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

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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 ($\sim$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_2; 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_2) 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

2

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_2 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

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

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

Materials and Methods

Sea urchin source and maintenance

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

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 ℃

149 for 24 h then homogenised into a fine powder and stored in an air tight container at -20 ℃

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 ℃ until use. Diets were defrosted prior to introduction to adult *P. miliaris*.

155

156 *CO_2 microcosm*

157 A flow-through CO_2 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_2 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 ± 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 (ºC; YSI Model 63), salinity (YSI Model 63), pH_{NIST} and

166   TCO_2 (mmol L^{-1}; Ciba Corning TCO_2 Analyzer 965, Olympic Analytical, UK) were measured

167   and recorded for three replicate experimental tanks per treatment. The TCO_2 analyzer was

168   calibrated with 2 g L^{-1} CO_2 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 ºC 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_2 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 CO_2 (~ 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 persitomal membrane into the coelom until 5 females were successfully

223   spawned for each treatment exposure. After injection urchins were stored in ~ 300 mL glass

224   jars filled with 5-µm filtered treatment seawater until gametes were shed and then they were

225   returned to flow-through aquaria. Jars were kept closed during spawning and prior to

226   fertilisation to prevent crossing of gametes/fertilisation prior to targeted fertilisation. Clean

227   gloves and pipettes were utilised for handling of each *P. miliaris* and transfer of gametes as a

228   further measure to prevent non-target fertilisation/gamete transfer from occurring. This

229   ensured that samples for fertilisation success were within a precise time. Eggs from the 5

230   females for each treatment were combined and divided equally into three replicate volumes,

231   rinsed with filtered seawater and placed as a monolayer on the bottom of sealed buckets

232   containing 12 L of filtered treatment seawater. Sperm concentration was not calculated but

233   was subject to the same dilution series from concentrated sperm across all treatments (1:5;

234   Kelly et al., 2000). Eggs were fertilised by adding 1-2 mL of diluted sperm from the 2 males

235   from each treatment. Forty-five minutes later the seawater in each bucket was gently well

236   mixed and five 15 ml sub-samples were fixed to determine egg density and fertilisation rate.

237   To ensure that the pH of the seawater remained relatively uniform over the initial

238   development period when no aeration can occur, premixed CO_2 gas was introduced at a rate

239   of 0.2 L min^{-1} into the header space between the seawater surface and lid for 24 hours.

240   Twenty four hours after fertilisation hatched blastulae densities were quantified for

241   each treatment (three pseudoreplicates) using a 1 mL sedgewick rafter cell and compound

242   microscope. Blastulae were decanted into their respective treatment buckets at a density of ~ 1

243   individual mL^{-1}. Glass tubes were used to deliver relevant treatment gases (at ~ 200 mL min^{-1})

244   to the bottom of the buckets. Complete seawater exchanges were carried out every two days,

245   with larvae retained in a 40 µm mesh sieve in a water bath while buckets were cleaned and

246   filled.

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 ºC. To maintain a constant high CO_2

261   environment for larvae reared in reduced pH, Petri dishes were housed in propagators. Pre-

262   mixed CO_2 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 'CO$_2$ 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

10

Larval morphometrics

275

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

Statistical analysis

292

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 Bonferronni'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

13

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

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

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

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

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

15

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 *Heliodaris 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**

