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

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

Abstract

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

Keywords

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

Abbreviations

ASY: asymmetry index; BL: body length; BW: body width; BRL: left body rod length; BRR right body rod length; FSW: filtered seawater; POL: left post-oral rod length; POR: right post-oral rod length; SUR: survival rate; TOC: time of culture.

1. Introduction

Global changes due to increased atmospheric CO_2 emissions are altering ocean ecosystems, though there is considerable uncertainty about the spatial and temporal details (Hoegh-Guldberg and Bruno, 2010). Major physicochemical changes in marine ecosystems come in two different ways: ocean warming and acidification. In the Mediterranean Sea, long-term datasets have revealed temperature increases of 0.8–1.4 ºC over the last 30 years (Lejeusne et al., 2010 and references therein) and a further 2 ºC increase is expected by 2100 (Meehl et al., 2007; IPCC, 2007). On the other hand, the average pH of surface seawater has declined worldwide by approximately 0.1 units since the industrial revolution and future reductions are expected to be around 0.3–0.5 units by 2100 (Caldeira and Wickett, 2003, 2005; Royal Society, 2005).

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

70 Only a few previous works have studied the combined effects of increased
71 temperature and ocean acidification on echinoderm larvae (Sheppard Brennand 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

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

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

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

2. Materials and methods

2.1. Adult sea urchins collection

Adult *Arbacia lixula* individuals were collected by SCUBA diving at Tossa de Mar (NE Spain, 41º43'16" N, 2º56'24" E) in September 2012, kept in a 10 L plastic tank with seawater aerated by oxygen tablets and transported by airplane within 24 h to the Sven Lovén Centre for Marine Sciences - Kristineberg (Sweden). Induced spawning and *in vitro* fecundation were carried out shortly upon arrival.

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 µL of a 1 L egg dilution. Sperm stock solution in FSW was added to a final
127 concentration of ~ 1,000 sperm 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 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 (~ 60 bubbles
133 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 ºC. We compared four
139 different scenarios: (i) Treatment I (16 ºC, pH_T 8.1), corresponding to the lower range of
140 the present temperature variability; (ii) Treatment II (17.5 ºC, pH_T 8.1), an intermediate
141 temperature; (iii) Treatment III (19 ºC, pH_T 8.1), corresponding to the higher range of
142 temperature presently experienced by the autumnal larvae; (iv) Treatment IV (19 ºC,
143 pH_T 7.7), corresponding to near-future ocean acidification scenario. Two replicates were
144 used per treatment.

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

2.3. Larval morphology measures

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

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

2.4. Experiments with settled post-larvae

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

2.5. Seawater chemistry

Temperature was monitored daily. Total alkalinity (A_T) and pH_T were measured twice a week. A_T was determined on filtered samples with a titration system (TitroLine alpha plus, SI Analytics). pH_T (henceforth "pH") was measured with a Metrohm 827 pH-electrode adjusted for pH measurements at the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions (provided by Unité d'Océanographie Chimique, Université de Liège, Belgium). Total carbon (C_T) and the carbonate system speciation (p_{CO2}, Ω_{Ca} and Ω_{Ar}) were calculated from temperature, pH and A_T using CO2CALC (Robbins et al., 2010), an application based on CO2SYS (Lewis and Wallace, 1998), 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_2
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): $Y = \mu + \beta_1 \text{Ln(TOC)} + \beta_2 X + \beta_3 \text{Ln(TOC)} \times X + \beta_4$
209 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 λ is the scale parameter and β 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 ºC 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 ºC
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 β 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 ± 0.035).

267

268 *3.3. Larval morphology*

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

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

Fig. 5 graphically compares the size and morphology of average larvae reared using the four different treatments at two different times. Overall, we found developmental delay in all treatments when compared to pH 8.1 and 19 ºC. The growth rate and morphology of the larvae was remarkably affected by changes in temperature, but the effects of pH change were subtler and almost all the morphological differences 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 ºC, both at pH 8.1
297 and 7.7, whereas only a few settlers appeared at day 48-50 in the cultures at 16 ºC.
298 These cultures were stopped at day 52 and all the living settlers were counted. Overall,
299 we obtained 480 ± 341 (mean ± SE) settlers in the cultures at 19 ºC and pH 8.1, 149 ±
300 117 settlers in the cultures at 19 ºC and pH 7.7 and only 12 ± 12 settlers in the cultures
301 at 16 ºC. The settlers reared at 19 ºC and pH 8.1 had diameters of 489 ± 5 μm (mean ±
302 SE) and were significantly bigger (t_{58} = 6.62; p < 0.0001) than those reared at pH 7.7
303 (diameter = 433 ± 7 μm; Fig.6).

