Author's post print version

1   **Experimental influence of pH on the early life-stages of sea urchins I: different**

2   **rates of introduction give rise to different responses**

3

4   Suckling, C.C.[1,2, 3*], Clark, M.S.[1], Peck, L.S.[1], Cook, E.J.[3]

5   [1] British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley

6   Road, Cambridge, CB3 0ET, UK;

7   [2] Department of Earth Sciences, University of Cambridge, Downing Street, Cambridge, CB2

8   3EQ, UK;

9   [3] Scottish Association for Marine Sciences, Scottish Marine Institute, Oban, Argyll, Scotland,

10   PA37 1QA, UK;

11

12   *Corresponding author: telephone +44-1223-221459, fax +44-1223-362616, e-mail address:

13   coleen.suckling@bangor.ac.uk / coleen.suckling@cantab.net; current address: School of

14   Biological Sciences, Bangor University, Deiniol Road, Bangor, Gwynedd, Wales LL57 2UW

15   (C.C. Suckling)

16

17   **Abstract**

18

19   Many early life-stage response studies to ocean acidification utilise gametes/offspring

20   obtained from ambient-sourced parents, which are then directly introduced to experimentally

21   altered seawater-pH. This approach may produce a stress response potentially impacting

22   development and survival. Hence, this study determined whether this approach is suitable by

23   subjecting embryos/larvae to different rates of introduction to lowered seawater-pH to assess

24   larval success under acute and staggered experimental pH scenarios. Embryos and 4-armed

25   larvae of the sea urchin *Psammechinus miliaris* were introduced to pH-conditions, widely

26   used in ocean acidification studies, from ambient conditions utilizing 380, 470, 560, 700 and

27   840ppm CO_2 changed at incremental steps at two rates: fast (every 3rd hour) or slow (every

28   48th hour). Direct transfers from ambient to low seawater-pH gave rise to dramatic negative

29   impacts (smaller size and low survival), but slower rates of introductions gave rise to lesser

30   negative responses (low survival). There was no treatment effect on settled juveniles. Fast

31   introductions utilised in many studies are likely not ideal approaches when assessing pre-

32   settlement larval developmental responses. Therefore careful consideration of the pattern of

33   response is needed when studies report the responses of offspring, derived from ambient

34   conditions, introduced directly to forecasted ocean acidification conditions.

35

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

37

38   **Introduction**

39   Currently large efforts are being made to predict the responses of marine organisms to

40   rapid climate change. One area of particular focus is ocean acidification. Oceanic surface

41   waters have acidified by approximately 0.1 pH units since the onset of the industrial

42   revolution (~250 years ago; Royal Society, 2005) and are forecasted to decrease even further

43   and more rapidly by 0.3-0.5 units over the next 90 years (Houghton et al., 2001; Caldeira and

44   Wickett, 2003; 2005, Canadell et al., 2007; IPCC, 2013). Consequently surface waters may

45   become undersaturated with respect to calcium carbonate ($CaCO_3$) which may have

46   significant consequences for marine organisms (Doney et al., 2009).

47   Laboratory based experiments assess the responses of these marine organisms by

48   exposing them to altered seawater pH conditions. Such conditions are achieved through

49   technically controlled mixing of seawater and carbon dioxide gas (CO_2). Studies have

50   confirmed that reduced pH conditions can have a range of impacts (positive to negative) on

51   adult marine calcifying organisms (e.g. Ries et al., 2009) highlighting that responses can be

52   species specific. An increasing number of laboratory studies have emerged in recent years

53   predicting the impacts of forecasted ocean acidification scenarios on the early life-stages of a

54   range of marine organisms (e.g. Munday et al., 2009; Gazeau et al., 2010; Caldwell et al.,

2

55   2011). Early life-stages are often identified as the most sensitive part of an organism's life-

56   cycle, and therefore are potentially more susceptible to change in pH compared to adults.

57   More importantly this area of the life cycle is a key driver to the success of populations

58   (Martin et al., 2011). Information is therefore vital in enabling the scientific community to

59   predict organismal responses, and potentially population dynamics, by measuring the impact

60   on larval success.

61   Much of the information generated so far has however been gained from offspring

62   derived from ambient sources. By this we mean that adults have been sourced from present-

63   day coastal areas, spawned under ambient laboratory conditions and the subsequent gametes /

64   offspring used for experimental investigation. These ambient sourced gametes / offspring

65   have generally been directly introduced to low target pH conditions and their responses

66   reported. Such studies are certainly interesting because they demonstrate the physiological

67   flexibility which present-day organisms exhibit and possibly highlight the underlying

68   mechanisms which might be used to deal with change in the future. Such approaches may also

69   be representative of dramatic CO_2 sequestration leak scenarios resulting in reduced pH in

70   localized areas of the water column. In a wider global context, however, and with respect to

71   the real-time scale of ocean acidification (decadal changes), organisms might acclimate,

72   produce and deposit gametes, reproduce and adapt to change (Dam, 2013). Therefore the

73   method of direct transfer of ambient sourced gametes/ offspring to low seawater pH may

74   drive experiments to certain responses which are likely to be chronic and acute. These in turn

75   could produce misleading predictions for organismal responses under future ocean

76   acidification.

77   Previous studies have shown that different rates of introduction of marine organisms

78   to other laboratory induced environmental parameters can result in very different responses.

79   In one example study, early life-stages of tropical crabs (*Armases miersii*, Rathbun) were

80   introduced directly to acute salinities (Anger, 1996). This rapid introduction resulted in

81   offspring displaying prolonged development times and lower survival compared to controls.

82   In another example, Peck et al. (2009) reported marked differences in the temperature

83   tolerances of Antarctic marine invertebrates when warmed at different rates. Faster rates of

84   warming (1 °C d^{-1}) resulted in organismal thermal limits that were 5-10°C higher than those

85   warmed more gradually (1-2 °C wk^{-1} or m^{-1}). Although this study followed well-established

86   thermal biology protocols and not pCO$_2$, it certainly highlights, along with Anger's (1996)

87   study, the unpredictable influence that rates of change in laboratory induced environmental

88   factors can have on organismal responses. Comparable studies have not yet been reported for

89   the introduction of organisms to reduced pH conditions. We therefore directed this study

90   towards a technical approach that should be considered for experimental designs assessing

91   early life-stage responses towards altered seawater pH conditions emulating ocean

92   acidification. This is an aspect not considered in widely available guidelines on ocean

93   acidification research (e.g. EPOCA; Riebesell et al., 2010) which could be used to validate

94   previously published larval responses.

95   In this study we assessed the responses of early-life stages of sea urchins when

96   introduced to reduced seawater pH at different rates. This is not an effort to make organismal

97   predictions to ocean acidification because realistically gametes of echinoids will deposit and

98   develop under the gradual reduction of seawater pH. In this study we are following the

99   methods taken by previous studies and comparing it to slower rates of introduction purely to

100   determine whether responses of offspring differ or not under laboratory conditions. Early and

101   later development stages of offspring were also assessed under reduced seawater pH to

102   determine the impact of rapid exposure at different stages of development. We also extended

103   the assessment of larval development and survival to include settling juvenile stages to

104   determine whether pre-settlement stage outcomes are continued through to post-settling

105   juvenile stages. The sea urchin (*Psammechinus miliaris* (P.L.S. Müller, 1771)), was utilised

106   due to these organisms being proposed as a suitable model organism for toxicity studies (e.g.

107   Matranga et al., 2000) with well-established reproductive and larval rearing methodology

108   (McEdward and Herrera, 1999; Kelly et al., 2000; Liu et al., 2007).

109

110   **Materials and Methods**

111 *Sea urchin source and maintenance*

112   *Psammechinus miliaris* (total n = 30; 29.3 ± 3.0 mm test diameter) were collected by

113 scuba divers in August 2009 during the low spring tide from 3-10 m depth at Rubha Garbh,

114 Loch Creran, Scotland (Symonds et al., 2009; Suckling et al., 2011). Animals were

115 transferred to aquaria held at the Scottish Association for Marine Science, Oban. *P. miliaris*

116 were maintained in one 100 L aquarium and fed *Saccharina latissima* ((Linnaeus) C.E. Lane,

117 C. Mayes, Druehl and G.W. Saunders, 2006; formerly *Laminaria saccharina*, (Linnaeus) J.V.

118 Lamouroux, 1813) for 48 h until spawning was induced. Seawater supplied to adults was UV

119 sterilized and 5-μm mechanically filtered at ambient temperature (14.6 ºC) and salinity (32

120 psu). Animals were held under a sixteen hours light and eight hours dark cycle for the

121 duration of the experiment as described in Kelly et al. (2000).

