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Experimental influence of pH on the early life-stages of sea urchins I: different rates of introduction give rise to different responses

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Abstract

Many early life-stage response studies to ocean acidification utilise gametes/offspring obtained from ambient-sourced parents, which are then directly introduced to experimentally altered seawater-pH. This approach may produce a stress response potentially impacting development and survival. Hence, this study determined whether this approach is suitable by subjecting embryos/larvae to different rates of introduction to lowered seawater-pH to assess larval success under acute and staggered experimental pH scenarios. Embryos and 4-armed larvae of the sea urchin *Psammechinus miliaris* were introduced to pH-conditions, widely used in ocean acidification studies, from ambient conditions utilizing 380, 470, 560, 700 and

840ppm CO_2 changed at incremental steps at two rates: fast (every 3rd hour) or slow (every 48th hour). Direct transfers from ambient to low seawater-pH gave rise to dramatic negative impacts (smaller size and low survival), but slower rates of introductions gave rise to lesser negative responses (low survival). There was no treatment effect on settled juveniles. Fast introductions utilised in many studies are likely not ideal approaches when assessing pre-settlement larval developmental responses. Therefore careful consideration of the pattern of response is needed when studies report the responses of offspring, derived from ambient conditions, introduced directly to forecasted ocean acidification conditions.

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

Introduction

Currently large efforts are being made to predict the responses of marine organisms to rapid climate change. One area of particular focus is ocean acidification. Oceanic surface waters have acidified by approximately 0.1 pH units since the onset of the industrial revolution (~250 years ago; Royal Society, 2005) and are forecasted to decrease even further and more rapidly by 0.3-0.5 units over the next 90 years (Houghton et al., 2001; Caldeira and Wickett, 2003; 2005, Canadell et al., 2007; IPCC, 2013). Consequently surface waters may become undersaturated with respect to calcium carbonate ($CaCO_3$) which may have significant consequences for marine organisms (Doney et al., 2009).

Laboratory based experiments assess the responses of these marine organisms by exposing them to altered seawater pH conditions. Such conditions are achieved through technically controlled mixing of seawater and carbon dioxide gas (CO_2). Studies have confirmed that reduced pH conditions can have a range of impacts (positive to negative) on adult marine calcifying organisms (e.g. Ries et al., 2009) highlighting that responses can be species specific. An increasing number of laboratory studies have emerged in recent years predicting the impacts of forecasted ocean acidification scenarios on the early life-stages of a range of marine organisms (e.g. Munday et al., 2009; Gazeau et al., 2010; Caldwell et al.,

2011). Early life-stages are often identified as the most sensitive part of an organism's life-cycle, and therefore are potentially more susceptible to change in pH compared to adults. More importantly this area of the life cycle is a key driver to the success of populations (Martin et al., 2011). Information is therefore vital in enabling the scientific community to predict organismal responses, and potentially population dynamics, by measuring the impact on larval success.

Much of the information generated so far has however been gained from offspring derived from ambient sources. By this we mean that adults have been sourced from present-day coastal areas, spawned under ambient laboratory conditions and the subsequent gametes / offspring used for experimental investigation. These ambient sourced gametes / offspring have generally been directly introduced to low target pH conditions and their responses reported. Such studies are certainly interesting because they demonstrate the physiological flexibility which present-day organisms exhibit and possibly highlight the underlying mechanisms which might be used to deal with change in the future. Such approaches may also be representative of dramatic CO_2 sequestration leak scenarios resulting in reduced pH in localized areas of the water column. In a wider global context, however, and with respect to the real-time scale of ocean acidification (decadal changes), organisms might acclimate, produce and deposit gametes, reproduce and adapt to change (Dam, 2013). Therefore the method of direct transfer of ambient sourced gametes/ offspring to low seawater pH may drive experiments to certain responses which are likely to be chronic and acute. These in turn could produce misleading predictions for organismal responses under future ocean acidification.

Previous studies have shown that different rates of introduction of marine organisms to other laboratory induced environmental parameters can result in very different responses. In one example study, early life-stages of tropical crabs (*Armases miersii*, Rathbun) were introduced directly to acute salinities (Anger, 1996). This rapid introduction resulted in offspring displaying prolonged development times and lower survival compared to controls. In another example, Peck et al. (2009) reported marked differences in the temperature

tolerances of Antarctic marine invertebrates when warmed at different rates. Faster rates of warming (1 ºC d^{-1}) resulted in organismal thermal limits that were 5-10°C higher than those warmed more gradually (1-2 ºC wk^{-1} or m^{-1}). Although this study followed well-established thermal biology protocols and not pCO_2, it certainly highlights, along with Anger's (1996) study, the unpredictable influence that rates of change in laboratory induced environmental factors can have on organismal responses. Comparable studies have not yet been reported for the introduction of organisms to reduced pH conditions. We therefore directed this study towards a technical approach that should be considered for experimental designs assessing early life-stage responses towards altered seawater pH conditions emulating ocean acidification. This is an aspect not considered in widely available guidelines on ocean acidification research (e.g. EPOCA; Riebesell et al., 2010) which could be used to validate previously published larval responses.

In this study we assessed the responses of early-life stages of sea urchins when introduced to reduced seawater pH at different rates. This is not an effort to make organismal predictions to ocean acidification because realistically gametes of echinoids will deposit and develop under the gradual reduction of seawater pH. In this study we are following the methods taken by previous studies and comparing it to slower rates of introduction purely to determine whether responses of offspring differ or not under laboratory conditions. Early and later development stages of offspring were also assessed under reduced seawater pH to determine the impact of rapid exposure at different stages of development. We also extended the assessment of larval development and survival to include settling juvenile stages to determine whether pre-settlement stage outcomes are continued through to post-settling juvenile stages. The sea urchin (*Psammechinus miliaris* (P.L.S. Müller, 1771)), was utilised due to these organisms being proposed as a suitable model organism for toxicity studies (e.g. Matranga et al., 2000) with well-established reproductive and larval rearing methodology (McEdward and Herrera, 1999; Kelly et al., 2000; Liu et al., 2007).

