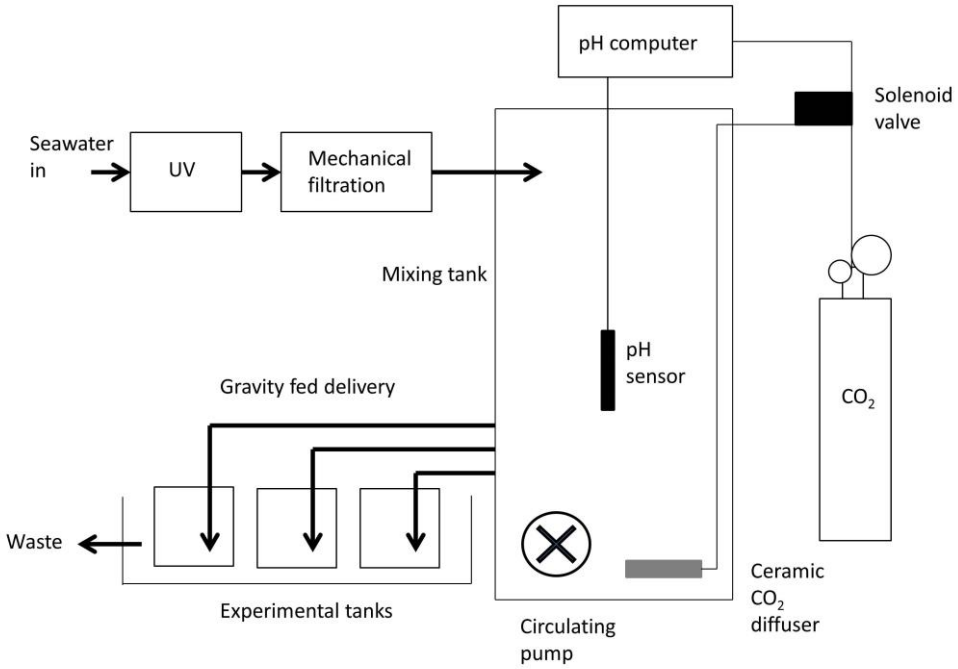
713 **List of figures**



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715 **Fig. 1:** Schematic diagram of the CO_2 microcosm adapted from Widdicombe and Needham's

716 seawater acidification tank system (2007).

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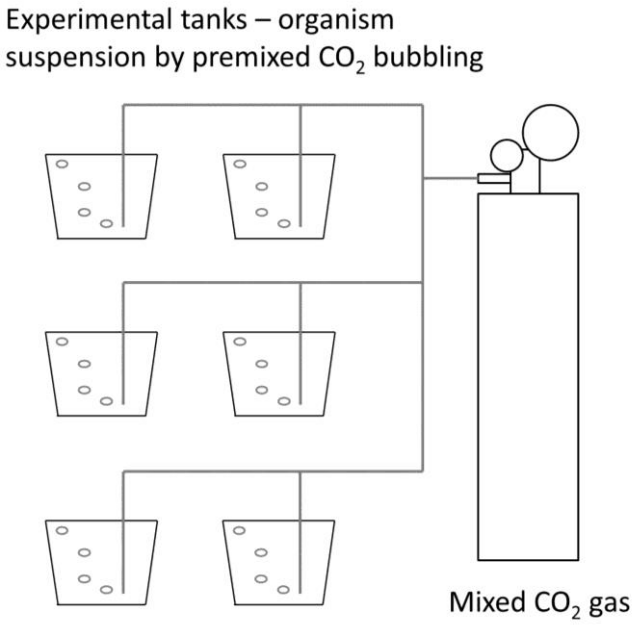

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732 **Fig. 2:** Schematic diagram of the premixed CO_2 larval system.