122

123 *Spawning and larval rearing*

124   Adult *P. miliaris* were spawned and reared following the methods described by Kelly

125 et al. (2000) and Liu et al. (2007). Animals were selected for spawning at random from the

126 100 L holding tank until the ratio of 15 females and 2 males was achieved. *P. miliaris* were

127 spawned using injection of 1-2 mL of 0.5 M KCl administered through the peristomal

128 membrane into the coelom (Kelly et al., 2000; Liu et al., 2007). After injection urchins were

129 stored in ~ 300 mL glass jars filled with 5-μm filtered ambient seawater until gametes were

130 shed and then adults were returned to their 100 L housing aquarium. Jars were kept closed

131 with lids during spawning and prior to fertilization to prevent accidental cross-contamination

132 of gametes and ensure targeted fertilization within a precise time. Clean lab gloves and

133 pipettes were utilized for the handling of every sea urchin and transfer of gametes as a further

134 measure to prevent non-target fertilization from occurring. Eggs from 15 females were

135 combined and distributed across all buckets (three replicates per treatment) until they formed

136 a monolayer at the bottom of each bucket containing 12 L of filtered seawater and sealed with

137 a lid. Following the protocols of Kelly et al. (2000) and Liu et al. (2007) eggs were fertilized

138 under control conditions by adding 1-2 mL of diluted (1:5) sperm from 2 males. After 45

139   minutes, seawater was gently mixed and samples were removed and viewed under a

140   compound light microscope to check for the successful fertilization of eggs (mean ± SE = 80

141   ± 3 %) by the presence of a fertilization membrane.   Gametes which are not quantified

142   could have important impacts on fertilisation success. For example polyspermy (eggs

143   fertilised by more than one sperm) can reduce rates of fertilisation (Levitan, 2006). However

144   fertilisation success was relatively high and was not under assessment in this study. Buckets

145   were then supplied with pre-mixed CO_2 (BOC, UK; see section 'Rates of introducing CO_2') or

146   ambient air at ~ 0.2 L min^{-1} via bubbling through glass rods (Kelly et al., 2000; Lui et al.,

147   2007).

148   Twenty four hours after fertilization, five 10 mL samples were removed and checked

149   for mortality and development stage (using a compound light microscope). Swimming

150   gastrulae were observed in all replicates and treatments and therefore could be progressed

151   further. Gas delivery was removed from all cultures and buckets were left static for up to half

152   an hour to allow swimming gastrulae to swim to the surface of the culture vessels. The first 2

153   L of all cultures were decanted into individual glass beakers to capture the swimming

154   gastrulae and to quantify their densities (Kelly et al., 2000; Liu et al., 2007). Densities were

155   quantified by gently mixing the beakers, decanting 1 mL samples onto a 1 mL Sedgewick

156   rafter cell and counted under a compound microscope. The cultures were then decanted into

157   their respective treatment and replicate buckets at a density of ~ 1 ind mL^{-1}. Complete

158   seawater exchanges with appropriately treated seawater were carried out every two days, with

159   larvae retained in a 40-µm mesh sieve in a water bath while buckets were cleaned and filled.

160   Once the stomach had fully formed (gastrula stage, 48 h after fertilization) feeding of

161   larvae was initiated. *Dunaliella tertiolecta* (Culture Collection of Algae and Protozoa, code

162   19/6B) was cultured following methods described in Liu et al. (2007). For larvae with two,

163   three and four pairs of arms, the daily feeding rate was 1500, 4500 and 7500 algal cells mL^{-1},

164   respectively (Kelly et al. 2000). Algal cells were quantified with a Neubauer double chamber

165   counter and then the appropriate volume of algal culture was decanted into larval cultures.

166   Larvae were considered competent for settlement when the rudiment was similar in

167 size to the stomach and spines and tube feet were visible in this structure. Their capacity to

168 settle was quantified by placing larvae into Petri dishes (diameter = 90 mm) conditioned with

169 a natural biofilm of marine bacteria and algae including diatoms (Hinegardner, 1969). Fifty

170 echinoplutei larvae were decanted in triplicate per replicate and assessed for settlement every

171 24 h for 3 days. Larvae that metamorphosed into juveniles were classed as successful settlers.

172 Settlement Petri dishes for the ambient treatment were placed on a small tray and left exposed

173 to the ambient air in a CT room at ~ 16ºC. For experimental treatments, a constant high CO_2

174 environment was maintained by housing Petri dishes in propagators (B&Q Verve 42 cm

175 unheated). Propagators were adapted by inserting three 0.5 mm HDPE hoses and sealing the

176 tray and cover together with silicone grease and bulldog clips, all other openings were sealed

177 closed with silicone sealant prior to the experiment (Fig. 1). Pre-mixed CO_2 gas was supplied

178 to the propagator at ~ 0.1 L min^{-1} via two of the 0.5 mm HDPE hose inlets, the third

179 functioned as a vent to inhibit back diffusion of ambient air (Fig. 1). Blank reduced pH

180 seawater samples were maintained in this environment simultaneously and were measured for

181 pH each day, which remained constant.

182   Temperature and pH (NIST certified) were recorded daily for all larval cultures.

183 Seawater parameters (see section 'Rates of introducing CO_2') were measured for new

184 treatment seawater and 2-day-old seawater prior to water exchange. Seawater samples in the

185 static samples were filtered through a 40-µm mesh basket to prevent the removal of larvae.

186

187 ***Rates of introducing CO$_2$***

188 Introduction of early *P. miliaris* life-stages to year 2100 scenarios (840 ppm

189 Houghton et al., 2001; Guinotte and Fabry, 2008; from ambient control starting conditions)

190 was achieved in a step-wise manner via increasing [CO_2] concentrations from 380, to 470,

191 560, 700 and finally 840 ppm at different rates of change and maintained at 840 ppm [CO_2]

192 until the experiment was terminated. Four experimental regimes were followed (Fig. 2): C:

193   Constant control conditions; F: Fast rates of introduction with a $[CO_2]$ change every 3 h until

194   reaching 840 ppm, with the application of increasing CO_2 being applied during the cleaving

195   (2-cell) embryo stage on Day 1; CF: Fast rates of introduction (as above, but applied from

196   Day 9 when larvae were at the 4-armed echinopluteus stage); S: Slow rate of introduction

197   where a $[CO_2]$ change was instigated every 48 h until 840 ppm $[CO_2]$ was achieved (during

198   the cleaving embryo stage; treatment S; Fig. 2). All air and premixed gas (840ppm ~ pH 7.7)

199   was supplied at a rate of $0.2\ L\ min^{-1}$ into buckets.

200   Daily temperature and pH_{NIST} (YSI Model 63) were recorded, along with twice

201   weekly salinity (psu; YSI Model 63) and TCO_2 ($mmol\ L^{-1}$; Ciba Corning TCO_2 Analyzer 965,

202   Olympic Analytical, UK) for all treatment replicates. The YSI Model 63 was calibrated every

203   second or third day with NIST certified pH buffer solutions. The TCO_2 analyzer was

204   calibrated with $2\ g\ L^{-1}\ CO_2$ standard prior to measurements. Sixty mL were also extracted

205   from each treatment mixing tanks, filtered through GFF filter papers, and stored at -20 ºC in a

206   light proof container until defrosted for nutrient analysis (phosphate and silicate; duplicate

207   samples; for methods see Nickell *et al.* (2003)). Seawater pH conditions under which *P.*

208   *miliaris* larvae were exposed in this study are presented in Fig. 3. Mean seawater parameters

209   under which sea urchins were exposed after reaching target pH levels are presented in Table

210   1. These values include data derived from pre- and post-seawater changes, the addition of

211   microalgal food and settlement plate seawater. Values reported for control conditions are

212   within the normally experienced range for shallow coastal seawater (Barry et al., 2010;

213   Gazeau et al., 2010; Suckling et al., submitted). Control seawater was supersaturated ($\Omega > 1$)

214   with respect to calcite and aragonite (Table 1). The reduced pH treatment was saturated with

215   respect to calcite and undersaturated ($\Omega < 1$) with respect to aragonite (Table 1).

