

1 **Some like it hot: temperature and pH modulate larval**  
2 **development and settlement of the sea urchin *Arbacia lixula***

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18 **Running headline:** Effect of temperature and pH on *Arbacia lixula* larvae

19 **Abstract**

20 We studied the effects of temperature and pH on larval development, settlement and  
21 juvenile survival of a Mediterranean population of the sea urchin *Arbacia lixula*. Three  
22 temperatures (16, 17.5 and 19 °C) were tested at present pH conditions (pH<sub>T</sub> 8.1). At 19  
23 °C, two pH levels were compared to reflect present average (pH<sub>T</sub> 8.1) and near-future  
24 average conditions (pH<sub>T</sub> 7.7, expected by 2100). Larvae were reared for 52-days to  
25 achieve the full larval development and complete the metamorphosis to the settler stage.  
26 We analysed larval survival, growth, morphology and settlement success. We also  
27 tested the carry-over effect of acidification on juvenile survival after 3 days. Our results  
28 showed that larval survival and size significantly increased with temperature.  
29 Acidification resulted in higher survival rates and developmental delay. Larval  
30 morphology was significantly altered by low temperatures, which led to narrower larvae  
31 with relatively shorter skeletal rods, but larval morphology was only marginally  
32 affected by acidification. No carry-over effects between larvae and juveniles were  
33 detected in early settler survival, though settlers from larvae reared at pH 7.7 were  
34 significantly smaller than their counterparts developed at pH 8.1. These results suggest  
35 an overall positive effect of environmental parameters related to global change on the  
36 reproduction of *Arbacia lixula*, and reinforce the concerns about the increasing negative  
37 impact on shallow Mediterranean ecosystems of this post-glacial colonizer.

38 **Keywords**

39 ocean acidification, temperature, sea urchin, larvae, settlers, Mediterranean

40

41 **Abbreviations**

42 ASY: asymmetry index; BL: body length; BW: body width; BRL: left body rod length;

43 BRR right body rod length; FSW: filtered seawater; POL: left post-oral rod length;

44 POR: right post-oral rod length; SUR: survival rate; TOC: time of culture.

## 45 **1. Introduction**

46 Global changes due to increased atmospheric CO<sub>2</sub> emissions are altering ocean  
47 ecosystems, though there is considerable uncertainty about the spatial and temporal  
48 details (Hoegh-Guldberg and Bruno, 2010). Major physicochemical changes in marine  
49 ecosystems come in two different ways: ocean warming and acidification. In the  
50 Mediterranean Sea, long-term datasets have revealed temperature increases of 0.8–1.4  
51 °C over the last 30 years (Lejeusne et al., 2010 and references therein) and a further 2 °C  
52 increase is expected by 2100 (Meehl et al., 2007; IPCC, 2007). On the other hand, the  
53 average pH of surface seawater has declined worldwide by approximately 0.1 units  
54 since the industrial revolution and future reductions are expected to be around 0.3–0.5  
55 units by 2100 (Caldeira and Wickett, 2003, 2005; Royal Society, 2005).

56 Much research effort has been devoted to elucidate the effects of ocean  
57 acidification on the development of echinoderms (see, e.g., reviews by Kurihara, 2008,  
58 Dupont et al., 2010c; Dupont and Thorndyke, 2013). Some species show a clear  
59 impairment when their larvae are grown at lowered pH conditions, either as increased  
60 mortality (e.g. *Ophiothrix fragilis*, Dupont et al., 2008), as delayed development (e.g.  
61 *Lytechinus pictus*, O'Donnell et al., 2010; *Strongylocentrotus purpuratus*, Stumpp et al.,  
62 2011) or as developmental malformations (e.g. *Sterechinus neumayeri*, Byrne et al.,  
63 2013). But in many other species the effects are neutral or undetectable (e.g. *Arbacia*  
64 *punctulata*, Carr et al., 2006; *Heliocidaris erythrogramma*, Byrne et al., 2009;  
65 *Paracentrotus lividus*, Martin et al., 2011; *Arbacia dufresnei*, Catarino et al., 2012) and  
66 a few species may even show enhanced development when grown at moderate levels of  
67 acidification (e.g. *Crossaster papposus*, Dupont et al., 2010b). Thus, with some  
68 exceptions, echinoderm larvae have shown to be robust to mild acidification (Dupont et  
69 al., 2010c).

70           Only a few previous works have studied the combined effects of increased  
71 temperature and ocean acidification on echinoderm larvae (Sheppard Brennan et al.,  
72 2010; Ericson et al., 2012; Foo et al., 2012; Nguyen et al., 2012; Padilla-Gamiño et al.,  
73 2013; Gianguzza et al., 2013) and all of them were limited to the first stages of early  
74 endotrophic development (2 to 3 days exposure). From this limited dataset, it appears  
75 that interaction between temperature and ocean acidification is complex, from  
76 temperature being the main driver of change to temperature amplifying or diminishing  
77 the negative effects of ocean acidification. Gianguzza et al. (2013) showed that  
78 temperature and pH had no significant effect on fertilization and larval survival (2 days)  
79 of *Arbacia lixula* for temperatures <27°C. However, both temperature and pH had  
80 effects on the developmental dynamics. Temperature appeared to modulate the impact  
81 of decreasing pH on the % of larvae reaching the pluteus stage, leading to a positive  
82 effect (faster growth compared to pH 8.2) of low pH at 20°C, a neutral effect at 24°C  
83 and a negative effect (slower growth) at 26°C.

84           The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the  
85 most abundant sea urchins in the Mediterranean (Benedetti-Cecchi et al., 1998; Palacín  
86 et al., 1998; Hereu et al., 2012) and tropical Eastern Atlantic (Hernández et al., 2013). It  
87 is recognized as a thermophilous species of tropical affinities (Stefanini, 1911;  
88 Mortensen, 1935; Tortonese, 1965) which probably spread through the Mediterranean  
89 in the Upper Pleistocene (Wangensteen et al., 2012) where it lives in suboptimal  
90 temperature conditions. Thus, it is a candidate species to be favoured by increased  
91 temperatures due to global change. *A. lixula* is an omnivore tending to carnivory  
92 (Wangensteen et al., 2011) which has a high potential to impact shallow rocky areas by  
93 originating or maintaining barren zones (Guidetti et al., 2003; Bonaviri et al., 2011).  
94 Despite its increasingly recognized ecological importance (Bulleri et al., 1999; Guidetti

95 et al., 2003; Guidetti and Dulcic, 2007; Bonaviri et al., 2011; Privitera et al., 2011;  
96 Gianguzza et al., 2011; Wangensteen et al., 2011), it has been traditionally understudied  
97 compared with the sympatric edible sea urchin *Paracentrotus lividus* and its actual  
98 potential to modify shallow rocky ecosystems may be currently underestimated.

99 *Arbacia lixula* has undergone population increases in the past (Petit et al., 1950;  
100 Boudouresque et al., 1989; Francour et al., 1994; Harmelin et al., 1995). Its  
101 reproductive potential in the Mediterranean may be boosted by increasing temperature  
102 (Gianguzza et al., 2011, Wangensteen et al., 2013) and some results suggest that their  
103 larval survival may also increase with temperature (Privitera et al., 2011), supporting  
104 the view that their populations in the Mediterranean could be presently constrained by  
105 larval mortality due to low temperatures or to phytoplankton shortage and may then  
106 benefit from ocean warming.

107 In this work, we studied the effect of temperature and acidification on the  
108 development (survival, growth, morphology and settlement success) of larvae from a  
109 northwestern Mediterranean population of *Arbacia lixula*. We also studied the carry-  
110 over effect of acidification on the 3-day survival of the settlers.

111

## 112 **2. Materials and methods**

### 113 *2.1. Adult sea urchins collection*

114 Adult *Arbacia lixula* individuals were collected by SCUBA diving at Tossa de  
115 Mar (NE Spain, 41°43'16" N, 2°56'24" E) in September 2012, kept in a 10 L plastic  
116 tank with seawater aerated by oxygen tablets and transported by airplane within 24 h to  
117 the Sven Lovén Centre for Marine Sciences - Kristineberg (Sweden). Induced spawning  
118 and *in vitro* fecundation were carried out shortly upon arrival.

