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*Published in:*  
Invertebrate Reproduction & Development

*Publication date:*  
2014

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*Citation for published version (APA):*

Suckling, C. C., Clark, M. S., Beveridge, C., Brunner, L., Hughes, A. D., Harper, E. M., Cook, E. J., Davies, A. J., & Peck, L. S. (2014). Experimental influence of pH on the early life-stages of sea urchins II: increasing parental exposure times gives rise to different responses. *Invertebrate Reproduction & Development*, 58(3), 161-175. <https://doi.org/10.1080/07924259.2013.875951>

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

3

4 Coleen C. Suckling<sup>1,2,3,4</sup>, Melody S. Clark<sup>1</sup>, Christine Beveridge<sup>2</sup>, Lars Brunner<sup>2</sup>, Adam D.  
5 Hughes<sup>2</sup>, Elizabeth M. Harper<sup>3</sup>, Elizabeth J. Cook<sup>2</sup>, Andrew J. Davies<sup>4</sup>, Lloyd S. Peck<sup>1</sup>.

6 <sup>1</sup> British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley  
7 Road, Cambridge, CB3 0ET, UK;

8 <sup>2</sup>Scottish Association for Marine Sciences, Scottish Marine Institute, Oban, Argyll, Scotland,  
9 PA37 1QA, UK;

10 <sup>3</sup> Department of Earth Sciences, University of Cambridge, Downing Street, Cambridge, CB2  
11 3EQ, UK;

12 <sup>4</sup> School of Ocean Sciences, Bangor University, Askew Street, Menai Bridge, Anglesey. LL59  
13 5AB.

14

15 \*Corresponding author: telephone +44-1248-383146, e-mail address:  
16 [coleen.suckling@bangor.ac.uk](mailto:coleen.suckling@bangor.ac.uk) / [coleen.suckling@cantab.net](mailto:coleen.suckling@cantab.net) (C.C. Suckling)

17

18 **Abstract**

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

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

36

37 **Keywords:** CO<sub>2</sub>; echinoderm; larvae; ocean acidification; *Psammechinus miliaris*; settlement.

38

### 39 **Introduction**

40 Predicting the responses of marine organisms to rapid climate change has been a  
41 focus in interdisciplinary science during the last decade. One area in particular is that of ocean  
42 acidification. The oceans have absorbed up to a third of anthropogenic atmospheric carbon  
43 dioxide (CO<sub>2</sub>) since the onset of the industrial revolution. As a consequence oceanic surface  
44 waters have reduced by 0.1 pH units during the last 250 years and are forecasted to reduce by  
45 a further 0.3-0.5 units by the year 2100 (Houghton et al., 2001; Caldeira and Wickett, 2003;  
46 2005; Royal Society, 2005; Canadell et al., 2007). This in turn, will likely cause the  
47 undersaturation of calcium carbonate which may have important consequences for marine  
48 organisms, particularly those which biomineralise (Doney et al., 2009).

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

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

58 An increasing number of laboratory based studies focussing on early life stages of  
59 marine organisms have emerged in recent years (e.g. Dupont et al., 2010; Byrne, 2011).  
60 Although these studies, like those on adults, show a range of responses, the majority report  
61 negative impacts (e.g. abnormality and mortality; Dupont et al., 2008; Kurihara, 2008; Doo et  
62 al., 2011). However, such studies largely utilise offspring derived from ambient sources; the  
63 adults were generally collected from present day coastal areas and then spawned under  
64 present day conditions in the laboratory, after which the subsequent gametes or offspring were  
65 then directly introduced into low seawater pH. The responses of offspring using this approach  
66 may reflect scenarios such as a CO<sub>2</sub> sequestration leak that causes reduced seawater pH in a  
67 localised area. However, ocean acidification occurs over many years and in a wider global  
68 context and at this pace, these organisms could acclimate, produce and deposit gametes,  
69 reproduce and perhaps even adapt to change (Suckling, 2012). These approaches utilise direct  
70 introduction of ambient sourced gametes/offspring into low pH conditions may therefore  
71 report responses that are likely to be acute and chronic demonstrating only the plasticity of  
72 present-day organisms.

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

82 Previous studies on echinoid early life stage responses have largely used ambient  
83 derived offspring, without considering parental pre-exposure or pre-conditioning to reduced  
84 pH conditions (e.g. Havenhand et al., 2008; Kurihara, 2008; Clark et al., 2009; Dupont et al.,  
85 2010a; Byrne et al., 2011), thus not addressing wider parts of the life-cycle. Species such as  
86 *Arbacia dufresnei* (Blainville, 1825), *A. punctulata* and *Paracentrotus lividus* (Lamarck,  
87 1816) appear to not be affected by near-future ocean acidification (Carr et al., 2006; Moulin et  
88 al., 2011; Martin et al., 2011; Catarino et al., 2012). However, negative impacts such as  
89 smaller larval sizes, have been reported for several species due to physiological stress under  
90 lower carbonate saturation states (e.g. *Evichinus chloroticus* (Havenhand et al., 2008);  
91 *Heliodaris erythrogramma* (Valenciennes, 1846): Kurihara & Shiryama, 2004; Kurihara et  
92 al., 2004); *Strongylocentrotus droebachiensis* (Müller, 1776): Stumpp et al., 2011)). These  
93 studies generally concluded that marine calcifiers will respond negatively when exposed to  
94 altered seawater pH at forecasted year 2100 conditions. However, organisms will develop,  
95 mature, deposit gametes and reproduce along with the gradual process of ocean acidification.  
96 Many studies have demonstrated the influence that external factors can have on gamete  
97 development and reproduction, especially in the area of aquaculture (e.g. Shpigel et al., 2004;  
98 Hammer et al., 2006). Therefore these studies, which conclude negative responses, likely  
99 demonstrate instead the physiological flexibilities of present day echinoids to altered seawater  
100 pH conditions.