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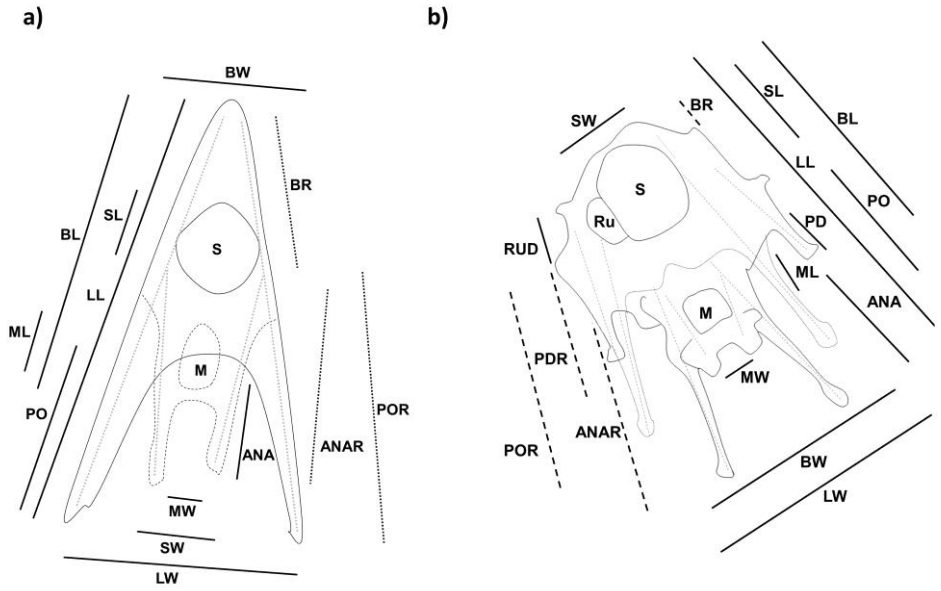

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746 **Fig. 3:** Morphometric parameters measured on gastrulae and echinoplutei (within the larval
747 structure outlines 'M' denotes the mouth, 'S' the stomach and 'Ru' the rudiment). Days 7 (a)
748 and 14 (b) depicted examples; day 2: length and width; day 7-11: larval length (LL) and larval
749 width (LW), body length (BL) and rod (BR), stomach length (SL) and width (SW), mouth
750 length (ML) and width (MW), postoral arm length (PO) and rod (POR), anterolateral arm
751 length (ANA) and rod (ANAR); day 14-17: parameters measured for days 7-11 and
752 posterodorsal arm length (PD), posterodorsal rod length (PDR), preoral arm length (P),
753 preoral rod length (PR) and rudiment length (RUD). Skeletal morphometrics are presented as
754 dashed lines.

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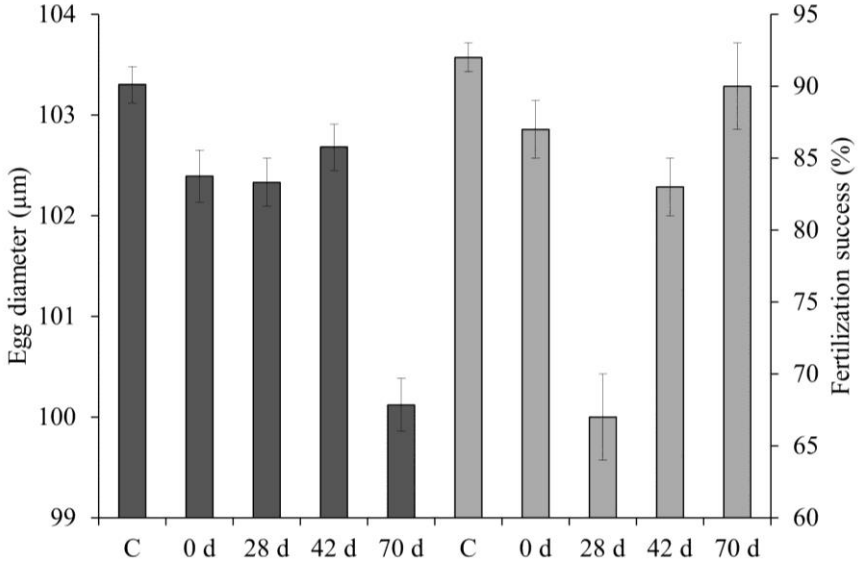

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761 **Fig. 4:** Mean egg diameters (■) and fertilization success rates (□; ± SE) from adults
762 exposed to control or reduced pH treatments for different periods of times (d = days). Note
763 ordinate axis do not start at zero.