216

217   ***Larval morphometrics***

218   Early larval stages were fixed for morphometric measures after 7 and 17 days, based

219   on the protocols of McEdward and Herrera (1999) and Kelly et al. (2000). Echinoplutei larvae

220   were photographed on an inverted compound microscope with a digital camera and a 1 mm

221   graticule at relevant magnifications. Photography was carried out within 25 days of the

222   experiment to avoid deterioration arising from long-term storage in unbuffered 4% formalin.

223   Calibrations and measurements were then quantified utilizing ImageJ Analysis software

224   (Abramoff et al., 2004). Approximately 30 larvae per replicate (3 replicates per treatment) in

225   each treatment were measured for various parameters dependant on developmental stages.

226   Parameters measured included larval length (LL), larval width (LW), body length (BL), body

227   rod (BR), postoral arm length (PO), postoral arm rod (POR) and rudiment length (RUD).

228   Development stages were recorded as the main categories of 2-armed, 4-armed, 6-

229   armed and 8-armed plutei described in McEdward and Herrera (1999). Larvae were classified

230   as abnormal developers when development diverged strongly away from the norm described

231   in McEdward and Herrera (1999) and Kurihara (2008). Examples of abnormal development

232   included missing arms, abnormal tissue growth and the presence of two rudiments (see Fig. 4

233   for example photographs).

234

235   *Statistical analyses*

236   Treatment differences were analysed using either Nested ANOVA via a General

237   Linear Model or One Way ANOVA (Kelly et al., 2000) after testing for normality (Anderson

238   Darling Test) and homogeneity of variance ($P > 0.05$) in Minitab (Statistical Software™

239   Version 15). After significant ANOVA results, Tukey's or Bonferronni's Pairwise

240   Comparisons were utilized to determine which treatments differed. The relationship between

241   body length and postoral arm length (natural logarithm transformed) were analyzed utilizing

242   Analysis of Covariance (ANCOVA; Sokal and Rohlf, 1995). Prior to ANCOVA, regression

243   analysis determined significant linear relationships between these morphometrics and

244   homogeneity of slopes established. Non-parametric Kruskal Wallis tests were carried out

245   where heterogeneous residual variability remained after transforming data following Sokal

246   and Rohlf (1995). Proportional data were arcsin transformed prior to analysis (Kelly *et al.*,

247   2000). Where Type I errors occurred the means and 95% confidence intervals were

248   graphically analyzed to illustrate the data under normal assumptions. Graphical and tabulated

249   representations of data were presented as the mean and standard errors of treatments unless

250   otherwise indicated.

251

252   **Results**

253   *Larval survival*

254   Decreasing survival occurred in larval cultures with time (Fig. 5), but there were no

255   significant differences between treatments until day 22 (One way ANOVA; day 6: $H_{(3)} = 3.81$,

256    $P = 0.278$; day 8: $F_{(3,8)} = 2.51$, $P = 0.133$; day 10: $F_{(3,8)} = 1.16$, $P = 0.383$; day 12: $F_{(3,8)} = 0.15$,

257    $P = 0.925$; day 14: $F_{(3,8)} = 1.38$, $P = 0.318$; day 16: $F_{(3,8)} = 2.24$, $P = 0.161$; day 18: $F_{(3,8)} =$

258   2.33, $P = 0.151$; Fig. 5). On day 22 the ANOVA probability value was close to the level of

259   acceptance ($P = 0.053$), therefore paired t-tests were utilized to elucidate treatment effects.

260   This showed that the numbers of larvae on day 22 which had been introduced to reduced pH

261   at fast (F) and slow (S) rates were significantly lower compared to the control held at ambient

262   pH (C vs S: $T_{(4)} = 5.77$, $P = 0.004$; C vs F: $T_{(4)} = 3.61$, $P = 0.023$; Fig. 5). Four-armed plutei

263   rapidly introduced to reduced pH (CF) showed large variation in the numbers of surviving

264   larvae and these did not significantly differ from the remaining treatments (C, S and F; C vs

265   CF: $T_{(4)} = 1.27$, $P = 0.273$; F vs S: $T_{(4)} = 0.21$, $P = 0.847$; F vs CF: $T_{(4)} = 1.08$, $P = 0.340$; S vs

266   CF: $T_{(4)} = 1.36$, $P = 0.245$; Fig. 5).

267

268   *Stage and abnormal development*

269   The larval development stage did not differ across treatments on days 7 and 17 and

270   were within the range of stages normally expected for *P. miliaris* reared under ambient

271   conditions (Kelly et al., 2000; Table 2). However, abnormal development was ~ 60% higher

272   in larvae rapidly introduced from the echinoplutei stage (D9) to reduced pH (CF) than other

273   treatments on day 17 (One way ANOVA: $F_{(3,8)} = 7.70$, $P = 0.010$; Table 2).

274

275   *Larval morphometrics and settlement*

276   Larval length measurements: At day 7 offspring in treatment CF were still held under

277   control conditions and had not yet introduced echinoplutei rapidly to low pH conditions (due

278   day 9). These two 'control' treatments (C and CF) were significantly longer (Nested

279   ANOVA: larval length: $F_{(3,314)}$ = 3.87, P = 0.010; body length: $F_{(3,314)}$ = 2.78, P = 0.041) than

280   larvae introduced to reduced pH at fast rates (F) during the cleaving embryo stage. However,

281   larvae which had been introduced to reduced pH conditions at a slow rate (S) did not differ in

282   length compared to those introduced as fast rates (F) or controls (C and CF; Table 3). Larvae

283   in controls (C) had longer Post oral arms and post oral arm rods (Nested ANOVA: PO: $F_{(3,314)}$

284   = 3.42, P = 0.018; POR: $F_{(3,314)}$ = 2.71, P = 0.045; Table 3) than those introduced rapidly (F)

285   during the embryo stage to reduced pH conditions. However, larvae which had been

286   introduced to reduced pH conditions at a slow rate (S) did not differ in post oral arm or post

287   oral arm rod lengths compared to those introduced as fast rates (F) or controls (C and CF;

288   Table 3). No other treatment effects were observed for larval width or body rods on day 7

289   (Table 3).

290   There were significant linear relationships between ln transformed larval body lengths

291   and postoral arms for all treatments (Table 4). The ratio of postoral arm length and larval

292   body length can be used as a size independent measure of larval shape (McEdward, 1984). On

293   day 7, no differences were found in larval shapes across treatments (Table 5).

294   Treatment effects on measured larval parameters were apparent in larval widths and

295   body lengths on day 17. Control (C) and 4-armed plutei rapidly introduced to reduced pH

296   (CF) were wider than those introduced during the cleaving embryo stage at slow (S) and fast

297   (F) rates to reduced pH conditions (Kruskal Wallis: $H_{(3)}$ = 11.91, P = 0.008; Table 3). Body

298   lengths retained the same treatment effects as day 7, with controls (C) longer than larvae

299   rapidly introduced (F) during the embryo stage (Nested ANOVA: $F_{(3,322)}$ = 2.95, P = 0.033;

300   Table 3).

301   Control (C), slow (S) and fast (F) introduction treatments displayed significant linear

302   relationships between ln transformed larval body lengths and postoral arms (Table 4). Data

303   from the treatment which rapidly introduced 4-armed plutei (CF) to reduced pH conditions

304   were not linear and therefore were excluded from the ANCOVA analysis (Table 4). No

305   differences were found in larval shapes across the control (C), slow (S) and fast (F)

306   introduction treatments (F and P values provided in Table 5).

307   Total numbers of larvae metamorphosed and settled as juveniles were similar (One

308   way ANOVA: $F_{(3,8)} = 3.03$, P = 0.093; Fig. 6) across treatments with a mean (± SE) for all

309   treatments of 1497 ± 160 juveniles.