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

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

121 In this study, we assessed the responses of early life stages of sea urchins (*P. miliaris*)  
122 when derived from parents previously exposed to either control or altered seawater pH for  
123 increasing periods of time (up to 70 days). This species was utilised as it has well-established  
124 reproductive and larval rearing methodology (McEdward and Herrera, 1999; Kelly et al.  
125 2000; Liu et al, 2007). We follow established methods undertaken by earlier studies and also  
126 take into consideration the effects of parental exposure of different durations on offspring. In  
127 contrast to some studies, we also assessed the full cycle of larval development and survival to  
128 include settling juvenile stages.

129

## 130 **Materials and Methods**

### 131 *Sea urchin source and maintenance*

132 Subtidal *Psammechinus miliaris* (total n = 192) were hand collected by scuba divers  
133 in March 2009 from a site at 3-10m depth at Rubha Garbh, Loch Creran (56°30'36.5088" N  
134 5°22'57.1791" W), Scotland described by Symonds et al. (2009). Animals were transferred to  
135 aquaria at the Scottish Association for Marine Science, Oban. The urchins ( $30.74 \pm 0.14$  mm  
136 test diameter (TD  $\pm$  SE)) were selected at random and distributed between 24 three-litre  
137 aquaria (n = 8 urchins per aquaria =  $164 \text{ cm}^2 \text{ urchin}^{-1}$ ). Each aquarium had an independent

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

145

#### 146 ***Artificial diet preparation***

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

155

#### 156 ***CO<sub>2</sub> microcosm***

157 A flow-through CO<sub>2</sub> microcosm (132 L; Fig. 1) was developed and adapted from a  
158 seawater acidification tank system similar to that of Widdicombe and Needham (2007). UV  
159 sterilized and 250 µm filtered seawater was delivered to 60 L closed cylindrical mixing tanks.  
160 This was mixed with CO<sub>2</sub> gas via a ceramic diffuser using an Aquamedic pH controlled  
161 computer and electrode system circulated by a 2000 L h<sup>-1</sup> pump. The computer was set to a  
162 designated pH level ± 0.02 pH units hysteresis. Seawater of set pH was fed by gravity to 12  
163 experimental tanks in parallel, at a rate of 0.92 ± 0.6 L min<sup>-1</sup> per tank. A control system used  
164 an identical set up but excluded the pH control system.

165 Twice weekly, temperature (°C; YSI Model 63), salinity (YSI Model 63), pH<sub>NIST</sub> and  
166 TCO<sub>2</sub> (mmol L<sup>-1</sup>; Ciba Corning TCO<sub>2</sub> Analyzer 965, Olympic Analytical, UK) were measured  
167 and recorded for three replicate experimental tanks per treatment. The TCO<sub>2</sub> analyzer was  
168 calibrated with 2 g L<sup>-1</sup> CO<sub>2</sub> standard prior to measurements. Aquamedic pH probes were  
169 calibrated every second day with NIST certified pH buffer solutions. Sixty mL were also  
170 extracted from each treatment mixing tanks, filtered through GFF filter paper, and stored at -  
171 20 °C in a light proof container until defrosted for nutrient analysis (Phosphate and silicate;  
172 duplicate samples; for methods see Nickell et al. (2003)).

173

#### 174 ***Adult exposure treatments***

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

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



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

204

#### 205 ***Adult data collection***

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

213

#### 214 ***Spawning and larval rearing***

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

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

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

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

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

252           Larval settlement success was assessed following the methods described by Suckling  
253 et al. (submitted). In summary, larvae were considered competent for settlement when the  
254 rudiment was similar in size to the stomach and spines and tube feet were visible in the  
255 rudiment. Capacity to settle was quantified by placing larvae into Petri-dishes conditioned  
256 with a natural biofilm of marine bacteria, algae including diatoms (Hinegardner, 1969). Thirty  
257 echinoplutei were decanted into pre-fouled 90 mm Petri-dishes in triplicate and assessed for  
258 settlement every 24 h for 3 days. Larvae that metamorphosed into juveniles were classed as  
259 successful settlers. Settlement Petri-dishes for the control were placed on a small tray and left  
260 exposed to the ambient air in the CT room at ~ 16 °C. To maintain a constant high CO<sub>2</sub>  
261 environment for larvae reared in reduced pH, Petri dishes were housed in propagators. Pre-  
262 mixed CO<sub>2</sub> gas was supplied to the propagator ~ 0.1 L min<sup>-1</sup>. A 0.5 m HDPE hose vent was  
263 installed to inhibit back diffusion of ambient air. Blank reduced pH seawater samples were  
264 maintained in this environment simultaneously and were measured for pH each day and were  
265 found to remain constant. Numbers of successfully settled juveniles were counted directly and  
266 calculated as a percentage utilising the last larval survival density count. Absolute numbers  
267 settled per 1000 eggs were calculated by utilising an artificial count of egg numbers (base of  
268 bucket surface area divided by measured egg area) because true egg numbers were not  
269 recorded.

270           Temperature and pH<sub>NIST</sub> were recorded daily for the static larval-systems. Seawater  
271 parameters (see ‘CO<sub>2</sub> microcosm’) were measured for fresh seawater and existing seawater  
272 prior to water exchange. Seawater samples in the static cultures were filtered through a 40 µm  
273 mesh to prevent the uptake of larvae into the sampling devices.

274

275 ***Larval morphometrics***

276 Survival was assessed every 2-5 days in triplicate (per treatment replicate, n = 3).  
277 Cultures were gently mixed prior to each 15 mL pseudoreplicate being removed and counted  
278 under a dissection microscope and then returned to respective cultures. Early life stages were  
279 fixed for morphometric measures after 2, 7, 11, 14 and 17 days, based on the protocol of  
280 McEdward and Herrera (1999) and Kelly et al. (2000). Blastulae, gastrulae and echinopluteus  
281 larvae were photographed on an inverted compound microscope with digital camera and 1  
282 mm graticules at relevant magnifications. Calibrations and measurements were then  
283 quantified using ImageJ Analysis software (Abramoff et al., 2004). Approximately 30 larvae  
284 per replicate in each treatment were measured for the parameters described in Fig. 3,  
285 dependent upon developmental stage. Development stages were recorded under the main  
286 categories of 2-armed, 4-armed, 6-armed and 8-armed plutei as described in McEdward and  
287 Herrera (1999). Larvae were classified as abnormal developers when development diverged  
288 strongly away from the norm described in McEdward and Herrera (1999) and Kurihara  
289 (2008). Examples of abnormal development included missing arms, abnormal tissue growth  
290 and the presence of two rudiments (Suckling et al. submitted).