119

120 2.2. *In vitro* fecundation and larval cultures

121 All filtered seawater (FSW) used in the experiments was supplied with sea salts  
122 to achieve a salinity of 38 (comparable to Mediterranean water). Spawning was induced  
123 by intracoelomic injection of 1 mL of 0.5 M KCl in FSW. Seven females and one male  
124 were used for the fecundation. Eggs were collected in FSW, and sperm was collected  
125 dry and kept on ice until use. The number of eggs was estimated as the average of five  
126 counts of 50  $\mu$ L of a 1 L egg dilution. Sperm stock solution in FSW was added to a final  
127 concentration of  $\sim 1,000$  sperm  $\text{mL}^{-1}$ , allowing a fertilization success  $>80\%$ . After  
128 fertilization, embryos were rinsed with FSW, after 2 hours they were aliquoted and  
129 inoculated in 5-L bottles filled with FSW at a density of 6000 embryos  $\text{L}^{-1}$  and the  
130 relevant temperature and pH. Bottles were maintained in chambers with controlled  
131 temperature and continuously aerated to maintain oxygen concentrations close to air  
132 saturation by the slow convective current of a stream of single bubbles ( $\sim 60$  bubbles  
133  $\text{min}^{-1}$ ).

134 In the northwestern Mediterranean, the planktotrophic *A. lixula* larvae may be  
135 found in the water column between June and November and can be exposed to a wide  
136 range of temperatures (15 to 24°C; Fenaux, 1968; Pedrotti, 1993). Nevertheless,  
137 Pedrotti's (1993) results suggest that the highest planktonic concentrations occur in  
138 October-November, when the temperature ranges from 16 to 19 °C. We compared four  
139 different scenarios: (i) Treatment I (16 °C,  $\text{pH}_T$  8.1), corresponding to the lower range of  
140 the present temperature variability; (ii) Treatment II (17.5 °C,  $\text{pH}_T$  8.1), an intermediate  
141 temperature; (iii) Treatment III (19 °C,  $\text{pH}_T$  8.1), corresponding to the higher range of  
142 temperature presently experienced by the autumnal larvae; (iv) Treatment IV (19 °C,  
143  $\text{pH}_T$  7.7), corresponding to near-future ocean acidification scenario. Two replicates were  
144 used per treatment.

145           After three days, larvae were fed daily with the cryptophyte algae *Rhodomonas*  
146 sp., which were raised in B1 medium (Guillard and Ryther, 1962) at 20 °C under a  
147 12:12 h light:dark cycle. Algal strains were provided by the Marine Algal Culture  
148 Centre at Gothenburg University (GUMACC). The carbon content of the algae was  
149 estimated based on volume measurements as equivalent spherical diameter with an  
150 electronic particle analyzer (Elzone 5380, Micrometrics, Aachen, Germany) and  
151 equations provided by Mullin et al. (1966). Algae concentration and size were checked  
152 daily using the same analyzer and then adjusted in the experimental bottles to a  
153 concentration of 150 µg C L<sup>-1</sup>. The FSW of all cultures was changed twice a week,  
154 coinciding with chemistry measurements (see section 2.5 below). Larval densities were  
155 monitored daily for the first 15-day post-fertilization, and every second day thereafter  
156 until day 36. Every sampling day, four subsamples of 10 mL of each replicate were  
157 counted. Density at time t ( $N_t$ , number of larvae L<sup>-1</sup>) was estimated as the mean of this  
158 four measures. Daily survival (SUR) was calculated as:  $SUR = (N_t/N_0)*100$ . Cultures  
159 were run until day 52 in order to get settlers to be used in the following experiment,  
160 except Treatment II, which was discontinued at day 26 due to logistical issues.

161

### 162 2.3. Larval morphology measures

163           For each treatment, 10 larvae, fixed in buffered 4% paraformaldehyde in FSW,  
164 were photographed every two days (2 to 8 days post-fertilization) or every three days  
165 (11 to 20 days post-fertilization) using a digital camera mounted on a dissecting  
166 microscope with polarized light to visualize the skeleton. Six morphometric lengths:  
167 body length (BL), body width (BW), body rod lengths (right BRR and left BRL) and  
168 post-oral rod lengths (right POR and left POL) were measured for each larva (Fig. 1)  
169 using ImageJ 1.46r image analyzing software (Schneider et al. 2012). An asymmetry



170 index (ASY) was calculated as the ratio between the shortest and the longest maximum  
171 total length (MTL=BR+PO at each side of the body).

172

#### 173 *2.4. Experiments with settled post-larvae*

174 After 40-42 days of culture, settlers appeared spontaneously in the experimental  
175 bottles kept at 19 °C, both at pH<sub>T</sub> 8.1 and pH<sub>T</sub> 7.7. Living settlers were then recovered  
176 and the test diameter of 30 individuals from each treatment was measured. A survival  
177 experiment was performed in order to test the effect of pH on the survival of the settlers.  
178 For this experiment, we used a crossed design (pH during larval growth x pH during  
179 settler growth) with settlers grown at pH<sub>T</sub> 8.1 or 7.7, transferred to plastic plates with 3-  
180 mL wells and kept in FSW at 19 °C and pH<sub>T</sub> 8.1 or 7.7. We used three replicates for  
181 each treatment, with 18 settlers (6 wells; 3 individuals per well) per replicate (a total of  
182 54 settlers per treatment). After three days, we counted the settlers which remained alive  
183 and calculated the survival rate as the % of surviving juveniles.

184

#### 185 *2.5. Seawater chemistry*

186 Temperature was monitored daily. Total alkalinity (A<sub>T</sub>) and pH<sub>T</sub> were measured  
187 twice a week. A<sub>T</sub> was determined on filtered samples with a titration system (TitroLine  
188 alpha plus, SI Analytics). pH<sub>T</sub> (henceforth “pH”) was measured with a Metrohm 827  
189 pH-electrode adjusted for pH measurements at the total scale using Tris/HCl and 2-  
190 aminopyridine/HCl buffer solutions (provided by Unité d’Océanographie Chimique,  
191 Université de Liège, Belgium). Total carbon (C<sub>T</sub>) and the carbonate system speciation  
192 (pCO<sub>2</sub>, Ω<sub>Ca</sub> and Ω<sub>Ar</sub>) were calculated from temperature, pH and A<sub>T</sub> using CO2CALC  
193 (Robbins et al., 2010), an application based on CO2SYS (Lewis and Wallace, 1998),  
194 using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and

195 Millero (1987). pH was maintained in each experimental bottle using a computerized  
196 feedback system (AquaMedic) that regulated pH by addition of pure gaseous CO<sub>2</sub>  
197 directly into the seawater (±0.02 pH units).

198

## 199 2.6. *Statistical analyses*

200 One-way ANOVA followed by SNK post hoc test was used to confirm that  
201 differences between measured temperatures and pH were as expected between the four  
202 treatments.

203 The effects of temperature and pH on larval size (BL) and on survival rate  
204 (SUR) at a given time of culture (TOC) were tested using separate ANCOVAs for each  
205 factor, to avoid problems arising from our not fully crossed experimental design; TOC  
206 (Ln-transformed) was the covariate. The following lineal model was used for each  
207 variable Y, where Y represents the dependent variable (BL or SUR) and X represents  
208 the factor (either temperature or pH):  $Y = \mu + \beta_1 \text{Ln}(\text{TOC}) + \beta_2 X + \beta_3 \text{Ln}(\text{TOC}) \times X + \beta_4$   
209  $\text{Replicate}(X)$ . X was considered as a fixed factor and the replicate was nested within it.  
210 Similar linear models were used to assess the effects of the two physicochemical factors  
211 in the relations between SUR and BL as a covariate (also Ln-transformed).

212 The effects of temperature and pH in the morphological variables of the larvae  
213 were also tested separately using BL as a covariate. Linear regressions (not shown) were  
214 used for each experimental treatment to check the linearity of the relationships between  
215 morphological variables and BL. The following lineal model was used for each variable  
216 Y, where Y represents a morphological variable and X represents either temperature or  
217 pH:  $Y = \mu + \beta_1 \text{BL} + \beta_2 X + \beta_3 \text{BL} \times X + \beta_4 \text{Replicate}(X)$ .