304 The survival experiment was carried out using only settlers grown at 19 ºC, 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 ºC 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 ºC 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 ºC, 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 β. All values obtained for β 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 β 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 ºC. 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 β, 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 ºC 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 ºC and at days 48-50 at 16 ºC. 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 ºC suffered 100%
367 mortality at 7 days, while the same larvae reared at 22 ºC 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

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

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

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

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

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

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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

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

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

643 **Table 1**

644 Physicochemical variables measured in the four experimental treatments (mean ± SD).
645 Partial pressure of carbon dioxide (p_{CO2}), total dissolved inorganic carbon (C_T) and
646 calcium carbonate saturation state for calcite and aragonite (Ω_{Ca}, Ω_{Ar}) were calculated
647 from temperature, pH_T and total alkalinity (A_T).

648

Treatment	**T (ºC)**	**pH_T**	**A_T (µmol/kg)**	**p_{CO2} (µatm)**	**C_T (µmol/kg)**	**Ω_{Ca}**	**Ω_{Ar}**
I. 16 ºC pH 8.1	16.3±0.4	8.09±0.05	2638±39	547±79	2403±56	4.13±0.38	2.67±0.24
II. 17.5 ºC pH 8.1	17.5±0.3	8.08±0.03	2637±78	548±53	2384±77	4.45±0.29	2.88±0.19
III. 19 ºC pH 8.1	18.8±0.3	8.09±0.04	2630±44	548±60	2379±53	4.42±0.27	2.87±0.17
IV. 19 ºC pH 7.7	18.8±0.3	7.69±0.04	2658±61	1575±153	2590±56	1.95±0.15	1.27±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

a. BL ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
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. BL ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
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

a. SUR ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
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. SUR ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
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		
c. SUR vs Ln(BL) + T + Ln(BL) x T + Replicate(T)			
Source	**d.f.**	***F***	***P***
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		
d. SUR vs Ln(BL) + pH + Ln(BL) x pH + Replicate(pH)			
Source	**d.f.**	***F***	***P***
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

Treatment	**λ ($day^{-\beta}$)**	**β**	**SSR**	**R^2**
I: 16.0ºC pH 8.1	0.304 ± 0.034	0.642 ± 0.050	0.223	0.87
II: 17.5ºC pH 8.1	0.313 ± 0.025	0.572 ± 0.035	0.050	0.95
III: 19.0ºC pH 8.1	0.301 ± 0.026	0.531 ± 0.035	0.149	0.89
IV: 19.0ºC pH 7.7	0.434 ±0.039	0.338 ±0.035	0.200	0.72

