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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

27 840ppm CO_2 changed at incremental steps at two rates: fast (every 3rd hour) or slow (every
28 48th hour). Direct transfers from ambient to low seawater-pH gave rise to dramatic negative
29 impacts (smaller size and low survival), but slower rates of introductions gave rise to lesser
30 negative responses (low survival). There was no treatment effect on settled juveniles. Fast
31 introductions utilised in many studies are likely not ideal approaches when assessing pre-
32 settlement larval developmental responses. Therefore careful consideration of the pattern of
33 response is needed when studies report the responses of offspring, derived from ambient
34 conditions, introduced directly to forecasted ocean acidification conditions.

35

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

37

38 **Introduction**

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

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

55 2011). Early life-stages are often identified as the most sensitive part of an organism's life-
56 cycle, and therefore are potentially more susceptible to change in pH compared to adults.
57 More importantly this area of the life cycle is a key driver to the success of populations
58 (Martin et al., 2011). Information is therefore vital in enabling the scientific community to
59 predict organismal responses, and potentially population dynamics, by measuring the impact
60 on larval success.

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

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

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

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

109

110 **Materials and Methods**

111 ***Sea urchin source and maintenance***

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

122

123 ***Spawning and larval rearing***

124 Adult *P. miliaris* were spawned and reared following the methods described by Kelly
125 et al. (2000) and Liu et al. (2007). Animals were selected for spawning at random from the
126 100 L holding tank until the ratio of 15 females and 2 males was achieved. *P. miliaris* were
127 spawned using injection of 1-2 mL of 0.5 M KCl administered through the peristomal
128 membrane into the coelom (Kelly et al., 2000; Liu et al., 2007). After injection urchins were
129 stored in ~ 300 mL glass jars filled with 5-µm filtered ambient seawater until gametes were
130 shed and then adults were returned to their 100 L housing aquarium. Jars were kept closed
131 with lids during spawning and prior to fertilization to prevent accidental cross-contamination
132 of gametes and ensure targeted fertilization within a precise time. Clean lab gloves and
133 pipettes were utilized for the handling of every sea urchin and transfer of gametes as a further
134 measure to prevent non-target fertilization from occurring. Eggs from 15 females were
135 combined and distributed across all buckets (three replicates per treatment) until they formed
136 a monolayer at the bottom of each bucket containing 12 L of filtered seawater and sealed with
137 a lid. Following the protocols of Kelly et al. (2000) and Liu et al. (2007) eggs were fertilized
138 under control conditions by adding 1-2 mL of diluted (1:5) sperm from 2 males. After 45

139 minutes, seawater was gently mixed and samples were removed and viewed under a
140 compound light microscope to check for the successful fertilization of eggs (mean ± SE = 80
141 ± 3 %) by the presence of a fertilization membrane. Gametes which are not quantified
142 could have important impacts on fertilisation success. For example polyspermy (eggs
143 fertilised by more than one sperm) can reduce rates of fertilisation (Levitan, 2006). However
144 fertilisation success was relatively high and was not under assessment in this study. Buckets
145 were then supplied with pre-mixed CO_2 (BOC, UK; see section 'Rates of introducing CO_2') or
146 ambient air at ~ 0.2 L min^{-1} via bubbling through glass rods (Kelly et al., 2000; Lui et al.,
147 2007).

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

160 Once the stomach had fully formed (gastrula stage, 48 h after fertilization) feeding of
161 larvae was initiated. *Dunaliella tertiolecta* (Culture Collection of Algae and Protozoa, code
162 19/6B) was cultured following methods described in Liu et al. (2007). For larvae with two,
163 three and four pairs of arms, the daily feeding rate was 1500, 4500 and 7500 algal cells mL^{-1},
164 respectively (Kelly et al. 2000). Algal cells were quantified with a Neubauer double chamber
165 counter and then the appropriate volume of algal culture was decanted into larval cultures.

