

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

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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:** CO₂; 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 (CO₂) 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 CO₂ 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 ± 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 ***CO₂ microcosm***

157 A flow-through CO₂ 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 CO₂ gas via a ceramic diffuser using an Aquamedic pH controlled
161 computer and electrode system circulated by a 2000 L h⁻¹ pump. The computer was set to a
162 designated pH level \pm 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⁻¹ 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_{NIST} and
166 TCO₂ (mmol L⁻¹; Ciba Corning TCO₂ Analyzer 965, Olympic Analytical, UK) were measured
167 and recorded for three replicate experimental tanks per treatment. The TCO₂ analyzer was
168 calibrated with 2 g L⁻¹ CO₂ 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 pCO₂ 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 (± 0.01 g) and maximum horizontal test diameter (TD, vernier
208 callipers ± 0.1 mm) were recorded before dissection. Gonad wet weight (± 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 CO₂ (~ 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- μ 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 CO₂ gas was introduced at a rate
239 of 0.2 L min⁻¹ into the header space between the seawater surface and lid for 24 hours.

240 Twenty four hours after fertilisation hatched blastulae densities were quantified for
241 each treatment (three pseudoreplicates) using a 1 mL sedgewick rafter cell and compound
242 microscope. Blastulae were decanted into their respective treatment buckets at a density of ~ 1
243 individual mL⁻¹. Glass tubes were used to deliver relevant treatment gases (at ~ 200 mL min⁻¹)
244 to the bottom of the buckets. Complete seawater exchanges were carried out every two days,
245 with larvae retained in a 40 μ 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⁻¹, 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 CO₂
261 environment for larvae reared in reduced pH, Petri dishes were housed in propagators. Pre-
262 mixed CO₂ gas was supplied to the propagator ~ 0.1 L min⁻¹. 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_{NIST} were recorded daily for the static larval-systems. Seawater
271 parameters (see ‘CO₂ 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

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530

531 **References**

532 Abramoff, MD, Magelhaes, PJ, Ram, SJ. 2004. Image Processing with ImageJ. *Biophotonics*
533 *International*, 11: 36-42.

534 Anger, K. 1996. Salinity tolerance of the larvae and first juveniles of a semiterrestrial grapsid
535 crab, *Armases miersii* (Rathbun). *Journal of Experimental Marine Biology and*
536 *Ecology*, 202: 205-223.

537 Barry, JP, Tyrell, T, Hansson, L, Plattner, G, Gattuso, J. 2010. Atmospheric CO₂ targets for
538 ocean acidification perturbation experiments. Publications Office of the European
539 Union.

540 Benjamini, Y, Hochberg, Y. 1995. Controlling the false discovery rate: A practical and
541 powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series*
542 *B (Methodological)*, 57: 289-300.

543 Byrne, M. 2011. Impact of ocean warming and ocean acidification on marine invertebrate life
544 history stages: vulnerabilities and potential for persistence in a changing ocean.
545 *Oceanography and Marine Biology: An Annual Review*, 49: 1-42.

546 Byrne, M, Ho, M, Wong, E, Soars, NA, Selvakumaraswamy, P, Shepard-Brennan, H,
547 Dworjanyn, SA, Davis, AR. 2011. Unshelled abalone and corrupted urchins:
548 development of marine calcifiers in a changing ocean. *Proceedings of the Royal*
549 *Society of London Series B – Biological Society*, 278: 2376-2383.

550 Caldeira, K, Wickett, ME, 2003. Oceanography: Anthropogenic carbon and ocean pH.
551 *Nature*, 425: 365-365

552 Caldeira, K, Wickett, ME. 2005. Ocean model predictions of chemistry changes from carbon
553 dioxide emissions to the atmosphere and ocean. *Journal of Geophysical Research*,
554 doi:10.1029/2004JC002671.

555 Caldwell, GS, Fitzner, S, Gillespie, CS, Pickavance, G, Turnbull, E, Bentley, MG. 2011. Ocean
556 acidification takes sperm back in time. *Invertebrate Reproduction and*
557 *Development*, 55: 217-221

558 Canadell, JG, Le Quéré, C, Raupach, MR, Field, CB, Buitenhuis, ET, Ciais, P, Conway, TJ,
559 Gillett, NP, Houghton, RA, Marland, G. 2007. Contributions to accelerating
560 atmospheric CO₂ growth from economic activity, carbon intensity, and efficiency of
561 natural sinks. *Proceedings of the National Academy of Sciences*, 104, 18866-18870

562 Carr, RS, Biedenbach, JM, Nipper, M. 2006. Influence of potentially confounding factors on
563 sea urchin porewater toxicity tests. *Archives of Environmental Contamination and*
564 *Toxicology*, 51: 573–579.

565 Catarino, A, De Ridder, C, Gonzalez, M, Gallardo, P, Dubois, P. 2012. Sea urchin *Arbacia*
566 *dufresnei* (Blainville 1825) larvae response to ocean acidification. *Polar Biology*, 35:
567 455-461.

568 Clark, D, Lamare, M, Barker, M. 2009. Response of sea urchin pluteus larvae
569 (Echinodermata: Echinoidea) to reduced seawater pH: a comparison among a
570 tropical, temperate, and a polar species. *Marine Biology*, 156: 1125-1137.