310

311   **Discussion**

312   The aim of this study was to determine whether the direct transfer of ambient-derived

313   offspring to reduced pH conditions is a suitable technical design to use in ocean acidification

314   studies. This is particularly important to investigate since forecasts on organism responses to

315   future climate change are largely based on results gathered from these direct transfer

316   approaches. The rationale was based on previous studies showing an effect of differing rates

317   of introduction of salinity (Anger, 1996) and temperature (Peck et al., 2009) on organism

318   response to laboratory manipulated environmental perturbation. There is also anecdotal

319   evidence from previous ocean acidification work indicating that there may be a complexity of

320   response underlying experimental manipulations, which need further investigation; a range of

321   impacts have been reported from introducing larvae to ocean acidification conditions

322   equivalent to year 2100 forecasts. An example of a positive impact of the effect of pH

323   includes larvae of the orange clownfish, *Amphiprion percula* (Lacepède, 1802), where larvae

324   grew faster than controls (Munday et al., 2009). Some organisms have shown no observed

325   impacts on early life-stages e.g. *Crassostrea ariakensis* (Fujita, 1913) and *C. virginica*

326   (Gmelin, 1791): Miller et al., 2009. However, negative impacts including abnormal

327   development, reduced survival and reduced biomineralization have been reported for a wide

328   range of organisms (e.g. *Saccostrea glomerata* (Gould, 1850): Watson et al., 2009;

329   *Triptneustes gratilla* (Linnaeus, 1758): Sheppard-Brennand et al., 2010; *Haliotis*

330   *kamtschatkana* Jonas, 1845: Crim et al., 2011. Given that many studies have shown negative

331   impacts, this raises the question of whether these negative responses are a product of exposure

332   to forecasted ocean acidification scenarios or potentially an acute stress response of larvae

333   when transferred directly to low seawater pH conditions.

334   Different developmental stages during early life histories have been shown to vary in

335   their responses towards forecasted ocean acidification conditions. Many studies (e.g. Kurihara

336   et al., 2004; Kurihara et al., 2008; Gazeau et al., 2010) have focused on pre-settlement stages,

337   and do not extend to include settled juveniles. The few studies which do include settlement

338   (e.g. Byrne et al., 2011; Dupont et al., 2012) either do not report pre-settlement stage

339   responses in great detail or the post-settlement responses are unclear due to large mortalities

340   within treatment replicates. It has been suggested that there is an increasing sensitivity of sea

341   urchin larvae towards ocean acidification with increasing development time after rapid

342   introduction to altered conditions (e.g. *Paracentrotus lividus* (Lamarck, 1816): Martin et al.,

343   2011; *Strongylocentrotus purpuratus* (Stimpson, 1857): Stummp et al., 2011). In this study

344   pre-settlement (planktotrophic) larvae showed increased mortality in acidic seawater, when

345   acidified from the embryonic stage (at fast (F) and slow (S) rates only), however this was

346   contrasted when post-settlement stages were also considered. This indicates that the former

347   statement (increasing sensitivity with increasing time) may be true for pre-settlement

348   planktotrophic stages but not for metamorphosed juveniles indicating the different levels of

349   sensitivity at different development stages of the life cycle for *P. miliaris*.

350   Various rates of introduction (fast and slow) were utilised to determine whether the

351   response of *P. miliaris* was a direct result of exposure to reduced pH or whether the response

352   was complicated by the rate of introduction. In this study fast introduction (F) to reduced pH

353   conditions gave rise to the most pronounced negative responses from the larvae of ambient

354   sourced sea urchins. These larvae were consistently the smallest in size compared to controls.

355   Furthermore fast rates (F) of introduction resulted in increased mortality 22 days after

356   fertilization compared to controls. The responses of larvae introduced to reduced pH

357   conditions at a slow rate (S) resulted in fewer, less severe negative responses. On day 7 the

358   sizes of larvae introduced to reduced seawater pH slowly (S) were not statistically smaller

359   than controls nor were they statistically larger than their counterparts introduced at a rapid

360 rate. It is later in their development when the negative impacts of reduced pH were

361 manifested. For example, 17 days after fertilization slowly introduced (S) larvae, similarly to

362 those introduced rapidly (F), were smaller in width compared to controls. Finally, slow

363 introductions, (S) also resulted in increased mortality 22 days into the experiment. Therefore,

364 when considering pre-settlement stages of offspring, in this case with the sea urchin *P.*

365 *miliaris*, there is clear evidence that there are variable responses when offspring are exposed

366 to reduced pH at different rates of introduction. The effect on embryos receiving lower pH

367 during early stages of development was more pronounced than offspring experiencing lower

368 pH during later stages of development (see below).

369   If only the pre-metamorphosis results of our study are considered, then larval

370 responses to reduced pH conditions were similar to those reported in other studies (e.g.

371 Watson et al., 2009; Sheppard-Brennand et al., 2010). Although the fast and slow rates of

372 introduction utilised in this study both resulted in negative responses, which is in agreement

373 with the litereature, when compared to each other they resulted in significantly different

374 responses. Control *P. miliaris* were generally larger than counterparts reared under reduced

375 pH treatments, which is in agreement with other echinoderm studies (e.g. Kurihara and

376 Shirayama, 2004; O'Donnell et al., 2010; Stummp et al., 2011). Studies also support reduced

377 larval survival (by day 22) and abnormal development (Dupont et al., 2008; Doo et al., 2011).

378 Similar findings have been reported for other calcifying groups in terms of size, survival or

379 abnormal development (i.e. molluscs: Watson et al., 2009; Gazeau et al., 2010; Talmage and

380 Gobler, 2010; Crim et al., 2011). These studies generally concluded that marine calcifiers will

381 respond negatively when exposed to altered seawater pH at forecasted year 2100 conditions.

382   When this study was extended to include post-settlement stages, there was no overall

383 impact on numbers of individuals successfully reaching the metamorphosed juvenile stage. In

384 comparison to previous research on early life-stage responses to reduced pH conditions, the

385 study described here, is one of only a few (e.g. Byrne et al., 2011; Dupont et al., 2012) to take

386 echinoderm larvae through to settlement, or at least settlement stages. The post-settlement

387 results in this study contrast with the pre-settlement stage results and may indicate that in

388 terms of reaching the settled juvenile stage in *P. miliaris*, the rates of introduction of CO_2

389 when manipulating ambient derived offspring are not relevant. Furthermore any increased

390 exposure to increased CO_2 levels had no effect on numbers successfully developing to settled

391 juveniles. This suggests that a subtle altered selection process (removal of less fit larvae at

392 earlier stages) under reduced pH conditions, and greater mortality at settlement and

393 metamorphosis in controls, may be responsible for the observed negative impacts during

394 development. This subtle altered process of selection may have selected for larvae with

395 specific alleles that improve performance under these conditions as discussed by Pespeni et al.

396 (2013). However, this requires further research to assess how this may impact recruitment and

397 populations in the long term.

398   It has been suggested that ambient-sourced sea urchin larvae rapidly introduced to

399 reduced seawater pH conditions develop morphologically at a slower rate compared to

400 controls (i.e. smaller at the same sample time post fertilization; Martin et al., 2011; Stummp et

401 al., 2011). However, in this study, no significant stage delay was observed when quantifying

402 development stage directly but smaller larval sizes were observed, and indeed if these

403 represent delays in development then they were subtle. Therefore the approach of analysing

404 larvae at similar development stages rather than at the same sample times, as utilised by

405 Stummp et al. (2011), was not employed in the current study.

406   In the current study the resilience of larval stages was also investigated. Ambient-

407 derived larvae were introduced to reduced pH conditions from early (cleaving embryo; F and

408 S) and later (4-armed; CF) development stages. Introduction from early stages (cleaving

409 embryos; F and S) generally gave rise to well pronounced negative responses (lower survival

410 and smaller size) compared to later stages (4-armed; CF). The rapid introduction of the 9-day-

411 old 4-armed larvae (CF) resulted in significantly lower negative impacts of increased

412 abnormal development by day 17 but remained similar in size to controls. This could indicate

413 that later stages are more resilient to reduced pH conditions than those introduced at the

414 embryo stage. However this also may be due to the shorter period of time exposed to reduced

415 pH. Stummp et al. (2011) suggest that there is an increase in metabolic rate of feeding larval

416   development stages under reduced pH conditions compared to controls, likely due to

417   increased maintenance of cellular homeostasis and calcification rates. Therefore our results of

418   abnormal development for larvae introduced during the 4-armed stage (CF) to reduced pH

419   may be an indication of later induced metabolic cost compared to those introduced from the

420   embryonic stage (F and S). The latter were exposed for a longer period to reduced pH and

421   therefore likely confronted with higher metabolic costs resulting in subtle growth differences

422   and mortalities.

