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1 **Optimising stocking density for the commercial cultivation of sea urchin larvae.**

2
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13
14 **Abstract**

15
16 Increased pressure on wild stocks of sea urchins had led to a requirement for aquaculture based
17 production. However, effective and efficient methodologies still remain under development. The effects
18 of stocking density on *Psammechinus miliaris* and *Paracentrotus lividus* were investigated in order to
19 evaluate optimum stocking densities for large scale production. Larvae were reared at stocking densities
20 of 1, 2, 3 and 4 larvae mL⁻¹ and the effects on survival, development, abnormality and morphology were
21 recorded. Additional cultures were maintained at a high density of 3 larvae mL⁻¹ and then displaced to
22 a lower density of 1 larvae mL⁻¹ part way through the larval life cycle ('displacement treatment'; day
23 13), to evaluate whether negative effects of high stocking densities could be mitigated. Responses from
24 each species differed. *P. miliaris* demonstrated the highest growth at 1 larvae mL⁻¹, resulting in larger
25 larval and rudiment sizes by the end of the experiment (day 16). Rearing at 2 larvae mL⁻¹ also
26 demonstrated good growth performance, but only up to day 12. Higher densities of 3 and 4 larvae mL⁻¹
27 ¹ did not affect survival or development, but significantly negatively impacted growth. There was no
28 significant impact on survival, development, and morphology at any of the tested stocking densities for
29 *P. lividus*. However, of note is that *P. lividus* reared at a high density of 4 larvae mL⁻¹ had 25% lower
30 survival than controls by the end of the experimental period (day 16). Displacement (larvae transferred
31 from 3 to 1 larvae mL⁻¹ on day 13) was effective for both *P. miliaris* and *P. lividus* with survival and
32 rudiment sizes similar to larvae stocked continuously at low densities of 1 larvae mL⁻¹. Although, *P.*
33 *lividus* generally performed well at high densities, this demonstrates that displacement approaches could
34 be possible for this species if required. However, of note is that displaced *P. lividus* had 30% lower
35 survival than controls by the end of the experimental period (day 16). Therefore, this cultivation

36 approach may be a generally viable option for large scale cultivation of these species. This study
37 highlights that species responses can be different when reared at differing stocking densities
38 highlighting a need to expand this approach to a wider range of marketable species. It also demonstrates
39 that more efficient means of production (e.g. displacing larval densities part way through the production
40 process) might be possible for some species (e.g. *P. miliaris*).

41

42 **Keywords:** aquaculture; echinoderm; echinoculture; market; rearing; shellfish.

43

44

45 1. Introduction

46 Global harvesting of sea urchins has substantially increased in recent decades. Rising from
47 48,000 tonnes in 1982 to 120,000 tonnes in 1995, this has caused sharp declines of wild stocks as a
48 direct result of overexploitation, with harvesting currently at 75,000 tonnes (Pearce, 2010; Stefánsson
49 et al., 2017). Consequently, there has been an increased effort into the development of successful rearing
50 techniques for a variety of edible species (e.g. Fernandez & Caltagirone, 1994; de Jong-Westman *et al.*
51 1995; Grosjean *et al.* 1998) with rapid advances in research into intensive culture systems, especially
52 in Europe (Carboni *et al.* 2014).

53 Sea urchin larval cultivation techniques are reasonably well established with some studies
54 focussed on optimising methodology, examples include investigating the effects of feed types
55 (Hinegardner, 1969; Fenaux *et al.* 1985; Leighton *et al.* 1994; Cook *et al.* 1998; Kelly *et al.* 2000; Liu
56 *et al.* 2007), salinity (Metaxas 1998; George & Walker 2007), and temperature (Hart & Scheibling
57 1988; Sewell & Young 1999). However, optimum stocking densities for larval cultures have not yet
58 been satisfactorily identified for all species. Experimental studies commonly maintain cultures at 1
59 larvae mL⁻¹ (e.g. Fenaux *et al.* 1994; Leighton 1995; Kelly *et al.* 2000; Liu *et al.* 2007), but within a
60 commercial setting it would be more economical and efficient to rear larvae at higher densities, as long
61 as larval quality is not compromised. To date there are only two studies which have directly investigated
62 the effect of larval stocking density on echinoid species. Buitrago *et al.* (2005) assessed the response of
63 larvae of the sea urchin *Lytechinus variegatus* to extremely low stocking densities (equivalent to 0.25,
64 0.50 and 1 larvae mL⁻¹). Larval masses reared at a density of 1 larvae mL⁻¹ were 50% lower than larvae
65 reared at the lower densities of 0.25 and 0.50 larvae mL⁻¹. However, the authors concluded that stocking
66 densities of 1 larvae mL⁻¹ were suitable for cultivation. Azad *et al.* (2012) used higher stocking densities
67 equivalent to 0.5, 1, 2 and 4 larvae mL⁻¹ on *Strongylocentrotus purpuratus* and concluded that larval
68 survival and growth was greatest when stocked at low densities of ≤ 1 larvae mL⁻¹ compared to higher
69 densities (>2 larvae mL⁻¹). These two studies are in agreement, suggesting that an optimal stocking
70 density for sea urchins may be around 1 larvae mL⁻¹, but are based on only two species. It is widely
71 known that responses to different holding conditions can be species specific (e.g. Fujisawa, 1989; Liu
72 & Chang, 2015). Therefore, more resilient species could display commercially acceptable tolerances,
73 allowing for an intensification of stocking density practices.

74 Larvae stocked at higher densities will have less relative space per individual and subsequently
75 crowding, competition for space, food and other resources will be more pronounced. These will be
76 exacerbated as the larvae grows and occupies more space (Forsythe *et al.* 2002). The interaction
77 between conspecifics, competitors and prey can affect growth directly. For example, by affecting food
78 intake, or indirectly, by diverting energy from somatic growth (Forsythe & Heaukelem, 1987;
79 Siikavuopio, *et al.* 2007). Overcrowding can also restrict oxygen supply and increase collisions
80 resulting in physical damage (Buitrago *et al.* 2005; Azad *et al.* 2010). Subsequently these factors of
81 influence can negatively impact survival, growth and quality in many studied species (e.g. sea

82 cucumbers (Li & Li, 2009), shrimp (Martin *et al.* 1998) and fish (Paspatis *et al.* 2003)). Introducing
83 additional feed into systems can alleviate competition but also results in increased waste production,
84 which can introduce dangerous levels of toxins, causing malformation or mortality (Cho *et al.* 1994;
85 Gomes *et al.* 2000; Ebeling *et al.* 2006; McEdward & Miner, 2007). Some negative effects can largely
86 be mitigated by appropriate cultivation techniques. However, the effects of space limitations caused by
87 high stocking densities cannot, unless these densities are reduced, and this could be implemented part
88 way through the larval development cycle (e.g. from high to low stocking density). During the early
89 stages of larval development larvae are typically small, occupy less space and subsequently may be less
90 prone to damage compared to later developmental stages. No studies have yet investigated this approach
91 on larval quality during cultivation and this approach may enhance sea urchin cultivation success.

92 The aim of this study was to determine an optimal larval stocking density for sea urchin species
93 where this has not yet been previously assessed. Additionally, larvae reared at high stocking densities
94 during the early stages of larval development were later transferred to lower densities, to determine
95 whether larval survival, growth and development could be improved. In this study, two sea urchin
96 species were investigated, *Paracentrotus lividus* and *Psammechinus miliaris*. *P. lividus* is a well-
97 established commercially harvested species with substantial commercial appeal (Bourdouresque &
98 Verlaque, 2007). Whilst, *P. miliaris* has demonstrated resilience to future climate change, shows
99 generally positive responses with respect to marketability and is a potential candidate for human
100 consumption (e.g. Kelly *et al.* 1998; Suckling *et al.* 2011; 2014a,b).