530

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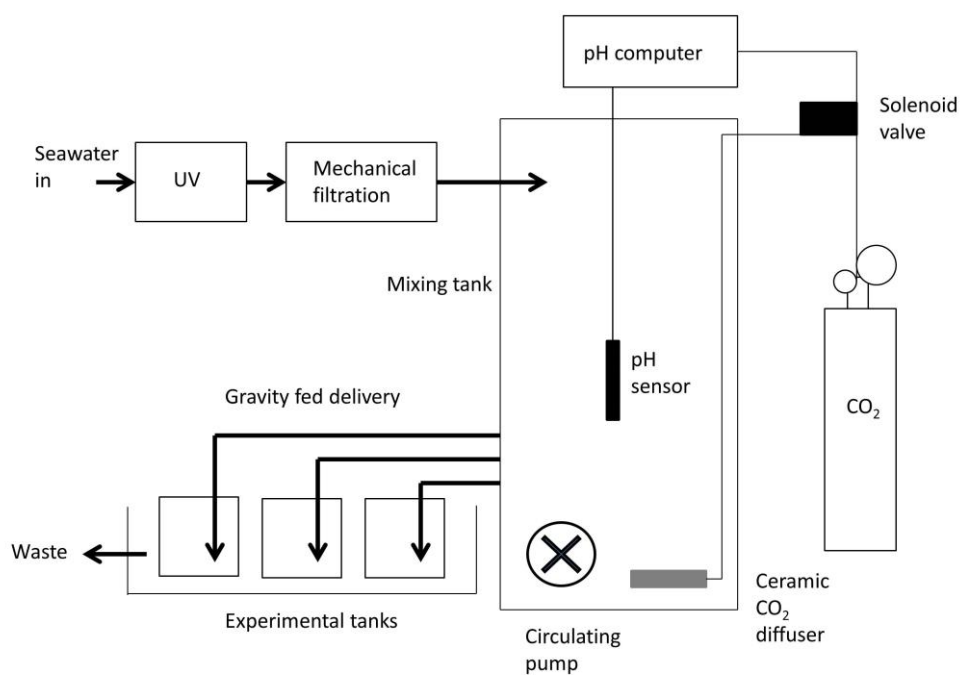
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715 **Fig. 1:** Schematic diagram of the CO_2 microcosm adapted from Widdicombe and Needham's

716 seawater acidification tank system (2007).

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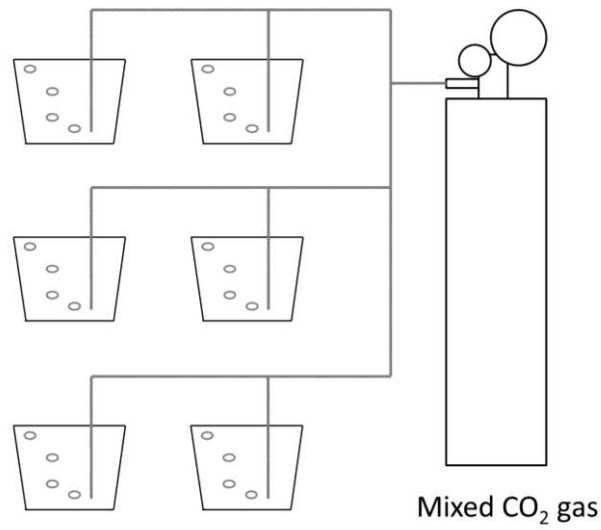
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Experimental tanks – organism suspension by premixed CO_2 bubbling

Mixed CO_2 gas

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

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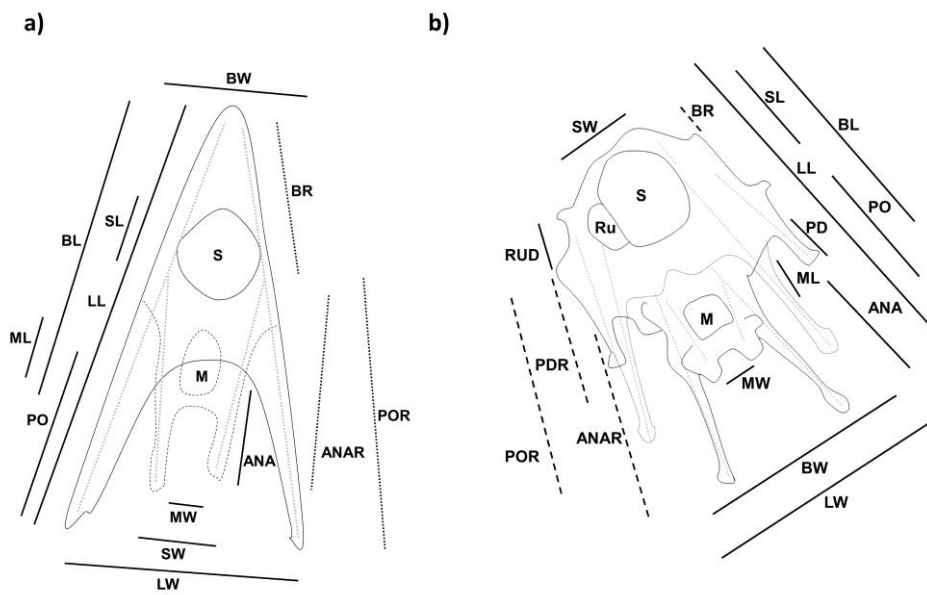
Fig. 3: Morphometric parameters measured on gastrulae and echinoplutei (within the larval structure outlines 'M' denotes the mouth, 'S' the stomach and 'Ru' the rudiment). Days 7 (a) and 14 (b) depicted examples; day 2: length and width; day 7-11: larval length (LL) and larval width (LW), body length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