423   Coelomic fluid in regular sea urchins is the principal circulatory medium (Ruppert

424   and Barnes, 1994). Subsequently this will reflect changes which occur in the external

425   environment. Therefore if oceanic pH decreases, the pH within sea urchins will also decrease

426   (Spicer et al., 1988; Miles et al., 2007; Spicer et al., 2011; Dupont and Thorndyke, 2012) so

427   that gametogenesis will take place at lower pH. Therefore widening the life-cycle focus to

428   include the adults (parents) should be considered next in studies observing offspring

429   responses to ocean acidification. This approach would not only provide more realistic insight

430   into organismal responses but it would also provide another validation against studies which

431   have previously used methods directly transferring ambient-sourced offspring into low

432   seawater pH conditions. Recently studies have emerged and improved our understanding of F_1

433   generation organisms by pre-exposing parents prior to spawning. Parker et al. (2012) showed

434   that after exposing adult oysters, *Saccostrea glomerata*, to laboratory induced ocean

435   acidification conditions, offspring demonstrated capacities to acclimate (and possibly adapt).

436   Similarly Dupont et al. (2012) showed similar responses in the offspring of the sea urchin

437   *Strongylocentrotus droebachiensis* (Müller, 1776) which had been exposed to ocean

438   acidification conditions prior to spawning. In contrast however, Uthicke et al. (2012) found

439   that the responses of the offspring of the sea urchin *Echinometra mathaei* (Blainville, 1825)

440   were not more resilient to ocean acidification conditions after pre-exposing the parents. This

441   requires further investigation for *P. miliaris* and has been reported by Suckling (2012) and

442   Suckling et al. (Submitted).

443   Ideally multigenerational studies would be used to determine when responses change

444   from physiological flexibility, acclimation to adaptation. However this is difficult to achieve

445   with respect to slow growing invertebrates, such as *P. miliaris* which would take numerous

446   years to achieve. Organisms with rapid life-cycles, such as copepods, therefore provide

447   excellent models for intergenerational responses to altered seawater pH (Dam, 2013). Fitzer et

448   al. (2012) used such an approach and found that *Tisbe battagliai* reallocated resources

449   towards maintaining reproductive output at the expense of somatic growth across three

450   generations. It was concluded that *T. battagliai* has the capacity for phenotypic plasticity but

451   it was unclear on the aspects of acclimation and adaptation (Fitzer et al., 2012). Further work

452   is therefore required to pick apart these types of responses.

453   We conclude that the use of fast introductions of larvae derived from ambient

454   acclimated adults to altered sea water pH, as utilised in many ocean acidification studies, may

455   potentially result in an acute stress response, manifested as increased numbers of abnormally

456   developed larvae and reduced survival rates. Whilst the current study was only conducted on

457   the sea urchin *P. miliaris*, this methodology clearly needs to be repeated on other sea urchins

458   and a wider range of organisms to determine the prevalence of these effects. Our data show

459   that careful consideration is needed when studies report the responses of offspring

460   development, derived from ambient conditions, introduced directly to forecasted ocean

461   acidification. Furthermore, this study highlights the importance of wider life-cycle approaches

462   when forecasting organismal responses to ocean acidification (e.g. including settlement

463   success following larval development).

464

473   **References**

474   Abramoff, M D, Magelhaes, PJ, Ram, SJ. 2004. Image Processing with ImageJ

475   Biophotonics International 11, 36-42

476   Anger, K. 1996. Salinity tolerance of the larvae and first juveniles of a semiterrestrial grapsid

477   crab, *Armases miersii* (Rathbun). Journal of experimental Marine Biology and

478   Ecology 202, 205-223

479   Barry, JP, Tyrell, T, Hansson, L, Plattner, G, Gattuso, J. 2010. Atmospheric CO_2 targets for

480   ocean acidification pertubation experiments. Publications Office of the European

481   Union

482   Byrne, M, Ho, M, Wong, E, Soars, NA, Selvakumaraswamy, P, Sheppard-Brennand, H,

483   Dworjanyn, SA, Davis, AR. 2011. Unshelled abalone and corrupted urchins:

484   development of marine calcifiers in a changing ocean. Proceedings of the Royal

485   Society of London Series B – Biological Society 278: 2376-2383.

486   Caldeira, K, Wickett, ME, 2003. Oceanography: Anthropogenic carbon and ocean pH. Nature

487   425, 365-365

488   Caldeira, K, Wickett, ME. 2005. Ocean model predictions of chemistry changes from carbon

489   dioxide emissions to the atmosphere and ocean. Journal of Geophysical Research

490   doi:10.1029/2004JC002671

491   Caldwell, GS, Fitzer, S, Gillespie, CS, Pickavance, G, Turnball, E, Bentley, MG. 2011. Ocean

492   acidification takes sperm back in time. Invertebrate Reproduction and Development

493   55, 217-221

494   Canadell, JG, Le Quéré, C, Raupach, MR, Field, CB, Buitenhuis, ET, Ciais, P, Conway, TJ,

495   Gillett, NP, Houghton, RA, Marland, G. 2007. Contributions to accelerating

496   atmospheric CO_2 growth from economic activity, carbon intensity, and efficiency of

497   natural sinks. Proceedings of the National Academy of Sciences 104, 18866-18870

498   Crim, RN, Sunday, JM, Harley, CDG. 2011. Elevated seawater CO_2 concentrations impair

499   larval development and reduce larval survival in endangered northern abalone

500   (*Haliotis kamtschatkana*). Journal of Experimental Marine Biology and Ecology 400:

501   272-277.

502   Dam, HG. 2013. Evolutionary adaptation of marine zooplankton to global change. Annual

503   Review of Marine Science, 5: 349-70

504   Dickson, AG, Millero, FJ, 1987. A comparison of the equilibrium-constants for the

505   dissociation of carbonic-acid in seawater media. Deep-Sea Research 34, 1733-1743.

506   Doney, SC, Fabry, VJ, Feely, RA, Kleypas, JA. 2009. Ocean acidification: The Other CO_2

507   Problem. Annual Review of Marine Science 1, 169-192

508   Doo, SS, Dworjanyn, SA, Foo, SA, Soars, NA, Byrne, M. 2011. Impacts of ocean

509   acidification on development of the meroplanktonic larval stage of the sea urchin

510   *Centrostephanus rodgersii*. ICES Journal of Marine Science doi:

511   10.1093/icesjms/fsr123.

512   Dupont, S, Havenhand, J, Thorndyke, W, Peck, LS, Thorndyke, M. 2008. Near-future level of

513   CO_2-driven ocean acidification radically affects larval survival and development in

514   the brittlestar *Ophiothrix fragilis*. Marine Ecology Progress Series 373: 285–294.

515   Dupont, S, Dorey, N, Stumpp, M, Melzner, F, Throndyke, M. 2012. Long-term and trans-life-

516   cycle effects of exposure to ocean acidification in the green sea urchin

517   *Strongylocentrotus droebachiensis*. Marine Biology, 1-9. doi: 10.1007/s00227-012-

518   1921-x

519   Dupont, S, Thorndyke, M. 2012. Relationship between CO_2-driven changes in extracellular

520   acid–base balance and cellular immune response in two polar echinoderm species.