218 The survival curves for the larvae were considered to be derived from a hazard  
219 function following a 2-parameter Weibull distribution (Cox and Oakes, 1984). Thus, the

220 ratio of surviving larvae (SUR) at a given TOC, is given by  $SUR = \exp(-\lambda \cdot TOC^\beta)$ ,  
221 where  $\lambda$  is the scale parameter and  $\beta$  is the shape parameter. We calculated both  
222 parameters separately for every replicate using non-linear least-squares regressions  
223 (Bates and Watts, 1988), and pooled the replicates for each treatment, after verifying the  
224 absence of significant differences.

225 Differences in the diameter of settlers derived from larvae reared under pH 8.1  
226 and pH 7.7 were tested using a t-test and differences in settler survival were tested using  
227 one-way ANOVA. Homogeneity of variances and normality of residuals were tested in  
228 all models using the Bartlett and Shapiro-Wilk tests respectively. All statistical analyses  
229 were performed in R using the RStudio interface (RStudio Inc., Boston, MA, USA).

230

### 231 **3. Results**

#### 232 *3.1. Physicochemical variables*

233 The experimental means and standard deviations of the measured  
234 physicochemical parameters for the four treatments are summarized in Table 1. As  
235 expected, ANOVA followed by SNK post hoc test found significant differences for  
236 temperatures between treatments I, II and III (all  $P < 0.001$ ) but not between treatments  
237 III and IV ( $P = 0.67$ ). Concerning pH, ANOVA followed by SNK found no differences  
238 between treatments I, II and III (all  $P > 0.33$ ), whereas treatment IV was significantly  
239 different from the former three treatments (all  $P < 0.001$ ).

240

#### 241 *3.2. Larval growth and survival*

242 The variation over time of larval size at different temperatures and pH is  
243 displayed in Fig. 2 and the ANCOVAs are listed in Table 2. No significant differences  
244 between replicates were found for any variable throughout all analyses, so replicates

245 have been pooled for clarity in the graphical representations. The larval size, measured  
246 as body length (BL) grew significantly faster with increasing temperatures (treatments I,  
247 II and III, Table 2a). The effect of a pH decrease from 8.1 to 7.7 at 19 °C produced no  
248 appreciable difference in BL during the first eight days of culture, but originated  
249 significantly smaller larvae from then on (treatments III and IV, Table 2b).

250 The survival curves are shown in Fig. 3 for the four treatments tested. The  
251 results of the ANCOVAs are listed in Table 3. Temperature increase from 16 to 19 °C  
252 had a positive significant effect on larval survival (Table 3a). The effect of pH on  
253 survival was more complex, as reflected by the significant Ln(TOC) x pH interaction of  
254 the ANCOVA (Table 3b). The survival was similar at pH 8.1 and 7.7 during the first 14  
255 days, but it was significantly higher from then on at the lower pH. The significant  
256 ANCOVAs of survival rate (SUR) with BL as covariate suggest that the differences in  
257 survival may be ascribed to the effects of temperature (Table 3c) and pH (Table 3d), and  
258 are not attributable to a hidden effect of body length due to developmental delay. The  
259 significant Ln(BL) x pH interaction (Table 3d) proves that at smaller sizes the survival  
260 rate was higher at pH 8.1, but at bigger sizes the survival rate was higher at pH 7.7.

261 The calculated values for the parameters of the hazard functions for the four  
262 different treatments are listed in Table 4. The values of the shape parameter  $\beta$  were  $< 1$   
263 in all cases, showing that the survival curves departed from the exponential function.  
264 That is, the hazard rates were not constant and were higher during the first days of  
265 development. The hazard rate variation was most apparent in the pH 7.7 treatment ( $\beta =$   
266  $0.338 \pm 0.035$ ).

267

### 268 3.3. Larval morphology

269 The variation of larval morphology (allometry) using body length as covariate at

270 different temperatures and pH is summarized in Fig. 4 and the results of the ANCOVAs  
271 for the studied variables are listed in Table 5. Changes in temperature affected  
272 significantly to all the morphological variables studied (Tables 5a, 5c, 5e and 5g).  
273 Maximum total length (Fig. 4A) varied similarly with body length for treatments II, III  
274 and IV, but a significant BL x T interaction proves that, in treatment I, larvae at 16 °C  
275 tended to have significantly smaller post-oral rods when reaching BL > 250 µm. The  
276 variation of body rod length (Fig. 4B) and of body width (Fig. 4C) with body size was  
277 similar at 16 and 17.5 °C, but was significantly different at 19 °C, implying that larvae  
278 grown at the higher temperature were relatively wider and with longer body rods than  
279 those grown at colder temperatures, for similar values of BL. All BL x T interaction  
280 terms were significant for these variables, thus the observed effects of temperature on  
281 larval morphology were complex and changing over the size range. Conversely, the  
282 effects of pH were nonsignificant for almost all morphological variables (Tables 5b, 5d  
283 and 5h), and thus larvae grown at 19 °C had the same overall morphology independently  
284 of pH, except for a significant BL x pH interaction effect on body width (Table 5f).  
285 Larvae grown at pH 8.1 and 19 °C tend to grow wider than those grown at pH 7.7 and  
286 the same temperature, when BL > 400 µm. The asymmetry index showed a high degree  
287 of dispersion for BL > 150 µm (Fig. 4D) and these results (a slightly significant effect  
288 of temperature, Table 5g) must then be taken with caution.

289 Fig. 5 graphically compares the size and morphology of average larvae reared  
290 using the four different treatments at two different times. Overall, we found  
291 developmental delay in all treatments when compared to pH 8.1 and 19 °C. The growth  
292 rate and morphology of the larvae was remarkably affected by changes in temperature,  
293 but the effects of pH change were subtler and almost all the morphological differences  
294 between treatments III and IV may be attributable to the delay in the development.

295 3.4. *Settlers count, size and survival*

296 The first settlers appeared at day 40-42 in the cultures at 19 °C, both at pH 8.1  
297 and 7.7, whereas only a few settlers appeared at day 48-50 in the cultures at 16 °C.  
298 These cultures were stopped at day 52 and all the living settlers were counted. Overall,  
299 we obtained  $480 \pm 341$  (mean  $\pm$  SE) settlers in the cultures at 19 °C and pH 8.1,  $149 \pm$   
300  $117$  settlers in the cultures at 19 °C and pH 7.7 and only  $12 \pm 12$  settlers in the cultures  
301 at 16 °C. The settlers reared at 19 °C and pH 8.1 had diameters of  $489 \pm 5 \mu\text{m}$  (mean  $\pm$   
302 SE) and were significantly bigger ( $t_{58} = 6.62$ ;  $p < 0.0001$ ) than those reared at pH 7.7  
303 (diameter =  $433 \pm 7 \mu\text{m}$ ; Fig.6).

304 The survival experiment was carried out using only settlers grown at 19 °C, in  
305 pH 8.1 or 7.7 (treatments III and IV), which were recovered on day 45 and transferred  
306 to FSW at 19 °C and pH 8.1 or 7.7 (all combinations) and cultured for three days. The  
307 survival rate did not differ between the four treatments (ANOVA  $F=2.43$ ,  $P = 0.14$ ; Fig.  
308 7).

309

310 **4. Discussion**

311 The main conclusion arising from our results is that temperature is a main factor  
312 affecting the developmental timing and survival rate of *Arbacia lixula* larvae  
313 (temperature increases from 16 to 17.5 to 19 °C improved their survival and accelerated  
314 their growth), whereas a moderate drop in pH (such as that predicted for 2100) affected  
315 the development only to a lesser degree.