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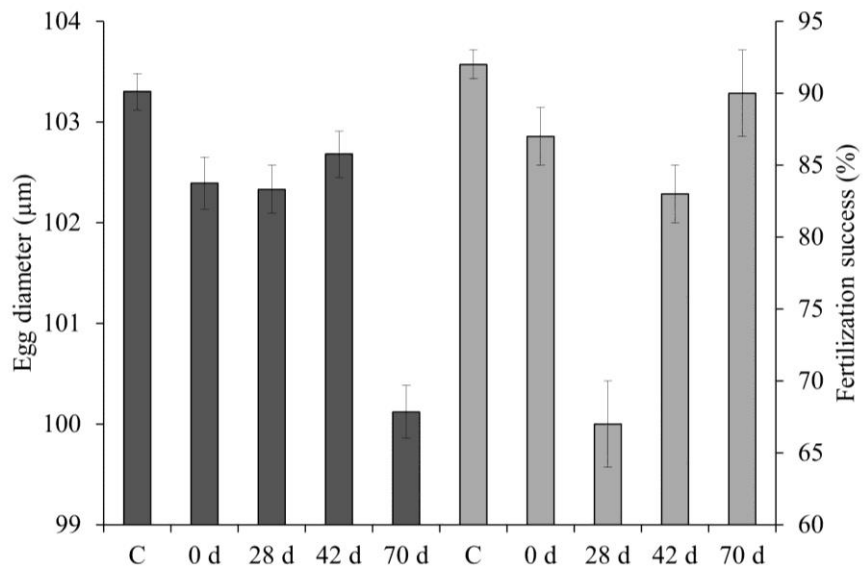
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Fig. 4: Mean egg diameters (■) and fertilization success rates (■ ; ± SE) from adults exposed to control or reduced pH treatments for different periods of times (d = days). Note ordinate axis do not start at zero.
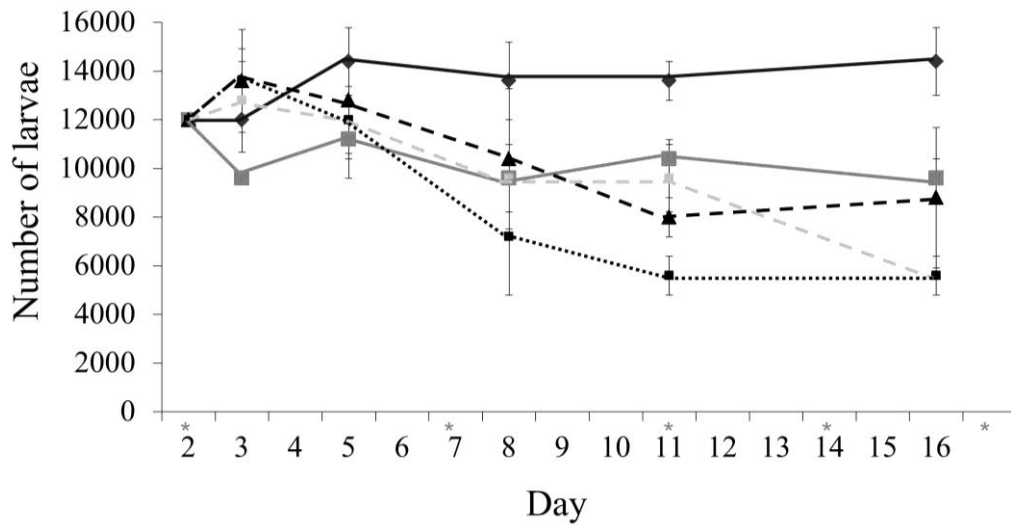
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Fig. 5: Mean larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (± SE) derived from adults exposed for differing periods of time to control and reduced pH conditions. Settlement tests commenced from ~day 20; therefore, no density data are presented after day 16. = Control, = 0d, = 28d, = 42 d, = 70 d,* = Morphometric measurement sample times.