291

292 ***Statistical analysis***

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

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

305

## 306 **Results**

### 307 *Adults*

308 One hundred percent survival was observed for adult *P. miliaris* held under the two  
309 treatment conditions (Control and low pH) from April until July 2009. There were no  
310 significant differences between treatment exposures for test diameter (TD, One-way ANOVA,  
311  $F_{(3,51)} = 0.49$ ,  $P = 0.693$ ), total wet weight (One-way ANOVA,  $F_{(3,51)} = 0.93$ ,  $P = 0.435$ ) or  
312 gonad index (GI, One-way ANOVA,  $F_{(3,51)} = 0.44$ ,  $P = 0.727$ ) amongst adult *P. miliaris*.  
313 Adult data separated by sex showed no significant differences between treatment exposures  
314 (TD, ♂, Kruskal-Wallis,  $H_{(3)} = 1.94$ ,  $P = 0.584$ , ♀, One-way ANOVA,  $F_{(3,27)} = 0.49$ ,  $P =$   
315  $0.690$ ; total wet weight, ♂, One-way ANOVA,  $F_{(3,20)} = 1.95$ ,  $P = 0.154$ , ♀, One-way  
316 ANOVA,  $F_{(3,27)} = 0.69$ ,  $P = 0.568$ ; GI, ♂, One-way ANOVA,  $F_{(3,20)} = 1.04$ ,  $P = 0.395$ , ♀, One-  
317 way ANOVA,  $F_{(3,27)} = 0.46$ ,  $P = 0.712$ ).

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

322

### 323 *Early life stages*

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

330

331 ***Stage and abnormal development***

332           Significantly less larvae from controls developed preoral arms compared to larvae  
333 reared under reduced pH on day 17 (One-way ANOVA,  $F_{(4,10)} = 27.42$ ,  $P = 0.002$ ). All other  
334 development stages were not significantly different across samples days (One-way ANOVAs,  
335  $P > 0.05$ ; Table 4). On day 11 Control larvae had significantly fewer rods protruding from  
336 tissues compared to larvae reared under reduced pH (One-way ANOVA,  $F_{(4,10)} = 77.35$ ,  $P =$   
337  $0.047$ ). Although no other significant abnormalities were found, there was a notable increase  
338 in abnormal development with exposure time across all treatments (Table 5).

339

340 ***Larval morphometrics and settlement***

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

347           During earlier development stages (days 2-7) offspring in controls were larger (larval  
348 length (LL) and width (LW)) than larvae reared in reduced pH treatments (day 2: length One-  
349 way ANOVA,  $F_{(4,605)} = 18.92$ ,  $P < 0.001$ ; width, One-way ANOVA,  $F_{(4,605)} = 41.86$ ,  $P <$   
350  $0.001$ ; day 7: length, Kruskal-Wallis,  $H_{(4)} = 109.87$ ,  $P = 0.002$ , width, One-way ANOVA,  
351  $F_{(4,336)} = 19.89$ ,  $P = 0.002$ ; Fig. 6a and b). However, from day 14 onwards the trend in the size  
352 of offspring appeared to reverse, with controls being smaller than those larvae in reduced pH,  
353 and persisted until metamorphosis and settlement (day 14: length, Kruskal-Wallis,  $H_{(4)} =$   
354  $92.10$ ,  $P = 0.002$ , width, Kruskal-Wallis,  $H_{(4)} = 13.70$ ,  $P = 0.015$ ; day 17: length, Kruskal-  
355 Wallis,  $H_{(4)} = 58.61$ ,  $P = 0.002$ , width, One-way ANOVA,  $F_{(4,385)} = 5.18$ ,  $P = 0.002$ ; Fig. 6a  
356 and b). On day 17 the differing parental pre-exposure times demonstrated varying impacts on  
357 offspring morphometrics. The offspring derived from parents exposed longest to low seawater  
358 pH (42 and 70 d) were significantly larger than those pre-exposed for 28 days (28 d) and

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

367 Body lengths (BL) were longer in controls compared to reduced pH treatments during  
368 early-stages of development (day 7: One-way ANOVA,  $F_{(4,339)} = 12.48$ ,  $P = 0.002$ ; day 11:  
369 Kruskal-Wallis,  $H_{(4)} = 23.46$ ,  $P = 0.003$ ; Fig. 8a). Towards metamorphosis body lengths (BL)  
370 were similar across all treatments (day 14: Kruskal-Wallis,  $H_{(4)} = 4.45$ ,  $P = 0.460$ ; day 17:  
371 One-way ANOVA,  $F_{(4,387)} = 2.79$ ,  $P = 0.040$ ; Fig. 8a). The skeletal body rods (BR)  
372 demonstrated a contrasting response to seawater treatments. During earlier development  
373 stages, 28 d and 42 d offspring generally had longer body rod lengths (BR) than controls (day  
374 7: Kruskal-Wallis,  $H_{(4)} = 43.05$ ,  $P = 0.002$ ; day 11: One-way ANOVA,  $F_{(4,370)} = 8.47$ ,  $P =$   
375  $0.004$ ; Fig. 8b). During later development, controls comprised of longer body rod lengths  
376 (BR) than echinoplutei reared under reduced pH conditions (day 14: One-way ANOVA,  
377  $F_{(4,388)} = 17.64$ ,  $P = 0.002$ ; day 17: One-way ANOVA,  $F_{(4,384)} = 7.40$ ,  $P = 0.002$ ; Fig. 8b).