571 Crim, RN, Sunday, JM, Harley, CDG. 2011. Elevated seawater CO₂ concentrations impair
572 larval development and reduce larval survival in endangered northern abalone
573 (*Haliotis kamtschatkana*). *Journal of Experimental Marine Biology and Ecology*, 400:
574 272-277.

575 Dam, HG. 2013. Evolutionary adaptation of marine zooplankton to global change. *Annual*
576 *Review of Marine Science*, 5: 349-70.

577 Dickson, AG, Millero, FJ. 1987. A comparison of the equilibrium-constants for the
578 dissociation of carbonic-acid in seawater media. *Deep-Sea Research*, 34: 1733-1743.

579 Doney, SC, Fabry, VJ, Feely, RA, Kleypas, JA. 2009. Ocean Acidification: The Other CO₂
580 Problem. Annual Review of Marine Science, 1: 169-192.

581 Doo, SS, Dworjanyn, SA, Foo, SA, Soars, NA, Byrne, M. 2011. Impacts of ocean
582 acidification on development of the meroplanktonic larval stage of the sea urchin
583 *Centrostephanus rodgersii*. ICES Journal of Marine Science, Doi:
584 10.1093/icesjms/fsr123.

585 Dupont, S, Havenhand, J, Thorndyke, W, Peck, L, Thorndyke, M. 2008. Near-future level of
586 CO₂-driven ocean acidification radically affects larval survival and development in
587 the brittlestar *Ophiothrix fragilis*. Marine Ecology Progress Series, 373: 285-294.

588 Dupont, S, Ortega-Martínez, O, Thorndyke, M. 2010a. Impact of near-future ocean
589 acidification on echinoderms. Ecotoxicology, 19: 449-462.

590 Dupont, S, Lundve, B, Thorndyke, M. 2010b. Near future ocean acidification increases
591 growth rate of the lecithotrophic larvae and juveniles of the sea star *Crossaster*
592 *papposus*. Journal of Experimental Zoology Part B, 314B.

593 Dupont, S, Dorey, N, Stumpp, M, Melzner, F, Throndyke, M. 2012. Long-term and trans-life-
594 cycle effects of exposure to ocean acidification in the green sea urchin
595 *Strongylocentrotus droebachiensis*. Marine Biology, 1-9. doi: 10.1007/s00227-012-
596 1921-x

597 Fitzer, SC, Caldwell, GS, Close, AJ, Clare, AS, Upstill-Goddard, RC, Bentley, MG. 2012.
598 Ocean acidification induces multi-generational decline in copepod naupliar
599 production with possible conflict for reproductive resource allocation. Journal of
600 experimental Marine Biology and Ecology, 418-419: 30-36.

601 Fitzer, SC, Caldwell, GS, Clare, AS, Upstill-Goddard, RC, Bentley, MG. 2013. Response of
602 copepods to elevated pCO₂ and environmental copper as co-stressors – A
603 multigenerational study. PLOS ONE, 8: e71257.

604 Gazeau, F, Gattuso, J-P, Dawber, C, Pronker, AE, Peene, F, Peene, J, Heip, CHR,
605 Middelburg, JJ. 2010. Effect of ocean acidification on the early life stages of the blue
606 mussel *Mytilus edulis*. Biogeosciences, 7: 2051-2060.

607 Gezelius, G. 1962. Adaptation of the sea urchin *Psammechinus miliaris* to different salinities.
608 Zoologiska Bidrag Fran Uppsala, 35: 329-337.

609 Guinotte, JM, Fabry, VJ. 2008. Ocean acidification and its potential effects on marine
610 ecosystems. Annals of the New York Academy of Sciences, 1134: 320-342.

611 Hammer, H, Hammer, B, Watts, S, Lawrence, A, Lawrence, J. 2006. The effect of
612 dietary protein and carbohydrate concentration on the biochemical composition and
613 gametogenic condition of the sea urchin *Lytechinus variegates*. Journal of
614 Experimental Marine Biology and Ecology, 334: 109-121.

615 Havenhand, JN, Buttler, F-R, Thorndyke, MC, Williamson, JE. 2008. Near-future levels of
616 ocean acidification reduce fertilization success in a sea urchin. Current Biology, 18:
617 R651-R652.

618 Hinegardner, RT. 1969. Growth and development of the laboratory cultured sea urchin.
619 Biological Bulletin, 137: 465-475.

620 Hintz, JL, Lawrence, JM. 1994. Acclimation of gametes to reduced salinity prior to spawning
621 in *Luidia clathrata* (Echinodermata: Asteroidea). Marine Biology, 120: 443-446.

622 Houghton, JT, Ding, Y, Griggs, DJ, Noguer, M, Van der Linden, PJ, Dai, X, Maskell, K,
623 Johnson, CA. 2001. Climate change 2001: The scientific basis, Cambridge, UK.

624 IPCC. The 4th Assessment Report of the IPCC. (Cambridge Univeristy Press, Cambridge,
625 UK, 2007).

626 Kelly, M.S., 2000. The reproductive cycle of the sea urchin *Psammechinus miliaris*
627 (Echinodermata: Echinoidea) in a Scottish sea loch. Journal of the Marine Biological
628 Association, UK, 80: 909-919.

629 Kelly, MS, Hunter, AJ, Scholfield, CL, McKenzie, JD. 2000. Morphology and survivorship of
630 larval *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) in response to
631 varying food quantity and quality. Aquaculture, 183: 223-240.