101

102 **2. Materials and methods**

103 *2.1 Animal collection and maintenance*

104 Broodstock of *P. lividus* were sourced from laboratory reared animals from Aquaculture Ltd.,
105 Ardtoe Marine Laboratory, Ardtoe, Scotland in November 2014. These were transported in coolboxes
106 with aerated seawater from Ardtoe to the Scottish Association for Marine Science aquaria within 4
107 hours and held in these facilities overnight. The following day the animals were transported under
108 similar conditions to Bangor University's School of Ocean Sciences within 8 hours with a 70 %
109 seawater change every 3rd hour. Broodstock of *Psammechinus miliaris* were initially sourced from Loch
110 Creran (Symonds *et al.*, 2009), transported under similar protocols and laboratory reared within Bangor
111 University's School of Ocean Science's aquarium following the methods described by Kelly *et al.*
112 (2000) and Suckling *et al.* (2014a,b). These broodstock were maintained at ambient temperature (6.1-
113 16.7 °C), salinity (35-36), and ambient photoperiod until the experimental period (June to July 2015).
114 *P. miliaris* were fed a diet of *Laminaria digitata* and *Mytilus edulis* and *P. lividus* were fed on diets of
115 *Laminaria digitata* and *Palmaria palmata ab libitum*.

116

117 *2.2. Spawning and larval rearing*

118 Culture methods used throughout the experiment were based on techniques used by Kelly *et al.*
119 (2000) and Suckling *et al.* (2014a,b) for rearing of *P. miliaris* and *P. lividus*. Spawning was induced
120 by injecting 0.5-1 mL of 0.5 M KCl into the haemocoel via the peristomal membrane and individuals
121 spawned into separate 200 mL jars filled with 1 μm filtered and UV sterilised seawater. Using a gamete
122 ratio of 250 ♂:1 ♀ (collected from four females and two males), gametes were mixed in two replicate
123 8 L buckets. After 45 minutes fertilisation success was > 92 % and after 24 hours hatching success >
124 90 % for both species indicating that the eggs used were viable. Successful larvae were then decanted
125 into 12 L buckets containing gently aerated 1 μm filtered and UV sterilised seawater to achieve four
126 stocking density treatments of 1, 2, 3 and 4 larvae mL^{-1} , each with three independent replicates.

127 Larvae were maintained at an ambient temperature of ~ 12 °C and under a photoperiod of 16
128 hours light and 8 hours dark. Every 2 to 3 days a full water change was carried out by carefully filtering
129 larvae through a 47 μm sieve in a water bath to reduce aerial exposure of larvae. The culture buckets
130 were then cleaned with freshwater and a non-abrasive sponge, and larvae washed off the sieve into the
131 relevant culture buckets containing fresh seawater. Total volume of filtered seawater in each treatment
132 were adjusted to ensure that targeted larval densities were maintained throughout the experiment. After
133 the stomach had formed (48 hours after fertilisation) larvae were fed at a rate of 1500, 4500 and 7500
134 cells $\text{mL}^{-1} \text{day}^{-1}$ of the alga *Dunaliella tertiolecta* (quantified using a haemocytometer) for larval
135 development stages with two, three and four pairs of arms respectively (Kelly *et al.* 2000). This
136 concentration of feed was scaled with larval density (e.g. cultures of 2 larvae mL^{-1} received 3000, 9000
137 and 15000 cells $^{-1} \text{mL}^{-1} \text{day}^{-1}$ for respective development stages).

138

139 2.3. Larval Survival, development and morphology

140 Changes in larval survival were calculated by dividing the number of larvae present in the
141 sample by the initial numbers stocked during the start of the experiment and then expressed as a
142 percentage. Each culture was gently agitated to evenly distribute the larval populations and three 5 mL
143 samples were then taken to assess the density of larvae with a Sedgewick Rafter cell. Larval
144 development was assessed by analysing the proportion of larvae in each stage (stage 1 = 2 pairs of arms,
145 stage 2 = 3 pairs of arms and stage 3 = 4 pairs of arms).

146 To assess the effects of culture density on morphology of larvae three 25 mL samples were
147 taken every 2-4 days from each replicate and fixed in 4% formaldehyde. Fifteen larvae were selected at
148 random for morphological analysis. Under a fume hood, larvae were photographed using a UMCO U-
149 series digital light microscope camera and analysed using the software ImageJ. Photos were scaled
150 using a 1 mm graticule photographed under the same magnifications. Five morphological measurements
151 were taken from each larva: larval length, body length, body width, post-oral arm length and rudiment
152 length as described by Kelly *et al.* (2002) and Suckling *et al.* (2014a). The rudiment, located by the
153 stomach, is where microscopic tube feet and spines appear when the individual is close to
154 metamorphosis for settlement (McEdward & Herrera, 1999). Larvae with deformities such as irregular

155 or additional growth, missing or damaged arms were considered abnormally developed (Okazaki,
156 1960). Where the skeletal rods protruded from the external membrane of the larvae or unusual
157 thickness/thinness was seen, larvae were considered malnourished (Kelly *et al.* 2000).

158

159 2.4. Density change trials

160 To determine whether the negative responses of highly stocked larvae could be avoided, larvae
161 reared under high stocking density were moved to lower densities part way through larval cultivation.
162 Larvae of *P. miliaris* and *P. lividus* were reared, following the protocols outlined above, initially at a
163 density of 3 larvae mL⁻¹. Cultures were then displaced to a lower density of 1 mL⁻¹ on day 13 (when the
164 majority of larvae display 6 pairs of arms) and maintained at this density for the remainder of the
165 experiment (to day 16). These cultures were maintained in the same 12 L culture buckets to maintain
166 experimental control conditions, and the excess larvae removed from this displacement were discarded
167 due to space limitations. The high stocking density of 3 larvae mL⁻¹ was selected based from preliminary
168 trials that showed that densities of 4 larvae mL⁻¹ resulted in lower larval survival than those reared at 3
169 larvae mL⁻¹. This displacement treatment (D) was compared directly to low stocking density controls
170 of 1 larvae mL⁻¹.

171

172 2.5. Seawater parameters

173 Seawater temperature and salinity was recorded daily using a FRN-3000 digital aquarium
174 thermometer and refractometer respectively. Samples for nitrate analysis were collected before and after
175 each water change and assessed using a Nutrafin nitrate test kit. Significantly higher temperatures were
176 observed for *P. miliaris* compared to *P. lividus* (Kruskal-Wallis, $P < 0.001$). Therefore, species
177 specific responses were analysed separately in this study. Mean nitrate levels were maintained at 2.9
178 mg L⁻¹ across all treatments, a level that is considered unharmed to larvae (Table.1; Gomes *et al.* 2000).

179

180 2.6. Statistical analysis

181 Larval survival and development data were analysed using the SPSS statistical software (IBM;
182 version 20). All data were initially tested for departures from normality using the Shapiro-Wilk test and
183 for homogeneity using the Levene's test. One-way Analysis of Variance (ANOVA) was used to
184 examine water quality parameters, larval survival, morphology, and development data (Sokal & Rohlf,
185 1995). Where significant differences occurred ($p < 0.05$), a post hoc Tukey's test was performed. All
186 percentage data were transformed prior to analysis using an arcsine transformation (Dytham, 1999).
187 Where data failed homogeneity testing analysis was carried out using the non-parametric Kruskal-
188 Wallace test (Dytham, 1999). Where type II errors occurred, the means and confidence intervals were
189 graphically analysed to illustrate the data under normal assumptions.

190 Morphological data were initially tested for normal distribution using the Shapiro-Wilk test and
191 log transformed followed by regression analyses with larval body length as the independent variable.

192 Significant relationships between total larval length and body width were tested for. Relationships were
193 then examined using Analysis of Covariance (ANCOVA) (Sokal & Rohlf, 1995). If the data failed the
194 Levene's test prior to ANCOVA, data were bootstrapped to allow for best estimation of actual results.

195

1963. 3. Results

1974. 3.1. Stocking density trials

198 3.1.1. Survival

199 *Psammochinus miliaris* survival showed a gradual decline across time but no statistically
200 significant differences were identified between stocking densities (day 2: $F_{(4,10)} = 1.351$, $p = 0.318$; day
201 4: $H_{(4)} = 4.633$, $p = 0.327$; day 7: $H_{(4)} = 0.645$, $p = 0.567$; day 10: $F_{(4,10)} = 0.140$, $p = 0.964$; day 13: $F_{(4,10)}$
202 $= 1.082$, $p = 0.416$; day 16: $F_{(4,10)} = 2.025$, $p = 0.167$; Figure 1a). *Paracentrotus lividus* demonstrated
203 no significant effect of stocking density on survival (day 2: $F_{(4,10)} = 0.748$, $p = 0.581$; day 4: $F_{(4,10)} =$
204 2.068 , $p = 0.160$; day 7: $H_{(4)} = 3.833$, $p = 0.429$; day 10: $F_{(4,10)} = 1.082$, $p = 0.416$; day 13: $H_{(4)} = 8.315$,
205 $p = 0.081$; day 16: $H_{(4)} = 0.048$, $P = 0.048$; Figure 1b).