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

382 Analyses of the percentage of successfully settled echinoplutei show that 28 d, 42 d  
383 and 70 d had significantly higher settlement compared to controls (One-way ANOVA,  $F_{(4,10)} =$   
384  $5.81$ ,  $P = 0.002$ ; Table 6). Further analyses of total numbers of larvae settled in the context of  
385 Day 16 densities and absolute numbers settled per 1000 fertilized eggs revealed no significant

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

388

### 389 **Discussion**

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

395 In this study offspring derived from adults pre-exposed to altered seawater did  
396 respond differently to offspring directly transferred from ambient conditions to low seawater  
397 pH. This is evident from our results for survival and morphometrics during pre-settlement  
398 stages. Controls had highest survival from day 11 compared to offspring derived from parents  
399 previously exposed to low seawater pH (28, 42 and 70 d). However, offspring that were  
400 sourced from ambient reared parents and directly transferred to low seawater pH conditions (0  
401 d) were not significantly different from controls (C) nor from offspring from pre-exposed  
402 parents (28, 42 and 70 d). As offspring reached later stages of development (day 17+)  
403 different responses in morphometrics were observed across experimental treatments.  
404 Offspring sourced from parents pre-exposed to low seawater pH for longest periods of time  
405 (42 and 70 d) were larger than those derived from parents pre-exposed for only 28 days (28 d)  
406 and offspring from ambient sourced parents directly transferred to low pH (0 d).

407 Twenty-eight days of parental pre-exposure appears to be an insufficient amount of  
408 time to demonstrate any observable impact on offspring morphometrics but extending this up  
409 to 42 days gives rise to different responses. Therefore, a minimum period of time of parental  
410 pre-exposure may be required to observe effects on subsequent offspring that would therefore  
411 indicate that these sea urchins have acclimated. Other studies have also shown that parents  
412 have capacities to acclimate when pre-exposed to other environmental changes. Gezelius  
413 (1962) demonstrated that *P. miliaris* has physiological plasticity with regards to salinity



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

421 Acclimation of parents in the longest exposure treatments was also demonstrated by  
422 improved fertilisation success. After 28 days (28 d) exposure to reduced pH fertilization  
423 success was lowest. Increasing the exposure time further (up to 70 days), however, showed  
424 fertilization success levels increased back towards control levels. Low fertilization success  
425 after 28 days could be a result of reduced sperm swimming speed or due to a delay as eggs  
426 guard themselves against polyspermy (becoming fertilised by more than one sperm; Levitan,  
427 2006; Havenhand et al., 2008). However, Caldwell et al. (2011) have shown that sperm  
428 swimming speeds increase for *P. miliaris* under low pH conditions. These data therefore need  
429 to be approached carefully since in these experiments sperm were diluted and not quantified  
430 which may have affected the results, despite relatively high levels of fertilisation observed for  
431 most of the treatments. It is still possible that acclimation underpins the increased fertilisation  
432 success observed with increased exposure time, similarly seen with this species when exposed  
433 to altered salinities (Gezelius, 1962) as discussed above. In conjunction eggs were smallest  
434 from urchins exposed for the longest time period (70 d) to reduced pH.

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

442 2011). Two studies also support our results of reduced larval survival (from day 11; Dupont et  
443 al., 2008; Doo et al., 2011). Similar findings have been reported for other calcifying groups in  
444 terms of size and survival development (i.e. molluscs: Watson et al., 2009; Gazeau et al.,  
445 2010; Talmage & Gobler, 2010; Crim et al., 2011). These studies generally concluded that  
446 marine calcifiers will respond negatively when exposed to altered seawater pH at forecasted  
447 year 2100 conditions.

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

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

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

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

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

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

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

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

523

524 **Acknowledgements**

525 This project was partially financed by core funding to the British Antarctic Survey  
526 (BAS) Adaptations and Physiology group under the BAS Polar Sciences for Planet Earth  
527 programme; a NERC (Natural Environment Research Council) algorithm studentship  
528 (NE/G523539/1) and a SAMS (Scottish Association for Marine Science) Research Bursary.  
529 The authors would like to thank CCAP for use of their facilities.

530

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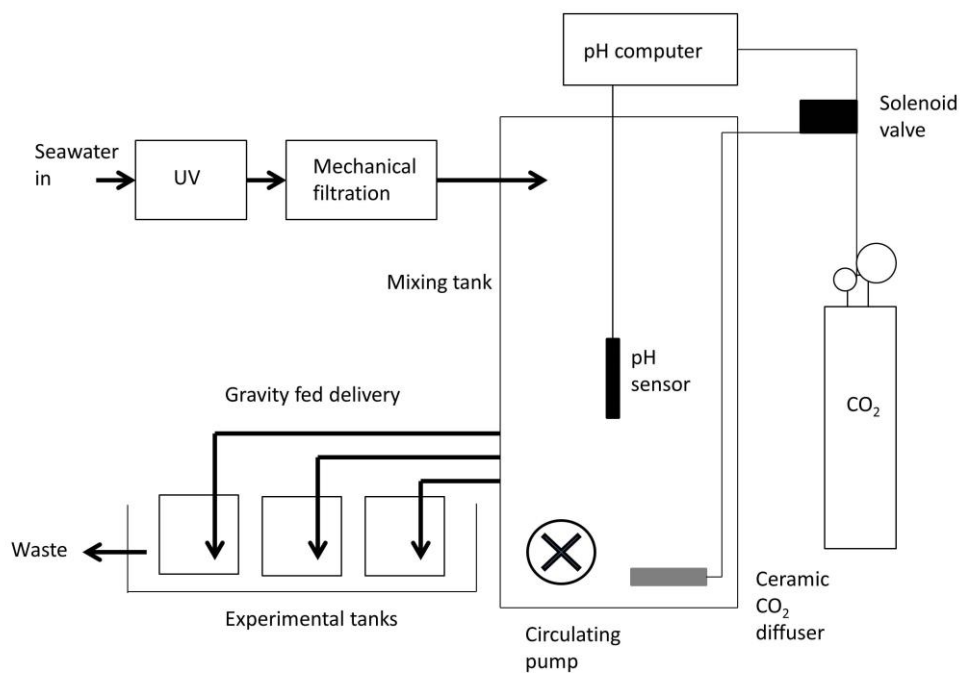
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713 **List of figures**



714

715 **Fig. 1:** Schematic diagram of the CO<sub>2</sub> microcosm adapted from Widdicombe and Needham's  
716 seawater acidification tank system (2007).