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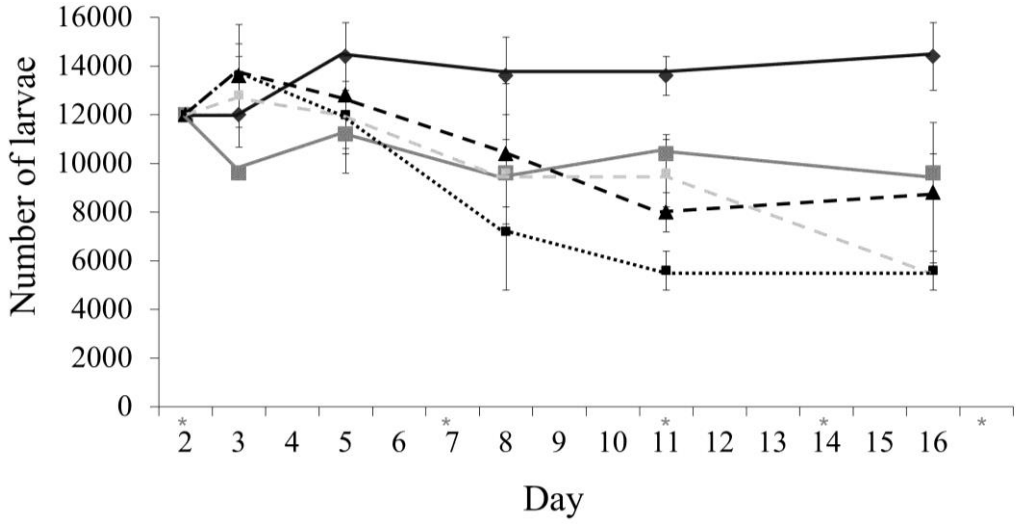


Fig. 5: Mean larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (± SE) derived from adults exposed for differing periods of time to control and reduced pH conditions. Settlement tests commenced from ~day 20; therefore, no density data are presented after day 16. = Control, = 0d, = 28d, = 42 d, = 70 d,* = Morphometric measurement sample times.

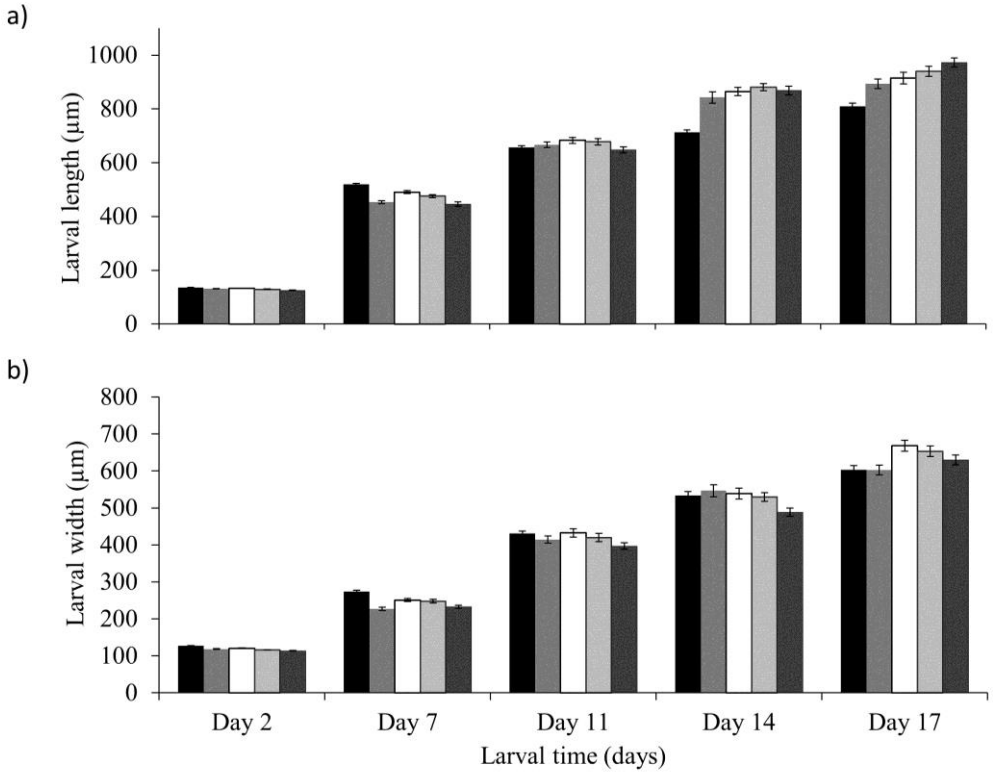


Fig. 6: Mean larval lengths (a) and widths (b; ± SE; µm) for *Psammechinus miliaris* echinoplutei derived from adults exposed for differing periods of time to control and reduced pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