166 Larvae were considered competent for settlement when the rudiment was similar in
167 size to the stomach and spines and tube feet were visible in this structure. Their capacity to
168 settle was quantified by placing larvae into Petri dishes (diameter = 90 mm) conditioned with
169 a natural biofilm of marine bacteria and algae including diatoms (Hinegardner, 1969). Fifty
170 echinoplutei larvae were decanted in triplicate per replicate and assessed for settlement every
171 24 h for 3 days. Larvae that metamorphosed into juveniles were classed as successful settlers.
172 Settlement Petri dishes for the ambient treatment were placed on a small tray and left exposed
173 to the ambient air in a CT room at ~ 16ºC. For experimental treatments, a constant high CO_2
174 environment was maintained by housing Petri dishes in propagators (B&Q Verve 42 cm
175 unheated). Propagators were adapted by inserting three 0.5 mm HDPE hoses and sealing the
176 tray and cover together with silicone grease and bulldog clips, all other openings were sealed
177 closed with silicone sealant prior to the experiment (Fig. 1). Pre-mixed CO_2 gas was supplied
178 to the propagator at ~ 0.1 L min^{-1} via two of the 0.5 mm HDPE hose inlets, the third
179 functioned as a vent to inhibit back diffusion of ambient air (Fig. 1). Blank reduced pH
180 seawater samples were maintained in this environment simultaneously and were measured for
181 pH each day, which remained constant.

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

186

187 ***Rates of introducing CO_2***

188 Introduction of early *P. miliaris* life-stages to year 2100 scenarios (840 ppm
189 Houghton et al., 2001; Guinotte and Fabry, 2008; from ambient control starting conditions)
190 was achieved in a step-wise manner via increasing [CO_2] concentrations from 380, to 470,
191 560, 700 and finally 840 ppm at different rates of change and maintained at 840 ppm [CO_2]
192 until the experiment was terminated. Four experimental regimes were followed (Fig. 2): C:

193 Constant control conditions; F: Fast rates of introduction with a [CO_2] change every 3 h until
194 reaching 840 ppm, with the application of increasing CO_2 being applied during the cleaving
195 (2-cell) embryo stage on Day 1; CF: Fast rates of introduction (as above, but applied from
196 Day 9 when larvae were at the 4-armed echinopluteus stage); S: Slow rate of introduction
197 where a [CO_2] change was instigated every 48 h until 840 ppm [CO_2] was achieved (during
198 the cleaving embryo stage; treatment S; Fig. 2). All air and premixed gas (840ppm ~ pH 7.7)
199 was supplied at a rate of 0.2 L min^{-1} into buckets.

200 Daily temperature and pH_{NIST} (YSI Model 63) were recorded, along with twice
201 weekly salinity (psu; YSI Model 63) and TCO_2 (mmol L^{-1}; Ciba Corning TCO_2 Analyzer 965,
202 Olympic Analytical, UK) for all treatment replicates. The YSI Model 63 was calibrated every
203 second or third day with NIST certified pH buffer solutions. The TCO_2 analyzer was
204 calibrated with 2 g L^{-1} CO_2 standard prior to measurements. Sixty mL were also extracted
205 from each treatment mixing tanks, filtered through GFF filter papers, and stored at -20 ºC in a
206 light proof container until defrosted for nutrient analysis (phosphate and silicate; duplicate
207 samples; for methods see Nickell *et al.* (2003)). Seawater pH conditions under which *P.*
208 *miliaris* larvae were exposed in this study are presented in Fig. 3. Mean seawater parameters
209 under which sea urchins were exposed after reaching target pH levels are presented in Table
210 1. These values include data derived from pre- and post-seawater changes, the addition of
211 microalgal food and settlement plate seawater. Values reported for control conditions are
212 within the normally experienced range for shallow coastal seawater (Barry et al., 2010;
213 Gazeau et al., 2010; Suckling et al., submitted). Control seawater was supersaturated ($\Omega > 1$)
214 with respect to calcite and aragonite (Table 1). The reduced pH treatment was saturated with
215 respect to calcite and undersaturated ($\Omega < 1$) with respect to aragonite (Table 1).