316 Nevertheless, our results show that *A. lixula* larvae can be cultured and complete  
317 their development at temperatures between 16 and 19 °C, though the survival curve  
318 showed quite elevated mortality rates, especially during the first days of culture. The  
319 advantage of using Weibull distributions to describe the survival curve is their

320 flexibility for modelling both increasing and decreasing hazard functions, depending on  
321 the value of the shape parameter  $\beta$ . All values obtained for  $\beta$  in our study were smaller  
322 than 1 (Table 3), implying that the hazard functions decreased over time; that is, in the  
323 conditions of our experiments, the larval mortality was higher during the first days of  
324 the development and it diminished over time. Also, the parameter  $\beta$  showed a clear  
325 trend to decrease with warming (Table 3), which suggests that the mortality remained  
326 more constant over time at low temperatures.

327 Gianguzza et al. (2013) reported that mild acidification could have a positive  
328 effect in the early developmental dynamics (two days) of *A. lixula* larvae raised at 20  
329 °C. Our results did not detect any positive effect of lowered pH on the growth rate of the  
330 early larvae, but showed that a decrease of pH from 8.1 to 7.7 led to an enhancement of  
331 survival rate of the larvae in the long-term. Actually, the difference with the survival at  
332 natural pH improved over time, as reflected by the low value of parameter  $\beta$ , the shape  
333 of the survival curve (Fig. 3A) and the significant Ln(TOC) x pH interaction term in the  
334 ANCOVA (Table 3b). However, this increase in the survival rate by lowered pH is  
335 accompanied by a significant decrease in body length (Fig. 2) and body width (Fig. 4C).

336 The overall shape of *A. lixula* larvae was remarkably affected by changes in  
337 temperature (Fig. 5). Lower temperatures produced smaller larvae (Fig. 2) with  
338 relatively shorter post-oral and body rods (Fig 4A, 4B) and narrower bodies (Fig. 4C).  
339 These morphological changes associated with temperature cannot be attributed to a  
340 hidden effect due to a developmental delay (Table 5). Conversely, pH affected larval  
341 morphology to a lesser degree (Table 5), and only the body width showed some  
342 dependence of pH (Table 5f).

343 Our results also demonstrate that, despite the significant differences in body size,  
344 the survival of early settlers of *Arbacia lixula* is resilient against changes induced by

345 slight acidification, either if exposed to it as larvae, as settlers, or both. No significant  
346 difference in the survival after 3 days was found between treatments. One previous  
347 work (Dupont et al., 2013) studied the possible carry-over effect of ocean acidification  
348 from sea urchin (*Strongylocentrotus droebachiensis*) larvae to settlers. Their results  
349 with this cold water species are not in good agreement with our results with *A. lixula*.  
350 They found that the combined exposition to pH 7.7 during larval development,  
351 continued as settlers, led to a higher mortality than that observed in individuals exposed  
352 to pH 8.1 as larvae, as settlers or both. These experiments were run for 3 months and the  
353 settlers were fed, which could explain the differences with our results. The difficulty to  
354 find a suitable food source for *Arbacia lixula* settlers prevented us from running a  
355 longer survival experiment. Further research is needed to produce robust evidence, as  
356 settlers are probably one of the most sensitive life-history stages to ocean acidification  
357 (Dupont and Thorndyke, 2013).

358         George et al. (1990) cultured Mediterranean *A. lixula* larvae at 22 °C which  
359 achieved metamorphosis at 26-30 days after fertilization. Their results also suggest the  
360 existence of natural variability in developmental growth rates, depending on the initial  
361 quality of the eggs (egg size and protein and lipids content). In our experiments, the first  
362 settlers appeared at days 40-42 at 19 °C and at days 48-50 at 16 °C. Thus, temperature  
363 may be a main factor affecting the developmental time of *A. lixula* in natural  
364 environments.

365         Another recent work studied the culture and settlement of *A. lixula*. Privitera et  
366 al. (2011) reported that larvae from Genoa populations cultured at 18 °C suffered 100%  
367 mortality at 7 days, while the same larvae reared at 22 °C survived and reached the  
368 competent stage at approximately 20 days. Our results show that *A. lixula* larvae from  
369 northwestern Mediterranean are indeed able to develop at lower temperatures, down to



370 16 °C, and even achieve metamorphosis and reach the settler stage, albeit with reduced  
371 survival and slower growth. This discrepancy in the results may arise from differences  
372 in the culture methods (container volume, algal species, feeding dose and timing,  
373 sterilization of FSW by autoclaving or the use of agitation by swinging paddles), since it  
374 is hardly attributable to genetic differences between Ligurian and Catalan populations  
375 (Wangensteen et al. 2012; Pérez-Portela et al., unpublished results).

376 On the other hand, Gianguzza et al. (2013) recently studied the development of  
377 *A. lixula* during the early endotrophic stages (up to 2 days) using temperatures from 20  
378 to 27 °C at two different pH values. They reported an interesting interaction between pH  
379 and temperature. Thus, slightly acidic pH accelerated growth at 20 °C, while it has a  
380 neutral effect at 24 °C and a negative effect at 26 °C. Our results showing enhanced  
381 survival rates using pH 7.7 at 19 °C are in accordance with a positive effect of slight  
382 acidification for *A. lixula* at temperatures around 20 °C, but we found a detectable  
383 enhanced survival rate only after approximately 14 days of culture and this change was  
384 concurrent with developmental delay.

385 Delay in the development is the most documented effect of ocean acidification  
386 on echinoderm larvae, with 16 out of 19 tested species showing some degree of retarded  
387 development (Dupont and Thorndyke, 2013). More sophisticated experiments have to  
388 be conducted in order to test the outcomes of this delay in natural ecosystems. It can be  
389 argued that larvae suffering delayed growth would have to develop for longer time and  
390 thus be more vulnerable to predation, drastically affecting their fitness (Dupont et al.,  
391 2010a). Interestingly, in our case this delayed development did not translate into longer  
392 larval periods, as settlers appeared at about the same time in cultures kept at natural and  
393 slightly acidic conditions, though the latter had lower settlement success and smaller  
394 size after metamorphosis (Fig. 6).

395 In the present work we report data of experiments spanning the whole larval  
396 development and the early post-settlement period of the thermophilous species *Arbacia*  
397 *lixula*. Further laboratory experiments, using a wider range of pH and temperature  
398 conditions and longer follow-up of settlers, supported by thorough field monitoring of  
399 larval and adult densities throughout several years should be carried out in order to  
400 acquire a full view of the possible impact of ocean acidification and global warming on  
401 the ecology of this significant species. A plethora of physical and biological factors  
402 other than temperature or acidification may modulate larval development and survival  
403 of sea urchins in natural environments, and many of them are subject to unpredictable  
404 changes in the near future. Some recent works have also proved that sea urchins feature  
405 high levels of genetic and larval phenotypic variability and thus show a high potential  
406 for adaptation to changing environmental conditions (Sunday et al., 2011; Pespeni et al.,  
407 2013).

408 Although the conditions of any experimental setup may be too simplistic to  
409 accurately predict the behaviour of complex systems, our results so far suggest that  
410 warming will contribute to enhance the reproductive success of *A. lixula* and that a mild  
411 acidification, coherent with the foreseeable situation in the near future, would reduce  
412 larval growth rates but improve larval survival. Overall, then, the impact of *A. lixula* on  
413 Mediterranean communities may be expected to increase in the forthcoming decades.

414

#### 415 **Acknowledgements**

416 We are indebted to Bengt Lundve for skilful technical assistance with culturing  
417 system and to Narimane Dorey for fruitful discussions and helping with IVF. We also  
418 thank Alex García-Cisneros and Fabiana Saporiti for help with sampling, Ramón  
419 Roqueta (from Andrea's Diving Center at Tossa de Mar) for field assistance and Sandra

420 Garcés and Valentí Rull (Institut Botànic de Barcelona, CSIC) for granting access to  
421 microscopy facilities. This work was funded by projects CTM2010-22218 from the  
422 Spanish Government, BIOCON 08-187/09 from BBVA Foundation, EPOCA (European  
423 Project on Ocean Acidification) N211384 from the European Community's Seventh  
424 Framework Programme (FP7/2007-2013) and grants from ASSEMBLE (Association of  
425 European Marine Biology Laboratories) and KVA (The Royal Swedish Academy of  
426 Sciences). OSW was funded by a grant from AGAUR BE-DGR 2012 (Generalitat de  
427 Catalunya). SD is funded by the CeMEB and supported by a Linnaeus-grant from the  
428 Swedish Research Councils VR and Formas.