Materials and Methods

Sea urchin source and maintenance

Psammechinus miliaris (total n = 30; 29.3 ± 3.0 mm test diameter) were collected by scuba divers in August 2009 during the low spring tide from 3-10 m depth at Rubha Garbh, Loch Creran, Scotland (Symonds et al., 2009; Suckling et al., 2011). Animals were transferred to aquaria held at the Scottish Association for Marine Science, Oban. *P. miliaris* were maintained in one 100 L aquarium and fed *Saccharina latissima* ((Linnaeus) C.E. Lane, C. Mayes, Druehl and G.W. Saunders, 2006; formerly *Laminaria saccharina*, (Linnaeus) J.V. Lamouroux, 1813) for 48 h until spawning was induced. Seawater supplied to adults was UV sterilized and 5-µm mechanically filtered at ambient temperature (14.6 ºC) and salinity (32 psu). Animals were held under a sixteen hours light and eight hours dark cycle for the duration of the experiment as described in Kelly et al. (2000).

Spawning and larval rearing

Adult *P. miliaris* were spawned and reared following the methods described by Kelly et al. (2000) and Liu et al. (2007). Animals were selected for spawning at random from the 100 L holding tank until the ratio of 15 females and 2 males was achieved. *P. miliaris* were spawned using injection of 1-2 mL of 0.5 M KCl administered through the peristomal membrane into the coelom (Kelly et al., 2000; Liu et al., 2007). After injection urchins were stored in ~ 300 mL glass jars filled with 5-µm filtered ambient seawater until gametes were shed and then adults were returned to their 100 L housing aquarium. Jars were kept closed with lids during spawning and prior to fertilization to prevent accidental cross-contamination of gametes and ensure targeted fertilization within a precise time. Clean lab gloves and pipettes were utilized for the handling of every sea urchin and transfer of gametes as a further measure to prevent non-target fertilization from occurring. Eggs from 15 females were combined and distributed across all buckets (three replicates per treatment) until they formed a monolayer at the bottom of each bucket containing 12 L of filtered seawater and sealed with a lid. Following the protocols of Kelly et al. (2000) and Liu et al. (2007) eggs were fertilized under control conditions by adding 1-2 mL of diluted (1:5) sperm from 2 males. After 45

minutes, seawater was gently mixed and samples were removed and viewed under a compound light microscope to check for the successful fertilization of eggs (mean ± SE = 80 ± 3 %) by the presence of a fertilization membrane. Gametes which are not quantified could have important impacts on fertilisation success. For example polyspermy (eggs fertilised by more than one sperm) can reduce rates of fertilisation (Levitan, 2006). However fertilisation success was relatively high and was not under assessment in this study. Buckets were then supplied with pre-mixed CO_2 (BOC, UK; see section 'Rates of introducing CO_2') or ambient air at ~ 0.2 L min^{-1} via bubbling through glass rods (Kelly et al., 2000; Lui et al., 2007).

Twenty four hours after fertilization, five 10 mL samples were removed and checked for mortality and development stage (using a compound light microscope). Swimming gastrulae were observed in all replicates and treatments and therefore could be progressed further. Gas delivery was removed from all cultures and buckets were left static for up to half an hour to allow swimming gastrulae to swim to the surface of the culture vessels. The first 2 L of all cultures were decanted into individual glass beakers to capture the swimming gastrulae and to quantify their densities (Kelly et al., 2000; Liu et al., 2007). Densities were quantified by gently mixing the beakers, decanting 1 mL samples onto a 1 mL Sedgewick rafter cell and counted under a compound microscope. The cultures were then decanted into their respective treatment and replicate buckets at a density of ~ 1 ind mL^{-1}. Complete seawater exchanges with appropriately treated seawater were carried out every two days, with larvae retained in a 40-µm mesh sieve in a water bath while buckets were cleaned and filled.

Once the stomach had fully formed (gastrula stage, 48 h after fertilization) 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, the daily feeding rate was 1500, 4500 and 7500 algal cells mL^{-1}, respectively (Kelly et al. 2000). Algal cells were quantified with a Neubauer double chamber counter and then the appropriate volume of algal culture was decanted into larval cultures.

Larvae were considered competent for settlement when the rudiment was similar in size to the stomach and spines and tube feet were visible in this structure. Their capacity to settle was quantified by placing larvae into Petri dishes (diameter = 90 mm) conditioned with a natural biofilm of marine bacteria and algae including diatoms (Hinegardner, 1969). Fifty echinoplutei larvae were decanted in triplicate per replicate 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 ambient treatment were placed on a small tray and left exposed to the ambient air in a CT room at ~ 16ºC. For experimental treatments, a constant high CO_2 environment was maintained by housing Petri dishes in propagators (B&Q Verve 42 cm unheated). Propagators were adapted by inserting three 0.5 mm HDPE hoses and sealing the tray and cover together with silicone grease and bulldog clips, all other openings were sealed closed with silicone sealant prior to the experiment (Fig. 1). Pre-mixed CO_2 gas was supplied to the propagator at ~ 0.1 L min^{-1} via two of the 0.5 mm HDPE hose inlets, the third functioned as a vent to inhibit back diffusion of ambient air (Fig. 1). Blank reduced pH seawater samples were maintained in this environment simultaneously and were measured for pH each day, which remained constant.

Temperature and pH (NIST certified) were recorded daily for all larval cultures. Seawater parameters (see section 'Rates of introducing CO_2') were measured for new treatment seawater and 2-day-old seawater prior to water exchange. Seawater samples in the static samples were filtered through a 40-µm mesh basket to prevent the removal of larvae.

Rates of introducing CO_2

Introduction of early *P. miliaris* life-stages to year 2100 scenarios (840 ppm Houghton et al., 2001; Guinotte and Fabry, 2008; from ambient control starting conditions) was achieved in a step-wise manner via increasing [CO_2] concentrations from 380, to 470, 560, 700 and finally 840 ppm at different rates of change and maintained at 840 ppm [CO_2] until the experiment was terminated. Four experimental regimes were followed (Fig. 2): C:

Constant control conditions; F: Fast rates of introduction with a [CO_2] change every 3 h until reaching 840 ppm, with the application of increasing CO_2 being applied during the cleaving (2-cell) embryo stage on Day 1; CF: Fast rates of introduction (as above, but applied from Day 9 when larvae were at the 4-armed echinopluteus stage); S: Slow rate of introduction where a [CO_2] change was instigated every 48 h until 840 ppm [CO_2] was achieved (during the cleaving embryo stage; treatment S; Fig. 2). All air and premixed gas (840ppm ~ pH 7.7) was supplied at a rate of 0.2 L min^{-1} into buckets.