632 Kurihara, H. 2008. Effects of CO₂-driven ocean acidification on the early developmental
633 stages of invertebrates. Marine Ecology Progress Series, 373: 275-284.

634 Kurihara, H, Shimode, S, Shirayama, Y. 2004. Sub-lethal effects of elevated concentration of
635 CO₂ on planktonic copepods and sea urchins. *Journal of Oceanography*, 60, 743-750
636 Kurihara, H, Shirayama, Y. 2004. Effects of increased atmospheric CO₂ on sea urchin early
637 development. *Marine Ecology Progress Series*, 274: 161-169.
638 Levitan, DR. 2006. The relationship between egg size and fertilization success in broadcast-
639 spawning marine invertebrates. *Integrative and Comparative Biology*, 46: 298-311.
640 Lewis, E, Wallace, DWR. 1998. Carbon Dioxide Information Analysis Center, Oak Ridge
641 National Laboratory, US Department of Energy, Oak Ridge, Tennessee, US.
642 Liu, H, Kelly, MS, Cook, EJ, Black, K, Orr, H, Zhu, JX, Dong, SL. 2007. The effect of diet
643 type on growth and fatty acid composition of the sea urchin larvae, II. *Psammechinus*
644 *miliaris* (Gmelin). *Aquaculture*, 264: 263–278.
645 Martin, S, Richier, S, Pedrotti, ML, Dupont, S, Castejon, C, Gerakis, Y, Kerros, ME,
646 Oberhansli, F, Teyssie, JL, Jeffree, R, Gattuso, JP. 2011. Early development and
647 molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to
648 CO₂-driven acidification. *Journal of Experimental Biology*, 214: 1357-1368.
649 McEdward, LR, Herrera, JC. 1999. Body form and skeletal morphometrics during larval
650 development of the sea urchin *Lytechinus variegatus* Lamarck. *Journal of*
651 *Experimental Marine Biology and Ecology*, 232: 151-176.
652 Mehrbach, C, Culberson, CH, Hawley, JE, Pytkowicz, RM. 1973. Measurement of apparent
653 dissociation constants of carbamic acid in seawater at atmospheric pressure.
654 *Limnology and Oceanography*, 18: 897-907.
655 Moulin, L., Catarino, AI, Claessens T, Dubois, P. 2011. Effects of seawater acidification on
656 early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816).
657 *Marine Pollution Bulletin*, 62: 48-54.
658 Nickell, LA, Black, KD, Hughes, DJ, Overnell, J, Brand, T, Nickell, TD, Breuer, E, Martyn,
659 HS. 2003. Bioturbation, sediment fluxes and benthic community structure around a
660 salmon cage farm in Loch Creran, Scotland. *Journal of Experimental Marine Biology*
661 *and Ecology*, 285: 221-233.

662 O'Donnell, MJ, Todgham, AE, Sewell, MA, Hammond, LM, Ruggiero, K, Fangué, NA,
663 Zippay, ML, Hofmann, GE. 2010. Ocean acidification alters skeletogenesis and gene
664 expression in larval sea urchins. *Marine Ecology Progress Series*, 398: 157-171.

665 Parker, LM, Ross, PM, O'Conner, WA, Borysko, L, Raftos, DA, Portner, H-O. 2012. Adult
666 exposure influences offspring response to ocean acidification in oysters. *Global*
667 *Change Biology*, 18: 82-92.

668 Peck, LS, Clark, MS, Morley, SA, Massey, A, Rossetti, H. 2009. Animal temperature limits
669 and ecological relevance: effects of size, activity and rates of change. *Functional*
670 *Ecology*, 23: 248-256.

671 Pespeni, MH, Sanford, E, Gaylord, B, Hill, TM, Hosfelt, JD, Jaris, HK, LaVigne, M, Lenz,
672 EA, Russell, AD, Young, MK, Palumbi, SR. 2013. Evolutionary change during
673 experimental ocean acidification. *Proceedings of the National Academy of Sciences*,
674 110: 6937-6942.

675 Ries, JB, Cohen, AL, McCorkle, DC. 2009. Marine calcifiers exhibit mixed responses to CO₂-
676 induced ocean acidification. *Geology*, 37, 1131-1134.

677 Royal Society 2005. *Ocean acidification due to increasing atmospheric carbon dioxide*.
678 London: The Royal Society, 223 pp.

679 Shirayama, Y, Thornton, H. 2005. Effect of increased atmospheric CO₂ on shallow water
680 marine benthos. *Journal of Geophysical Research*, 110: C09S08.

681 Shpigel, M, McBride, SC, Marciano, S, Lupatsch, I. 2004. The effect of photoperiod and
682 temperature on the reproduction of European sea urchin *Paracentrotus lividus*.
683 *Aquaculture*, 232: 343-355.

684 Sokal, RR, Rohlf, FJ. 1995. *Biometry: The principles and practice of statistics in biological*
685 *research*. W.H. Freeman, New York.