206

207 3.1.2 Development

208 There were no significant differences observed between stocking density treatments for the
209 responses of development stage, number of abnormally developed larvae and percentage of
210 malnourished larvae in *P. miliaris* across the experimental period ($p > 0.05$; Tables 2 and 3). Differences
211 in development stages and the percentage of malnourished larvae for *P. lividus* were not affected by
212 stocking density treatments across the experimental period ($p > 0.05$; Tables 2 and 3). However, on day
213 8, the percentage of abnormally developed larvae in the control group (1 larvae mL^{-1}) was significantly
214 higher compared to *P. lividus* reared at the highest density (4 larvae mL^{-1} ; Table 3).

215

216 3.1.3. Morphology

217 With respect to *P. miliaris* larval lengths and post oral arm lengths, on day 4 (first observation
218 for these parameters) no initial significant differences were observed between the different stocking
219 density treatments (larval length: day 4: $F_{(4,70)} = 1.922$, $p = 0.166$; post oral arm length: $H_{(4)} = 5.012$, p
220 $= 0.286$; Figure 2a and c). However, from day 8 onwards an effect of stocking density became evident.
221 On days 8 and 12 larval lengths and post oral arm lengths for lower stocking densities (1 and 2 larvae
222 mL^{-1}) were significantly larger than higher stocking density reared larvae (3, 4 mL^{-1} larval lengths: day
223 8: $F_{(4,70)} = 10.782$, $p < 0.001$; day 12: $F_{(4,70)} = 25.941$, $p < 0.001$; post oral arm lengths: day 8: $F_{(4,63)} =$
224 19.671 , $p < 0.001$; day 12: $H_{(4)} = 42.929$, $p < 0.001$; Figure 2a and c). On day 16, larvae at 1 mL^{-1} density
225 had significantly larger larval lengths and post oral arm lengths compared to all other stocking density
226 treatments (2, 3, and 4 mL^{-1} ; larval lengths: $F_{(4,70)} = 19.588$, $p < 0.001$; post oral arm lengths: $H_{(4)} =$
227 32.989 , $p < 0.001$; Figure 2a and c). Additionally, larvae stocked at 2 mL^{-1} had significantly larger larval
228 lengths compared to larvae stocked at the highest density (4 mL^{-1} ; Figure 2a). The rudiments in larvae

229 stocked at a low density (1 mL⁻¹) were significantly larger than larvae stocked at the highest density (4
230 mL⁻¹; $F_{(4,69)} = 6.565, p < 0.001$; Figure 3a).

231 With respect to *P. lividus*, no significant effect of stocking density was found on larval lengths
232 or post oral lengths across the experimental period (larval length: day 4: $H_{(4)} = 7.965, p = 0.093$; day 8:
233 $F_{(4,70)} = 0.318, p = 0.865$; day 12: $F_{(4,70)} = 1.096, p = 0.366$; day 16: $F_{(4,70)} = 3.071, P = 0.023$ (no
234 significant effects found in Tukey's post-hoc test); Post oral arm length: day 4: $F_{(4,70)} = 1.553, p = 0.196$;
235 day 8: $F_{(4,70)} = 1.609, p = 0.182$; day 12: $F_{(4,70)} = 1.962, p = 0.110$; day 16: $H_{(4)} = 8.190, p = 0.085$; Figure
236 2b and d) or on rudiment lengths during the experimental period ($F_{(4,60)} = 0.617, p = 0.652$; Figure 3).

237

238 3.1.4. Larval shape

239 Significant linear relationships were found across treatments for *P. miliaris* larval body lengths
240 and larval body widths ($p < 0.05$, Table 4) except for larvae reared at the highest density (4 larvae mL⁻¹
241 $; p > 0.05$; Table 4). Therefore, the highest stocking density treatment (4 larvae mL⁻¹) was excluded
242 from regression analysis. Regression analysis demonstrated no significant effect of stocking density on
243 larval body width ($p > 0.05$; Table 5). Significant linear relationships between larval body lengths and
244 post oral arm lengths were found only in the 3 larvae mL⁻¹ treatment (Table 4), with a progressive
245 shortening of the post-oral arms.

246 Significant linear relationships were found across treatments in *P. lividus* ($p < 0.05$; Table 4).
247 Regression analysis showed no significant effect of stocking density on the relationship between larval
248 body length and larval body width ($p > 0.05$; Table 5). Significant linear relationships between larval
249 body length and post oral arm lengths were seen across all treatments ($p < 0.05$; Table 4). *Paracentrotus*
250 *lividus* larvae reared at 2 larvae mL⁻¹ had lower post oral arm lengths relative to body size ratios in
251 comparison to the lowest density treatments (1 larvae mL⁻¹) ($p < 0.05$; Table 5) reflecting an increase
252 in larval body length and a reduction in post oral arm length. This irregular development may be caused
253 by external factors, such as differing larval development speeds between treatments which are more
254 subtle than the development stages recorded above.

255

256 3.2. Displacement trials

257 3.2.1. *Psammechinus miliaris*

258 No significant differences in survival or development were found between the low density
259 control larvae (1 mL⁻¹) and the displacement treatment (D) across the experimental period (day 2: $F_{(4,10)}$
260 $= 1.351, p = 0.318$; day 4: $H_{(4)} = 4.633, p = 0.327$; day 7: $H_{(4)} = 0.645, p = 0.567$; day 10: $F_{(4,10)} = 0.140,$
261 $p = 0.964$; day 13: $F_{(4,10)} = 1.082, p = 0.416$; day 16: $F_{(4,10)} = 2.025, p = 0.167$; Figure 4a; Tables 2 and
262 3).

263 From day 8 until the end of the experimental period, larval lengths and post oral arm lengths
264 for low stocking density larvae (1 mL⁻¹) were significantly larger than the displacement treatment (D;
265 larval lengths: day 8: $F_{(4,70)} = 10.782, p < 0.001$; day 12: $F_{(4,70)} = 25.941, p < 0.001$; day 16: $F_{(4,70)} =$

266 19.588, $p < 0.001$; post oral arm lengths: day 8: $F_{(4,63)} = 19.671$, $p < 0.001$; day 12: $H_{(4)} = 42.929$, $p <$
267 0.001 ; day 16: $H_{(4)} = 32.989$, $p < 0.001$; Figure 2a and c). However rudiment size was significantly
268 similar between the low density control larvae (1 mL^{-1}) and the displacement treatment (D; $F_{(4,69)} =$
269 6.565 , $p < 0.001$ (no significant effects found in Tukey's post-hoc test); Figure 3a).

270 Regression analysis demonstrated no significant effect of the low stocking density control
271 (1 mL^{-1}) or the displacement treatment (D) on larval body width ($p > 0.05$; Table 5) or between larval
272 body lengths and post oral arm lengths ($p > 0.05$; Table 4).

273

274 3.2.2. *Paracentrotus lividus*

275 No significant differences in survival were found between the low density control larvae (1 mL^{-1})
276 and the displacement treatment (D) across the experimental period (day 2: $F_{(4,10)} = 0.748$, $p = 0.581$;
277 day 4: $F_{(4,10)} = 2.068$, $p = 0.160$; day 7: $H_{(4)} = 3.833$, $p = 0.429$; day 10: $F_{(4,10)} = 1.082$, $p = 0.416$; day
278 13: $H_{(4)} = 8.315$, $p = 0.081$; day 16: $H_{(4)} = 0.048$, $P = 0.048$ (no significant effects found in Tukey's
279 post-hoc test); Figure 4b; Tables 2 and 3).