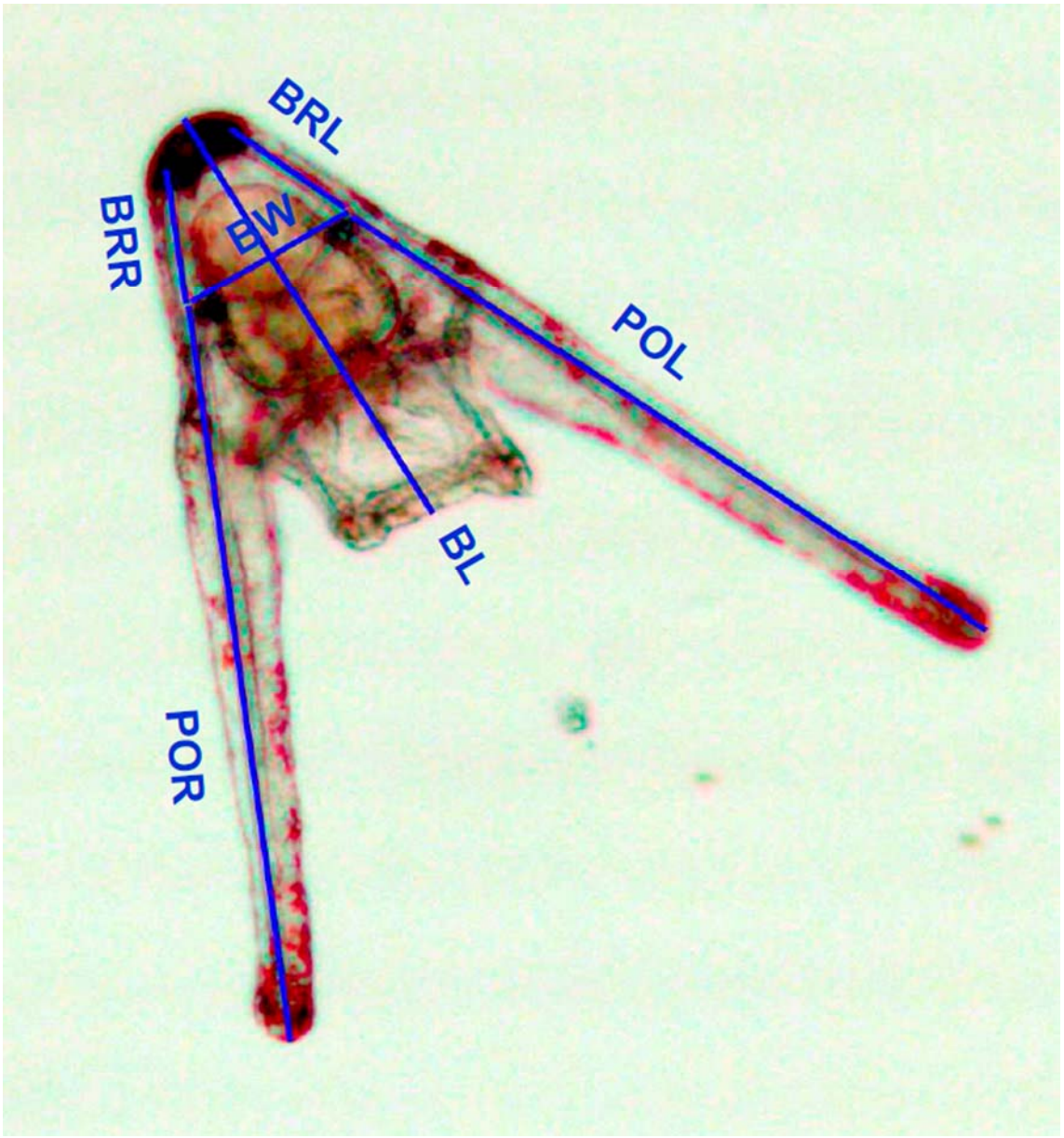
667

Table 5

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

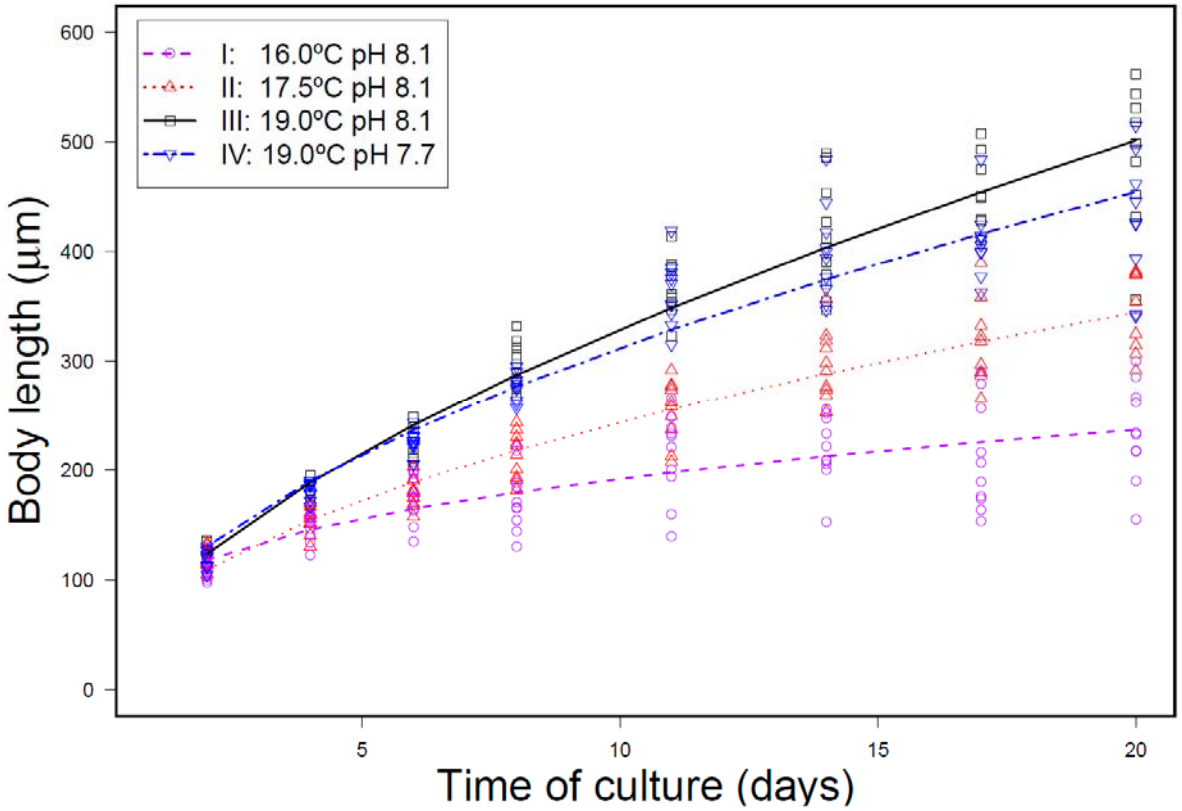
a. MTL ~ BL+ T + Replicate(T)				**b. MTL ~ BL+ pH + Replicate(pH)**			
Source	**d.f.**	***F***	***P***	**Source**	**d.f.**	***F***	***P***
BL	1	1951.94	< 0.0001	BL	1	779.96	< 0.0001
T	2	1.34	0.26	pH	1	1.19	0.28
BL x T	2	3.90	0.03	BL x pH	1	0.12	0.73
Replicate(T)	3	2.24	0.09	Replicate(pH)	2	1.29	0.28
Residuals	230			Residuals	153		
c. MBR ~ BL+ T + Replicate(T)				**d. MBR ~ BL+ pH + Replicate(pH)**			
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		
e. BW ~ BL+ T + Replicate(T)				**f. BW ~ BL+ pH + Replicate(pH)**			
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		
g. ASY ~ BL+ T + Replicate(T)				**h. ASY ~ BL+ pH + Replicate(pH)**			
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		