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611 **Legend to figures**

612

613 **Fig. 1.** Measured distances for the morphological study of *Arbacia lixula* pluteus larvae.

614 BL: body length. BW: Body width, BRL & BRR: Body rods lengths (left and right);

615 POL & POR: Post-oral rods lengths (left and right).

616

617 **Fig. 2.** Effect of temperature and pH on individual growth (body length) of *Arbacia*

618 *lixula* larvae. Since no differences were found between replicate cultures, replicates

619 have been pooled for clarity.

620

621 **Fig. 3.** Survival curves for *Arbacia lixula* larvae cultured at different temperatures and

622 pH in function of time of culture (A) or body length (B). The interpolation curves in A

623 were calculated assuming hazard functions following a Weibull distribution. Since no

624 differences were found between replicate cultures, replicates have been pooled for

625 clarity.

626

627 **Fig. 4.** Maximum total length (A), maximum body rod length (B), body width (C) and

628 asymmetry index (D) plotted against body length of *Arbacia lixula* larvae grown at

629 different conditions of temperature and pH. Since no differences were found between

630 replicate cultures, replicates have been pooled for clarity.

631

632 **Fig. 5.** Typical morphology and size of *Arbacia lixula* larvae grown under different

633 conditions, after eight (upper row) or fourteen (lower row) days of culture. The four

634 treatments tested are shown.

635

636 **Fig. 6.** Diameters of early settlers (n=30) reared from *Arbacia lixula* larvae grown at pH  
637 8.1 or pH 7.7.

638

639 **Fig 7.** Effect of water acidification on the survival of *Arbacia lixula* settlers reared from  
640 larvae grown at pH 8.1 or 7.7 and then transferred to either pH 8.1 or 7.7 after  
641 settlement. No significant differences between treatments were found.

642

643 **Table 1**

644 Physicochemical variables measured in the four experimental treatments (mean  $\pm$  SD).  
 645 Partial pressure of carbon dioxide ( $p_{CO_2}$ ), total dissolved inorganic carbon ( $C_T$ ) and  
 646 calcium carbonate saturation state for calcite and aragonite ( $\Omega_{Ca}$ ,  $\Omega_{Ar}$ ) were calculated  
 647 from temperature,  $pH_T$  and total alkalinity ( $A_T$ ).

648

Treatment	T (°C)	$pH_T$	$A_T$ ( $\mu\text{mol/kg}$ )	$p_{CO_2}$ ( $\mu\text{atm}$ )	$C_T$ ( $\mu\text{mol/kg}$ )	$\Omega_{Ca}$	$\Omega_{Ar}$
<b>I. 16 °C pH 8.1</b>	16.3 $\pm$ 0.4	8.09 $\pm$ 0.05	2638 $\pm$ 39	547 $\pm$ 79	2403 $\pm$ 56	4.13 $\pm$ 0.38	2.67 $\pm$ 0.24
<b>II. 17.5 °C pH 8.1</b>	17.5 $\pm$ 0.3	8.08 $\pm$ 0.03	2637 $\pm$ 78	548 $\pm$ 53	2384 $\pm$ 77	4.45 $\pm$ 0.29	2.88 $\pm$ 0.19
<b>III. 19 °C pH 8.1</b>	18.8 $\pm$ 0.3	8.09 $\pm$ 0.04	2630 $\pm$ 44	548 $\pm$ 60	2379 $\pm$ 53	4.42 $\pm$ 0.27	2.87 $\pm$ 0.17
<b>IV. 19 °C pH 7.7</b>	18.8 $\pm$ 0.3	7.69 $\pm$ 0.04	2658 $\pm$ 61	1575 $\pm$ 153	2590 $\pm$ 56	1.95 $\pm$ 0.15	1.27 $\pm$ 0.10

649

650

651 **Table 2**

652 Analysis of covariance testing the effects of temperature (a) and pH (b) on *Arbacia*

653 *lixula* larval growth. BL: body length, TOC: time of culture, T: temperature.

654

<b>a. BL ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(TOC)	1	1219.23	< 0.0001
T	2	299.62	< 0.0001
Ln(TOC) x T	2	115.90	< 0.0001
Replicate(T)	3	0.06	0.98
Residuals	230		
<b>b. BL ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(TOC)	1	1475.94	< 0.0001
pH	1	9.17	0.0031
Ln(TOC) x pH	1	6.68	0.011
Replicate(pH)	2	2.00	0.14
Residuals	153		

655 **Table 3**

656 Analysis of covariance for *Arbacia lixula* larvae survival data. SUR: Survival rate,

657 TOC: Time of culture, T: Temperature, BL: Body length.

658

<b>a. SUR ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(TOC)	1	849.33	< 0.0001
T	2	20.71	< 0.0001
Ln(TOC) x T	2	1.11	0.33
Replicate(T)	3	0.93	0.43
Residuals	102		
<b>b. SUR ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(TOC)	1	420.62	< 0.0001
pH	1	4.69	0.033
Ln(TOC) x pH	1	15.98	0.00014
Replicate(pH)	2	1.16	0.32
Residuals	82		
<b>c. SUR vs Ln(BL) + T + Ln(BL) x T + Replicate(T)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(BL)	1	283.90	< 0.0001
T	2	192.07	< 0.0001
Ln(BL) x T	2	13.70	0.0003
Replicate(T)	3	0.52	0.47
Residuals	230		
<b>d. SUR vs Ln(BL) + pH + Ln(BL) x pH + Replicate(pH)</b>			
<b>Source</b>	<b>d.f.</b>	<b>F</b>	<b>P</b>
Ln(BL)	1	490.20	< 0.0001
pH	1	1.37	0.24
Ln(BL) x pH	1	14.45	0.0002
Replicate(pH)	2	2.17	0.14
Residuals	153		

660



661 **Table 4**

662 Calculated values for the parameters of the hazard functions (Weibull distributions)  
 663 describing the survival of *Arbacia lixula* larvae raised at different temperature and pH.  
 664 SSR: sum of squared residuals of the nonlinear regression. The survival function against  
 665 time of culture can be modelled by  $SUR = \exp(-\lambda \cdot TOC^\beta)$ .

666

<b>Treatment</b>	<b><math>\lambda</math> (day<sup>-β</sup>)</b>	<b><math>\beta</math></b>	<b>SSR</b>	<b><math>R^2</math></b>
<b>I: 16.0°C pH 8.1</b>	0.304 ± 0.034	0.642 ± 0.050	0.223	0.87
<b>II: 17.5°C pH 8.1</b>	0.313 ± 0.025	0.572 ± 0.035	0.050	0.95
<b>III: 19.0°C pH 8.1</b>	0.301 ± 0.026	0.531 ± 0.035	0.149	0.89
<b>IV: 19.0°C pH 7.7</b>	0.434 ± 0.039	0.338 ± 0.035	0.200	0.72