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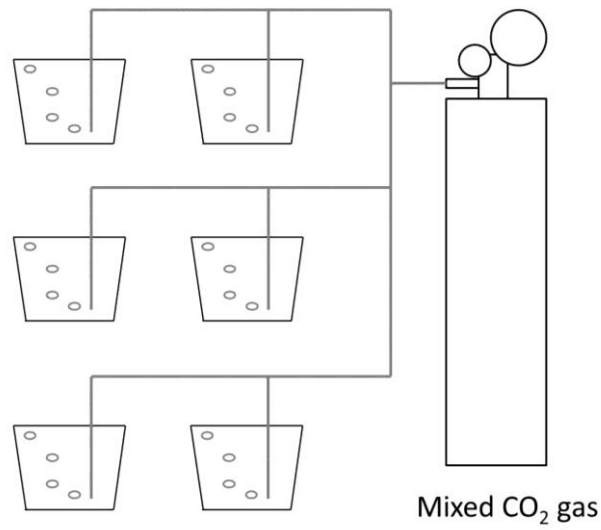
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Experimental tanks – organism  
suspension by premixed CO<sub>2</sub> bubbling



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732 **Fig. 2:** Schematic diagram of the premixed CO<sub>2</sub> larval system.

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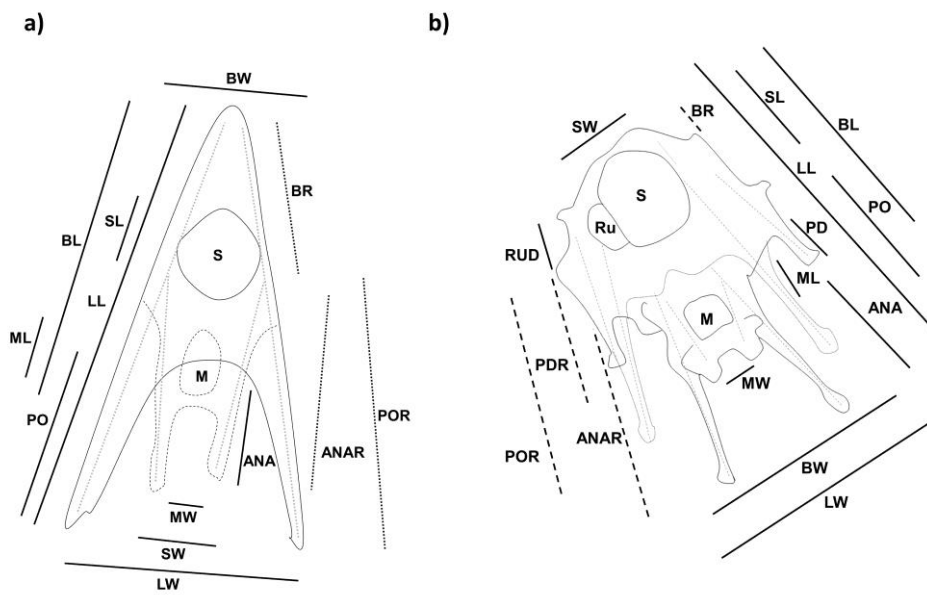
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746 **Fig. 3:** Morphometric parameters measured on gastrulae and echinoplutei (within the larval  
 747 structure outlines 'M' denotes the mouth, 'S' the stomach and 'Ru' the rudiment). Days 7 (a)  
 748 and 14 (b) depicted examples; day 2: length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

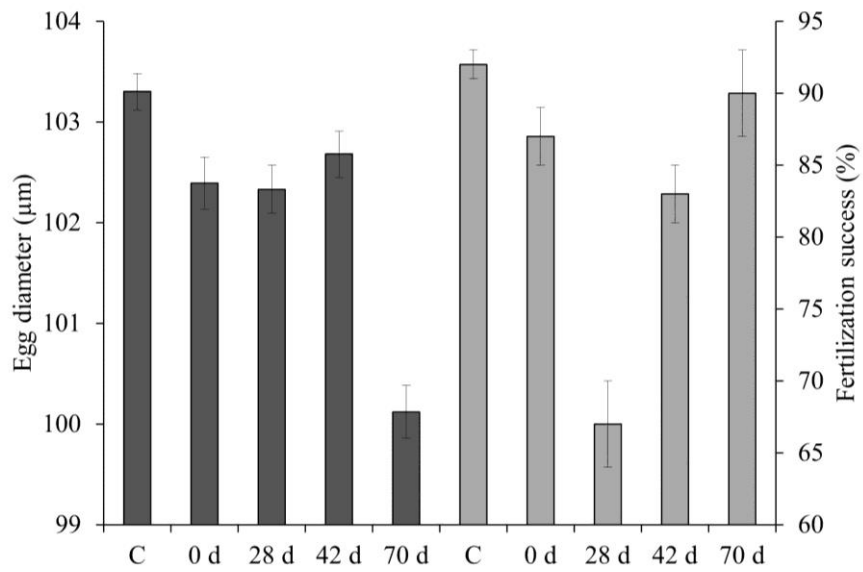
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761 **Fig. 4:** Mean egg diameters (■) and fertilization success rates (□; ± SE) from adults  
 762 exposed to control or reduced pH treatments for different periods of times (d = days). Note  
 763 ordinate axis do not start at zero.