686 Stumpp, M, Wren, J, Melzner, F, Thorndyke, MC, Dupont, ST. 2011. CO₂ induced seawater
687 acidification impacts sea urchin larval development I: Elevated metabolic rates
688 decrease scope for growth and induce developmental delay. *Comparative*

689 Biochemistry and Physiology A – Molecular and Integrative Physiology, 160: 331-
690 340.

691 Suckling, CC. 2012. Calcified marine invertebrates: the effects of ocean acidification. Phd
692 Thesis, University of Cambridge, UK

693 Suckling, CC, Clark, MS, Peck, LS, Cook, EJ. submitted. Experimental influence of pH on the
694 early life-stages of sea urchins I: different rates of introduction give rise to different
695 responses. Invertebrate Reproduction and Development.

696 Suckling, CC, Symonds, RC, Kelly, MS, Young, AJ. 2011. The effect of artificial diets on
697 gonad colour and biomass in the edible sea urchin *Psammechinus miliaris*.
698 Aquaculture, 318: 335-342.

699 Symonds, RS, Kelly, MS, Suckling, CC, Young, AJ. 2009. Carotenoids in the gonad and gut
700 of the edible sea urchin *Psammechinus miliaris*. Aquaculture, 288: 120-125.

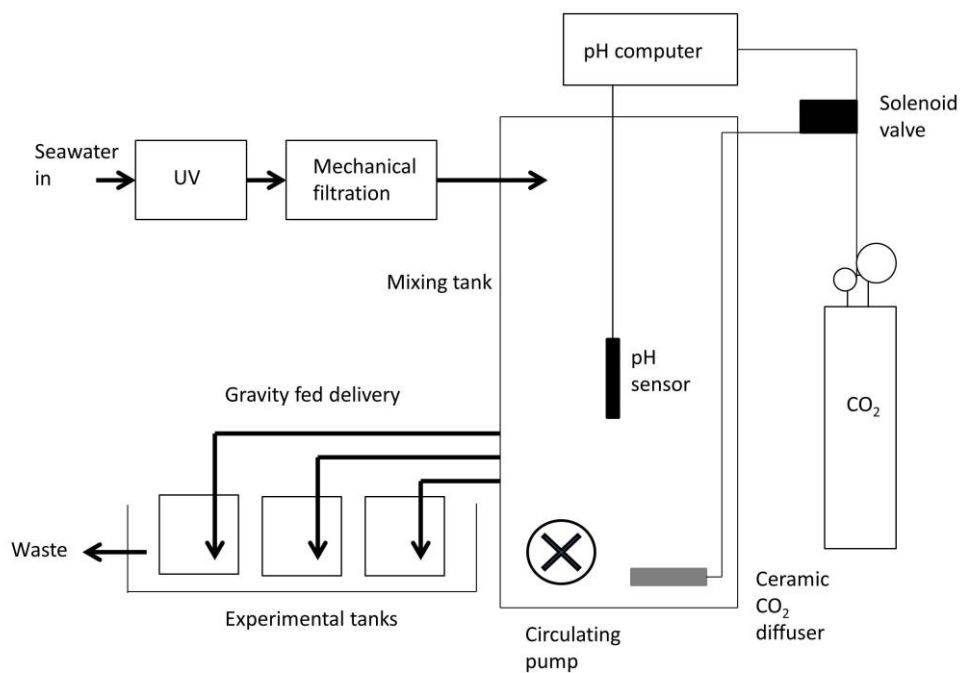
701 Talmage, SC, Gobler, CJ. 2010. Effects of past, present, and future ocean carbon dioxide
702 concentrations on the growth and survival of larval shellfish. Proceedings of the
703 National Academy of Sciences, 107: 17246-17251.

704 Uthicke, S, Soars, N, Foo, S, Byrne, M. 2012. Effects of elevated CO₂ and the effect of parent
705 acclimation on development in the tropical Pacific sea urchin *Echinometra mathaei*.
706 Marine Biology, 1-14 doi: 10.1007/s00227-012-2023-5

707 Watson, SA, Southgate, PC, Tyler, PA, Peck, LS. 2009. Early larval development of the
708 Sydney rock oyster *Saccostrea glomerata* under near-future predictions of CO₂-driven
709 ocean acidification. Journal of Shellfish Research, 28: 431-437.

710 Widdicombe, S, Needham, HR. 2007. Impact of CO₂ induced seawater acidification on the
711 burrowing activity of *Nereis virens* and sediment nutrient flux. Marine Ecology
712 Progress Series, 341: 111-122.

713 **List of figures**



714

715 **Fig. 1:** Schematic diagram of the CO₂ microcosm adapted from Widdicombe and Needham's
716 seawater acidification tank system (2007).

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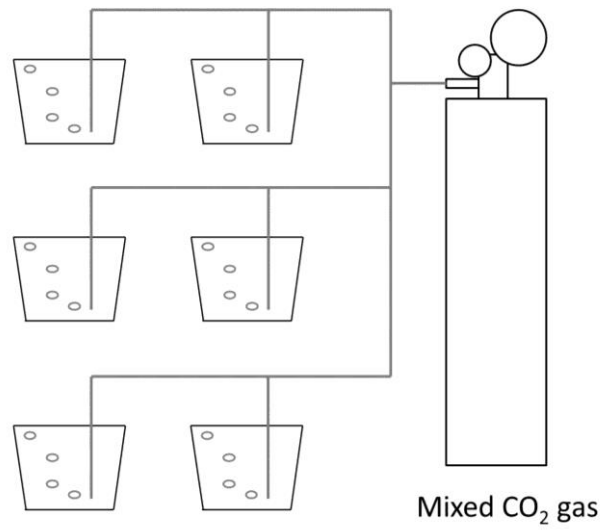
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Experimental tanks – organism
suspension by premixed CO₂ bubbling



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732 **Fig. 2:** Schematic diagram of the premixed CO₂ larval system.