Daily temperature and pH_{NIST} (YSI Model 63) were recorded, along with twice weekly salinity (psu; YSI Model 63) and TCO_2 (mmol L^{-1}; Ciba Corning TCO_2 Analyzer 965, Olympic Analytical, UK) for all treatment replicates. The YSI Model 63 was calibrated every second or third day with NIST certified pH buffer solutions. The TCO_2 analyzer was calibrated with 2 g L^{-1} CO_2 standard prior to measurements. Sixty mL were also extracted from each treatment mixing tanks, filtered through GFF filter papers, 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)). Seawater pH conditions under which *P. miliaris* larvae were exposed in this study are presented in Fig. 3. Mean seawater parameters under which sea urchins were exposed after reaching target pH levels are presented in Table 1. These values include data derived from pre- and post-seawater changes, the addition of microalgal food and settlement plate seawater. Values reported for control conditions are within the normally experienced range for shallow coastal seawater (Barry et al., 2010; Gazeau et al., 2010; Suckling et al., submitted). Control seawater was supersaturated ($\Omega > 1$) with respect to calcite and aragonite (Table 1). The reduced pH treatment was saturated with respect to calcite and undersaturated ($\Omega < 1$) with respect to aragonite (Table 1).

Larval morphometrics

Early larval stages were fixed for morphometric measures after 7 and 17 days, based on the protocols of McEdward and Herrera (1999) and Kelly et al. (2000). Echinoplutei larvae were photographed on an inverted compound microscope with a digital camera and a 1 mm

graticule at relevant magnifications. Photography was carried out within 25 days of the experiment to avoid deterioration arising from long-term storage in unbuffered 4% formalin. Calibrations and measurements were then quantified utilizing ImageJ Analysis software (Abramoff et al., 2004). Approximately 30 larvae per replicate (3 replicates per treatment) in each treatment were measured for various parameters dependant on developmental stages. Parameters measured included larval length (LL), larval width (LW), body length (BL), body rod (BR), postoral arm length (PO), postoral arm rod (POR) and rudiment length (RUD).

Development stages were recorded as the main categories of 2-armed, 4-armed, 6-armed and 8-armed plutei 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 (see Fig. 4 for example photographs).

Statistical analyses

Treatment differences were analysed using either Nested ANOVA via a General Linear Model or One Way ANOVA (Kelly et al., 2000) after testing for normality (Anderson Darling Test) and homogeneity of variance ($P > 0.05$) in Minitab (Statistical Software™ Version 15). After significant ANOVA results, Tukey's or Bonferronni's Pairwise Comparisons were utilized to determine which treatments differed. The relationship between body length and postoral arm length (natural logarithm transformed) were analyzed utilizing Analysis of Covariance (ANCOVA; Sokal and Rohlf, 1995). Prior to ANCOVA, regression analysis determined significant linear relationships between these morphometrics and homogeneity of slopes established. Non-parametric Kruskal Wallis tests were carried out where heterogeneous residual variability remained after transforming data following Sokal and Rohlf (1995). Proportional data were arcsin transformed prior to analysis (Kelly *et al.*, 2000). Where Type I errors occurred the means and 95% confidence intervals were graphically analyzed 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.

Results

Larval survival

Decreasing survival occurred in larval cultures with time (Fig. 5), but there were no significant differences between treatments until day 22 (One way ANOVA; day 6: $H_{(3)} = 3.81$, $P = 0.278$; day 8: $F_{(3,8)} = 2.51$, $P = 0.133$; day 10: $F_{(3,8)} = 1.16$, $P = 0.383$; day 12: $F_{(3,8)} = 0.15$, $P = 0.925$; day 14: $F_{(3,8)} = 1.38$, $P = 0.318$; day 16: $F_{(3,8)} = 2.24$, $P = 0.161$; day 18: $F_{(3,8)} = 2.33$, $P = 0.151$; Fig. 5). On day 22 the ANOVA probability value was close to the level of acceptance ($P = 0.053$), therefore paired t-tests were utilized to elucidate treatment effects. This showed that the numbers of larvae on day 22 which had been introduced to reduced pH at fast (F) and slow (S) rates were significantly lower compared to the control held at ambient pH (C vs S: $T_{(4)} = 5.77$, $P = 0.004$; C vs F: $T_{(4)} = 3.61$, $P = 0.023$; Fig. 5). Four-armed plutei rapidly introduced to reduced pH (CF) showed large variation in the numbers of surviving larvae and these did not significantly differ from the remaining treatments (C, S and F; C vs CF: $T_{(4)} = 1.27$, $P = 0.273$; F vs S: $T_{(4)} = 0.21$, $P = 0.847$; F vs CF: $T_{(4)} = 1.08$, $P = 0.340$; S vs CF: $T_{(4)} = 1.36$, $P = 0.245$; Fig. 5).

Stage and abnormal development

The larval development stage did not differ across treatments on days 7 and 17 and were within the range of stages normally expected for *P. miliaris* reared under ambient conditions (Kelly et al., 2000; Table 2). However, abnormal development was ~ 60% higher in larvae rapidly introduced from the echinoplutei stage (D9) to reduced pH (CF) than other treatments on day 17 (One way ANOVA: $F_{(3,8)} = 7.70$, $P = 0.010$; Table 2).

Larval morphometrics and settlement

Larval length measurements: At day 7 offspring in treatment CF were still held under control conditions and had not yet introduced echinoplutei rapidly to low pH conditions (due day 9). These two 'control' treatments (C and CF) were significantly longer (Nested ANOVA: larval length: $F_{(3,314)} = 3.87$, P = 0.010; body length: $F_{(3,314)} = 2.78$, P = 0.041) than larvae introduced to reduced pH at fast rates (F) during the cleaving embryo stage. However, larvae which had been introduced to reduced pH conditions at a slow rate (S) did not differ in length compared to those introduced as fast rates (F) or controls (C and CF; Table 3). Larvae in controls (C) had longer Post oral arms and post oral arm rods (Nested ANOVA: PO: $F_{(3,314)} = 3.42$, P = 0.018; POR: $F_{(3,314)} = 2.71$, *P* = 0.045; Table 3) than those introduced rapidly (F) during the embryo stage to reduced pH conditions. However, larvae which had been introduced to reduced pH conditions at a slow rate (S) did not differ in post oral arm or post oral arm rod lengths compared to those introduced as fast rates (F) or controls (C and CF; Table 3). No other treatment effects were observed for larval width or body rods on day 7 (Table 3).