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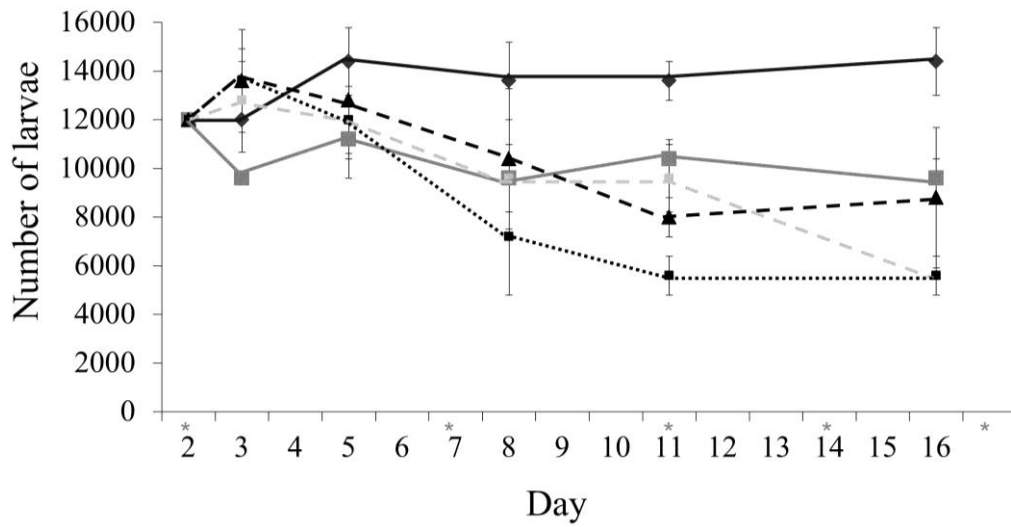
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777 **Fig. 5:** Mean larval survival (total experimental numbers) of *Psammechinus miliaris* larvae ( $\pm$   
 778 SE) derived from adults exposed for differing periods of time to control and reduced pH  
 779 conditions. Settlement tests commenced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