216

217 ***Larval morphometrics***

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

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

228 Development stages were recorded as the main categories of 2-armed, 4-armed, 6-
229 armed and 8-armed plutei described in McEdward and Herrera (1999). Larvae were classified
230 as abnormal developers when development diverged strongly away from the norm described
231 in McEdward and Herrera (1999) and Kurihara (2008). Examples of abnormal development
232 included missing arms, abnormal tissue growth and the presence of two rudiments (see Fig. 4
233 for example photographs).

234

235 ***Statistical analyses***

236 Treatment differences were analysed using either Nested ANOVA via a General
237 Linear Model or One Way ANOVA (Kelly et al., 2000) after testing for normality (Anderson
238 Darling Test) and homogeneity of variance ($P > 0.05$) in Minitab (Statistical Software™
239 Version 15). After significant ANOVA results, Tukey's or Bonferronni's Pairwise
240 Comparisons were utilized to determine which treatments differed. The relationship between
241 body length and postoral arm length (natural logarithm transformed) were analyzed utilizing
242 Analysis of Covariance (ANCOVA; Sokal and Rohlf, 1995). Prior to ANCOVA, regression
243 analysis determined significant linear relationships between these morphometrics and
244 homogeneity of slopes established. Non-parametric Kruskal Wallis tests were carried out
245 where heterogeneous residual variability remained after transforming data following Sokal
246 and Rohlf (1995). Proportional data were arcsin transformed prior to analysis (Kelly *et al.*,
247 2000). Where Type I errors occurred the means and 95% confidence intervals were
248 graphically analyzed to illustrate the data under normal assumptions. Graphical and tabulated

249 representations of data were presented as the mean and standard errors of treatments unless
250 otherwise indicated.
251
252 **Results**
253 ***Larval survival***
254 Decreasing survival occurred in larval cultures with time (Fig. 5), but there were no
255 significant differences between treatments until day 22 (One way ANOVA; day 6: $H_{(3)} = 3.81$,
256 $P = 0.278$; day 8: $F_{(3,8)} = 2.51$, $P = 0.133$; day 10: $F_{(3,8)} = 1.16$, $P = 0.383$; day 12: $F_{(3,8)} = 0.15$,
257 $P = 0.925$; day 14: $F_{(3,8)} = 1.38$, $P = 0.318$; day 16: $F_{(3,8)} = 2.24$, $P = 0.161$; day 18: $F_{(3,8)} =$
258 2.33, $P = 0.151$; Fig. 5). On day 22 the ANOVA probability value was close to the level of
259 acceptance ($P = 0.053$), therefore paired t-tests were utilized to elucidate treatment effects.
260 This showed that the numbers of larvae on day 22 which had been introduced to reduced pH
261 at fast (F) and slow (S) rates were significantly lower compared to the control held at ambient
262 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
263 rapidly introduced to reduced pH (CF) showed large variation in the numbers of surviving
264 larvae and these did not significantly differ from the remaining treatments (C, S and F; C vs
265 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
266 CF: $T_{(4)} = 1.36$, $P = 0.245$; Fig. 5).
267
268 ***Stage and abnormal development***
269 The larval development stage did not differ across treatments on days 7 and 17 and
270 were within the range of stages normally expected for *P. miliaris* reared under ambient
271 conditions (Kelly et al., 2000; Table 2). However, abnormal development was ~ 60% higher
272 in larvae rapidly introduced from the echinoplutei stage (D9) to reduced pH (CF) than other
273 treatments on day 17 (One way ANOVA: $F_{(3,8)} = 7.70$, $P = 0.010$; Table 2).
274
275 ***Larval morphometrics and settlement***