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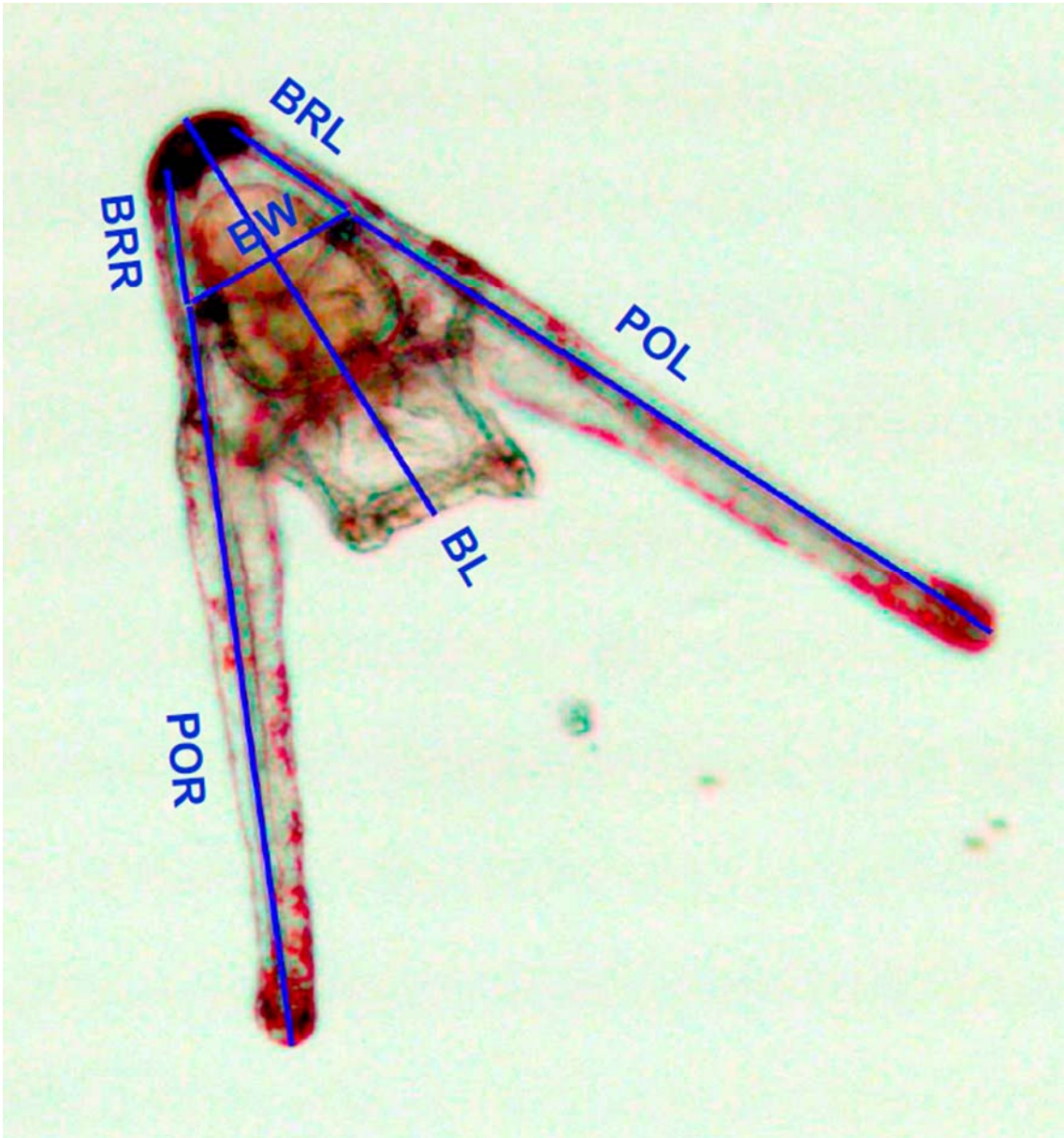
668 **Table 5**

669 Analysis of covariance for *Arbacia lixula* larval morphology against body length and  
 670 temperature or pH. BL: body length, T: temperature, MTL: maximum total length,  
 671 MBR: maximum body rod length, BW: body width, ASY: asymmetry index.  
 672

<b>a. MTL ~ BL+ T + Replicate(T)</b>				<b>b. MTL ~ BL+ pH + Replicate(pH)</b>			
Source	d.f.	F	P	Source	d.f.	F	P
BL	1	1951.94	< 0.0001	BL	1	779.96	< 0.0001
T	2	1.34	0.26	pH	1	1.19	0.28
BL x T	2	3.90	0.03	BL x pH	1	0.12	0.73
Replicate(T)	3	2.24	0.09	Replicate(pH)	2	1.29	0.28
Residuals	230			Residuals	153		
<b>c. MBR ~ BL+ T + Replicate(T)</b>				<b>d. MBR ~ BL+ pH + Replicate(pH)</b>			
Source	d.f.	F	P	Source	d.f.	F	P
BL	1	251.42	< 0.0001	BL	1	70.80	< 0.0001
T	2	13.16	< 0.0001	pH	1	0.54	0.46
BL x T	2	7.79	0.0005	BL x pH	1	0.68	0.41
Replicate(T)	3	0.80	0.50	Replicate(pH)	2	0.62	0.53
Residuals	230			Residuals	153		
<b>e. BW ~ BL+ T + Replicate(T)</b>				<b>f. BW ~ BL+ pH + Replicate(pH)</b>			
Source	d.f.	F	P	Source	d.f.	F	P
BL	1	801.67	< 0.0001	BL	1	390.67	< 0.0001
T	2	3.35	0.037	pH	1	3.83	0.052
BL x T	2	27.42	< 0.0001	BL x pH	1	7.44	0.007
Replicate(T)	3	0.36	0.78	Replicate(pH)	2	0.39	0.68
Residuals	230			Residuals	153		
<b>g. ASY ~ BL+ T + Replicate(T)</b>				<b>h. ASY ~ BL+ pH + Replicate(pH)</b>			
Source	d.f.	F	P	Source	d.f.	F	P
BL	1	37.65	< 0.0001	BL	1	32.01	< 0.0001
T	2	3.81	0.02	pH	1	0.60	0.44
BL x T	2	1.86	0.16	BL x pH	1	2.65	0.11
Replicate(T)	3	0.94	0.42	Replicate(pH)	2	1.26	0.29
Residuals	230			Residuals	153		