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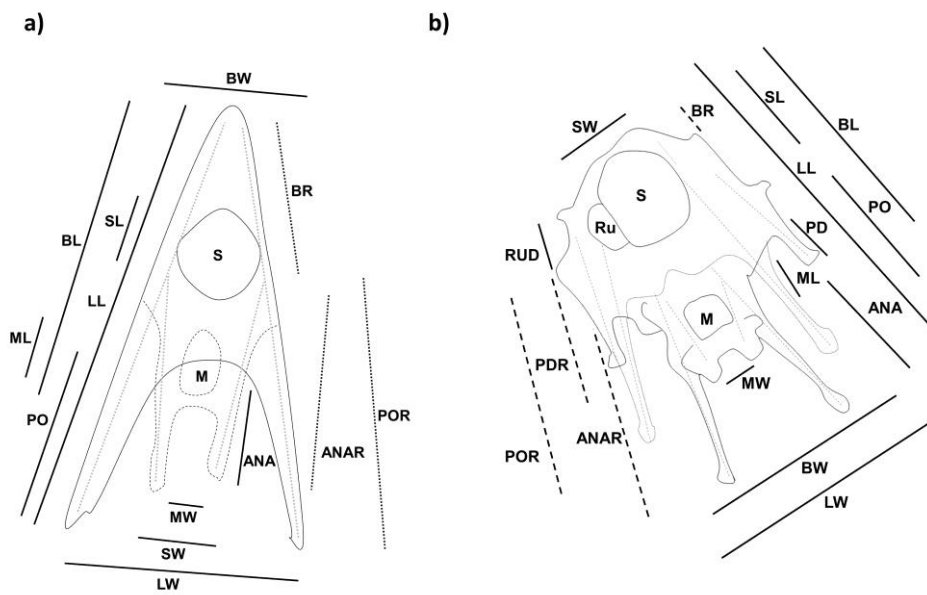
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746 **Fig. 3:** Morphometric parameters measured on gastrulae and echinoplutei (within the larval
 747 structure outlines ‘M’ denotes the mouth, ‘S’ the stomach and ‘Ru’ the rudiment). Days 7 (a)
 748 and 14 (b) depicted examples; day 2: length and 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.

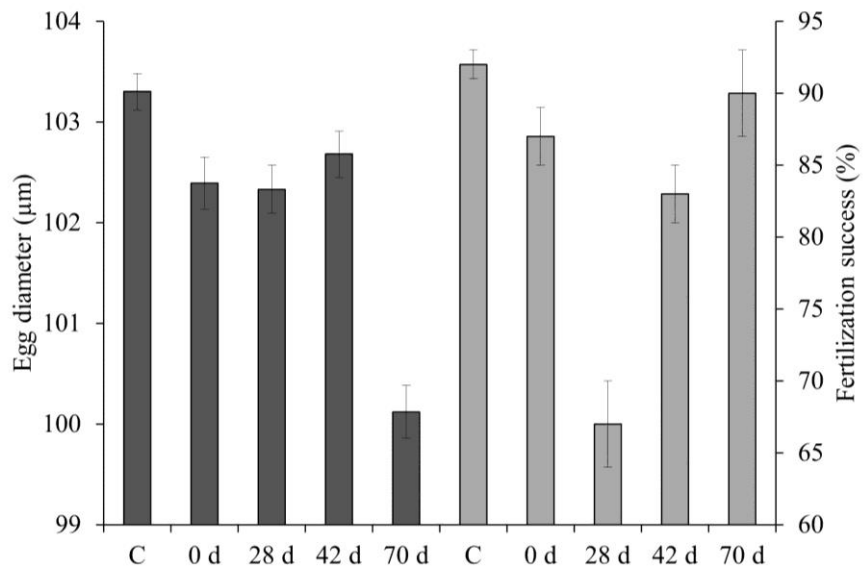
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761 **Fig. 4:** Mean egg diameters (■) and fertilization success rates (□; ± SE) from adults
 762 exposed to control or reduced pH treatments for different periods of times (d = days). Note
 763 ordinate axis do not start at zero.