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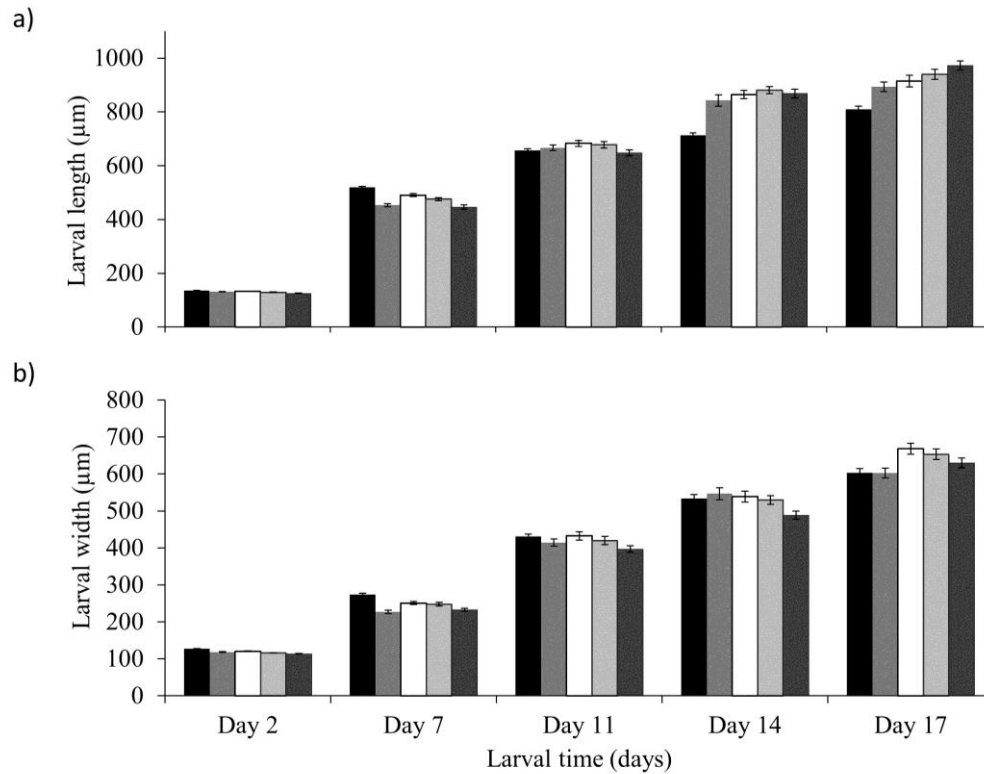
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794 **Fig. 6:** Mean larval lengths (a) and widths (b;  $\pm$  SE;  $\mu\text{m}$ ) for *Psammechinus miliaris*  
 795 echinoplutei derived from adults exposed for differing periods of time to control and reduced  
 796 pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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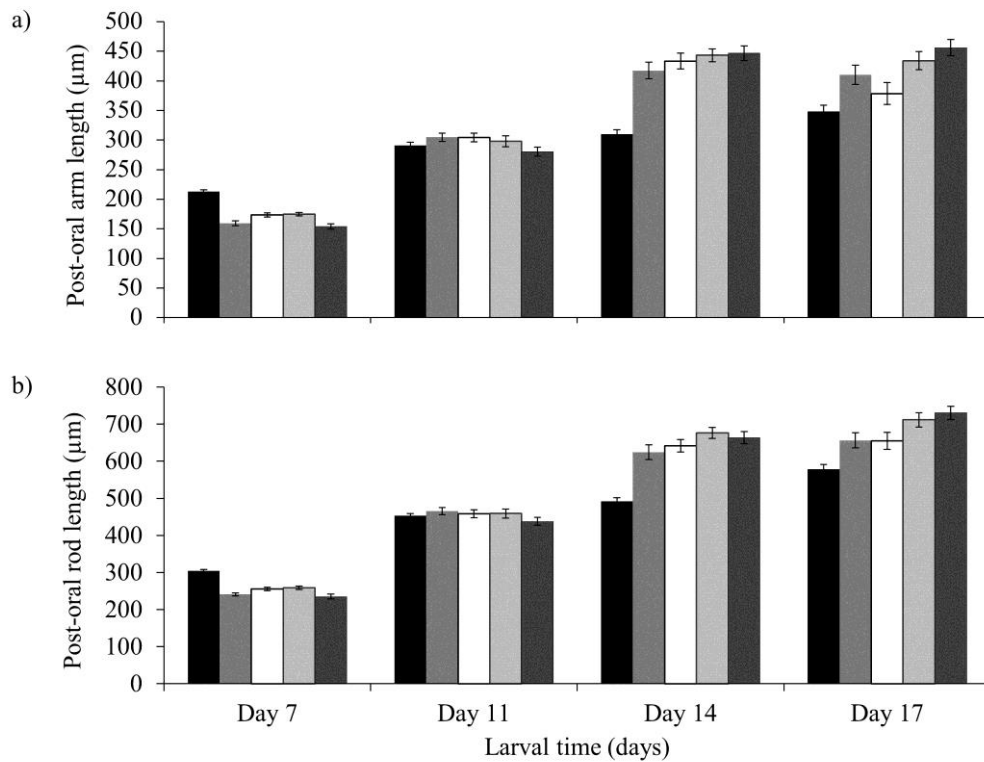
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809 **Fig. 7:** Mean larval post-oral arm (a) and rod lengths (b;  $\pm$  SE;  $\mu\text{m}$ ) for *Psammechinus*  
 810 *miliaris* echinoplutei derived from adults exposed for differing periods of time to control and  
 811 reduced pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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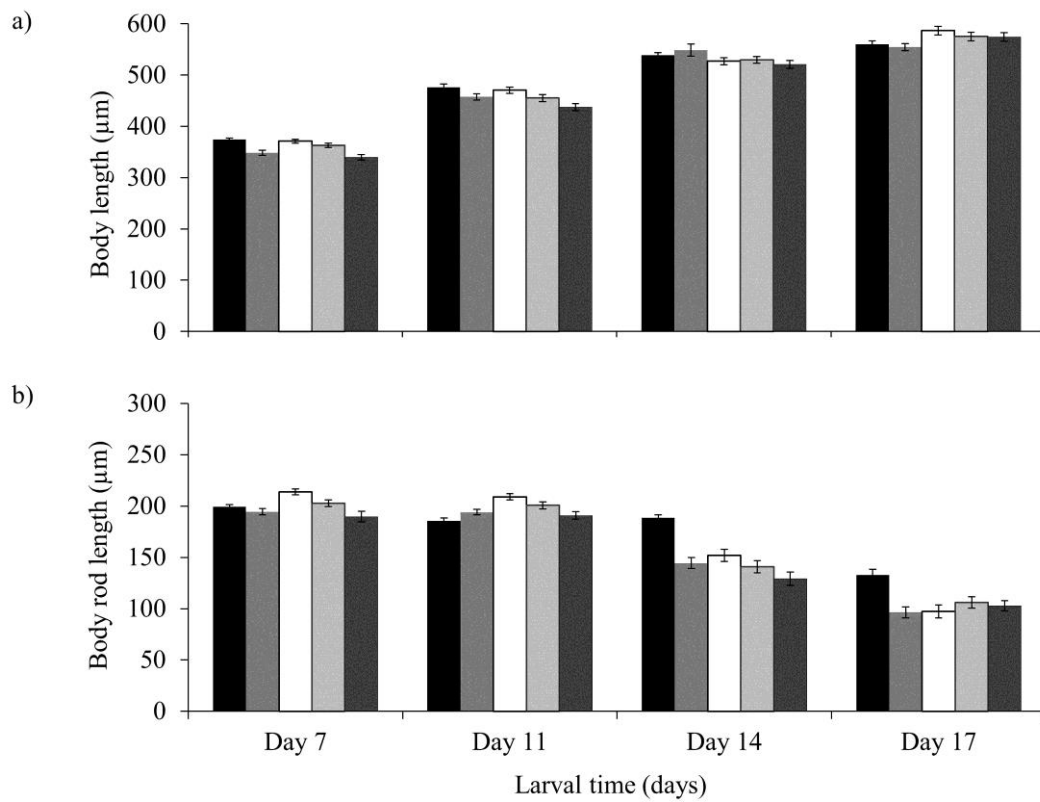
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824 **Fig. 8:** Mean larval body length (a) and rod lengths (b;  $\pm$  SE;  $\mu\text{m}$ ) for *Psammechinus miliaris*  
 825 echinoplutei derived from adults exposed for differing periods of time to control and reduced  
 826 pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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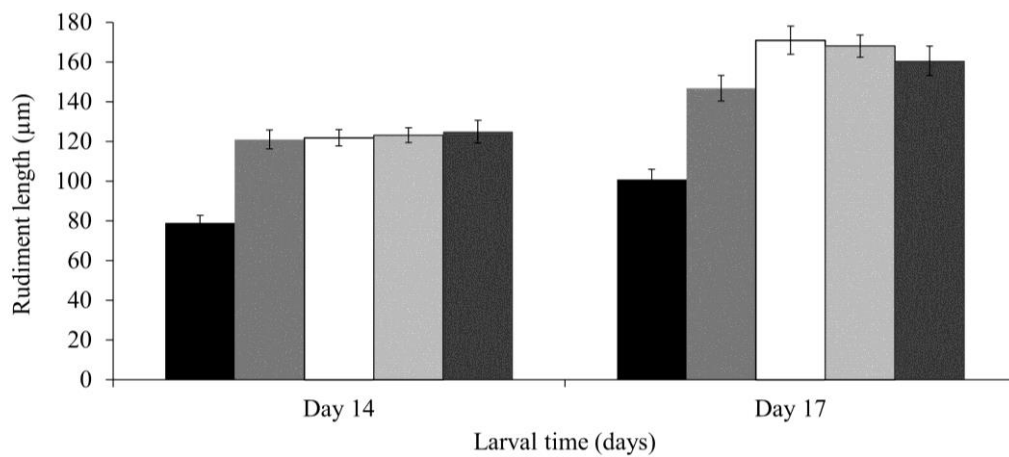
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839 **Fig. 9:** Mean rudiment lengths ( $\pm$  SE;  $\mu\text{m}$ ) for *Psammechinus miliaris* echinoplutei derived  
 840 from adults exposed for differing periods of time to control and reduced pH conditions. ■ =  
 841 Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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