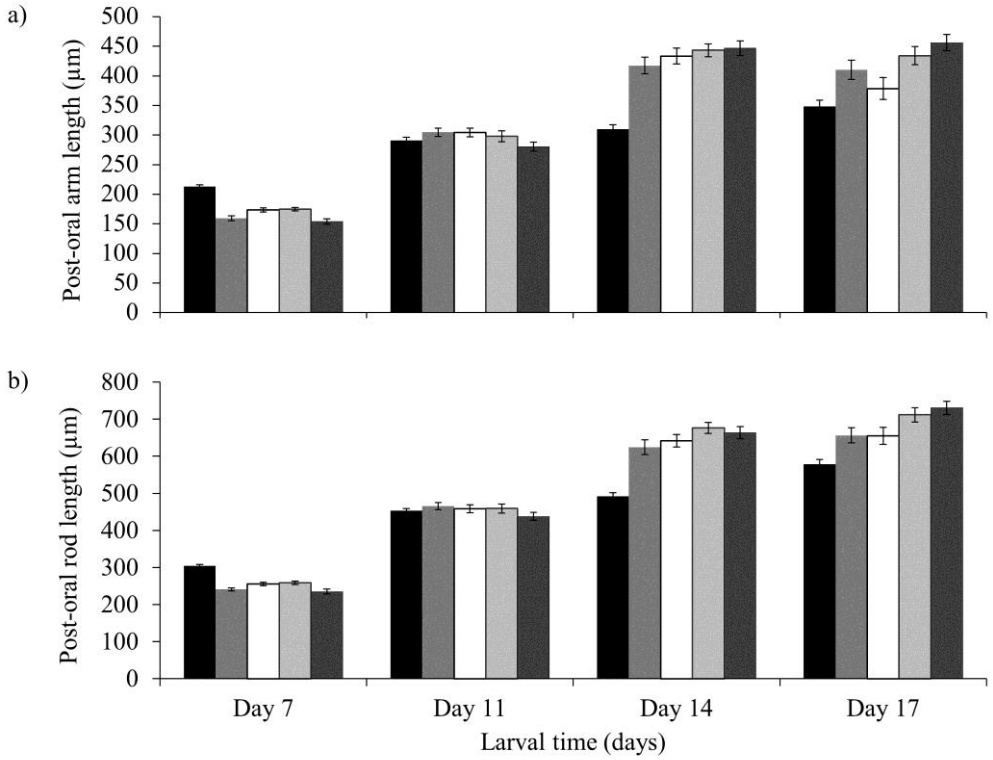


Fig. 7: Mean larval post-oral arm (a) and rod lengths (b; ± SE; µm) for *Psammechinus miliaris* echinoplutei derived from adults exposed for differing periods of time to control and reduced pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