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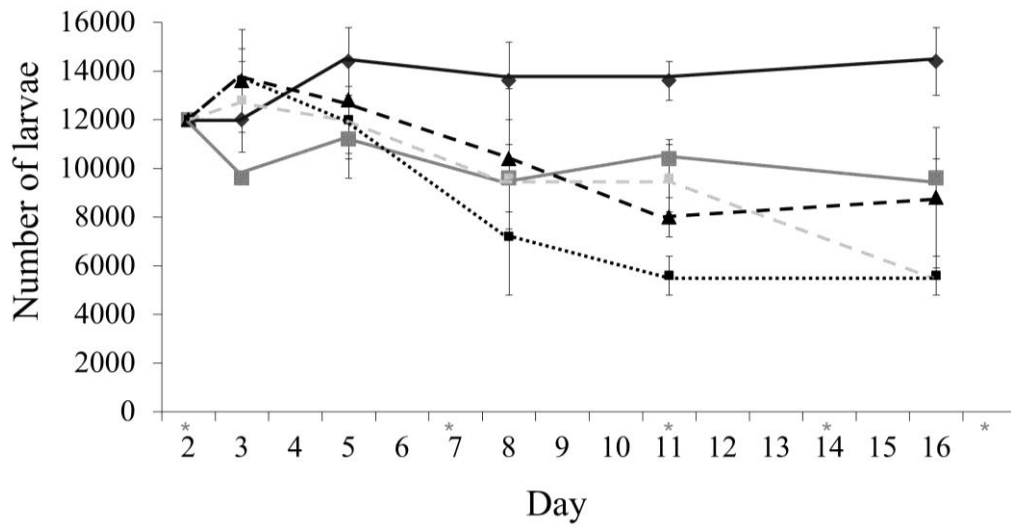
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777 **Fig. 5:** Mean larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (\pm
 778 SE) derived from adults exposed for differing periods of time to control and reduced pH
 779 conditions. Settlement tests commenced from ~day 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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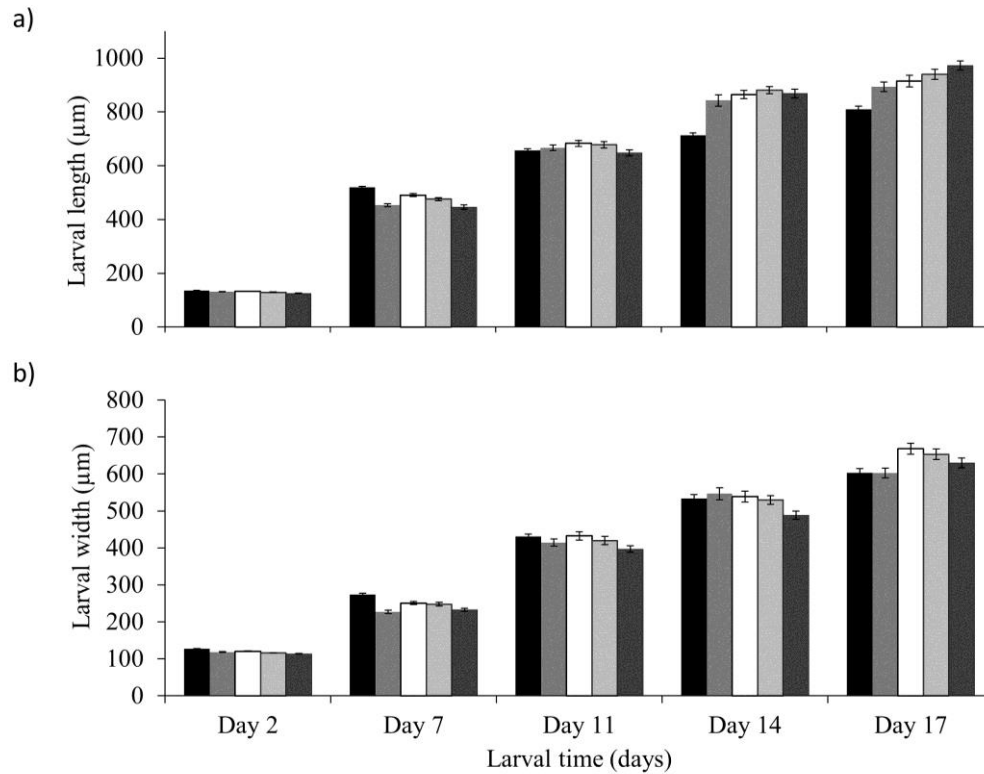
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794 **Fig. 6:** Mean larval lengths (a) and widths (b; \pm SE; μm) for *Psammechinus miliaris*
 795 echinoplutei derived from adults exposed for differing periods of time to control and reduced
 796 pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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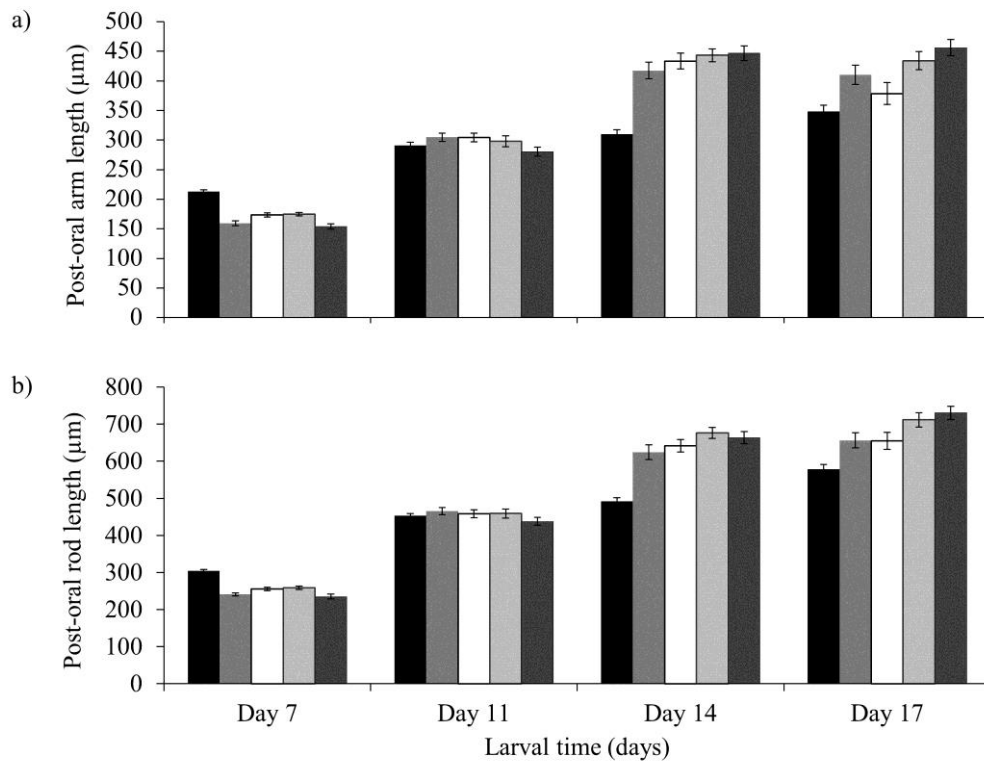
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809 **Fig. 7:** Mean larval post-oral arm (a) and rod lengths (b; \pm SE; μm) for *Psammechinus*
 810 *miliaris* echinoplutei derived from adults exposed for differing periods of time to control and
 811 reduced pH conditions. ■ = Control, ■ = 0 d,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