There were significant linear relationships between ln transformed larval body lengths and postoral arms for all treatments (Table 4). The ratio of postoral arm length and larval body length can be used as a size independent measure of larval shape (McEdward, 1984). On day 7, no differences were found in larval shapes across treatments (Table 5).

Treatment effects on measured larval parameters were apparent in larval widths and body lengths on day 17. Control (C) and 4-armed plutei rapidly introduced to reduced pH (CF) were wider than those introduced during the cleaving embryo stage at slow (S) and fast (F) rates to reduced pH conditions (Kruskal Wallis: $H_{(3)} = 11.91$, P = 0.008; Table 3). Body lengths retained the same treatment effects as day 7, with controls (C) longer than larvae rapidly introduced (F) during the embryo stage (Nested ANOVA: $F_{(3,322)} = 2.95$, P = 0.033; Table 3).

Control (C), slow (S) and fast (F) introduction treatments displayed significant linear relationships between ln transformed larval body lengths and postoral arms (Table 4). Data from the treatment which rapidly introduced 4-armed plutei (CF) to reduced pH conditions

were not linear and therefore were excluded from the ANCOVA analysis (Table 4). No differences were found in larval shapes across the control (C), slow (S) and fast (F) introduction treatments (F and P values provided in Table 5).

Total numbers of larvae metamorphosed and settled as juveniles were similar (One way ANOVA: $F_{(3,8)} = 3.03$, $P = 0.093$; Fig. 6) across treatments with a mean (± SE) for all treatments of 1497 ± 160 juveniles.

Discussion

The aim of this study was to determine whether the direct transfer of ambient-derived offspring to reduced pH conditions is a suitable technical design to use in ocean acidification studies. This is particularly important to investigate since forecasts on organism responses to future climate change are largely based on results gathered from these direct transfer approaches. The rationale was based on previous studies showing an effect of differing rates of introduction of salinity (Anger, 1996) and temperature (Peck et al., 2009) on organism response to laboratory manipulated environmental perturbation. There is also anecdotal evidence from previous ocean acidification work indicating that there may be a complexity of response underlying experimental manipulations, which need further investigation; a range of impacts have been reported from introducing larvae to ocean acidification conditions equivalent to year 2100 forecasts. An example of a positive impact of the effect of pH includes larvae of the orange clownfish, *Amphiprion percula* (Lacepède, 1802), where larvae grew faster than controls (Munday et al., 2009). Some organisms have shown no observed impacts on early life-stages e.g. *Crassostrea ariakensis* (Fujita, 1913) and *C. virginica* (Gmelin, 1791): Miller et al., 2009. However, negative impacts including abnormal development, reduced survival and reduced biomineralization have been reported for a wide range of organisms (e.g. *Saccostrea glomerata* (Gould, 1850): Watson et al., 2009; *Triptneustes gratilla* (Linnaeus, 1758): Sheppard-Brennand et al., 2010; *Haliotis kamtschatkana* Jonas, 1845: Crim et al., 2011. Given that many studies have shown negative impacts, this raises the question of whether these negative responses are a product of exposure

to forecasted ocean acidification scenarios or potentially an acute stress response of larvae when transferred directly to low seawater pH conditions.

Different developmental stages during early life histories have been shown to vary in their responses towards forecasted ocean acidification conditions. Many studies (e.g. Kurihara et al., 2004; Kurihara et al., 2008; Gazeau et al., 2010) have focused on pre-settlement stages, and do not extend to include settled juveniles. The few studies which do include settlement (e.g. Byrne et al., 2011; Dupont et al., 2012) either do not report pre-settlement stage responses in great detail or the post-settlement responses are unclear due to large mortalities within treatment replicates. It has been suggested that there is an increasing sensitivity of sea urchin larvae towards ocean acidification with increasing development time after rapid introduction to altered conditions (e.g. *Paracentrotus lividus* (Lamarck, 1816): Martin et al., 2011; *Strongylocentrotus purpuratus* (Stimpson, 1857): Stummp et al., 2011). In this study pre-settlement (planktotrophic) larvae showed increased mortality in acidic seawater, when acidified from the embryonic stage (at fast (F) and slow (S) rates only), however this was contrasted when post-settlement stages were also considered. This indicates that the former statement (increasing sensitivity with increasing time) may be true for pre-settlement planktotrophic stages but not for metamorphosed juveniles indicating the different levels of sensitivity at different development stages of the life cycle for *P. miliaris*.

Various rates of introduction (fast and slow) were utilised to determine whether the response of *P. miliaris* was a direct result of exposure to reduced pH or whether the response was complicated by the rate of introduction. In this study fast introduction (F) to reduced pH conditions gave rise to the most pronounced negative responses from the larvae of ambient sourced sea urchins. These larvae were consistently the smallest in size compared to controls. Furthermore fast rates (F) of introduction resulted in increased mortality 22 days after fertilization compared to controls. The responses of larvae introduced to reduced pH conditions at a slow rate (S) resulted in fewer, less severe negative responses. On day 7 the sizes of larvae introduced to reduced seawater pH slowly (S) were not statistically smaller than controls nor were they statistically larger than their counterparts introduced at a rapid

rate. It is later in their development when the negative impacts of reduced pH were manifested. For example, 17 days after fertilization slowly introduced (S) larvae, similarly to those introduced rapidly (F), were smaller in width compared to controls. Finally, slow introductions, (S) also resulted in increased mortality 22 days into the experiment. Therefore, when considering pre-settlement stages of offspring, in this case with the sea urchin *P. miliaris*, there is clear evidence that there are variable responses when offspring are exposed to reduced pH at different rates of introduction. The effect on embryos receiving lower pH during early stages of development was more pronounced than offspring experiencing lower pH during later stages of development (see below).