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

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897 **Table 2:** Mean ( $\pm$  SE) seawater treatment parameters for *Psammechinus miliaris*.  $p\text{CO}_2$ ,  $\Omega$   
 898 calcite and  $\Omega$  aragonite values modelled from CO2SYS (Lewis & Wallace, 1998) with  
 899 refitted constants (Mehrbach, *et al.*, 1973; Dickson & Millero, 1987).

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Seawater parameter	Control	Reduced pH
$\text{pH}_{\text{NIST}}$	$7.98 \pm 0.01$	$7.70 \pm 0.01$
$\text{pCO}_2$ ( $\mu\text{atm}$ )	$559 \pm 15$	$999 \pm 22$
$\Omega$ calcite	$2.01 \pm 0.06$	$0.98 \pm 0.13$
$\Omega$ aragonite	$1.28 \pm 0.04$	$0.63 \pm 0.08$
Temperature ( $^{\circ}\text{C}$ )	$12.9 \pm 0.4$	$12.8 \pm 0.4$
Salinity (psu)	$32 \pm 1$	$32 \pm 1$

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

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

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970 **Table 4:** Mean developmental stage (%;  $\pm$  SE) for *Psammechinus miliaris* echinoplutei.  
 971 Superscripts indicate where significant differences lie within each column in respective  
 972 development stage sections. Columns without superscripts indicate no treatment effects.  
 973

Stage	Treat	%			
		Day: 7	11	14	17
4 arms	C	78 $\pm$ 3	3 $\pm$ 2	2 $\pm$ 2	3 $\pm$ 2
	0d	81 $\pm$ 2	12 $\pm$ 1	5 $\pm$ 3	2 $\pm$ 2
	28d	79 $\pm$ 1	12 $\pm$ 3	4 $\pm$ 1	1 $\pm$ 1
	42d	78 $\pm$ 1	21 $\pm$ 8	4 $\pm$ 1	3 $\pm$ 1.9
	70d	87 $\pm$ 2	19 $\pm$ 3	2 $\pm$ 2	1 $\pm$ 1
			F <sub>(4,10)</sub> = 3.39 P = 0.059	H <sub>(4)</sub> = 8.99 P = 0.066	F <sub>(4,10)</sub> = 0.57, P = 0.492
6 arms	C	22 $\pm$ 3	97 $\pm$ 2	27 $\pm$ 12	49 $\pm$ 6 <sup>a</sup>
	0d	19 $\pm$ 2	88 $\pm$ 1	13 $\pm$ 4	16 $\pm$ 3 <sup>b</sup>
	28d	21 $\pm$ 1	88 $\pm$ 3	17 $\pm$ 5	13 $\pm$ 4 <sup>b</sup>
	42d	22 $\pm$ 1	79 $\pm$ 8	17 $\pm$ 3	12 $\pm$ 3 <sup>b</sup>
	70d	13 $\pm$ 2	81 $\pm$ 3	29 $\pm$ 6	13 $\pm$ 2 <sup>b</sup>
			F <sub>(4,10)</sub> = 3.39 P = 0.059	H <sub>(4)</sub> = 8.99 P = 0.066	F <sub>(4,10)</sub> = 1.16 P = 0.460
8 arms	C	0 $\pm$ 0	0 $\pm$ 0	71 $\pm$ 11	49 $\pm$ 4 <sup>a</sup>
	0d	0 $\pm$ 0	0 $\pm$ 0	85 $\pm$ 3	81 $\pm$ 3 <sup>b</sup>
	28d	0 $\pm$ 0	0 $\pm$ 0	82 $\pm$ 7	86 $\pm$ 4 <sup>b</sup>
	42d	3 $\pm$ 3	0 $\pm$ 0	80 $\pm$ 5	85 $\pm$ 3 <sup>b</sup>
	70d	0 $\pm$ 0	0 $\pm$ 0	69 $\pm$ 7	86 $\pm$ 2
			F <sub>(4,10)</sub> = 3.39 P = 0.071	-	F <sub>(4,10)</sub> = 0.97 P = 0.511

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1000 **Table 5:** Mean abnormal development types (%;  $\pm$  SE) for *Psammochinus miliaris*  
 1001 echinoplutei. Superscripts indicate where significant differences lie within each column in  
 1002 respective development stage sections. Columns without superscripts indicate no treatment  
 1003 effects.  
 1004

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

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1011 **Table 6:** Mean settled larvae ( $\pm$  SE) from adults exposed to control or reduced pH treatments  
 1012 for different periods of times (d = days). Columns without superscripts indicate no treatment  
 1013 effects, differing superscripts indicate where significant differences occur.

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Treat	Settlement (%)	Total # settled larvae	Absolute # settled larvae 1000 fertilized eggs <sup>-1</sup>
Control	40 $\pm$	6011 $\pm$	193 $\pm$
	10 <sub>a</sub>	2159	56
0d	55 $\pm$	5260 $\pm$	218 $\pm$
	7 <sub>ab</sub>	1077	53
28d	70 $\pm$	6235 $\pm$	377 $\pm$
	2 <sub>b</sub>	2212	125
42d	69 $\pm$	3780 $\pm$	189 $\pm$
	5 <sub>b</sub>	307	22
70d	82 $\pm$	4520 $\pm$	344 $\pm$
	4 <sub>c</sub>	467	70

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