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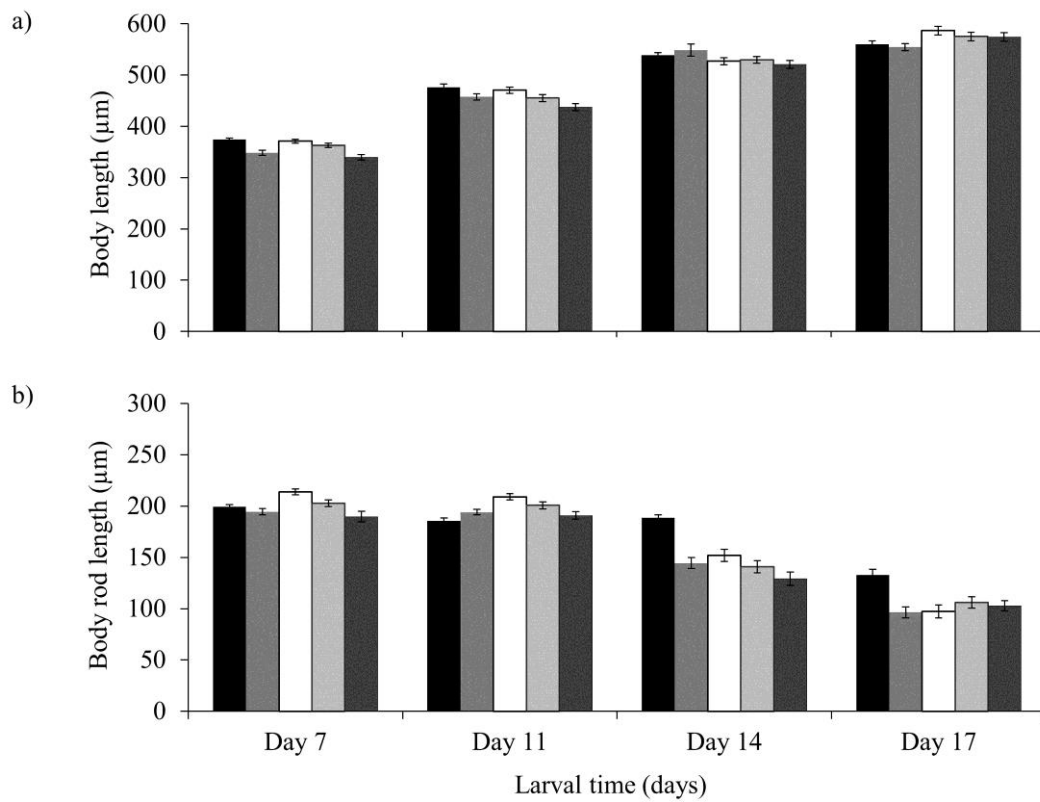
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824 **Fig. 8:** Mean larval body length (a) and rod lengths (b; \pm SE; μm) for *Psammechinus miliaris*
 825 echinoplutei derived from adults exposed for differing periods of time to control and reduced
 826 pH conditions. ■ = Control, ■ = 0 d, □ =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

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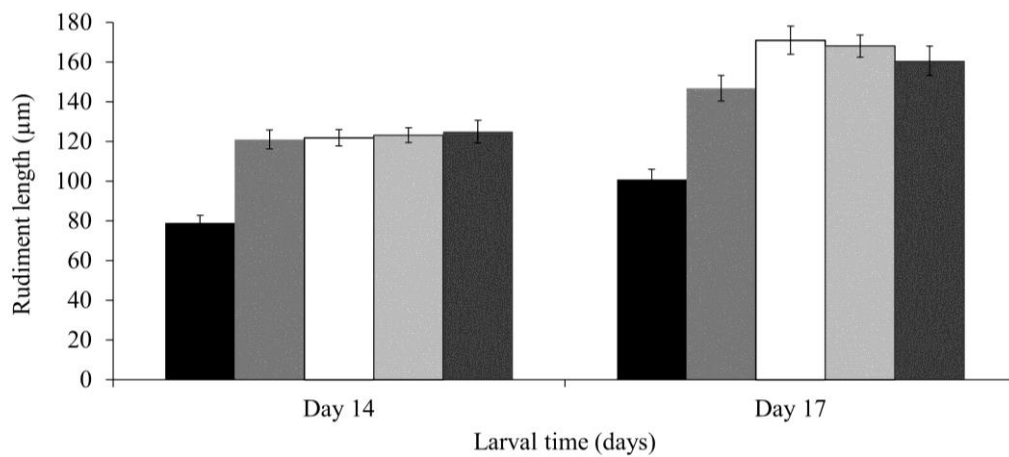
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839 **Fig. 9:** Mean rudiment lengths (\pm SE; μm) for *Psammechinus miliaris* echinoplutei derived
 840 from adults exposed for differing periods of time to control and reduced pH conditions. ■ =
 841 Control, ■ = 0 d, □ = 28 d, ■ = 42 d, ■ = 70 d.