280 On day 16, larval lengths in the low density control larvae (1 mL^{-1}) were significantly larger
281 than the displacement treatment (D; $F_{(4,70)} = 3.071$, $P = 0.023$; Figure 2b). Post oral arm and rudiment
282 lengths were significantly similar between these treatments across the experimental period (post oral
283 arms: day 4: $F_{(4,70)} = 1.553$, $p = 0.196$; day 8: $F_{(4,70)} = 1.609$, $p = 0.182$; day 12: $F_{(4,70)} = 1.962$, $p = 0.110$;
284 day 16: $H_{(4)} = 8.190$, $p = 0.085$; Figure 2; rudiment length: $F_{(4,60)} = 0.617$, $p = 0.652$; Figures 2d and 3b
285 respectively).

286 A significant linear relationship was not found between *P. lividus* larval body lengths and larval
287 body widths in the density change experiment (D; $p > 0.05$; Table 4). Therefore, a regression analysis
288 was not assessed for these morphometrics.

289 Significant linear relationships between larval body length and post oral arm lengths were seen
290 across both treatments ($p > 0.05$; Table 4). Regression analysis demonstrated no significant effect of
291 the low stocking density control (1 mL^{-1}) or the displacement treatment (D) on larval body width ($p >$
292 0.05 ; Table 5) or between larval body lengths and post oral arm lengths ($p > 0.05$; Table 4).

293

2943 4. Discussion

295 4.1. *Psammechinus miliaris*

296 This study shows that stocking larvae at low densities of 1 larvae mL^{-1} produce the best quality
297 larvae reflected by large larval and rudiment sizes. This result agrees with findings of Azad (2012) and
298 Buitrago *et al.* (2005) who proposed that a stocking density of 1 mL^{-1} is also best for *Strongylocentrotus*
299 *purpuratus* and *Lytechinus variegatus* respectively. This study also demonstrates that stocking *P.*
300 *miliaris* larvae at 2 larvae mL^{-1} for a large part of the cultivation process (up to day 12) produces
301 similarly performing larvae to those stocked at 1 mL^{-1} . Although at this density the rudiment size was
302 not significantly larger than counterparts stocked at higher densities (e.g. 3 and 4 larvae mL^{-1}), it was

303 similar in size to rudiments within low density (1 mL^{-1}) larvae. This contrasts with Azad *et al.* (2012)
304 who showed that larvae of *S. purpuratus* stocked at 2 mL^{-1} had significantly lower survival and growth
305 relative to counterparts reared at a lower density of 1 mL^{-1} , indicating that the latter species is likely to
306 be more sensitive in cultivation than *P. miliaris*.

307 Stocking *P. miliaris* larvae at higher densities (3 or 4 mL^{-1}) did not affect survival or abnormal
308 development. However, these densities did result in stunted growth leading to the development of
309 smaller rudiments. Our data show that previous developmental stages (e.g. number of arms) were not
310 impacted, therefore the smaller rudiment sizes were most likely due to stunted growth rather than
311 developmental delays. Shorter post-oral arm lengths were observed within these high stocking densities
312 which can indicate overabundant food supplies (Fenaux, 1994; Kelly *et al.* 2000) but food supplies were
313 controlled across all treatments and therefore unlikely to be the cause of this stunted growth. No
314 significant level of malnourishment was observed either, illustrating that these high stocking density
315 cultures had sufficient food supplies further supporting the notion that food supply was not the cause of
316 the stunted growth (Kelly *et al.* 2000; Liu *et al.* 2007). Buitrago *et al.* (2005) showed that larval
317 morphological changes occur within high stocking densities, even with appropriate controlled food
318 rations. Therefore, the morphological changes observed for *P. miliaris* stocked at high densities of 3
319 and 4 larvae mL^{-1} are likely to be density driven.

320 It is unknown how these morphological differences are caused, given that no significant effects
321 on abnormal developments were observed. It could be that interactions were increasing levels of stress
322 in larvae that in turn impacted metabolism, diverting energy away from somatic growth (Forsythe &
323 Heaukelem, 1987). Alternatively, increased levels of metabolically derived carbon dioxide, and
324 therefore a reduced quality of seawater, may have caused lower growth rates, a phenomenon observed
325 in ocean acidification studies (e.g. Azad *et al.*, 2010; Suckling *et al.*, 2014a). Therefore, impacts on the
326 seawater carbonate chemistry would require more focus in future stocking density trials.

327 The performance of *P. miliaris* within the density change experiment was encouraging, where
328 larvae were initially reared at a high density of 3 larvae mL^{-1} and then transferred to a lower density of
329 1 larvae mL^{-1} . These larvae, at the end of the experimental period, had similar survival rates and
330 rudiment lengths to those reared at low density (1 mL^{-1}) despite a smaller size. This indicates that a
331 density change approach to larval rearing may be a viable option in large scale cultivation of *P. miliaris*
332 as rudiment development is key to metamorphosis into a competent juvenile (Gosselin & Jangoux,
333 1998).

334

335 4.2. *Paracentrotus lividus*

336 *Paracentrotus lividus* appeared to be more resilient towards cultivation at higher stocking
337 densities when compared with *P. miliaris*. Survival, development and morphological performances
338 were all similar between larvae stocked at 1, 2, 3 and 4 larvae mL^{-1} , in direct contrast with observations
339 from other echinoid species (Azad, 2012; Buitrago *et al.* 2005). Although not a statistically significant

340 result, it is worth noting from a cultivation perspective that the survival of *P. lividus* larvae reared under
341 the higher stocking density of 4 larvae mL⁻¹ was approximately 25 % lower than controls which may
342 have influence on aquaculture approaches.

343 Similarly to *P. miliaris*, the displacement of larvae from a high (3 larvae mL⁻¹) to low stocking
344 density (1 mL⁻¹; D) showed benefits to larval success and rearing effort when directly compared to
345 larvae continuously reared at low control stocking densities (1 mL⁻¹). By the end of the experimental
346 period survival and rudiment sizes were similar despite smaller larval sizes found in displacement
347 larvae. Similar to our cautionary comment above, although not a statistically significant result, it is
348 worth noting that the survival of *P. lividus* larvae displaced from 3 to 1 larvae mL⁻¹ was approximately
349 30 % lower than controls which may have influence on aquaculture approaches.

350

351

352 4.3. Conclusions

353 *Psammechinus miliaris* is clearly best cultured at a density of 1 larvae mL⁻¹ to achieve largest
354 larval and rudiment sizes. The performance of *P. lividus* was similar across all densities of 1, 2, 3 and
355 4 larvae mL⁻¹, making this species somewhat more appealing in intensive cultivation efforts. However,
356 it must be noted that survival of high stocked larvae (4 larvae mL⁻¹) after 16 days was notably (but not
357 significantly) 25 % lower than those reared at lower densities of 1 and 2 larvae mL⁻¹. Displacing larvae
358 from a high density of 3 mL⁻¹ to a lower density of 1 mL⁻¹ at day 13 is a viable option for both species
359 if required by hatchery operators. However, it must be noted that survival of the displaced *P. lividus*
360 larvae after 16 days was notably (but not significantly) 30 % lower than those reared at a lower density
361 of 1 larvae mL⁻¹. This information is likely to be important for commercial hatcheries looking to utilise
362 higher stocking densities during the larval rearing process. Responses to fixed stocking densities
363 differed across the two species highlighting a need to take these approaches across a wider range of
364 commercially important species. Establishing the optimum and efficient larval stocking densities for
365 marketable species of sea urchins is crucial to the development of commercial scale hatcheries (Azad
366 *et al.*, 2010). This need is ever increasing in importance with natural fisheries facing over exploitation
367 and climate change challenges (Grosjean *et al.*, 1998; Suckling *et al.*, 2015).

368

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375

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514

515 **List of Tables**

516

517 **Table 1.** Temperature (°C), salinity (psu) and nitrate levels (ppm) of *Psammechinus miliaris* and
 518 *Paracentrotus lividus* rearing tanks. Larvae were raised at 1, 2, 3 and 4 larvae mL⁻¹ plus an additional
 519 culture where larvae were transferred from densities of 3 larvae mL⁻¹ to 1 larvae mL⁻¹ on day 13 (D).