276 Larval length measurements: At day 7 offspring in treatment CF were still held under
277 control conditions and had not yet introduced echinoplutei rapidly to low pH conditions (due
278 day 9). These two 'control' treatments (C and CF) were significantly longer (Nested
279 ANOVA: larval length: $F_{(3,314)} = 3.87$, P = 0.010; body length: $F_{(3,314)} = 2.78$, P = 0.041) than
280 larvae introduced to reduced pH at fast rates (F) during the cleaving embryo stage. However,
281 larvae which had been introduced to reduced pH conditions at a slow rate (S) did not differ in
282 length compared to those introduced as fast rates (F) or controls (C and CF; Table 3). Larvae
283 in controls (C) had longer Post oral arms and post oral arm rods (Nested ANOVA: PO: $F_{(3,314)}$
284 = 3.42, P = 0.018; POR: $F_{(3,314)} = 2.71$, *P* = 0.045; Table 3) than those introduced rapidly (F)
285 during the embryo stage to reduced pH conditions. However, larvae which had been
286 introduced to reduced pH conditions at a slow rate (S) did not differ in post oral arm or post
287 oral arm rod lengths compared to those introduced as fast rates (F) or controls (C and CF;
288 Table 3). No other treatment effects were observed for larval width or body rods on day 7
289 (Table 3).

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

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

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

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

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

310

311 **Discussion**

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

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

334 Different developmental stages during early life histories have been shown to vary in
335 their responses towards forecasted ocean acidification conditions. Many studies (e.g. Kurihara
336 et al., 2004; Kurihara et al., 2008; Gazeau et al., 2010) have focused on pre-settlement stages,
337 and do not extend to include settled juveniles. The few studies which do include settlement
338 (e.g. Byrne et al., 2011; Dupont et al., 2012) either do not report pre-settlement stage
339 responses in great detail or the post-settlement responses are unclear due to large mortalities
340 within treatment replicates. It has been suggested that there is an increasing sensitivity of sea
341 urchin larvae towards ocean acidification with increasing development time after rapid
342 introduction to altered conditions (e.g. *Paracentrotus lividus* (Lamarck, 1816): Martin et al.,
343 2011; *Strongylocentrotus purpuratus* (Stimpson, 1857): Stummp et al., 2011). In this study
344 pre-settlement (planktotrophic) larvae showed increased mortality in acidic seawater, when
345 acidified from the embryonic stage (at fast (F) and slow (S) rates only), however this was
346 contrasted when post-settlement stages were also considered. This indicates that the former
347 statement (increasing sensitivity with increasing time) may be true for pre-settlement
348 planktotrophic stages but not for metamorphosed juveniles indicating the different levels of
349 sensitivity at different development stages of the life cycle for *P. miliaris*.

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

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

369 If only the pre-metamorphosis results of our study are considered, then larval
370 responses to reduced pH conditions were similar to those reported in other studies (e.g.
371 Watson et al., 2009; Sheppard-Brennand et al., 2010). Although the fast and slow rates of
372 introduction utilised in this study both resulted in negative responses, which is in agreement
373 with the litereature, when compared to each other they resulted in significantly different
374 responses. Control *P. miliaris* were generally larger than counterparts reared under reduced
375 pH treatments, which is in agreement with other echinoderm studies (e.g. Kurihara and
376 Shirayama, 2004; O'Donnell et al., 2010; Stummp et al., 2011). Studies also support reduced
377 larval survival (by day 22) and abnormal development (Dupont et al., 2008; Doo et al., 2011).
378 Similar findings have been reported for other calcifying groups in terms of size, survival or
379 abnormal development (i.e. molluscs: Watson et al., 2009; Gazeau et al., 2010; Talmage and
380 Gobler, 2010; Crim et al., 2011). These studies generally concluded that marine calcifiers will
381 respond negatively when exposed to altered seawater pH at forecasted year 2100 conditions.

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

388 terms of reaching the settled juvenile stage in *P. miliaris*, the rates of introduction of CO_2
389 when manipulating ambient derived offspring are not relevant. Furthermore any increased
390 exposure to increased CO_2 levels had no effect on numbers successfully developing to settled
391 juveniles. This suggests that a subtle altered selection process (removal of less fit larvae at
392 earlier stages) under reduced pH conditions, and greater mortality at settlement and
393 metamorphosis in controls, may be responsible for the observed negative impacts during
394 development. This subtle altered process of selection may have selected for larvae with
395 specific alleles that improve performance under these conditions as discussed by Pespeni et al.
396 (2013). However, this requires further research to assess how this may impact recruitment and
397 populations in the long term.