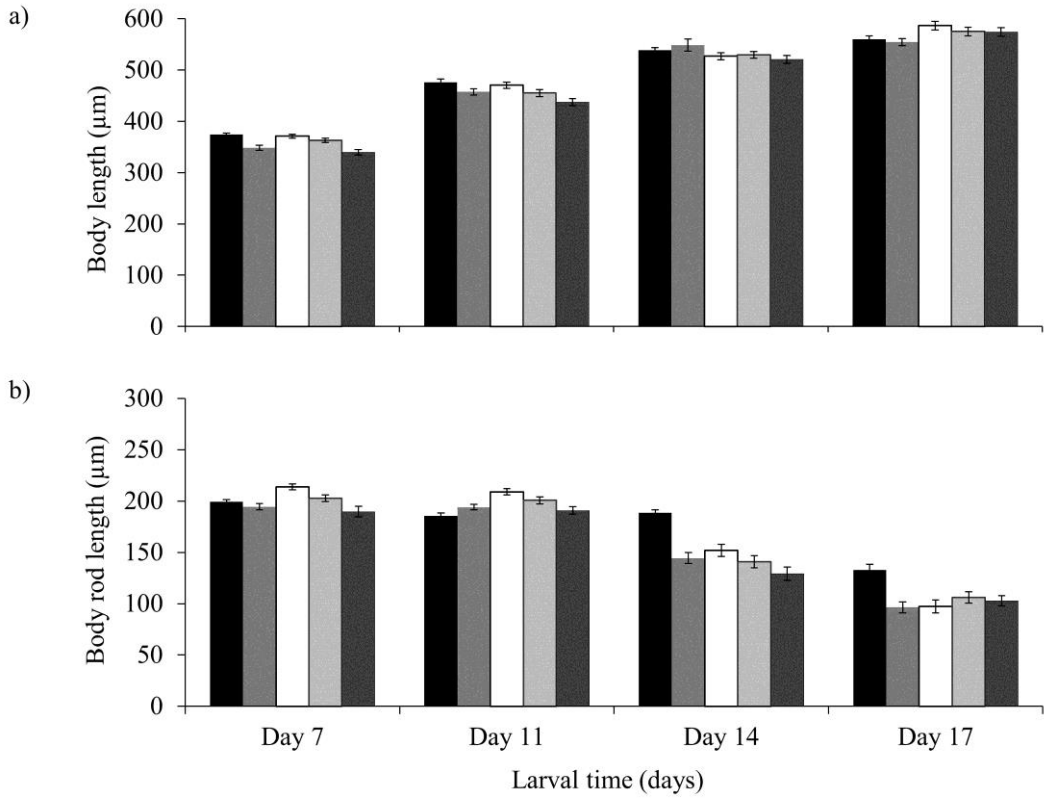
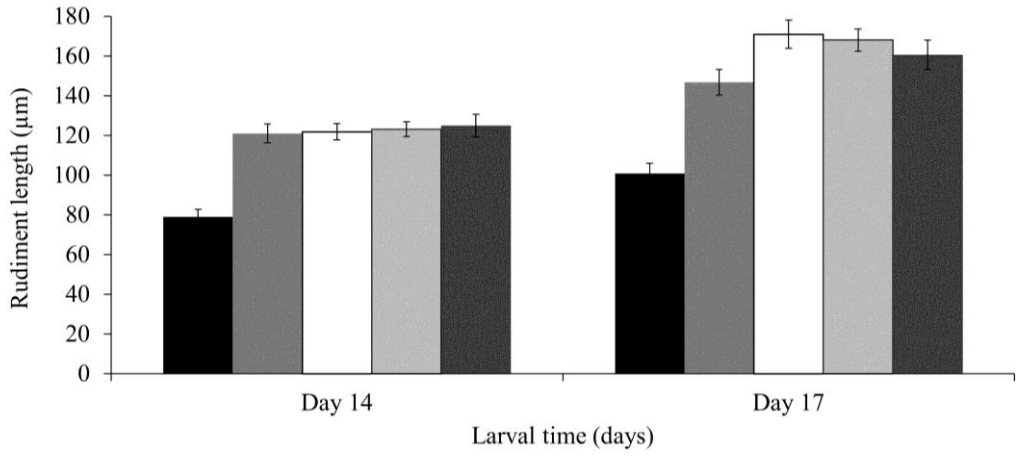


Fig. 8: Mean larval body length (a) and rod lengths (b; ± SE; µm) for *Psammechinus miliaris* echinoplutei derived from adults exposed for differing periods of time to control and reduced pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, □ = 42 d, ■ = 70 d.



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839 **Fig. 9:** Mean rudiment lengths (± SE; µm) for *Psammechinus miliaris* echinoplutei derived

840 from adults exposed for differing periods of time to control and reduced pH conditions. ■ =

841 Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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Table 1: Adult artificial diet composition (% wet mass). FW = freshwater.