520 Table 1.

Species	Treatment	(°C)	Salinity (psu)	Nitrate (ppm)
<i>Psammechinus miliaris</i>	1 mL ⁻¹	12.82 ± 0.20	33.92 ± 0.12	1.90 ± 0.16
	2 mL ⁻¹	12.51 ± 0.17	34.75 ± 0.11	3.19 ± 0.29
	3 mL ⁻¹	12.37 ± 0.18	34.56 ± 0.13	3.67 ± 0.28
	4 mL ⁻¹	12.25 ± 0.18	34.86 ± 0.13	4.52 ± 0.41
	D mL ⁻¹	12.45 ± 0.17	34.87 ± 0.13	3.05 ± 0.32
<i>Paracentrotus lividus</i>	1 mL ⁻¹	11.60 ± 0.06	34.47 ± 1.90	1.90 ± 0.15
	2 mL ⁻¹	11.23 ± 0.05	34.66 ± 0.08	2.52 ± 0.15
	3 mL ⁻¹	11.04 ± 0.04	34.78 ± 0.08	2.64 ± 0.19
	4 mL ⁻¹	10.85 ± 0.04	34.74 ± 0.10	3.19 ± 0.22
	D mL ⁻¹	11.14 ± 0.07	34.84 ± 0.09	2.50 ± 0.11

521

522

523 **Table 2.** Larval development stages (% \pm SE) of *Psammechinus miliaris* and *Paracentrotus lividus*.
 524 Larvae were raised at 1, 2, 3 and 4 larvae mL⁻¹ plus an additional culture where larvae were transferred
 525 from densities of 3 larvae mL⁻¹ to 1 larvae mL⁻¹ on day 13 (D). Differing letters as superscripts indicate
 526 where significant differences occur between treatments for each sample day row. DF = degrees of
 527 freedom, Statistic = statistical outcome, P = probability. NSD* indicates a Type II Error with a visual
 528 inspection of the means and confidence intervals showing no significant differences (NSD).

		Stocking density	Day				
			4	7	10	13	16
<i>Psammechinus miliaris</i>	Stage 1	1 mL ⁻¹	24.7 \pm 0.7	4.0 \pm 0.6	1.3 \pm 0.3	0.7 \pm 0.3	0.33 \pm 0.33
		2 mL ⁻¹	14.0 \pm 0.6	19.0 \pm 3.2	4.0 \pm 2.0	0.3 \pm 0.3	0.33 \pm 0.33
		3 mL ⁻¹	13.0 \pm 0.6	11.3 \pm 2.9	7.0 \pm 3.1	0.3 \pm 0.3	0.67 \pm 0.33
		4 mL ⁻¹	13.0 \pm 1.0	11.3 \pm 2.9	4.0 \pm 2.7	0.0 \pm 0.0	0.06 \pm 0.33
		D mL ⁻¹	11.7 \pm 0.3	17.3 \pm 2.3	4.3 \pm 1.9	0.3 \pm 0.3	0.00 \pm 0.00
		DF	14	14	14	12	12
		Statistic	F = 1.07	F = 2.537	F = 0.964	H = 3.721	H = 0.670
		P	0.421	0.106	0.468	0.293	0.880
	Stage 2	1 mL ⁻¹	1.3 \pm 0.7	2.3 \pm 0.8	8.7 \pm 1.7	0.7 \pm 0.3	0.7 \pm 0.3
		2 mL ⁻¹	1.0 \pm 0.6	13.0 \pm 3.1	10.7 \pm 3.0	1.7 \pm 0.9	0.7 \pm 0.7
		3 mL ⁻¹	2.0 \pm 0.6	14.0 \pm 2.1	22.3 \pm 4.9	5.3 \pm 3.9	2.0 \pm 1.2
		4 mL ⁻¹	2.0 \pm 1.0	20.0 \pm 2.9	30.3 \pm 3.5	2.3 \pm 1.2	0.0 \pm 0.0
		D mL ⁻¹	3.3 \pm 0.3	15.0 \pm 1.5	21.3 \pm 5.2	0.3 \pm 0.3	0.0 \pm 0.0
		DF	14	14	14	14	12
		Statistic	F = 1.07	F = 2.537	F = 0.964	F = 0.891	H = 3.026
		P	0.421	0.106	0.468	0.504	0.388
	Stage 3	1 mL ⁻¹	-	-	-	7.3 \pm 0.9	10.3 \pm 0.9
		2 mL ⁻¹	-	-	-	15.7 \pm 5.2	19.7 \pm 4.4
	3 mL ⁻¹	-	-	-	11.7 \pm 2.6	23.0 \pm 5.6	
	4 mL ⁻¹	-	-	-	27.0 \pm 2.5	19.0 \pm 11.2	
	D mL ⁻¹	-	-	-	21.7 \pm 0.3	10.3 \pm 0.7	
	DF	-	-	-	12	12	
	Statistic	-	-	-	H = 2.362	H = 3.521	
	P	-	-	-	0.501	0.318	
<i>Paracentrotus lividus</i>	Stage 1	1 mL ⁻¹	6.3 \pm 0.3	7.0 \pm 2.0	4.0 \pm 1.0	-	-
		2 mL ⁻¹	2.6 \pm 1.6	17.7 \pm 3.2	7.0 \pm 0.6	-	-
		3 mL ⁻¹	4.0 \pm 3.0	24.3 \pm 0.7	12.0 \pm 3.8	-	-
		4 mL ⁻¹	0.0 \pm 0.0	23.7 \pm 1.7	9.3 \pm 1.9	-	-
		D mL ⁻¹	0.0 \pm 0.0	19.3 \pm 2.2	5.7 \pm 2.2	-	-
		DF	-	12	14	-	-
		Statistic	-	H = 6.512	F = 0.511	-	-
		P	-	0.089	0.729	-	-
	Stage 2	1 mL ⁻¹	-	0.0 \pm 0.0	10.0 \pm 3.6	2.7 \pm 1.2	1.7 \pm 0.9
		2 mL ⁻¹	-	1.7 \pm 0.7	21.0 \pm 3.1	1.7 \pm 0.3	1.7 \pm 0.7
		3 mL ⁻¹	-	2.0 \pm 0.0	20.0 \pm 3.0	0.3 \pm 0.3	1.7 \pm 0.3
		4 mL ⁻¹	-	2.0 \pm 1.0	29.7 \pm 5.9	4.3 \pm 2.9	1.3 \pm 0.7
		D mL ⁻¹	-	0.3 \pm 0.3	13.7 \pm 1.3	0.7 \pm 0.3	0.0 \pm 0.0
		DF	-	12	14	14	12
		Statistic	-	H = 6.512	F = 0.598	F = 5.391	H = 4.198
		P	-	0.089	0.672	NSD*	0.241
	Stage 3	1 mL ⁻¹	-	-	0.0 \pm 0.0	7.3 \pm 0.9	8.3 \pm 3.2
		2 mL ⁻¹	-	-	0.0 \pm 0.0	19.7 \pm 1.8	13.7 \pm 1.3
	3 mL ⁻¹	-	-	0.7 \pm 0.7	22.3 \pm 1.2	12.3 \pm 0.9	
	4 mL ⁻¹	-	-	0.0 \pm 0.0	23.7 \pm 3.7	30.3 \pm 2.7	
	D mL ⁻¹	-	-	0.0 \pm 0.0	19.3 \pm 1.9	6.0 \pm 4.0	
	DF	-	-	12	14	12	
	Statistic	-	-	H = 3.000	F = 5.391	H = 4.198	

529

530

531 **Table 3.** Percentage of abnormally developed and malnourished larvae (% ± SE) of *Psammechinus*
 532 *miliaris* and *Paracentrotus lividus*. Larvae were raised at 1, 2, 3 and 4 larvae mL⁻¹ plus an additional
 533 culture where larvae were transferred from densities of 3 larvae mL⁻¹ to 1 larvae mL⁻¹ on day 13 (D).
 534 Differing letters as superscripts indicate where significant differences occur between treatments. DF =
 535 degrees of freedom, Statistic = statistical outcome, P = probability.