If only the pre-metamorphosis results of our study are considered, then larval responses to reduced pH conditions were similar to those reported in other studies (e.g. Watson et al., 2009; Sheppard-Brennand et al., 2010). Although the fast and slow rates of introduction utilised in this study both resulted in negative responses, which is in agreement with the litereature, when compared to each other they resulted in significantly different responses. Control *P. miliaris* were generally larger than counterparts reared under reduced pH treatments, which is in agreement with other echinoderm studies (e.g. Kurihara and Shirayama, 2004; O'Donnell et al., 2010; Stummp et al., 2011). Studies also support reduced larval survival (by day 22) and abnormal development (Dupont et al., 2008; Doo et al., 2011). Similar findings have been reported for other calcifying groups in terms of size, survival or abnormal development (i.e. molluscs: Watson et al., 2009; Gazeau et al., 2010; Talmage and 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.

When this study was extended to include post-settlement stages, there was no overall impact on numbers of individuals successfully reaching the metamorphosed juvenile stage. In comparison to previous research on early life-stage responses to reduced pH conditions, the study described here, is one of only a few (e.g. Byrne et al., 2011; Dupont et al., 2012) to take echinoderm larvae through to settlement, or at least settlement stages. The post-settlement results in this study contrast with the pre-settlement stage results and may indicate that in

terms of reaching the settled juvenile stage in *P. miliaris*, the rates of introduction of CO_2 when manipulating ambient derived offspring are not relevant. Furthermore any increased exposure to increased CO_2 levels had no effect on numbers successfully developing to settled juveniles. This suggests that a subtle altered selection process (removal of less fit larvae at earlier stages) under reduced pH conditions, and greater mortality at settlement and metamorphosis in controls, may be responsible for the observed negative impacts during development. This subtle altered process of selection may have selected for larvae with specific alleles that improve performance under these conditions as discussed by Pespeni et al. (2013). However, this requires further research to assess how this may impact recruitment and populations in the long term.

It has been suggested that ambient-sourced sea urchin larvae rapidly introduced to reduced seawater pH conditions develop morphologically at a slower rate compared to controls (i.e. smaller at the same sample time post fertilization; Martin et al., 2011; Stummp et al., 2011). However, in this study, no significant stage delay was observed when quantifying development stage directly but smaller larval sizes were observed, and indeed if these represent delays in development then they were subtle. Therefore the approach of analysing larvae at similar development stages rather than at the same sample times, as utilised by Stummp et al. (2011), was not employed in the current study.

In the current study the resilience of larval stages was also investigated. Ambient-derived larvae were introduced to reduced pH conditions from early (cleaving embryo; F and S) and later (4-armed; CF) development stages. Introduction from early stages (cleaving embryos; F and S) generally gave rise to well pronounced negative responses (lower survival and smaller size) compared to later stages (4-armed; CF). The rapid introduction of the 9-day-old 4-armed larvae (CF) resulted in significantly lower negative impacts of increased abnormal development by day 17 but remained similar in size to controls. This could indicate that later stages are more resilient to reduced pH conditions than those introduced at the embryo stage. However this also may be due to the shorter period of time exposed to reduced pH. Stummp et al. (2011) suggest that there is an increase in metabolic rate of feeding larval

development stages under reduced pH conditions compared to controls, likely due to increased maintenance of cellular homeostasis and calcification rates. Therefore our results of abnormal development for larvae introduced during the 4-armed stage (CF) to reduced pH may be an indication of later induced metabolic cost compared to those introduced from the embryonic stage (F and S). The latter were exposed for a longer period to reduced pH and therefore likely confronted with higher metabolic costs resulting in subtle growth differences and mortalities.

Coelomic fluid in regular sea urchins is the principal circulatory medium (Ruppert and Barnes, 1994). Subsequently this will reflect changes which occur in the external environment. Therefore if oceanic pH decreases, the pH within sea urchins will also decrease (Spicer et al., 1988; Miles et al., 2007; Spicer et al., 2011; Dupont and Thorndyke, 2012) so that gametogenesis will take place at lower pH. Therefore widening the life-cycle focus to include the adults (parents) should be considered next in studies observing offspring responses to ocean acidification. This approach would not only provide more realistic insight into organismal responses but it would also provide another validation against studies which have previously used methods directly transferring ambient-sourced offspring into low seawater pH conditions. Recently studies have emerged and improved our understanding of F_1 generation organisms by pre-exposing parents prior to spawning. Parker et al. (2012) showed that after exposing adult oysters, *Saccostrea glomerata*, to laboratory induced ocean acidification conditions, offspring demonstrated capacities to acclimate (and possibly adapt). Similarly Dupont et al. (2012) showed similar responses in the offspring of the sea urchin *Strongylocentrotus droebachiensis* (Müller, 1776) which had been exposed to ocean acidification conditions prior to spawning. In contrast however, Uthicke et al. (2012) found that the responses of the offspring of the sea urchin *Echinometra mathaei* (Blainville, 1825) were not more resilient to ocean acidification conditions after pre-exposing the parents. This requires further investigation for *P. miliaris* and has been reported by Suckling (2012) and Suckling et al. (Submitted).

Ideally multigenerational studies would be used to determine when responses change from physiological flexibility, acclimation to adaptation. However this is difficult to achieve with respect to slow growing invertebrates, such as *P. miliaris* which would take numerous years to achieve. Organisms with rapid life-cycles, such as copepods, therefore provide excellent models for intergenerational responses to altered seawater pH (Dam, 2013). Fitzer et al. (2012) used such an approach and found that *Tisbe battagliai* reallocated resources towards maintaining reproductive output at the expense of somatic growth across three generations. It was concluded that *T. battagliai* has the capacity for phenotypic plasticity but it was unclear on the aspects of acclimation and adaptation (Fitzer et al., 2012). Further work is therefore required to pick apart these types of responses.

We conclude that the use of fast introductions of larvae derived from ambient acclimated adults to altered sea water pH, as utilised in many ocean acidification studies, may potentially result in an acute stress response, manifested as increased numbers of abnormally developed larvae and reduced survival rates. Whilst the current study was only conducted on the sea urchin *P. miliaris*, this methodology clearly needs to be repeated on other sea urchins and a wider range of organisms to determine the prevalence of these effects. 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).