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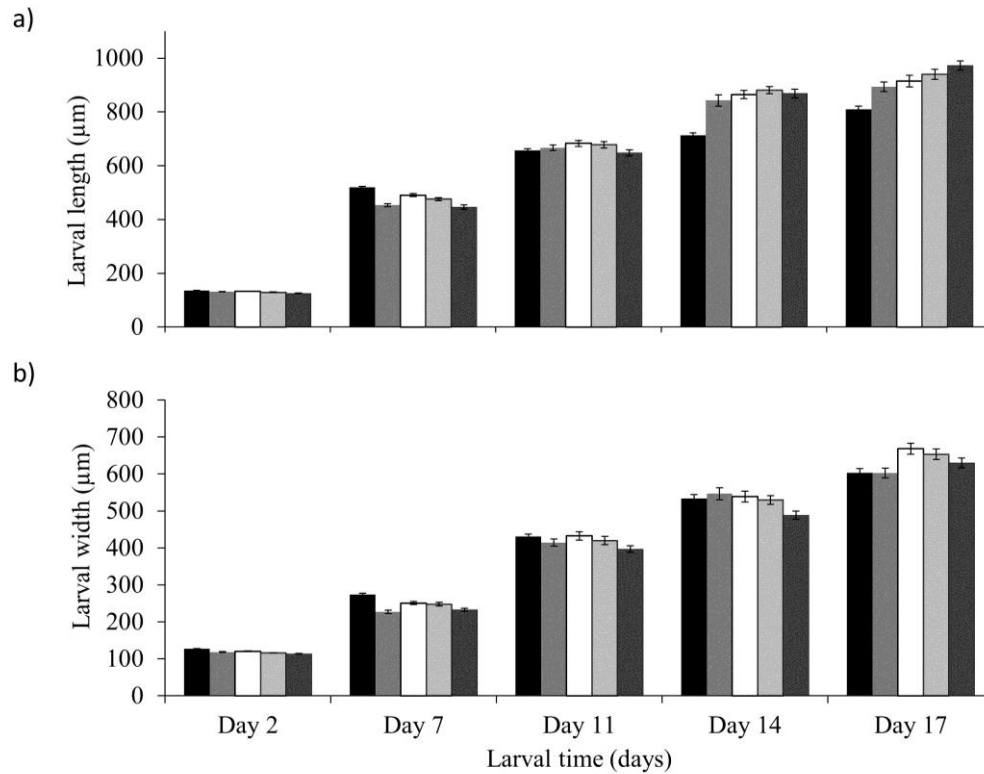
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Fig. 6: Mean larval lengths (a) and widths (b; ± SE; μm) for *Psammechinus miliaris* echinoplutei derived from adults exposed for differing periods of time to control and reduced pH conditions. ■ = Control, ■ = 0 d, □ = 28 d, ▢ = 42 d, ■ = 70 d.