		Stocking density	Day				
			4	8	12	16	
Abnormal development (%)	<i>Psammechinus miliaris</i>	1	6.7 ± 0.1	13.3 ± 0.1	13.3 ± 0.1	13.3 ± 0.1	
		2	0.0 ± 0.0	13.3 ± 0.1	13.3 ± 0.1	40.0 ± 0.1	
		3	0.0 ± 0.0	20.0 ± 0.1	53.3 ± 0.1	33.3 ± 0.1	
		4	0.0 ± 0.0	6.7 ± 0.03	13.3 ± 0.1	53.3 ± 0.1	
		D	0.0 ± 0.0	53.3 ± 0.1	20.0 ± 0.1	20.0 ± 0.1	
		DF	14	14	14	14	
		Statistic	H=0.406	F=2.700	F=1.915	F=1.374	
		P	0.452	0.092	0.185	0.310	
	<i>Paracentrotus lividus</i>	1	46.7 ± 0.1	46.7 ± 0.1 ^a	20.0 ± 0.1	0.0 ± 0.0	
		2	46.7 ± 0.1	6.7 ± 0.1 ^{ab}	6.7 ± 0.1	26.7 ± 0.1	
3		20.0 ± 0.1	26.7 ± 0.1 ^{ab}	20.0 ± 0.1	33.3 ± 0.1		
4		46.7 ± 0.1	0.0 ± 0.0 ^b	13.3 ± 0.1	33.3 ± 0.1		
D		53.3 ± 0.1	33.3 ± 0.1 ^{ab}	26.7 ± 0.1	36.7 ± 0.1		
		DF	14	14	14	14	
		Statistic	F=1.704	H=11.477	F=0.538	F=2.644	
		P	0.225	0.022	0.711	0.133	
Malnourished larvae (%)		<i>Psammechinus miliaris</i>	1	0.0 ± 0.0	6.7 ± 0.1	0.0 ± 0.0	13.3 ± 0.1
			2	13.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	3		0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
	4		13.3 ± 0.1	13.3 ± 0.1	6.7 ± 0.1	13.3 ± 0.1	
	D		0.0 ± 0.0	13.3 ± 0.1	0.0 ± 0.0	6.7 ± 0.1	
		DF	14	14	14	14	
		Statistic	H=5.709	H=4.056	H=4.000	H=4.056	
		P	0.222	0.399	0.406	0.399	
	<i>Paracentrotus lividus</i>	1	46.7 ± 0.1	46.7 ± 0.1	20.0 ± 0.1	0.0 ± 0.0	
		2	46.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1	26.7 ± 0.1	
3		20.0 ± 0.1	26.7 ± 0.1	20.0 ± 0.1	33.3 ± 0.1		
4		46.7 ± 0.1	0.0 ± 0.0	13.3 ± 0.1	33.3 ± 0.1		
D		53.3 ± 0.1	33.3 ± 0.1	26.7 ± 0.1	30.0 ± 0.1		
	DF	14	14	14	14		
	Statistic	F=2.146	H=4.056	F=0.538	F=0.382		
	P	0.149	0.399	0.711	0.816		

536

537

538

539 **Table 4.** Analysis of variance for linear regressions between larval body length with either larval body
540 width or post oral arm length, for *Psammechinus miliaris* and *Paracentrotus lividus*. Larvae were raised
541 at 1, 2, 3 and 4 larvae mL⁻¹ plus an additional culture where larvae were transferred from densities of 3
542 larvae mL⁻¹ to 1 larvae mL⁻¹ on day 13 (D). Significant regressions are indicated by numbers in bold.
543 DF = degrees of freedom, F = F-statistic, P = probability.

Species	Body shape metric	Treatment	Slope	R ²	DF	F	P
<i>Psammechinus miliaris</i>	Larval body width	1	-0.022	0.813	1, 53	230.411	<0.001
		2	-0.021	0.528	1, 55	61.548	<0.001
		3	-0.007	0.238	1, 57	17.784	<0.001
		4	0.014	0.064	1, 58	3.944	0.052
		D	-0.004	0.298	1, 58	24.584	<0.001
	Post oral arm length	1	0.020	0.022	1, 50	1.102	0.299
		2	0.015	0.000	1, 52	0.004	0.952
		3	0.019	0.090	1, 54	5.361	0.024
		4	0.005	0.028	1, 55	1.569	0.216
		D	0.010	0.004	1, 55	0.209	0.650
<i>Paracentrotus lividus</i>	Larval body width	1	0.000	0.373	1, 54	32.144	<0.001
		2	-0.006	0.602	1, 58	87.698	<0.001
		3	-0.005	0.552	1, 59	71.574	<0.001
		4	-0.013	0.705	1, 58	138.403	<0.001
		D	-0.021	0.064	1, 52	33.582	0.064
	Post oral arm length	1	0.000	0.382	1, 54	33.403	<0.001
		2	-0.003	0.465	1, 58	50.411	<0.001
		3	-0.001	0.327	1, 58	28.228	<0.001
		4	-0.002	0.400	1, 58	38.727	<0.001
		D	-0.001	0.335	1, 52	26.220	<0.001

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557 **Table 5.** Analysis of variance between stocking density on the a) larval body width and b) post oral arm
 558 length of *Psammechinus miliaris* and *Paracentrotus lividus* larvae. The covariate is larval body length
 559 and all data were log transformed. Significant treatment effects are indicated by numbers in bold. DF =
 560 degrees of freedom, MS = mean square, F = F statistic, P = probability.