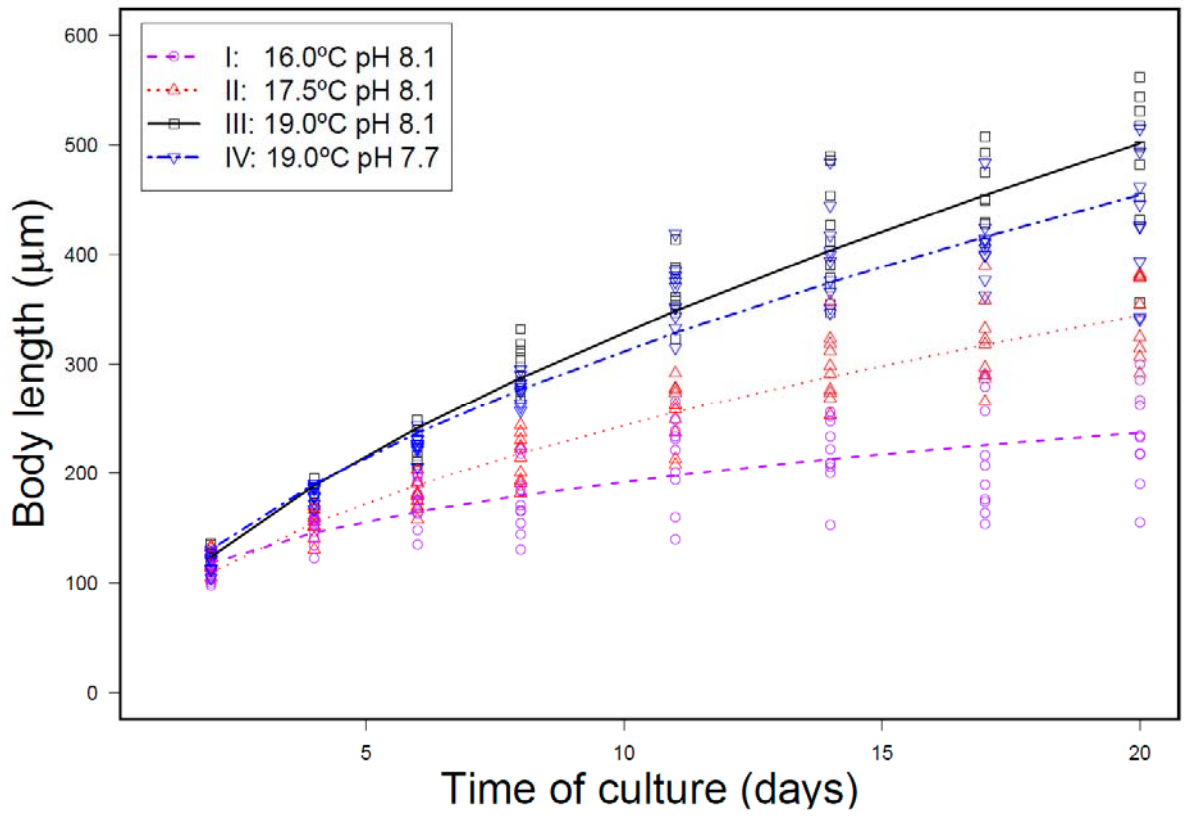
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674 Fig. 1.  
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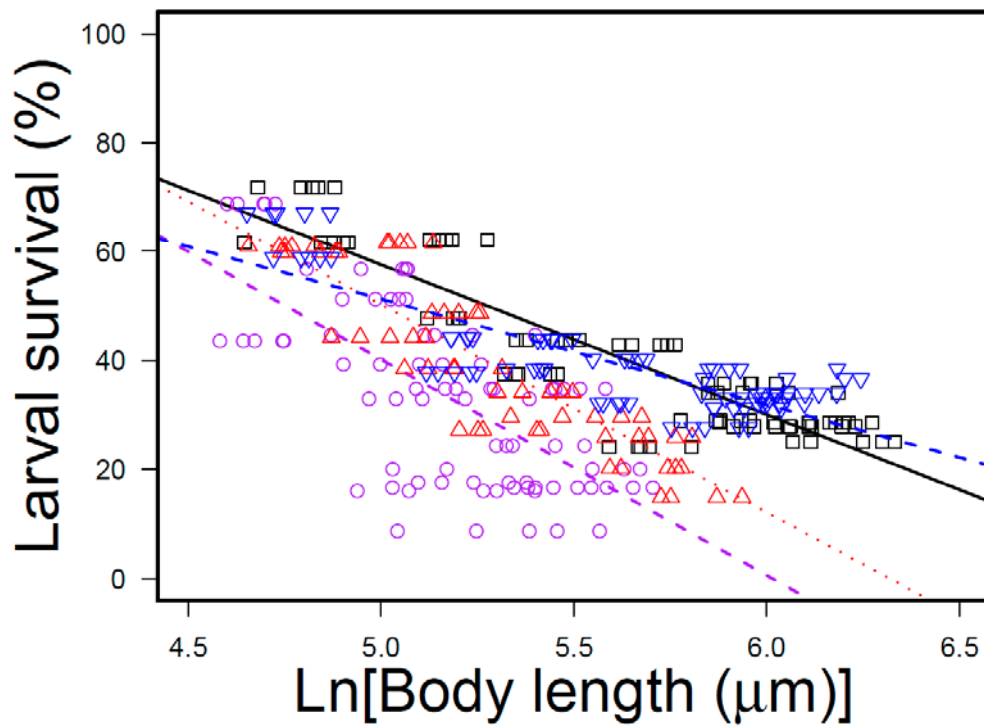
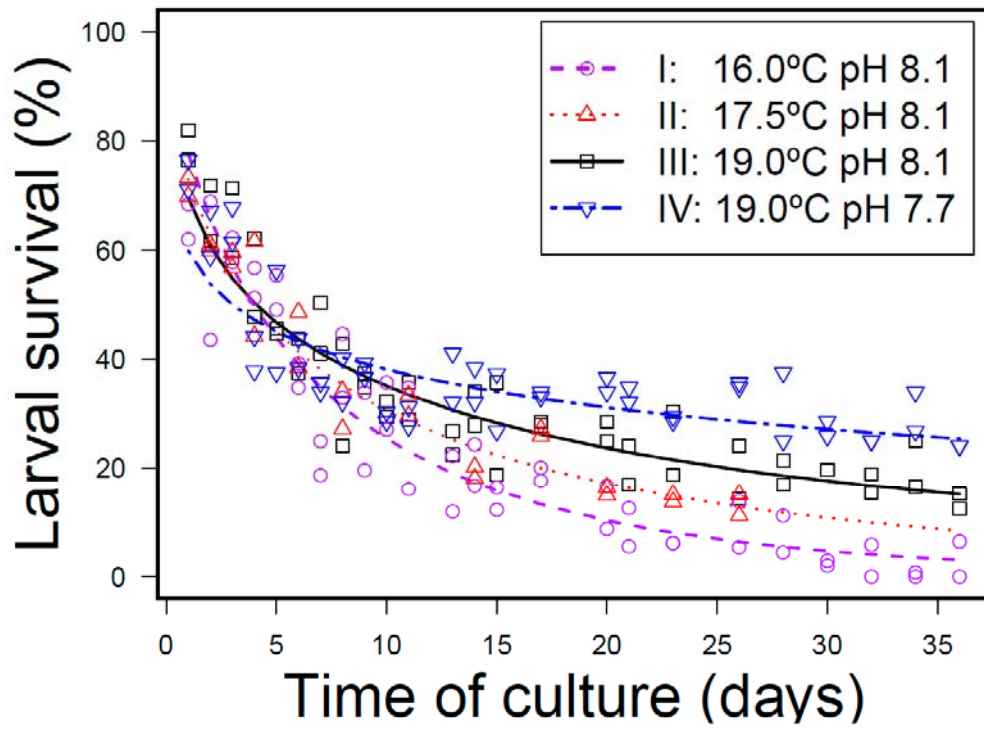
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677 Fig. 2.