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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*. $p\text{CO}_2$, Ω
 898 calcite and Ω 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
pH_{NIST}	7.98 ± 0.01	7.70 ± 0.01
pCO_2 (μatm)	559 ± 15	999 ± 22
Ω calcite	2.01 ± 0.06	0.98 ± 0.13
Ω aragonite	1.28 ± 0.04	0.63 ± 0.08
Temperature ($^{\circ}\text{C}$)	12.9 ± 0.4	12.8 ± 0.4
Salinity (psu)	32 ± 1	32 ± 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 *Psammochinus 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.
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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 _(4,10) = 3.39 P = 0.059	H ₍₄₎ = 8.99 P = 0.066	F _(4,10) = 0.57, P = 0.492
6 arms	C	22 \pm 3	97 \pm 2	27 \pm 12	49 \pm 6 ^a
	0d	19 \pm 2	88 \pm 1	13 \pm 4	16 \pm 3 ^b
	28d	21 \pm 1	88 \pm 3	17 \pm 5	13 \pm 4 ^b
	42d	22 \pm 1	79 \pm 8	17 \pm 3	12 \pm 3 ^b
	70d	13 \pm 2	81 \pm 3	29 \pm 6	13 \pm 2 ^b
			F _(4,10) = 3.39 P = 0.059	H ₍₄₎ = 8.99 P = 0.066	F _(4,10) = 1.16 P = 0.460
8 arms	C	0 \pm 0	0 \pm 0	71 \pm 11	49 \pm 4 ^a
	0d	0 \pm 0	0 \pm 0	85 \pm 3	81 \pm 3 ^b
	28d	0 \pm 0	0 \pm 0	82 \pm 7	86 \pm 4 ^b
	42d	3 \pm 3	0 \pm 0	80 \pm 5	85 \pm 3 ^b
	70d	0 \pm 0	0 \pm 0	69 \pm 7	86 \pm 2
			F _(4,10) = 3.39 P = 0.071	-	F _(4,10) = 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.

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Abnormality	Treat	%			
		Day: 7	11	14	17
Skeletal rods protruding (RP)	C	34 \pm 9	5 \pm 1 ^a	8 \pm 5	34 \pm 11
	0d	33 \pm 3	26 \pm 13 ^b	25 \pm 5	29 \pm 3
	28d	36 \pm 11	37 \pm 2 ^b	33 \pm 7	59 \pm 10
	42d	42 \pm 10	40 \pm 3 ^b	27 \pm 8	47 \pm 1
	70d	42 \pm 1	59 \pm 8 ^b	35 \pm 7	35 \pm 4
		H ₍₄₎ = 1.79 P = 0.476	F _(4,10) = 77.35 P = 0.047	F _(4,10) = 2.54 P = 0.112	F _(4,10) = 3.01 P = 0.111
Postoral arms (PO)	C	1 \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 _(4,10) = 2.39 P = 0.182	F _(4,10) = 1.27 P = 0.174	F _(4,10) = 1.93 P = 0.228	F _(4,10) = 3.24 P = 0.143
Anterolateral arms (ANA)	C	1 \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 _(4,10) = 0.71 P = 0.553	F _(4,10) = 1.31 P = 0.580	F _(4,10) = 0.43 P = 0.584	F _(4,10) = 1.27 P = 0.317
Posterodorsal arms (PD)	C	-	-	-	2 \pm 2
	0d	-	-	-	3 \pm 1
	28d	-	-	-	1 \pm 1
	42d	-	-	-	6 \pm 4
	70d	-	-	-	7 \pm 2
				F _(4,10) = 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 _(4,10) = 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 _(4,10) = 2.03 P = 0.241	F _(4,10) = 2.99 P = 0.121	F _(4,10) = 1.00 P = 0.493	F _(4,10) = 1.95 P = 0.411
General	C	2 \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 _(4,10) = 1.57 P = 0.281	F _(4,10) = 2.13 P = 0.111	F _(4,10) = 0.99 P = 0.493	F _(4,10) = 3.03 P = 0.087

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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 ⁻¹
Control	40 \pm	6011 \pm	193 \pm
	10 _a	2159	56
0d	55 \pm	5260 \pm	218 \pm
	7 _{ab}	1077	53
28d	70 \pm	6235 \pm	377 \pm
	2 _b	2212	125
42d	69 \pm	3780 \pm	189 \pm
	5 _b	307	22
70d	82 \pm	4520 \pm	344 \pm
	4 _c	467	70

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