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

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

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

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

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

453 We conclude that the use of fast introductions of larvae derived from ambient
454 acclimated adults to altered sea water pH, as utilised in many ocean acidification studies, may
455 potentially result in an acute stress response, manifested as increased numbers of abnormally
456 developed larvae and reduced survival rates. Whilst the current study was only conducted on
457 the sea urchin *P. miliaris*, this methodology clearly needs to be repeated on other sea urchins
458 and a wider range of organisms to determine the prevalence of these effects. Our data show
459 that careful consideration is needed when studies report the responses of offspring
460 development, derived from ambient conditions, introduced directly to forecasted ocean
461 acidification. Furthermore, this study highlights the importance of wider life-cycle approaches
462 when forecasting organismal responses to ocean acidification (e.g. including settlement
463 success following larval development).

464

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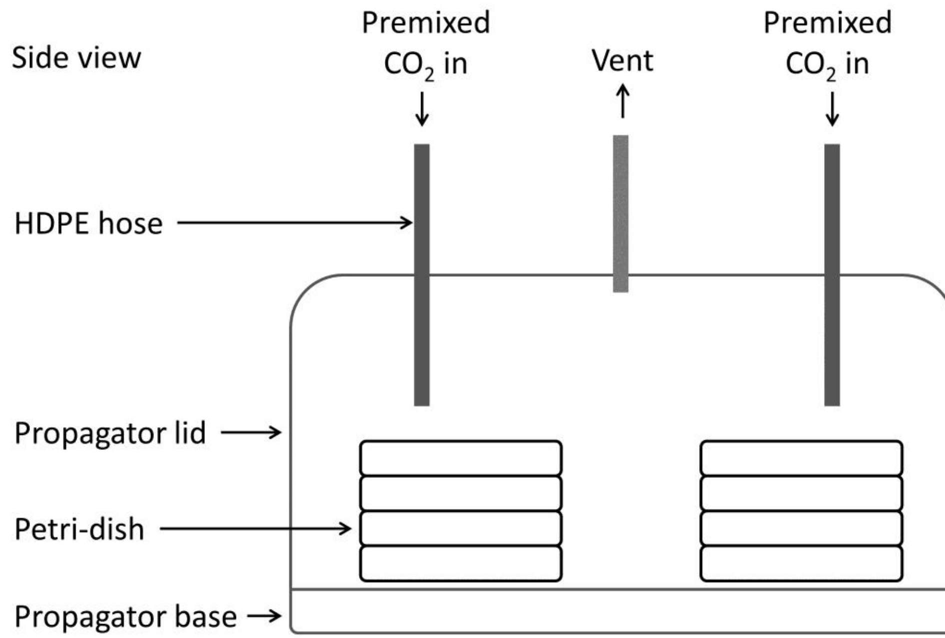

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658 **Fig. 1:** Schematic of settlement chambers comprised of adapted propagators.

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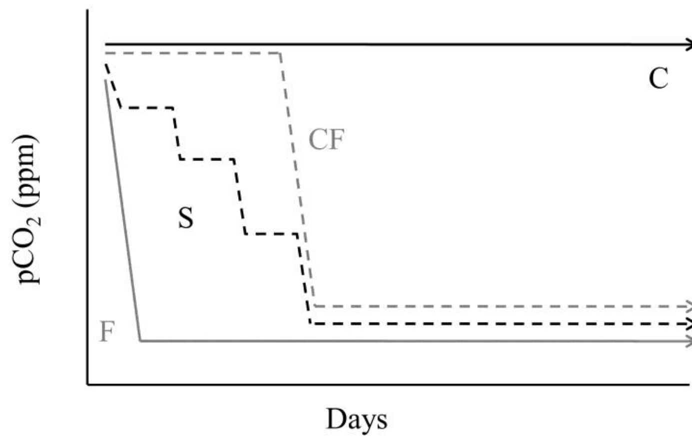

672
673 **Fig. 2:** Treatment rates of pH introduction on the early life-stages of *Psammechinus miliaris.*