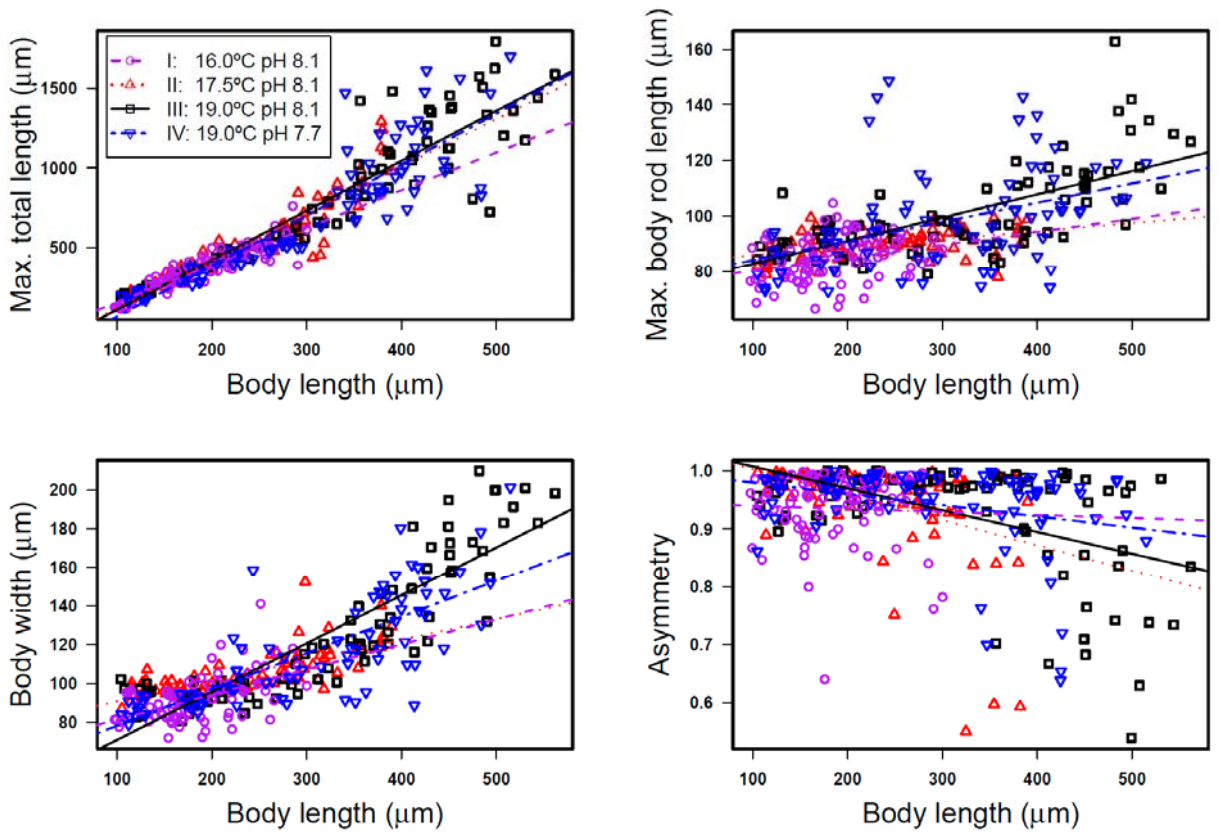
678

679 Fig. 3.  
680



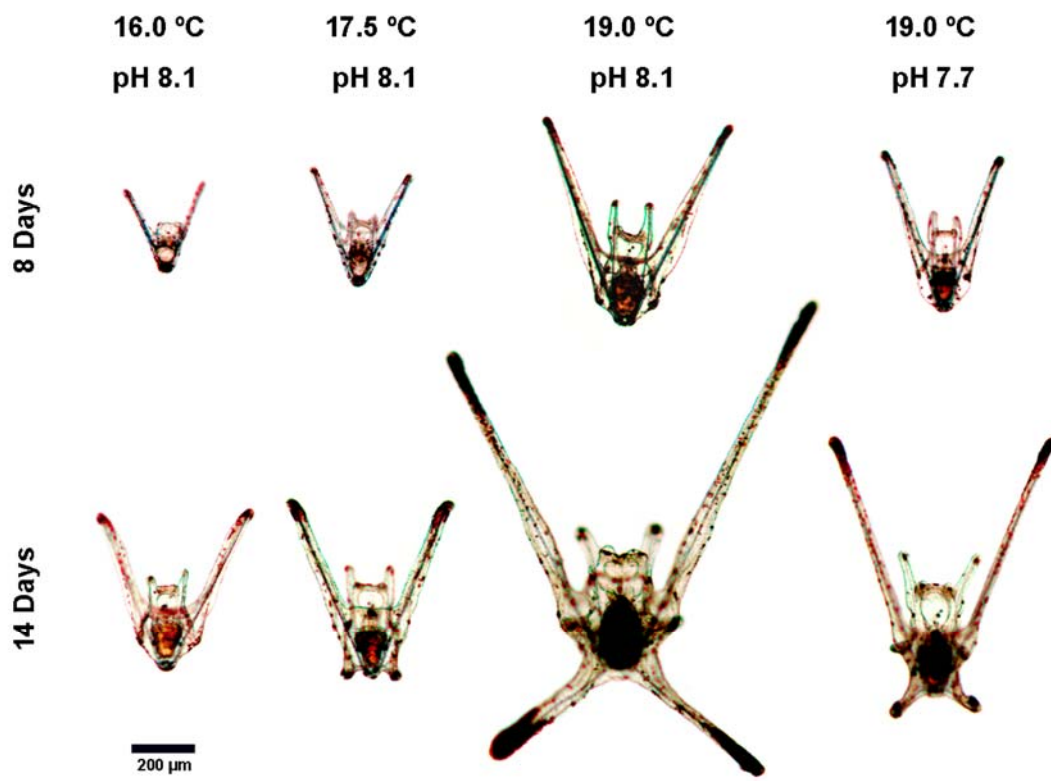
681

682 **Fig. 4.**  
683



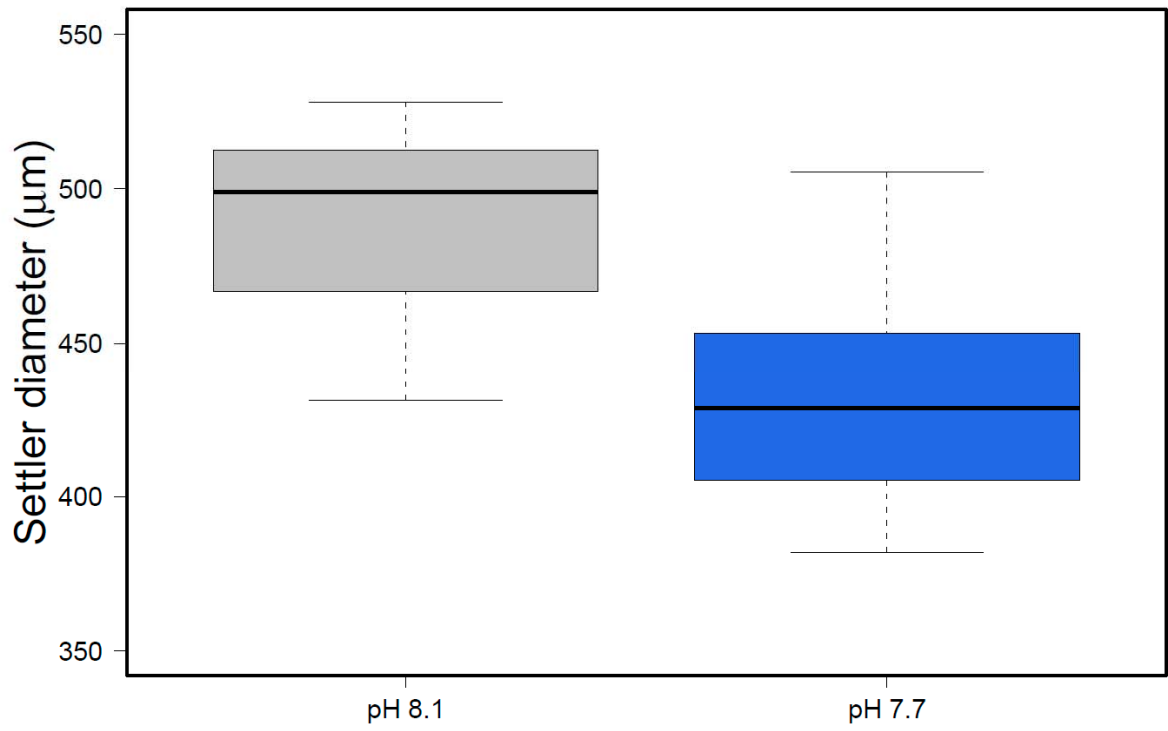
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685 Fig. 5.  
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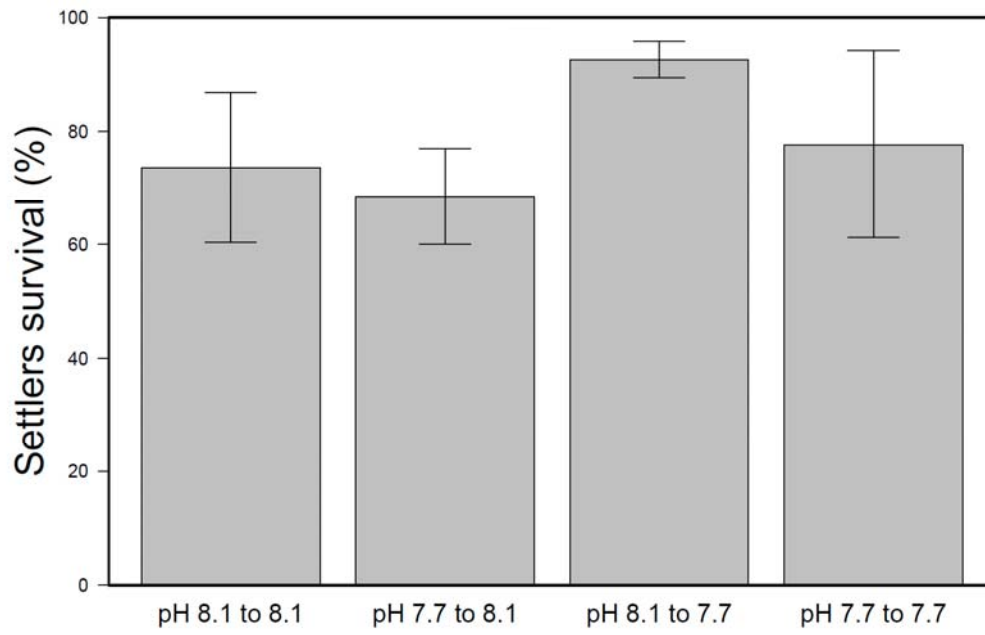
688 **Fig. 6.**  
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690



691 **Fig. 7.**



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