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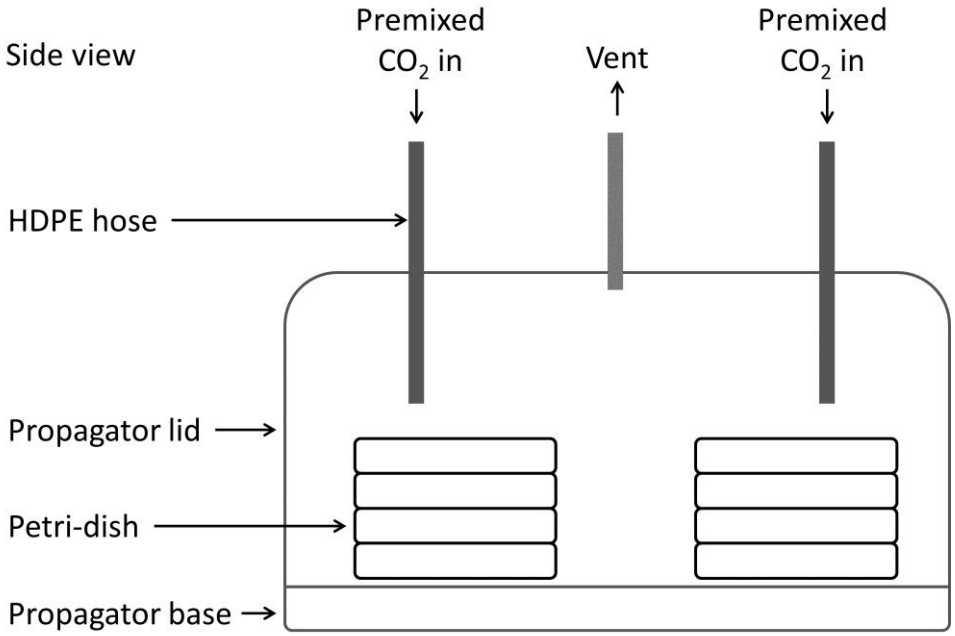

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658 **Fig. 1:** Schematic of settlement chambers comprised of adapted propagators.

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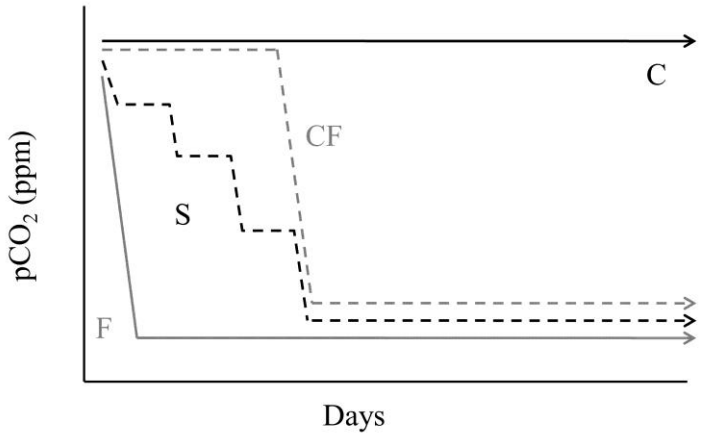

672
673 **Fig. 2:** Treatment rates of pH introduction on the early life-stages of *Psammechinus miliaris.*

674 C ——— = control larvae, CF - - - - = fast introduction of 9 day old control reared larvae

675 to reduced pH conditions, S - - - - = slow introduction of ambient cleaving embryos to

676 reduced pH conditions, F ——— = fast introduction of ambient cleaving embryos to reduced

677 pH conditions.

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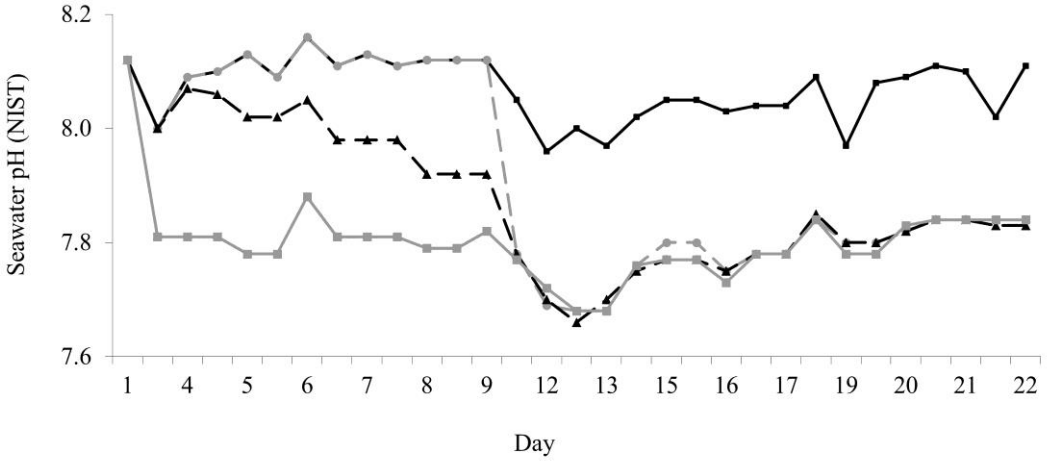

688
689 **Fig. 3:** Seawater pH introduced to *Psammechinus miliaris* larvae (± SE). C = control
690 larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH
691 conditions; S = slow introduction of control cleaving embryos to reduced pH
692 conditions; F = fast introduction of control cleaving embryos to reduced pH
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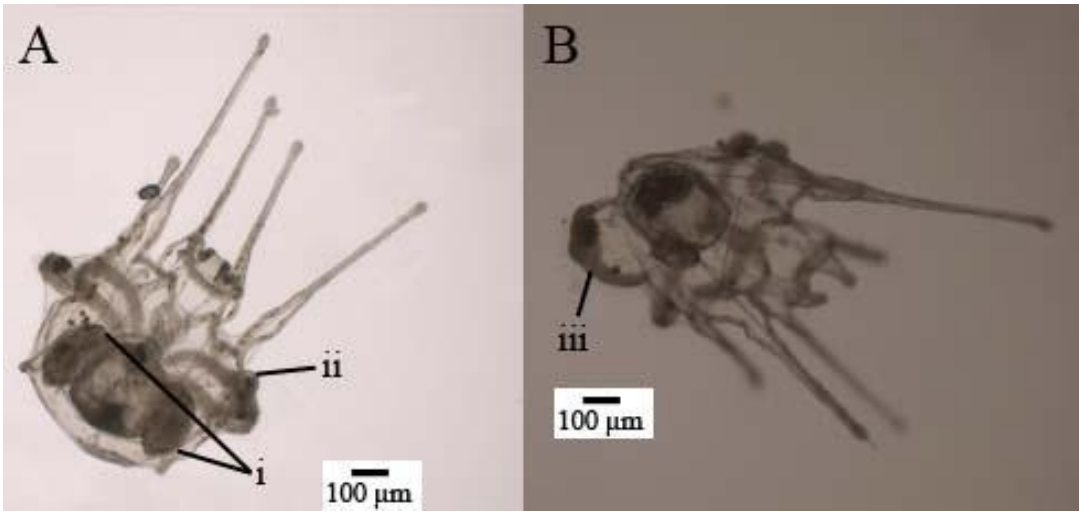


Fig. 4: Example photographs of abnormal development of larval *Psammechinus miliaris* are presented in a) and b): i) the presence of two rudiments, ii) a missing arm and iii) abnormal tissue growth.