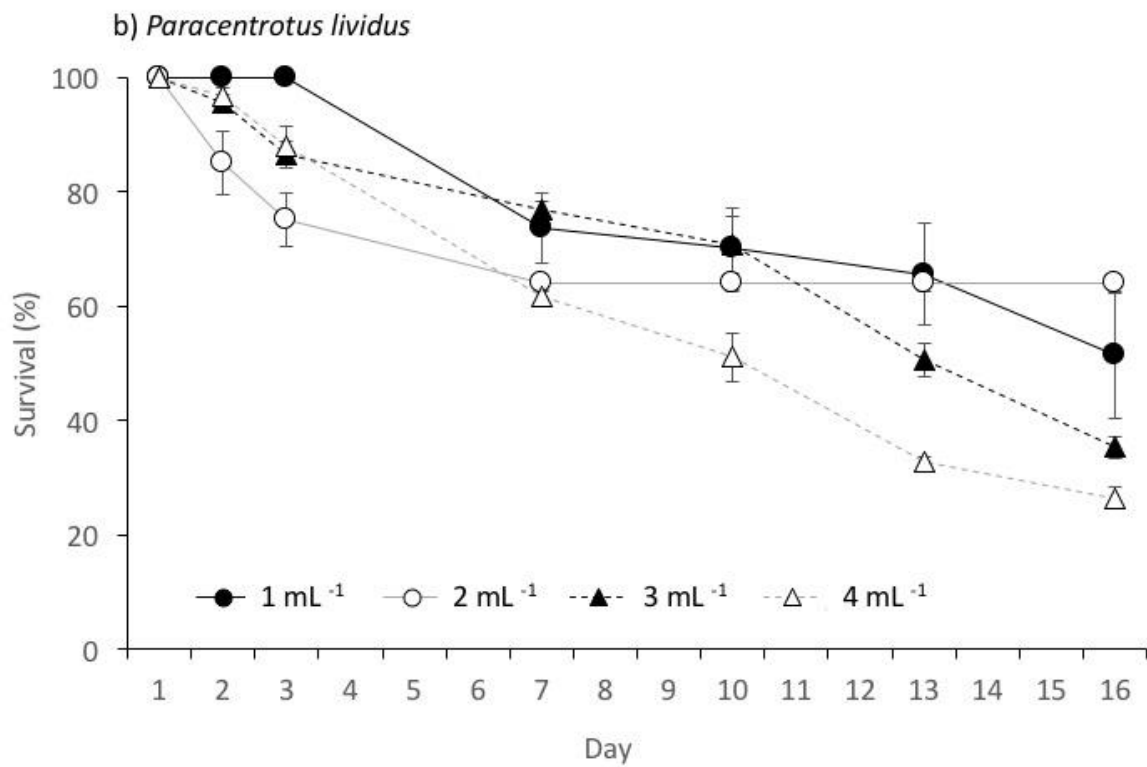
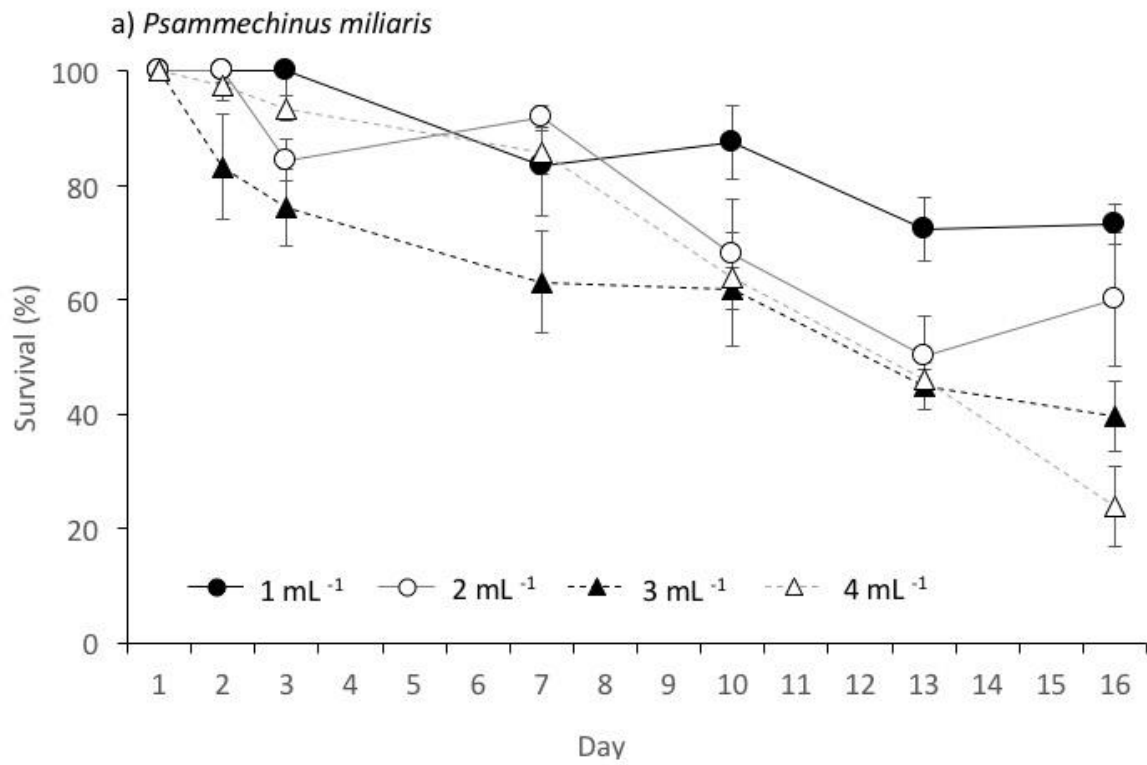
	Treatment	DF	MS	F	P
<i>Psammechinus miliaris</i>	Larval density	4	<0.001	0.620	0.610
	Larval width covaried with body length	1	0.014	32.580	<0.001
	Error	285	<0.001		
	Larval density	4	0.001	12.391	<0.001
	Post oral arm length covaried with body length	1	0.001	9.947	0.302
	Error	367	<0.001		
<i>Paracentrotus lividus</i>	Larval density	4	<0.001	0.642	0.585
	Larval width covaried with body length	1	0.015	69.354	<0.001
	Error	284	<0.001		
	Larval density	4	<0.001	5.999	<0.003
	Post oral arm length covaried with body length	1	0.002	123.881	<0.001
	Error	284	<0.001		

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583 **List of Figures**

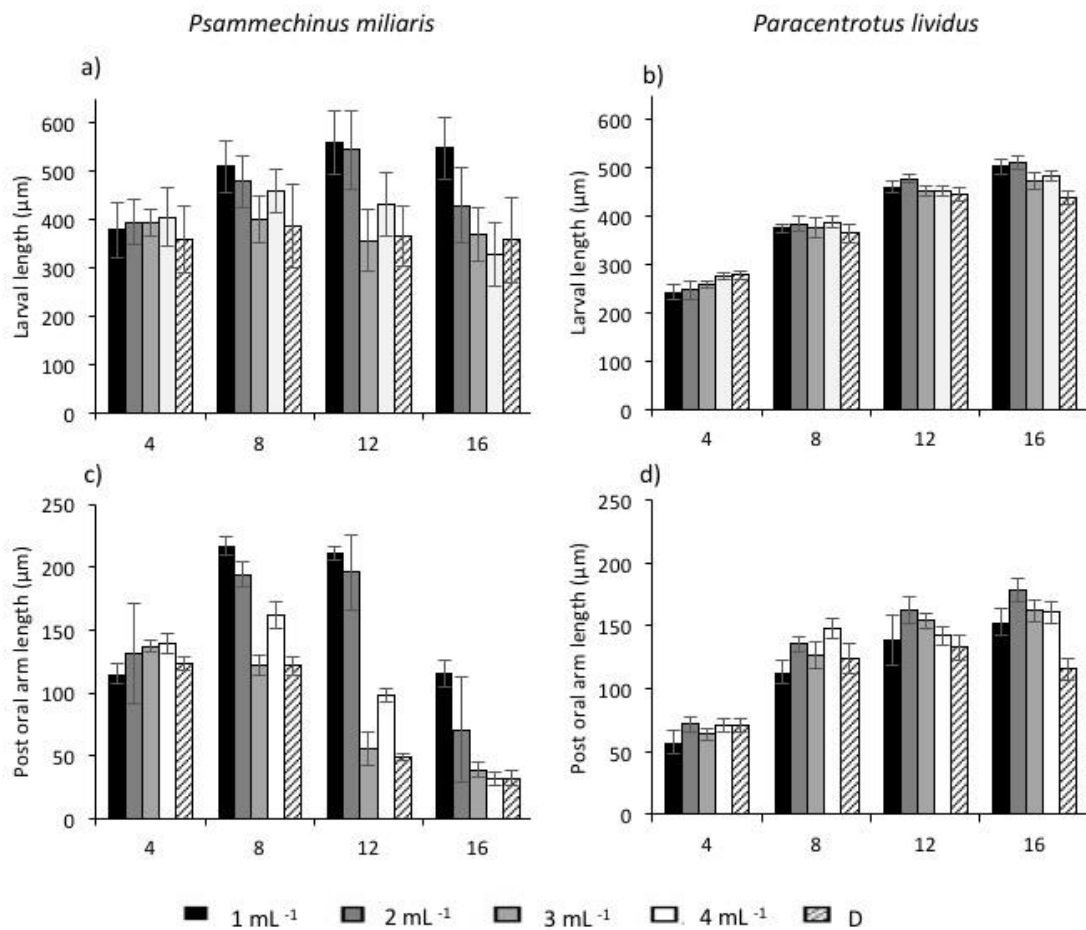
584

585 **Figure 1.** Mean (± 1 SE) survival of a) *Psammechinus miliaris* and b) *Paracentrotus lividus* raised at
586 stocking densities of 1, 2, 3 and 4 larvae mL⁻¹.