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

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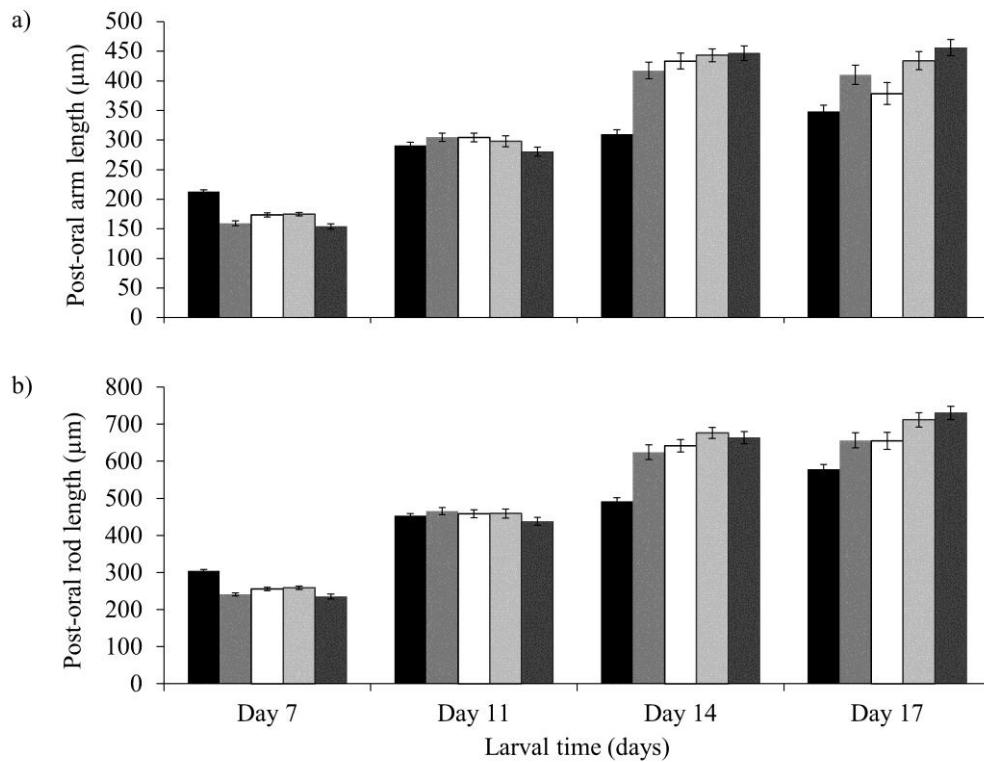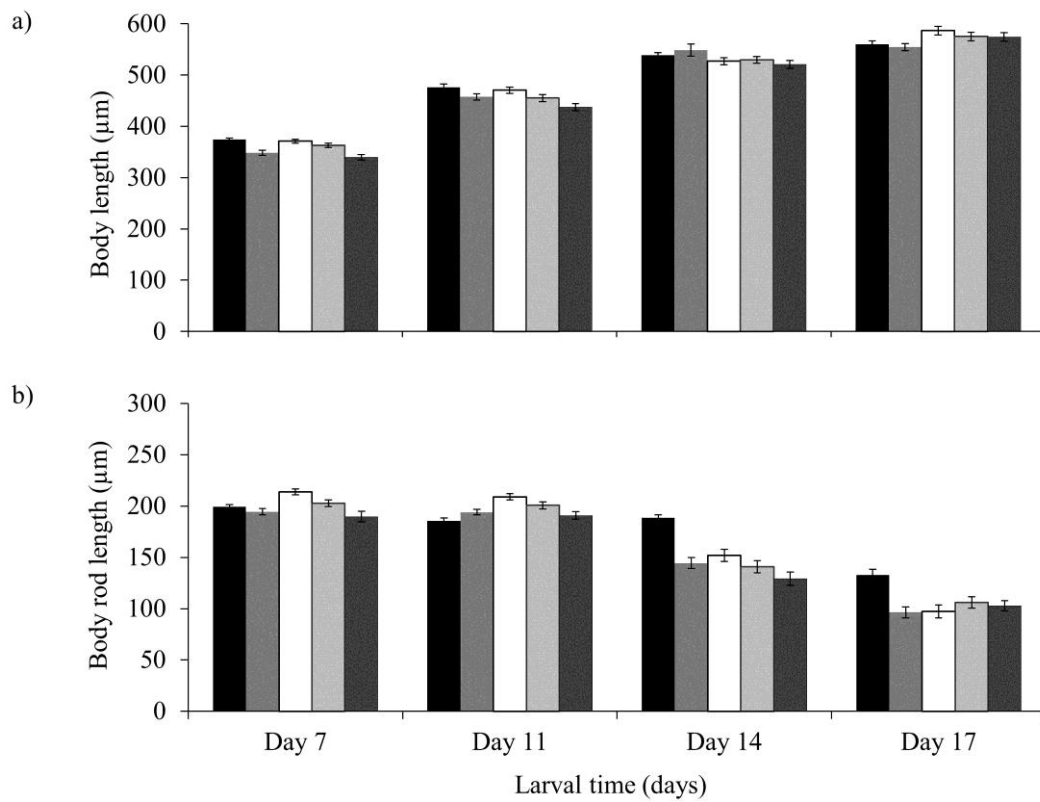
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Fig. 8: Mean larval body length (a) and rod lengths (b; ± SE; μm) for *Psammechinus miliaris* echinoplutei