674 **Fig. 1.**
675



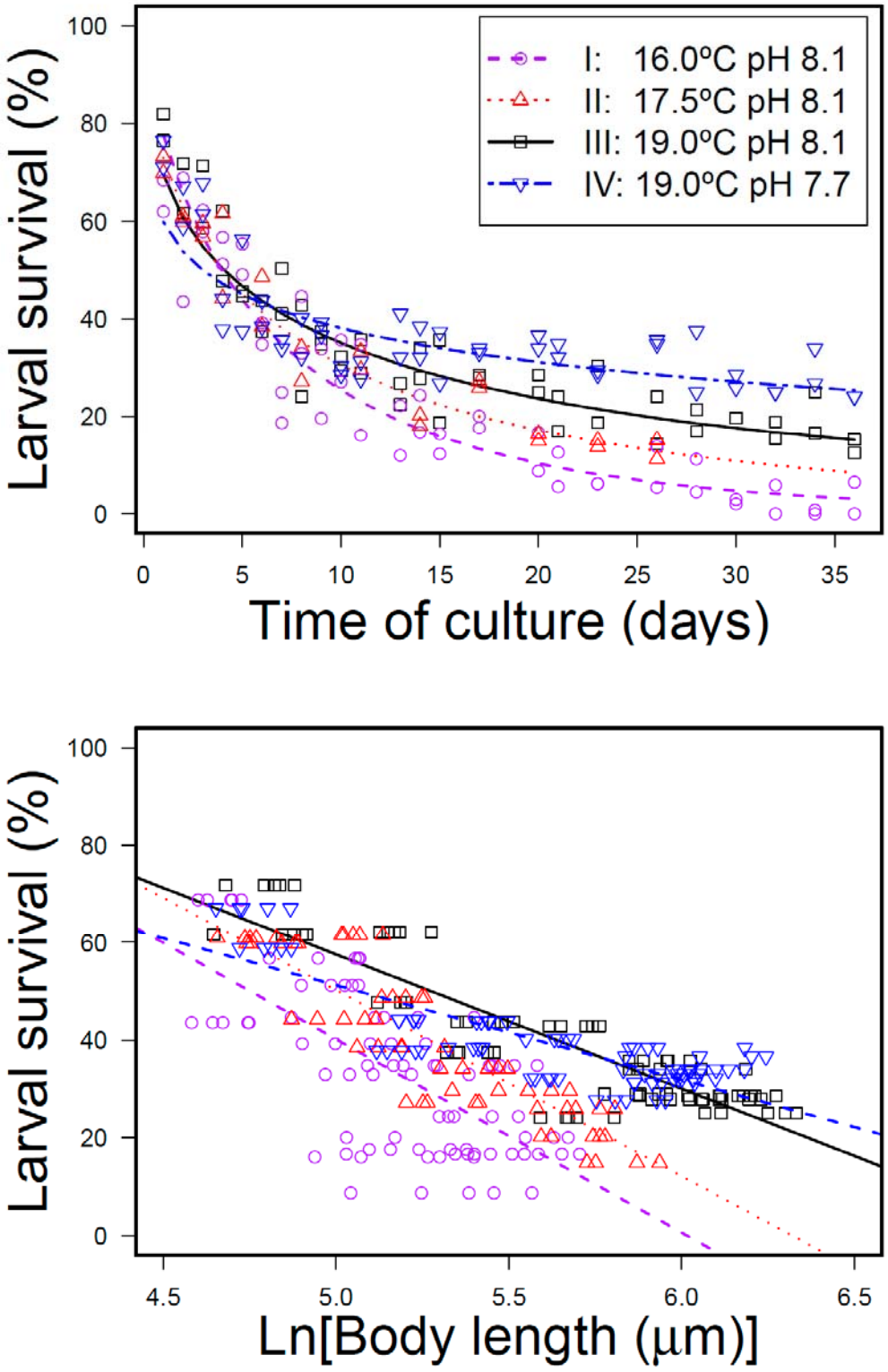
676

677 **Fig. 2.**


I: 16.0ºC pH 8.1
II: 17.5ºC pH 8.1
III: 19.0ºC pH 8.1
IV: 19.0ºC pH 7.7
Body length (µm)
0
100
200
300
400
500
600
5
10
15
20
Time of culture (days)