Ingredient	%	Composition per batch
Agar agar (Sigma)	38.7	12g agar: 400 mL FW
Homogenized Mussel Flesh	29.0	300g
Seaweed preparation	29.0	33.3g dried *L. digitata*: 267 mL FW
Alginic acid	3.3	1g: 28.57 mL FW

Table 2: Mean (± SE) seawater treatment parameters for *Psammechinus miliaris*. pCO_2, Ω calcite and Ω aragonite values modelled from CO2SYS (Lewis & Wallace, 1998) with refitted constants (Mehrbach, *et al.*, 1973; Dickson & Millero, 1987).

Seawater parameter	Control	Reduced pH
pH_{NIST}	7.98 ± 0.01	7.70 ± 0.01
pCO_2 (μatm)	559 ± 15	999 ± 22
Ω calcite	2.01 ± 0.06	0.98 ± 0.13
Ω aragonite	1.28 ± 0.04	0.63 ± 0.08
Temperature (°C)	12.9 ± 0.4	12.8 ± 0.4
Salinity (psu)	32 ± 1	32 ± 1

Table 3: Exposure time (days (d)) of adult *Psammechinus miliaris* to seawater treatments (Control and reduced pH = pH 7.98 and 7.70 respectively).

Treatment	Ambient (days)	Reduced pH (days)
Control	70	0
0 d (control)	70	0
28 d	-	28
42 d	-	42
70 d	-	70

Table 4: Mean developmental stage (%; ± SE) for *Psammechinus miliaris* echinoplutei. Superscripts indicate where significant differences lie within each column in respective development stage sections. Columns without superscripts indicate no treatment effects.

		%			
Stage	Treat	Day: 7	11	14	17
4 arms	C	78 ± 3	3 ± 2	2 ± 2	3 ± 2
	0d	81 ± 2	12 ± 1	5 ± 3	2 ± 2
	28d	79 ± 1	12 ± 3	4 ± 1	1 ± 1
	42d	78 ± 1	21 ± 8	4 ± 1	3 ± 1.9
	70d	87 ± 2	19 ± 3	2 ± 2	1 ± 1
		$F_{(4,10)}$ = 3.39 P = 0.059	$H_{(4)}$ = 8.99 P = 0.066	$F_{(4,10)}$ = 0.57, P = 0.492	$F_{(4,10)}$ = 0.35 P = 0.855
6 arms	C	22 ± 3	97 ± 2	27 ± 12	49 ± 6 [a]
	0d	19 ± 2	88 ± 1	13 ± 4	16 ± 3 [b]
	28d	21 ± 1	88 ± 3	17 ± 5	13 ± 4 [b]
	42d	22 ± 1	79 ± 8	17 ± 3	12 ± 3 [b]
	70d	13 ± 2	81 ± 3	29 ± 6	13 ± 2 [b]
		$F_{(4,10)}$= 3.39 P = 0.059	$H_{(4)}$ = 8.99 P = 0.066	$F_{(4,10)}$ = 1.16 P = 0.460	$F_{(4,10)}$ = 17.44 P = 0.002
8 arms	C	0 ± 0	0 ± 0	71 ± 11	49 ± 4 [a]
	0d	0 ± 0	0 ± 0	85 ± 3	81 ± 3 [b]
	28d	0 ± 0	0 ± 0	82 ± 7	86 ± 4 [b]
	42d	3 ± 3	0 ± 0	80 ± 5	85 ± 3 [b]
	70d	0 ± 0	0 ± 0	69 ± 7	86 ± 2
		$F_{(4,10)}$ = 3.39 P = 0.071	-	$F_{(4,10)}$ = 0.97 P = 0.511	$F_{(4,10)}$ = 27.42 P = 0.002

Table 5: Mean abnormal development types (%; ± SE) for *Psammechinus miliaris* echinoplutei. Superscripts indicate where significant differences lie within each column in respective development stage sections. Columns without superscripts indicate no treatment effects.