derived from adults exposed for differing periods of time to control and reduced pH conditions. ■ = Control, ▪ = 0 d, ▫ = 28 d, ▫ = 42 d, ■ = 70 d.
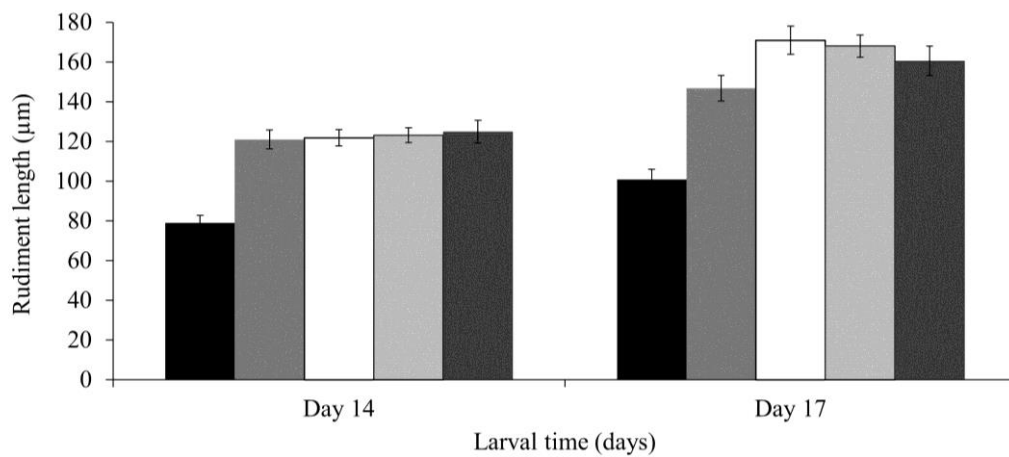
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839 **<u>Fig. 9:</u>** Mean rudiment lengths (± SE; μm) for *Psammechinus miliaris* echinoplutei derived