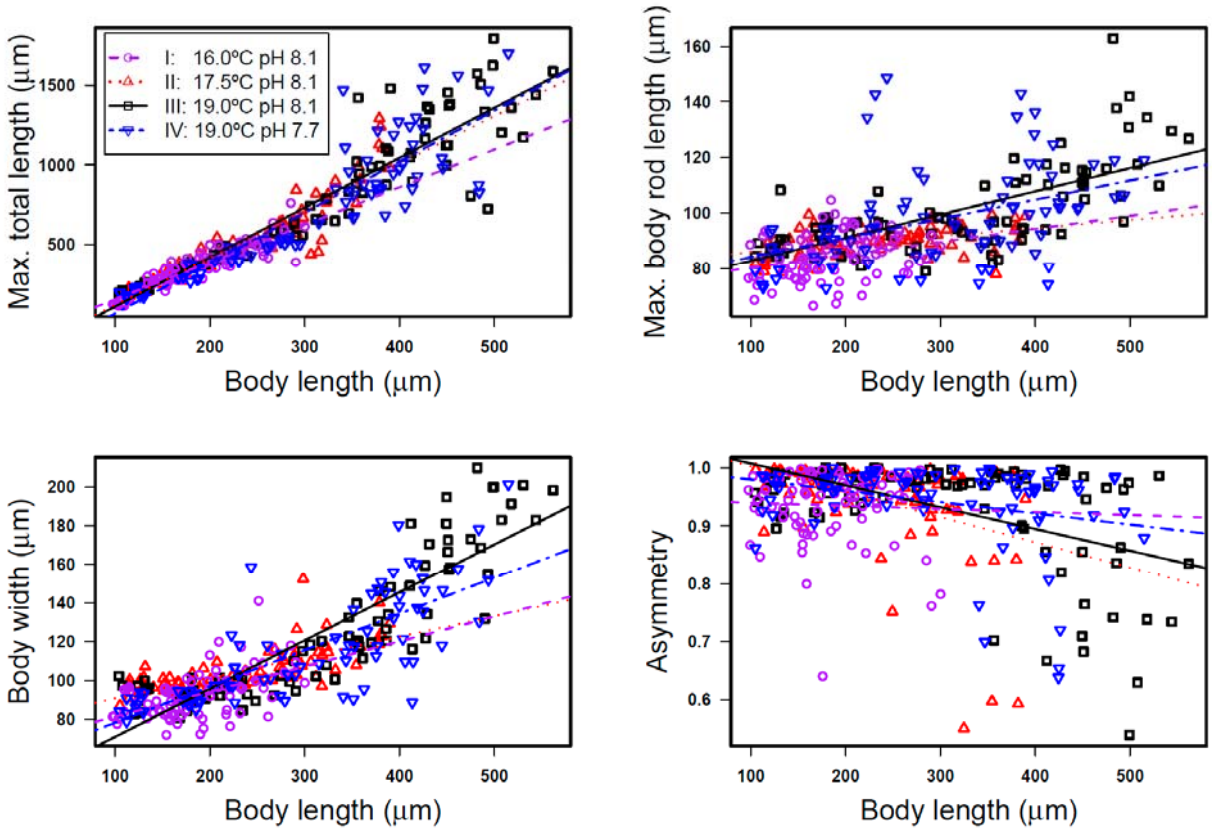
678

679 **Fig. 3.**
680



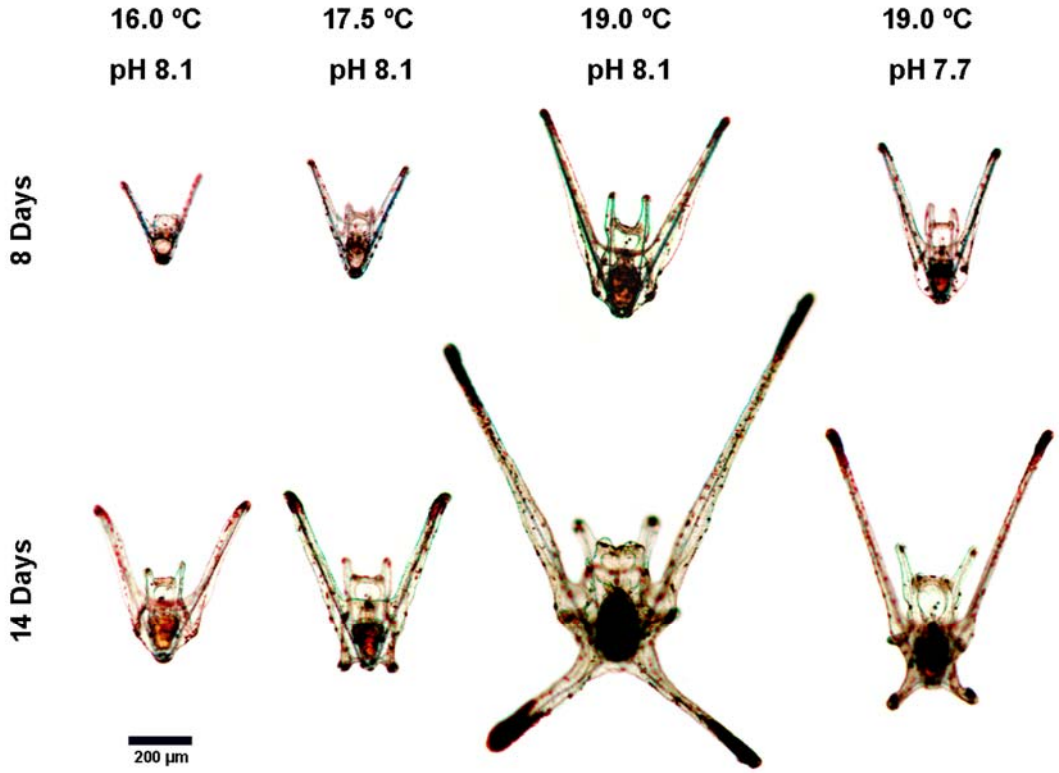
681

682 **Fig. 4.**
683



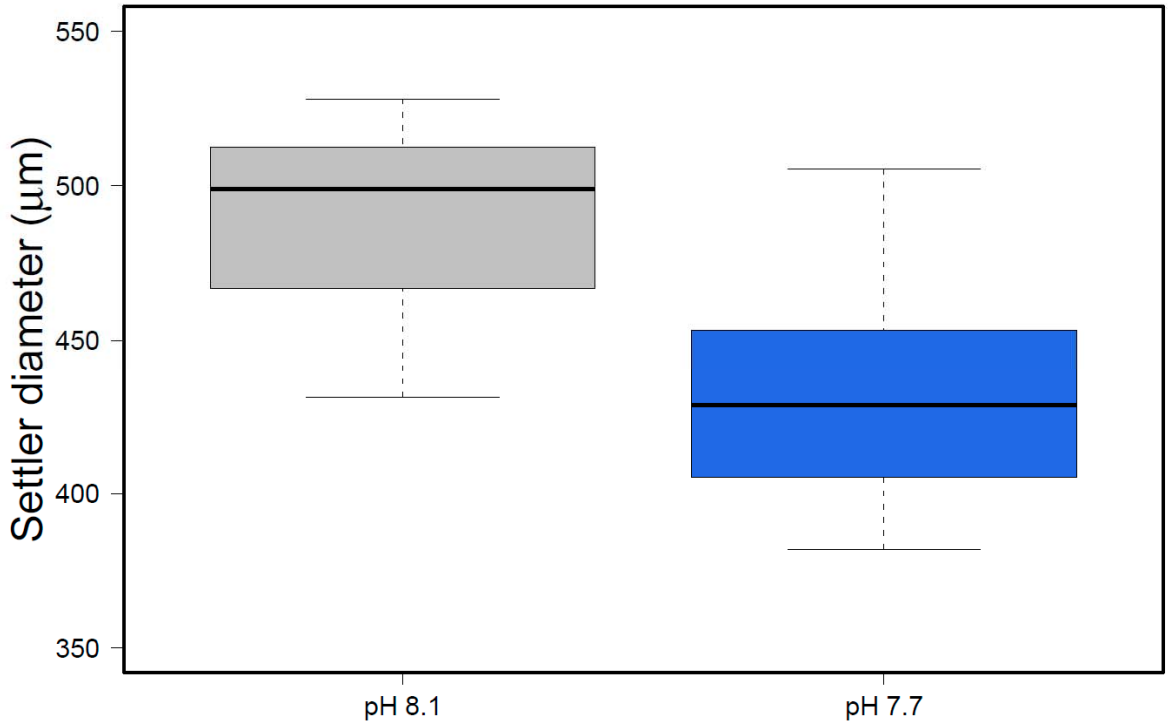
684

685 **Fig. 5.**
686