		%			
Abnormality	Treat	Day: 7	11	14	17
Skeletal rods protruding (RP)	C	34 ± 9	5 ± 1 [a]	8 ± 5	34 ± 11
	0d	33 ± 3	26 ± 13 [b]	25 ± 5	29 ± 3
	28d	36 ± 11	37 ± 2 [b]	33 ± 7	59 ± 10
	42d	42 ± 10	40 ± 3 [b]	27 ± 8	47 ± 1
	70d	42 ± 1	59 ± 8 [b]	35 ± 7	35 ± 4
		$H_{(4)} = 1.79$ P = 0.476	$F_{(4,10)} = 77.35$ P = 0.047	$F_{(4,10)} = 2.54$ P = 0.112	$F_{(4,10)} = 3.01$ P = 0.111
Postoral arms (PO)	C	1 ± 1	0 ± 0	12 ± 7	15 ± 6
	0d	5 ± 3	3 ± 2	5 ± 3	22 ± 6
	28d	0 ± 0	8 ± 5	17 ± 4	36 ± 4
	42d	0 ± 0	9 ± 2	21 ± 7	17 ± 4
	70d	9 ± 4	7 ± 5	5 ± 3	19 ± 3
		$F_{(4,10)} = 2.39$ P = 0.182	$F_{(4,10)} = 1.27$ P = 0.174	$F_{(4,10)} = 1.93$ P = 0.228	$F_{(4,10)} = 3.24$ P = 0.143
Anterolateral arms (ANA)	C	1 ± 1	1 ± 1	7 ± 2	12 ± 8
	0d	7 ± 3	2± 2	4 ± 2	28 ± 9
	28d	7 ± 3	6± 3	7± 3	33 ± 4
	42d	3± 3	4± 2	6± 2	20 ± 5
	70d	5± 3	10 ± 5	8 ± 2	33 ± 12
		$F_{(4,10)} = 0.71$ P = 0.553	$F_{(4,10)} = 1.31$ P = 0.580	$F_{(4,10)} = 0.43$ P = 0.584	$F_{(4,10)} = 1.27$ P = 0.317
Posterodorsal arms (PD)	C	-	-	-	2 ± 2
	0d	-	-	-	3 ± 1
	28d	-	-	-	1 ± 1
	42d	-	-	-	6 ± 4
	70d	-	-	-	7 ± 2
					$F_{(4,10)} = 1.43$ P = 0.142
Preoral arms (P)	C	-	-	-	5 ± 2
	0d	-	-	-	18 ± 3
	28d	-	-	-	17 ± 5
	42d	-	-	-	11 ± 4
	70d	-	-	-	13 ± 1
					$F_{(4,10)} = 2.33$ P = 0.150
Disintegrating body tissue	C	0 ± 0	0 ± 0	0 ± 0	17 ± 9
	0d	2 ± 1	0 ± 0	0 ± 0	7 ± 3
	28d	1 ± 1	0 ± 0	0 ± 0	3 ± 1
	42d	0 ± 0	3 ± 1	0 ± 0	4 ± 2
	70d	4 ± 2	3 ± 1	2 ± 2	1 ± 1
		$F_{(4,10)} = 2.03$ P = 0.241	$F_{(4,10)} = 2.99$ P = 0.121	$F_{(4,10)} = 1.00$ P = 0.493	$F_{(4,10)} = 1.95$ P = 0.411
General	C	2 ± 1	1 ± 1	20 ± 6	21 ± 16
	0d	12 ± 4	8 ± 5	12 ± 3	48 ± 7
	28d	8 ± 4	17 ± 8	19 ± 3	65 ± 6
	42d	6 ± 3	17 ± 6	22 ± 7	40 ± 4
	70d	14 ± 5	20 ± 6	25 ± 4	54 ± 10
		$F_{(4,10)} = 1.57$ P = 0.281	$F_{(4,10)} = 2.13$ P = 0.111	$F_{(4,10)} = 0.99$ P = 0.493	$F_{(4,10)} = 3.03$ P = 0.087

1011 **Table 6:** Mean settled larvae (± SE) from adults exposed to control or reduced pH treatments
1012 for different periods of times (d = days). Columns without superscripts indicate no treatment
1013 effects, differing superscripts indicate where significant differences occur.
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Treat	Settlement (%)	Total # settled larvae	Absolute # settled larvae 1000 fertilized eggs $^{-1}$
Control	40 ± 10 ^{a}	6011 ± 2159	193 ± 56
0d	55 ± 7 ^{ab}	5260 ± 1077	218 ± 53
28d	70 ± 2 ^{b}	6235 ± 2212	377 ± 125
42d	69 ± 5 ^{b}	3780 ± 307	189 ± 22
70d	82 ± 4 ^{c}	4520 ± 467	344 ± 70

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