521   Journal of Experimental Marine Biology and Ecology 424-425, 32-37.

522   Fitzer, SC, Caldwell, GS, Close, AJ, Clare, AS, Upstill-Goddard, RC, Bentley, MG. 2012.

523   Ocean acidification induces multi-generational decline in copepod naupliar

524   production with possible conflict for reproductive resource allocation. Journal of

525   experimental Marine Biology and Ecology, 418-419: 30-36

526   Gazeau, F, Gattuso, J-P, Dawber, C, Pronker, AE, Peene, F, Peene, J, Heip, CHR,

527   Middelburg, JJ. 2010. Effect of ocean acidification on the early life-stages of the blue

528   mussel *Mytilus edulis*. Biogeosciences 7, 2051-2060

529   Guinotte, JM, Fabry, VJ. 2008. Ocean Acidification and Its Potential Effects on Marine

530   Ecosystems. Annals of the New York Academy of Sciences 1134, 320-342

531   Hinegardner, RT. 1969. Growth and development of the laboratory cultured sea urchin.

532   Biological Bulletin 137, 465-475

533   Houghton, JT, Ding, Y, Griggs, DJ, Noguer, M, Van der Linden, PJ, Dai, X, Maskell K,

534   Johnson, CA. 2001. Climate change 2001: The scientific basis, Cambridge, UK

535   IPCC, 2013. Working Group I Contribution to the IPCC Fifth Assessment Report, Climate

536   Change 2013: The Physical Science Basis. Summary for Policymakers. [Cited 09-

537   Nov-13]. Available from: http://www.ipcc.ch/

538   Kelly, MS, Hunter, AJ, Scholfield, CL, McKenzie, JD, 2000. Morphology and survivorship of

539   larval *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) in response to

540   varying food quantity and quality. Aquaculture 183, 223-240

541   Kurihara, H, 2008. Effects of CO_2-driven ocean acidification on the early developmental

542   stages of invertebrates. Marine Ecology Progress Series 373, 275-284

543   Kurihara, H, Shimode, S, Shirayama, Y. 2004. Sub-lethal effects of elevated concentration of

544   CO_2 on planktonic copepods and sea urchins. Journal of Oceanography 60, 743-750

545   Kurihara, H, Shirayama, Y. 2004. Effects of increased atmospheric CO_2 on sea urchin early

546   development. Marine Ecology Progress Series 274, 161-169

547   Kurihara, H, Matsui, M, Furukawa, H, Hayashi, M, Ishimatsu, A. 2008. Long-term effects of

548   predicted future seawater CO2 conditions on the survival and growth of the marine

549   shrimp *Palaemon pacificus.* Journal of Experimental Marine Biology and Ecology

550   367, 41–46

551   Lane, CE, Mayes, C, Druehl, LD, Saunders, GW. 2006. A multi-gene molecular investigation

552   of the kelp (Laminariales, Phaeophyceae) supports substantial taxonomic re-

553   organization. Journal of Phycology 42, 493-512

554   Levitan, DR. 2006. The relationship between egg size and fertilization success in broadcast-
555   spawning marine invertebrates. Integrative and Comparative Biology, 46: 298-311

556   Lewis, E, Wallace, DWR, 1998. Carbon Dioxide Information Analysis Center, Oak Ridge
557   National Laboratory, US Department of Energy, Oak Ridge, Tennessee, US.

558   Liu, H, Kelly, MS, Cook, EJ, Black, K, Orr, H, Zhu, JX, Dong, SL. 2007. The effect of diet
559   type on growth and fatty acid composition of the sea urchin larvae, II. *Psammechinus*
560   *miliaris* (Gmelin). Aquaculture 264, 263-278

561   Martin, S, Richier, S, Pedrotti, ML, Dupont, S, Castejon, C, Gerakis, Y, Kerros, ME,
562   Oberhansli, F, Teyssie, JL, Jeffree, R, Gattuso, JP, 2011. Early development and
563   molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to
564   CO_2-driven acidification. Journal of Experimental Biology 214, 1357-1368

565   Matranga, V, Toia, G, Bonaventura, R, Muller, WG, 2000. Cellular and biochemical
566   responses to environmental and experimentally induced stress in sea urchin
567   coelomocytes. Cell Stress Chaperon. 5, 113-120

568   McEdward, LR, 1984. Morphometric and metabolic analysis of the growth and form of an
569   echinopluteus. Journal of Experimental Marine Biology and Ecology 82, 259-287

570   McEdward, LR, Herrera, JC, 1999. Body form and skeletal morphometrics during larval
571   development of the sea urchin *Lytechinus variegatus* Lamarck. Journal of
572   Experimental Marine Biology and Ecology 232, 151-176

573   Mehrbach, C, Culberson, CH, Hawley, JE, Pytkowicxz, RM, 1973. Measurement of apparent
574   dissociation constants of carbomic acid in seawater at armospheric pressure.
575   Limnology and Oceanography 18, 897-907

576   Miles, H, Widdicombe, S, Spicer, JI, Hall-Spencer, J. 2007. Effects of anthropogenic
577   seawater acidification on acid-base balance in the sea urchin *Psammechinus miliaris*.
578   Marine Pollution Bulletin 54, 89-96.

579   Miller, AW, Reynolds, AC, Sobrino, C, Riedel, GF, 2009. Shellfish face uncertain future in
580   high CO_2 world: Influence of acidification on oyster larvae calcificaiton and growth
581   in estuaries. PLoS ONE 4: e5661 doi: 10.1371/journal.pone.0005661

582 Munday, PL, Donelson, JM, Dixson, DL, Endo, GK. 2009. Effects of ocean acidification on

583   the early life history of a tropical marine fish. Proceedings of the Royal Society B:

584   Biological Sciences 276, 3275-3283.

585 Nickell, LA, Black, KD, Hughes, DJ, Overnell, J, Brand, T, Nickell, TD, Breuer, E, Martyn,

586   HS. 2003. Bioturbation, sediment fluxes and benthic community structure around a

587   salmon cage farm in Loch Creran, Scotland. Journal of Experimental Marine Biology

588   and Ecology 285: 221-233.

589 O'Donnell, MJ, Todgham, AE, Sewell, MA, Hammond, LM, Ruggiero, K, Fangue, NA,

590   Zippay, ML, Hofmann, GE. 2010. Ocean acidification alters skeletogenesis and gene

591   expression in larval sea urchins. Marine Ecology Progress Series 398, 157-171

592 Parker, LM, Ross, PM, O'Conner, WA, Borysko, L, Raftos, DA, Portner, H-O. 2012. Adult

593   exposure influences offspring response to ocean acidification in oysters. Global

594   Change Biology 18, 82-92.

595 Peck, LS, Clark, MS, Morley, SA, Massey, A, Rossetti, H. 2009. Animal temperature limits

596   and ecological relevance: effectsvof size, activity and rates of change. Functional

597   Ecology 23, 248-256.

598 Pespeni, MH, Sanford, E, Gaylord, B, Hill, TM, Hosfelt, JD, Jaris, HK, LaVigne, M, Lenz,

599   EA, Russell, AD, Young, MK, Palumbi, SR. 2013. Evolutionary change during

600   experirmntal ocean acidification. Proceedings of the National Academy of Sciences,

601   110: 6937-6942.

602 Riebesell U, Fabry VJ, Hansson L, Gattuso J-P. (Eds.) 2010. Guide to best practices for ocean

603   acidification research and data reporting, 260 p. Luxembourg: Publications Office of

604   the European Union.

605 Ries JB, Cohen AL, McCorkle DC. 2009. Marine calcifiers exhibit mixed responses to CO_2-

606   induced ocean acidification. Geology 37, 1131-1134.

607 Royal Society 2005. Ocean acidification due to increasing atmospheric carbon dioxide.

608   London: The Royal Society, 223 pp.

609 Ruppert, EE, Barnes, RD. 1996. Invertebrate Zoology. Thomson learning, UK.

610   Sheppard-Brennand, H, Soars, N, Dworjanyn, SA, Davis, AR, Byrne, M. 2010. Impact of

611   ocean warming and ocean acidification on larval development and calcification in the

612   sea urchin *Tripneustes gratilla*. PLoS ONE doi:10.1371/journal.pone.0011372

613   Sokal, RR, Rohlf, FJ. 1995. Biometry: The principles and practice of statistics in biological

614   research. W.H. Freeman, New York.

615   Spicer, JI, Taylor, AC, Hill, AD. 1988. Acid-base status in the sea urchins *Psammechinus*

616   *miliaris* and *Echinus esculentus* (Echinodermata: Echinoidea) during emersion.

617   Marine Biology 99, 527-534.

618   Spicer, JI, Widdicombe, S, Needham, HR, Berge, JA. 2011. Impact of CO_2-acidified seawater

619   on the extracellular acid-base balance of the northern sea urchin *Strongylocentrotus*

620   *dröebachiensis*. Journal of Experimental Marine Biology and Ecology 407, 19-25.