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
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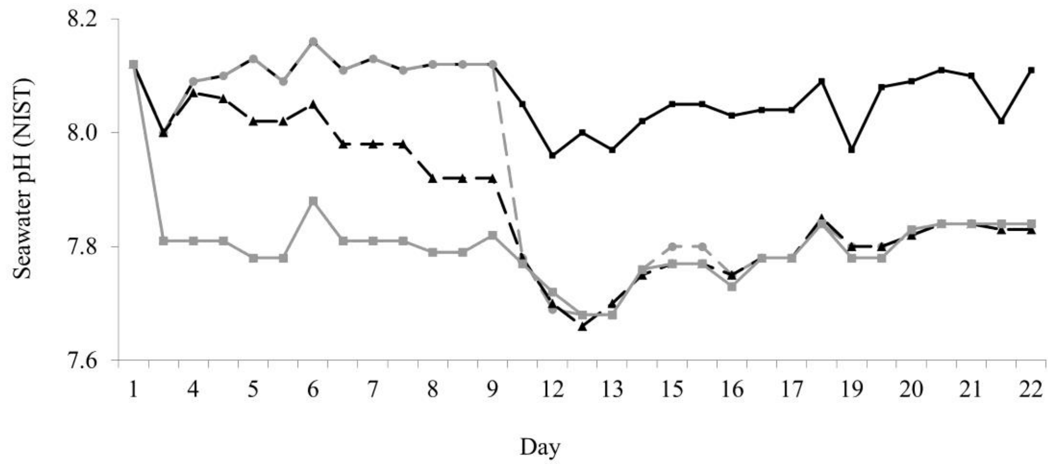

688
689 **Fig. 3:** Seawater pH introduced to *Psammechinus miliaris* larvae (± SE). C = control
690 larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH
691 conditions; S = slow introduction of control cleaving embryos to reduced pH
692 conditions; F = fast introduction of control cleaving embryos to reduced pH
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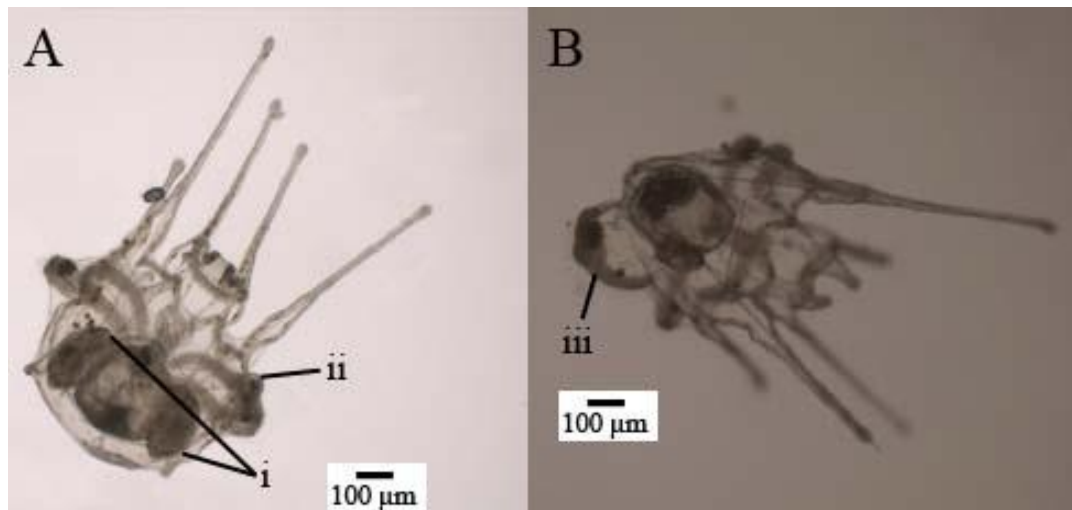
703 