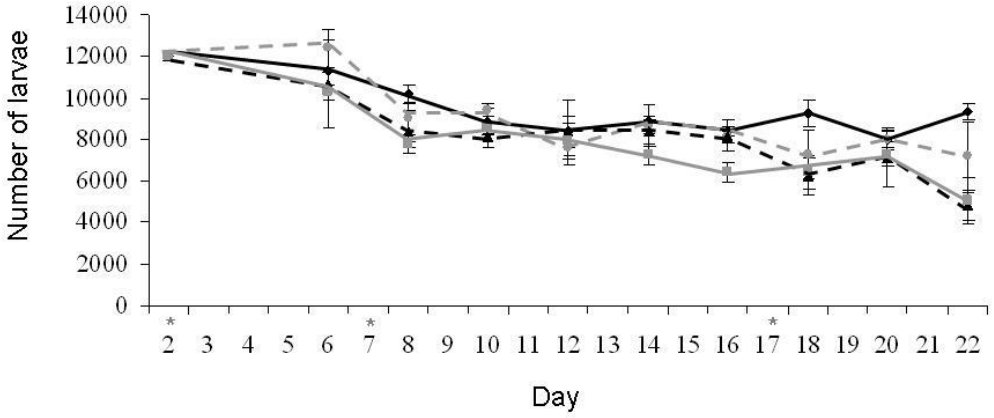
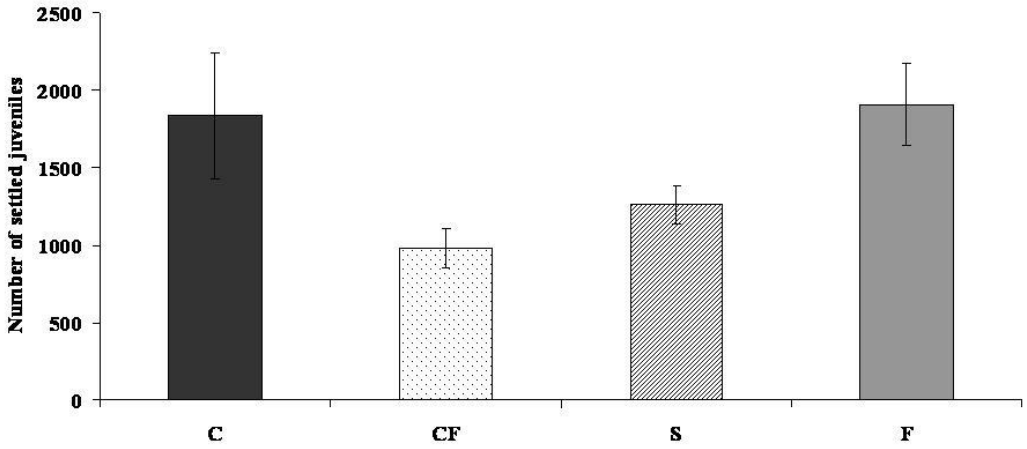


Fig. 5: Larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (± SE) introduced from control to reduced pH at different rates and from different development stages. Settlement tests commenced from ~Day 23, therefore no density data are presented after Day 22. C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F = fast introduction of control cleaving embryos to reduced pH conditions.* = Morphometric measurement sample times (See section 'Larval morphometrics').



739
740 **Fig. 6:** Mean numbers of settled juvenile *Psammechinus miliaris* (± SE) introduced from
741 control to reduced pH at different rates and from different development stages. C ■ =control
742 larvae; CF ⊡ = fast introduction of 9 day old control reared larvae to reduced pH conditions;
743 S ▨ = slow introduction of control cleaving embryos to reduced pH conditions; F ▩ = fast
744 introduction of control cleaving embryos to reduced pH conditions.
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Table 1: Mean (± SE) seawater treatment parameters introduced to *Psammechinus miliaris* larvae after target pH levels were reached. Ω calcite and Ω aragonite values modelled from CO2SYS (Lewis & Wallace, 1998) with refitted constants (Mehrbach, *et al.*, 1973; Dickson & Millero, 1987). C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F = fast introduction of control cleaving embryos to reduced pH conditions.

Seawater parameter	C	CF	S	F
pH_{NIST}	8.08 ± 0.01	7.78 ± 0.01	7.80 ± 0.02	7.79 ± 0.01
pCO_2 (µatm)	537 ± 15	862 ± 6	861 ± 6	867 ± 4
Ω calcite	2.41 ± 0.07	1.37 ± 0.08	1.34 ± 0.08	1.34 ± 0.05
Ω aragonite	1.53 ± 0.05	0.87 ± 0.05	0.85 ± 0.03	0.85 ± 0.03
Temperature (°C)	15.9 ± 0.1	16.0 ± 0.1	16.0 ± 0.1	16.1 ± 0.1
Salinity (psu)	31 ± 1	31 ± 1	31 ± 1	30 ± 1

777 **Table 2:** Mean development stages (± SE; larval numbers) of *Psammechinus miliaris* larvae
778 introduced from control to reduced pH at different rates and from different development
779 stages. C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced
780 pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F
781 = fast introduction of control cleaving embryos to reduced pH conditions. Superscripts
782 indicate where significant differences lie within each column in respective development stage
783 sections. Columns without superscripts indicate no treatment effects.