621   Stummp, M, Wren, J, Melzner, F, Thorndyke, MC, Dupont, ST. 2011. CO_2 induced seawater

622   acidification impacts sea urchin larval development I: Elevated metabolic rates

623   decrease scope for growth and induce developmental delay. Comparative

624   Biochemistry and Physiology A: Moloecular and Integrative Physiology 160, 331-

625   340.

626   Suckling, CC, Symonds, RC, Kelly, MS, Young, AJ. 2011. The effect of artificial diets on

627   gonad colour and biomass in the edible sea urchin *Psammechinus miliaris*.

628   Aquaculture 318, 335-342

629   Suckling, CC. 2012. Calcified marine invertebrates: the effects of ocean acidification. Phd

630   Thesis, University of Cambridge, UK

631   Suckling, CC, Clark, MS, Beveridge, C, Brunner, L, Hughes, AD, Harper, EM, Cook, EJ,

632   Davies, AJ, Peck, MS, submitted. Experimental influence of pH on the early life-

633   stages of sea urchins II: increasing parental exposure gives rise to different responses.

634   Invertebrate Reproduction and. Development.

635   Symonds, RS, Kelly, MS, Suckling ,CC, Young, AJ. 2009. Carotenoids in the gonad and gut

636   of the edible sea urchin *Psammechinus miliaris*. Aquaculture 288: 120-125.

637   Talmage, SC, Gobler, CJ. 2010. Effects of past, present, and future ocean carbon dioxide

638   concentrations on the growth and survival of larval shellfish. Proceedings of the

639   National Academy of Sciences doi 10.1073/pnas.0913804107

640   Uthicke, S, Soars, N, Foo, S, Byrne, M. 2012. Effects of elevated CO_2 and the effect of parent

641   acclimation on development in the tropical Pacific sea urchin *Echinometra mathaei*.

642   Marine Biology 1-14 doi: 10.1007/s00227-012-2023-5

643   Watson, SA, Southgate, PC, Tyler, PA, Peck, LS. 2009. Early larval development of the

644   sydney rock oyster *Saccostrea glomerata* under near-future predictions of CO_2-driven

645   ocean acidification. Journal of Shellfish Research 28, 431-437
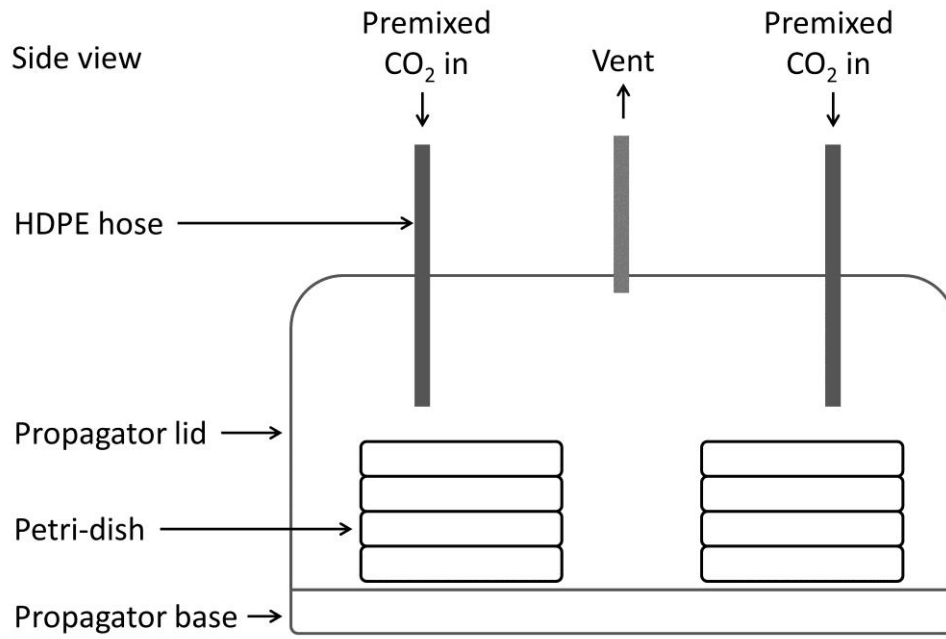
646

647

648

649

650

651

652

653

654

655

656

Side view

Premixed CO$_2$ in   Vent   Premixed CO$_2$ in

HDPE hose

Propagator lid

Petri-dish

Propagator base

657

658 **Fig. 1:** Schematic of settlement chambers comprised of adapted propagators.
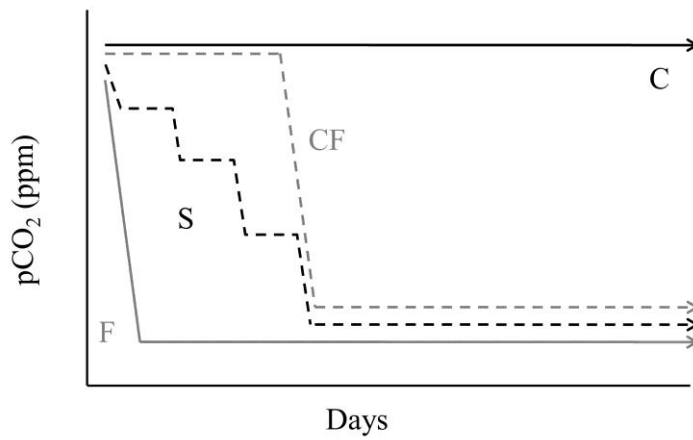
659

660

661

662

663

664

665

666

667

668

669

670

671

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

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

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

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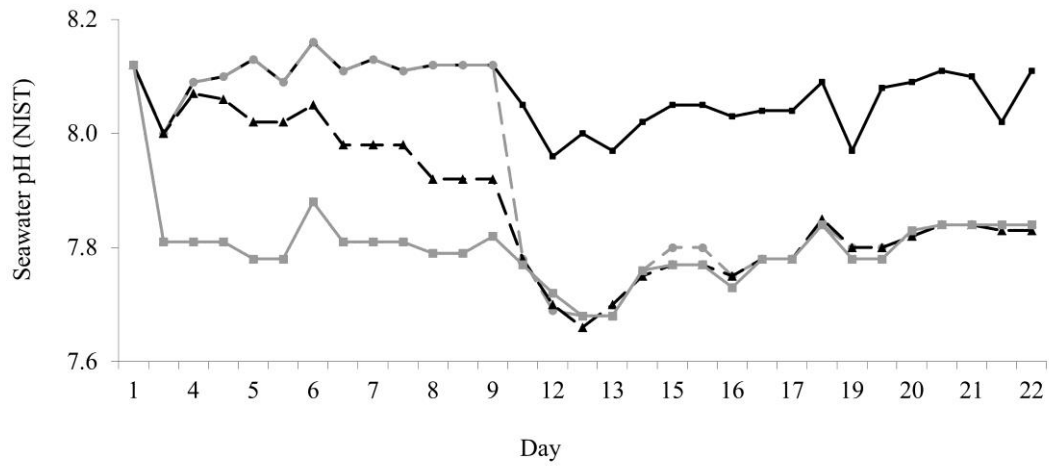
677   pH conditions.

678

679

680

681

682

683

684

685

686

687

688
689 **Fig. 3:** Seawater pH introduced to *Psammechinus miliaris* larvae (± SE). C ━━◆━━ = control

690 larvae; CF - -●- - = fast introduction of 9 day old control reared larvae to reduced pH

691 conditions; S - -▲- - = slow introduction of control cleaving embryos to reduced pH

692 conditions; F ━━■━━ = fast introduction of control cleaving embryos to reduced pH

693 conditions.