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

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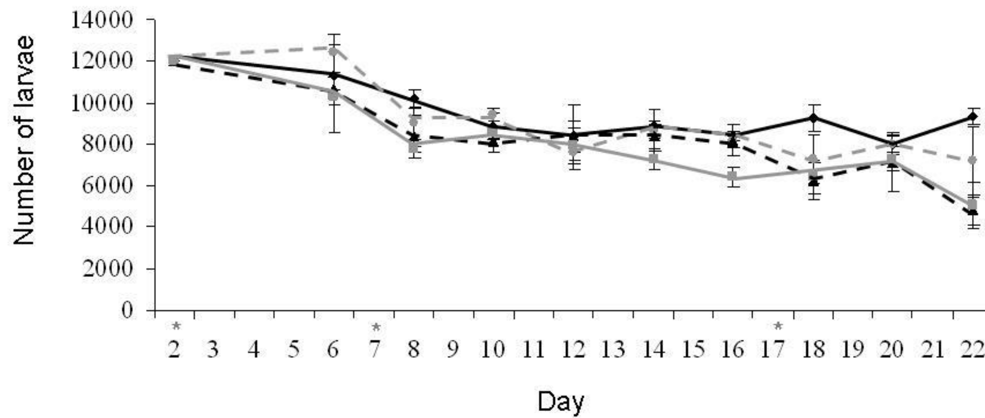

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725 **Fig. 5:** Larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (± SE)
726 introduced from control to reduced pH at different rates and from different development
727 stages. Settlement tests commenced from ~Day 23, therefore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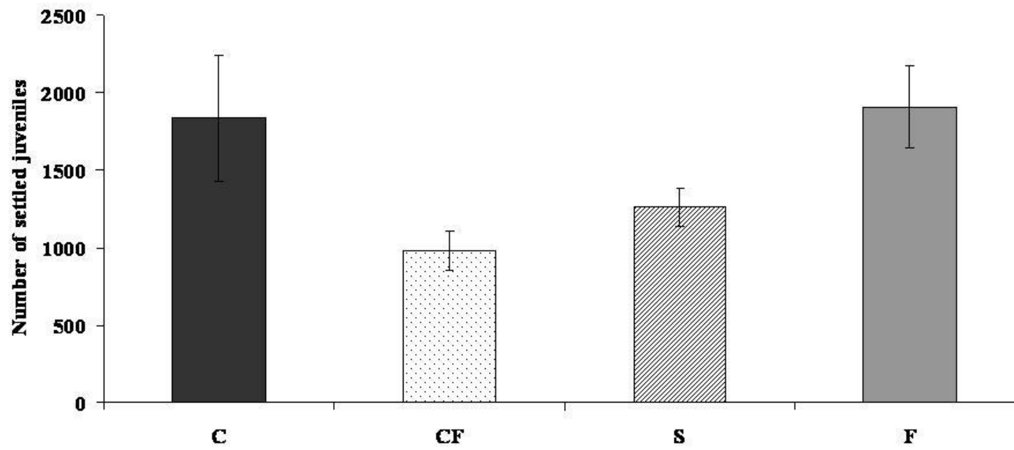

739
740 **Fig. 6:** Mean numbers of settled juvenile *Psammechinus miliaris* (± SE) introduced from
741 control to reduced pH at different rates and from different development stages. C ■ =control
742 larvae; CF ⬚ = fast introduction of 9 day old control reared larvae to reduced pH conditions;
743 S ▨ = 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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755 **Table 1:** Mean (± SE) seawater treatment parameters introduced to *Psammechinus miliaris*
756 larvae after target pH levels were reached. Ω calcite and Ω aragonite values modelled from
757 CO2SYS (Lewis & Wallace, 1998) with refitted constants (Mehrbach, *et al.*, 1973; Dickson
758 & Millero, 1987). C = control larvae; CF = fast introduction of 9 day old control reared larvae
759 to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH
760 conditions; F = fast introduction of control cleaving embryos to reduced pH conditions.

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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

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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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Stage	Treat	Number of larvae 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
	Treatment	3	0.065	1.15	0.327
7	Covariance (BL)	1	13.205	23..67	<0.001
	Error	320	0.057		
	Treatment	2	0.424	2.41	0.092
17 *	Covariance (BL)	1	24.947	41.43	<0.001
	Error	243	0.176		

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