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		Number of larvae	
Stage	Treat	Day: 7	17
4 arms	C	7660 ± 104	196 ± 196
	CF	9006 ± 615	0 ± 0
	S	7017 ± 1081	379 ± 213
	F	7054 ± 372	240 ± 240
		$F_{(3,8)}$ = 2.04, P=0.187	$F_{(3,8)}$ = 0.70, P = 0.580
6 arms	C	3673 ± 236	942 ± 491
	CF	3394 ±312	585 ± 374
	S	3255 ± 924	1017 ± 230
	F	3106 ± 313	727 ± 141
		$F_{(3,8)}$ = 0.21, P= 0.886	$F_{(3,8)}$ = 0.35, P= 0.794
8 arms	C	-	7126 ± 182
	CF	-	7486 ± 652
	S	-	6696 ± 400
	F	-	5353 ± 312
			$F_{(3,8)}$ = 3.80, P= 0.058
Abnormal	C	1370 ± 544	3117 ± 222[a]
	CF	3065 ± 801	5048 ± 535 [b]
	S	3075 ± 909	3035 ± 164 [a]
	F	2147 ± 621	3145 ± 364 [a]
		$F_{(3,8)}$ = 1.25, P= 0.353	$F_{(3,8)}$ = 7.70, P= 0.010

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Table 3: Mean morphometric and skeletal parameters (± SE; µm) of *Psammechinus miliaris* larvae introduced from control to reduced pH at different rates and from different development stages. C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F = fast introduction of control cleaving embryos to reduced pH conditions. LL = larval length; LW = larval width; BL = body length; PO = postoral arm length; BR = body rod; POR = postoral arm rod; RUD = rudiment. Superscripts indicate where significant differences lie. Columns without superscripts indicate no treatment effects.

Day		LL	LW	BL	PO	BR	POR	RUD
7	C	515.44 ± 9.07 ^{a}	257.39 ± 5.66	374.90 ± 6.16 ^{a}	196.35 ± 4.70 ^{a}	210.68 ± 3.75	284.71 ± 6.37 ^{a}	-
	CF	511.51 ± 7.74 ^{a}	267.91 ± 6.24	372.89 ± 5.30 ^{ab}	194.70 ± 4.75 ^{a}	205.34 ± 3.36	279.08 ± 6.38 ^{ab}	-
	S	504.70 ± 10.30 ^{ab}	248.98 ± 5.68	366.59 ± 6.65 ^{ab}	188.14 ± 6.09 ^{ab}	203.90± 4.21	272.60 ± 7.71 ^{ab}	-
	F	476.85 ± 8.53 ^{b}	251.70 ± 5.36	353.96 ± 5.52 ^{b}	176.28 ± 4.35 ^{b}	198.44± 3.70	260.49 ± 5.75 ^{b}	-
	Statistical result	$F_{(3,214)}$ = 3.87 P = 0.010	$F_{(3,314)}$ = 2.09 P = 0.101	$F_{(3,314)}$ = 2.78 P = 0.041	$F_{(3,314)}$ = 3.42 P = 0.018	$F_{(3,314)}$ = 1.87 P = 0.135	$F_{(3,314)}$ = 2.71 P = 0.045	- -
17	C	1087.70 ± 22.90	727.10± 17.10 ^{a}	642.69 ± 9.70 ^{a}	474.80 ± 19.20	152.57 ± 8.82	737.00 ± 22.40	171.81 ± 6.67
	CF	1097.50 ± 14.60	730.10 ± 10.60 ^{a}	637.36 ± 7.88 ^{ab}	463.40 ± 17.30	137.53 ± 6.03	723.20 ± 17.80	175.61 ± 5.47
	S	1036.90 ± 20.70	675.10 ± 15.60 ^{b}	618.14 ± 9.87 ^{ab}	437.30 ± 18.00	154.62 ± 5.62	673.20 ± 20.50	163.20 ± 10.20
	F	1049.30 ± 20.60	673.60 ± 15.20 ^{b}	609.62 ± 9.38 ^{b}	493.10 ± 18.60	144.80 ± 6.34	731.00 ± 22.00	156.93 ± 8.43
	Statistical result	$F_{(3,322)}$ = 2.07 P = 0.105	$H_{(3)}$ =11.91 P = 0.008	$F_{(3,322)}$ = 2.95 P = 0.033	$F_{(3,322)}$ = 1.75 P = 0.156	$F_{(3,322)}$ = 106 P = 0.368	$F_{(3,322)}$ = 206 P = 0.105	$F_{(3,288)}$ = 1.25 P = 0.293

Table 4: Regression analysis of *Psammechinus miliaris* larvae introduced from control to reduced pH at different rates and from different development stages. The data were ln transformed. C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F = fast introduction of control cleaving embryos to reduced pH conditions. PO = postoral arm length; BL = body length. * = Day 17 CF ratio not linear, therefore removed from subsequent ANCOVA analysis.

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Day	Treatment	Regression	R^2 (%)	DF	F	P
7	C	PO=-1.32+1.11BL	57.0	1, 76	100.76	<0.001
	CF	PO=-2.59+1.32BL	48.6	1, 84	79.48	<0.001
	S	PO=-4.17+1.58BL	37.0	1, 77	45.21	<0.001
	F	PO=-2.75+1.35BL	40.9	1, 80	55.47	<0.001
17	C	PO=-8.22+2.21BL	50.5	1, 84	85.54	<0.001
	CF	PO=6.06+0.001BL	0.0	1, 85	0.00	0.997*
	S	PO=-1.30+1.14BL	22.9	1, 72	21.34	<0.001
	F	PO=-7.45+2.11BL	33.4	1, 85	42.72	<0.001

840 **Table 5:** Analysis of covariance of *Psammechinus miliaris* larvae introduced from control to
841 reduced pH at different rates and from different development stages. Postoral arm lengths
842 were analysed with a covariate of larval body length (BL). The data were ln transformed. C =
843 control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH
844 conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F =
845 fast introduction of control cleaving embryos to reduced pH conditions. * Day 17 CF
846 treatment not linear, therefore not included in ANCOVA analysis.
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Day	Source	DF	MS	F	P
7	Treatment	3	0.065	1.15	0.327
	Covariance (BL)	1	13.205	23..67	<0.001
	Error	320	0.057		
17 *	Treatment	2	0.424	2.41	0.092
	Covariance (BL)	1	24.947	41.43	<0.001
	Error	243	0.176		

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