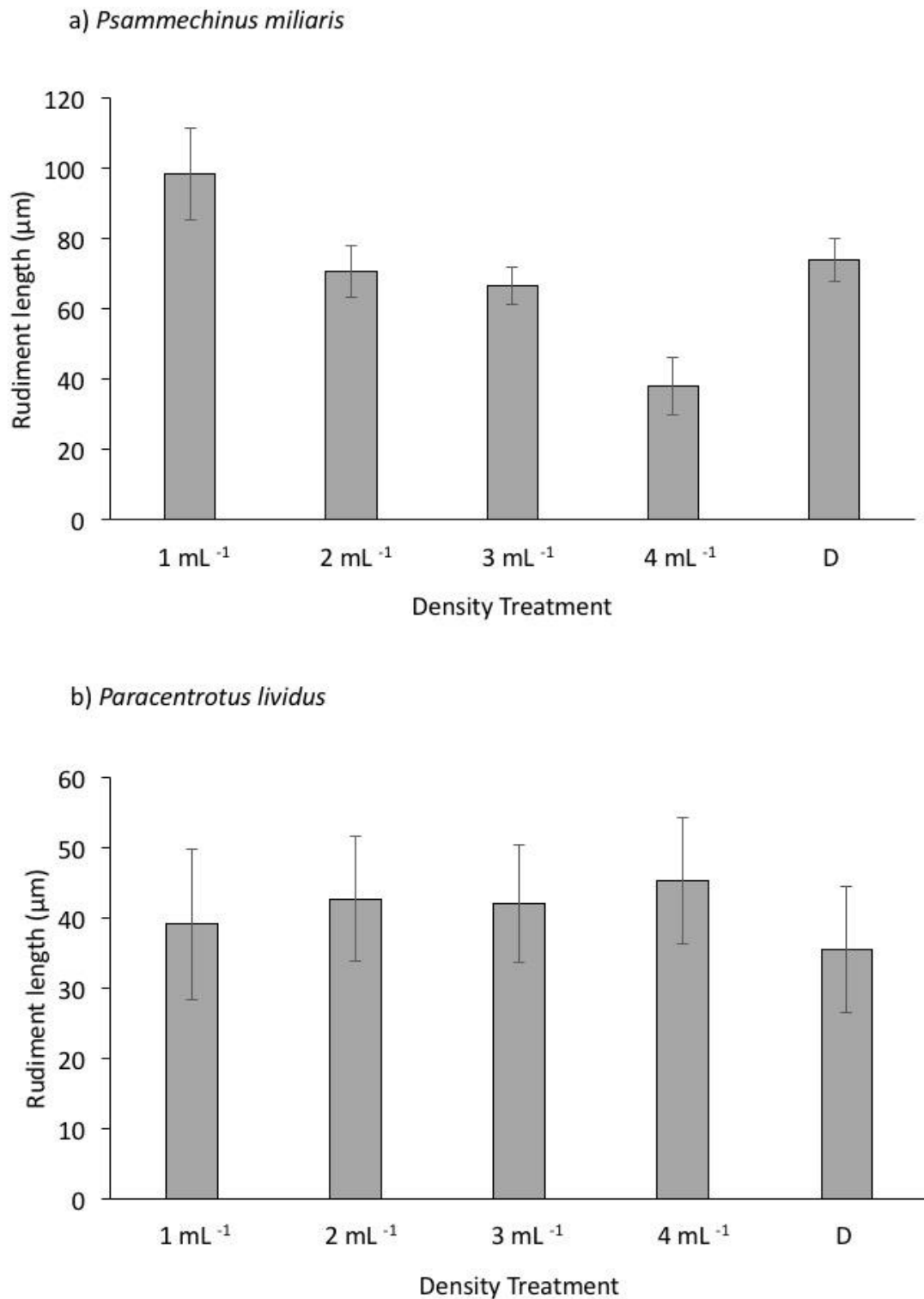


587

588 **Figure 2.** Morphological aspects of a) *Psammechinus miliaris* and b) *Paracentrotus lividus*; i) Larval
 589 length, ii) Post oral arm length as recorded throughout the larval life span (days) raised at stocking
 590 densities of 1, 2, 3 and 4 larvae mL⁻¹ plus an additional culture where larvae were transferred from
 591 densities of 3 larvae mL⁻¹ to 1 larvae mL⁻¹ on day 13 (D). Mean (\pm 1SE) values for pooled replicates (n
 592 = 15, total n = 45) are presented.

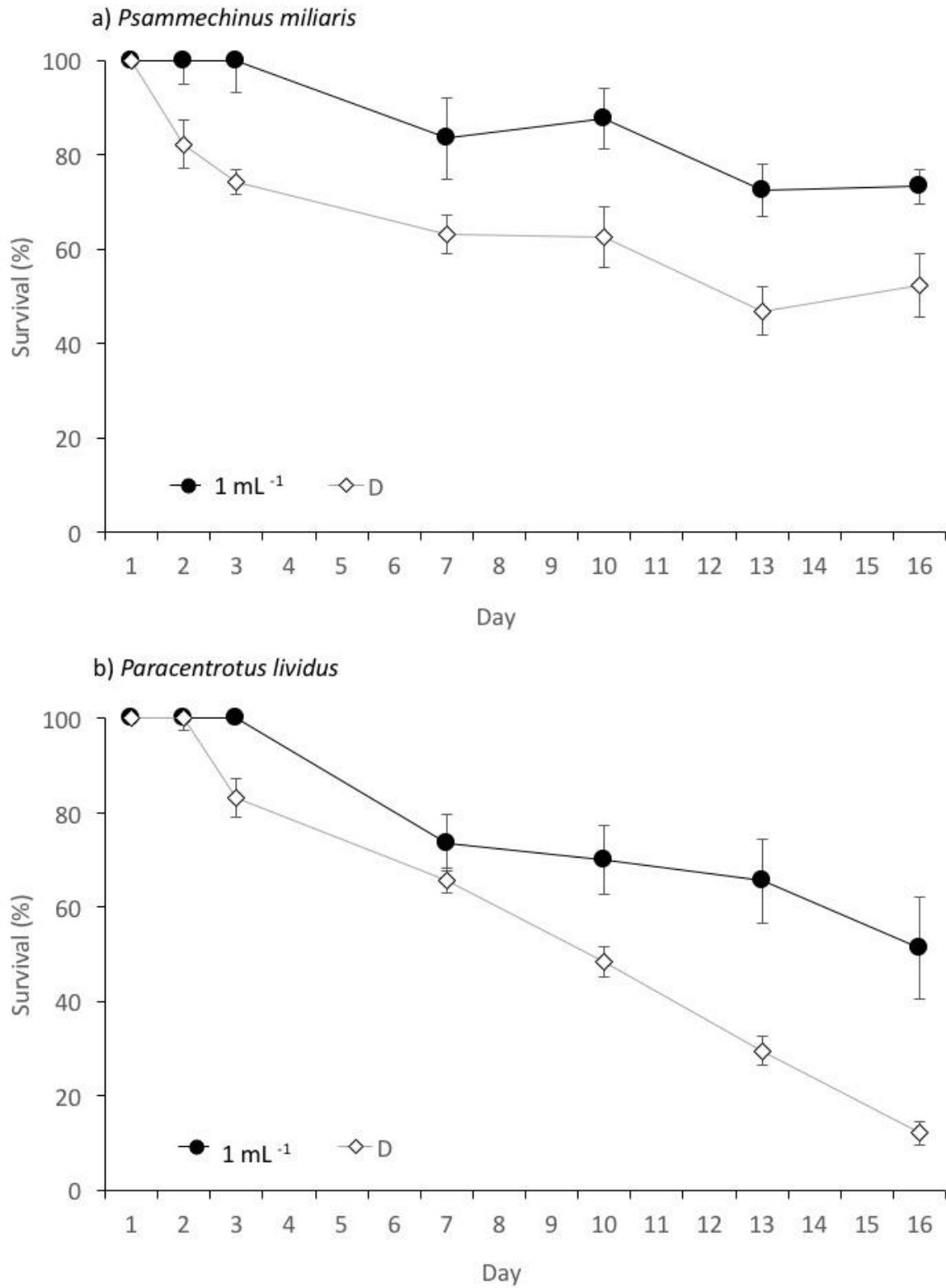


594 **Figure 3.** Rudiment lengths of a) *Psammechinus miliaris* and b) *Paracentrotus lividus*; length as
595 recorded throughout the larval life span (days) raised at stocking densities of 1, 2, 3 and 4 larvae mL⁻¹
596 plus an additional culture where larvae were transferred from densities of 3 larvae mL⁻¹ to 1 larvae mL⁻¹
597 on day 13 (D). Mean (\pm 1SE) values for pooled replicates ($n = 15$, total $n = 45$) are presented.



598

599 **Figure 4.** Mean (± 1 SE) survival of a) *Psammechinus miliaris* and b) *Paracentrotus lividus* raised at a
 600 low control stocking density of 1 larvae mL⁻¹ and larvae initially reared at a high density of 3 mL⁻¹ and
 601 then displaced to a low density of 1 mL⁻¹ on day 13 (D).



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