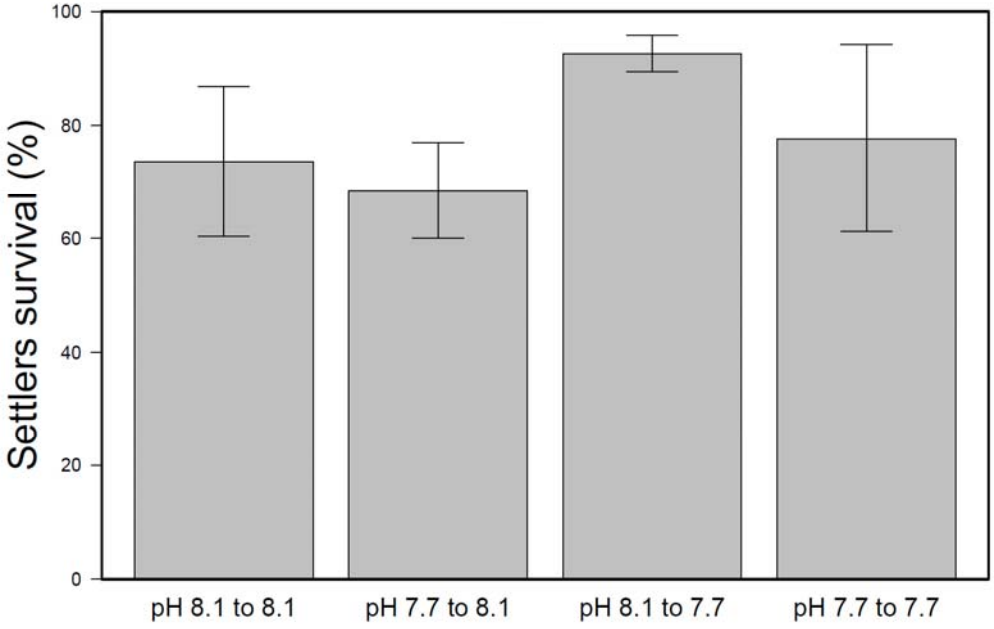
687

688 **Fig. 6.**
689



690

691 **Fig. 7.**


Settlers survival (%)
100
80
60
40
20
0
pH 8.1 to 8.1
pH 7.7 to 8.1
pH 8.1 to 7.7
pH 7.7 to 7.7

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