694

695

696

697

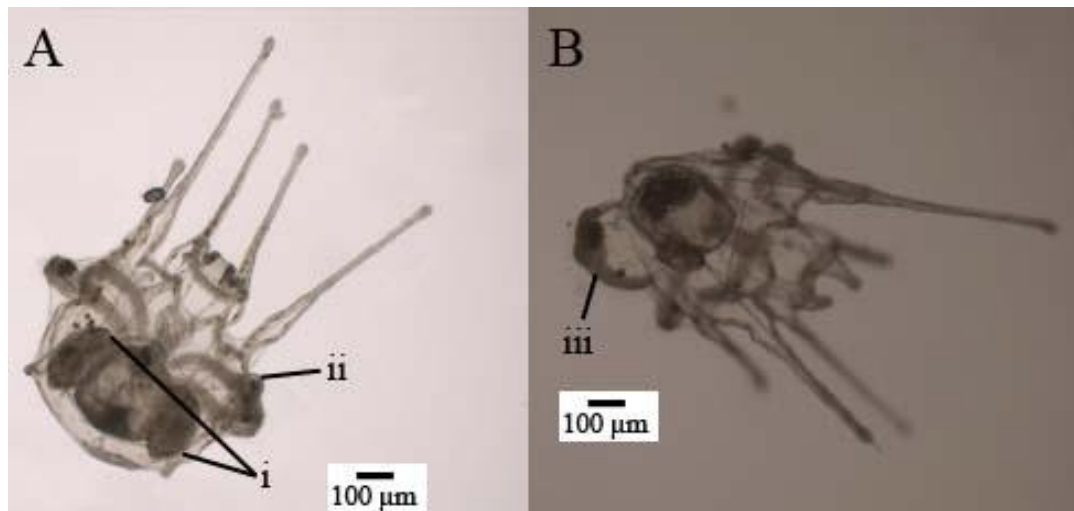698

699

700

701

702

Fig. 4: Example photographs of abnormal development of larval *Psammechinus miliaris* are presented in a) and b): i) the presence of two rudiments, ii) a missing arm and iii) abnormal tissue growth.
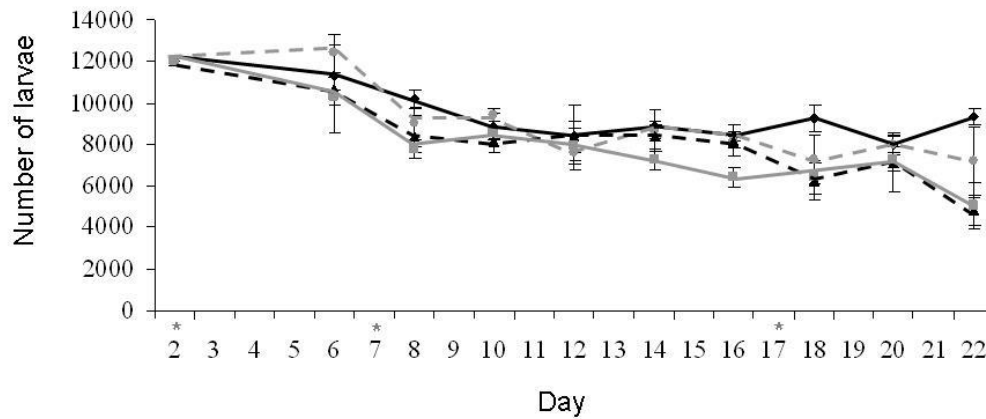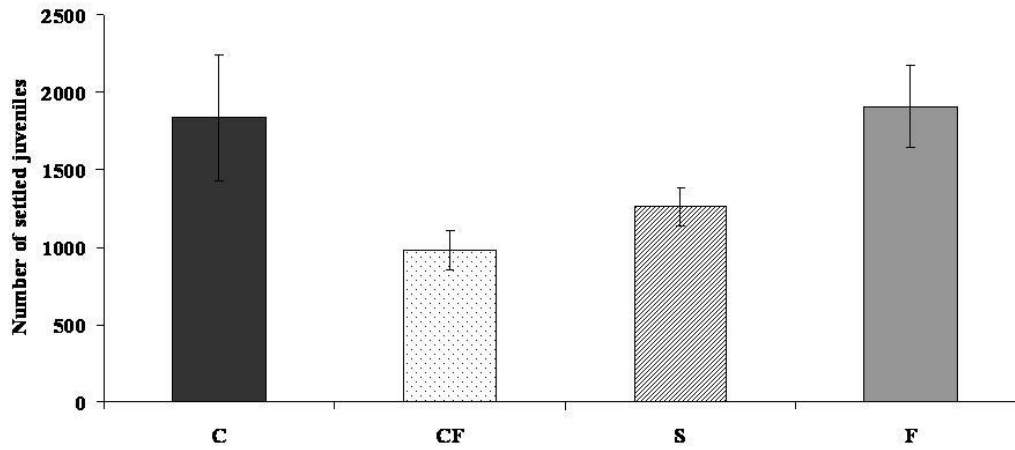
724
725 **Fig. 5:** Larval survival (total experimental numbers) of *Psammechinus miliaris* larvae (± SE)

726 introduced from control to reduced pH at different rates and from different development

727 stages. Settlement tests commenced from ~Day 23, therefore no density data are presented

728 after Day 22. C ●——— = control larvae; CF -- -●- - = fast introduction of 9 day old control

729 reared larvae to reduced pH conditions; S - -▲- - = slow introduction of control cleaving

730 embryos to reduced pH conditions; F ——■—— = fast introduction of control cleaving embryos

731 to reduced pH conditions.* = Morphometric measurement sample times (See section 'Larval

732 morphometrics').

733

734

735

736

737

738

739
740 **Fig. 6:** Mean numbers of settled juvenile *Psammechinus miliaris* (± SE) introduced from

741 control to reduced pH at different rates and from different development stages. C ■ =control

742 larvae; CF ▢ = fast introduction of 9 day old control reared larvae to reduced pH conditions;

743 S ▨ = slow introduction of control cleaving embryos to reduced pH conditions; F ▨ = fast

744 introduction of control cleaving embryos to reduced pH conditions.

745

746

747

748

749

750

751

752

753

754

755 **Table 1:** Mean (± SE) seawater treatment parameters introduced to *Psammechinus miliaris*

756 larvae after target pH levels were reached. Ω calcite and Ω aragonite values modelled from

757 CO2SYS (Lewis & Wallace, 1998) with refitted constants (Mehrbach, *et al.*, 1973; Dickson

758 & Millero, 1987). C = control larvae; CF = fast introduction of 9 day old control reared larvae

759 to reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH

760 conditions; F = fast introduction of control cleaving embryos to reduced pH conditions.

761

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

762
763

764

765

766

767

768

769

770

771

772

773

774

775

776

777 **Table 2:** Mean development stages (± SE; larval numbers) of *Psammechinus miliaris* larvae

778 introduced from control to reduced pH at different rates and from different development

779 stages. C = control larvae; CF = fast introduction of 9 day old control reared larvae to reduced

780 pH conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F

781 = fast introduction of control cleaving embryos to reduced pH conditions. Superscripts

782 indicate where significant differences lie within each column in respective development stage

783 sections. Columns without superscripts indicate no treatment effects.

784

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

785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801

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

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

818 **Table 4:** Regression analysis of *Psammechinus miliaris* larvae introduced from control to

819 reduced pH at different rates and from different development stages. The data were ln

820 transformed. C = control larvae; CF = fast introduction of 9 day old control reared larvae to

821 reduced pH conditions; S = slow introduction of control cleaving embryos to reduced pH

822 conditions; F = fast introduction of control cleaving embryos to reduced pH conditions. PO =

823 postoral arm length; BL = body length. * = Day 17 CF ratio not linear, therefore removed

824 from subsequent ANCOVA analysis.

825 .

826

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

827
828

829

830

831

832

833

834

835

836

837

838

839

Author's post print version

840   **Table 5:** Analysis of covariance of *Psammechinus miliaris* larvae introduced from control to

841   reduced pH at different rates and from different development stages. Postoral arm lengths

842   were analysed with a covariate of larval body length (BL). The data were ln transformed. C =

843   control larvae; CF = fast introduction of 9 day old control reared larvae to reduced pH

844   conditions; S = slow introduction of control cleaving embryos to reduced pH conditions; F =

845   fast introduction of control cleaving embryos to reduced pH conditions. * Day 17 CF

846   treatment not linear, therefore not included in ANCOVA analysis.

847

Day	Source	DF	MS	F	P
	Treatment	3	0.065	1.15	0.327
7	Covariance (BL)	1	13.205	23..67	<0.001
	Error	320	0.057		
	Treatment	2	0.424	2.41	0.092
17 *	Covariance (BL)	1	24.947	41.43	<0.001
	Error	243	0.176		

848
849
850
851
852

35