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

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

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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 *L. digitata*: 267 mL FW
Alginic acid	3.3	1g: 28.57 mL FW

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897 **Table 2:** Mean (± SE) seawater treatment parameters for *Psammechinus miliaris*. pCO_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).

900

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

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

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

1000 **Table 5:** Mean abnormal development types (%; ± SE) for *Psammechinus 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 ± 9	5 ± 1 [a]	8 ± 5	34 ± 11
	0d	33 ± 3	26 ± 13 [b]	25 ± 5	29 ± 3
	28d	36 ± 11	37 ± 2 [b]	33 ± 7	59 ± 10
	42d	42 ± 10	40 ± 3 [b]	27 ± 8	47 ± 1
	70d	42 ± 1	59 ± 8 [b]	35 ± 7	35 ± 4
		$H_{(4)} = 1.79$ $P = 0.476$	$F_{(4,10)} = 77.35$ $P = 0.047$	$F_{(4,10)} = 2.54$ $P = 0.112$	$F_{(4,10)} = 3.01$ $P = 0.111$
Postoral arms (PO)	C	1 ± 1	0 ± 0	12 ± 7	15 ± 6
	0d	5 ± 3	3 ± 2	5 ± 3	22 ± 6
	28d	0 ± 0	8 ± 5	17 ± 4	36 ± 4
	42d	0 ± 0	9 ± 2	21 ± 7	17 ± 4
	70d	9 ± 4	7 ± 5	5 ± 3	19 ± 3
		$F_{(4,10)} = 2.39$ $P = 0.182$	$F_{(4,10)} = 1.27$ $P = 0.174$	$F_{(4,10)} = 1.93$ $P = 0.228$	$F_{(4,10)} = 3.24$ $P = 0.143$
Anterolateral arms (ANA)	C	1 ± 1	1 ± 1	7 ± 2	12 ± 8
	0d	7 ± 3	2 ± 2	4 ± 2	28 ± 9
	28d	7 ± 3	6 ± 3	7 ± 3	33 ± 4
	42d	3 ± 3	4 ± 2	6 ± 2	20 ± 5
	70d	5 ± 3	10 ± 5	8 ± 2	33 ± 12
		$F_{(4,10)} = 0.71$ $P = 0.553$	$F_{(4,10)} = 1.31$ $P = 0.580$	$F_{(4,10)} = 0.43$ $P = 0.584$	$F_{(4,10)} = 1.27$ $P = 0.317$
Posterodorsal arms (PD)	C	-	-	-	2 ± 2
	0d	-	-	-	3 ± 1
	28d	-	-	-	1 ± 1
	42d	-	-	-	6 ± 4
	70d	-	-	-	7 ± 2
					$F_{(4,10)} = 1.43$ $P = 0.142$
Preoral arms (P)	C	-	-	-	5 ± 2
	0d	-	-	-	18 ± 3
	28d	-	-	-	17 ± 5
	42d	-	-	-	11 ± 4
	70d	-	-	-	13 ± 1
					$F_{(4,10)} = 2.33$ $P = 0.150$
Disintegrating body tissue	C	0 ± 0	0 ± 0	0 ± 0	17 ± 9
	0d	2 ± 1	0 ± 0	0 ± 0	7 ± 3
	28d	1 ± 1	0 ± 0	0 ± 0	3 ± 1
	42d	0 ± 0	3 ± 1	0 ± 0	4 ± 2
	70d	4 ± 2	3 ± 1	2 ± 2	1 ± 1
		$F_{(4,10)} = 2.03$ $P = 0.241$	$F_{(4,10)} = 2.99$ $P = 0.121$	$F_{(4,10)} = 1.00$ $P = 0.493$	$F_{(4,10)} = 1.95$ $P = 0.411$
General	C	2 ± 1	1 ± 1	20 ± 6	21 ± 16
	0d	12 ± 4	8 ± 5	12 ± 3	48 ± 7
	28d	8 ± 4	17 ± 8	19 ± 3	65 ± 6
	42d	6 ± 3	17 ± 6	22 ± 7	40 ± 4
	70d	14 ± 5	20 ± 6	25 ± 4	54 ± 10
		$F_{(4,10)} = 1.57$ $P = 0.281$	$F_{(4,10)} = 2.13$ $P = 0.111$	$F_{(4,10)} = 0.99$ $P = 0.493$	$F_{(4,10)} = 3.03$ $P = 0.087$

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1011 **Table 6:** Mean settled larvae (± SE) from adults exposed to control or reduced pH treatments

1012 for different periods of times (d = days). Columns without superscripts indicate no treatment

1013 effects, differing superscripts indicate where significant differences occur.

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

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