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 °C) were tested at present pH conditions (pH_T 8.1). At 19 °C, 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 CO₂ 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 °C over the last 30 years (Lejeusne et al., 2010 and references therein) and a further 2 °C 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).
Only a few previous works have studied the combined effects of increased temperature and ocean acidification on echinoderm larvae (Sheppard Brennand et al., 2010; Ericson et al., 2012; Foo et al., 2012; Nguyen et al., 2012; Padilla-Gamiño et al., 2013; Gianguzza et al., 2013) and all of them were limited to the first stages of early endotrophic development (2 to 3 days exposure). From this limited dataset, it appears that interaction between temperature and ocean acidification is complex, from temperature being the main driver of change to temperature amplifying or diminishing the negative effects of ocean acidification. Gianguzza et al. (2013) showed that temperature and pH had no significant effect on fertilization and larval survival (2 days) of *Arbacia lixula* for temperatures <27°C. However, both temperature and pH had effects on the developmental dynamics. Temperature appeared to modulate the impact of decreasing pH on the % of larvae reaching the pluteus stage, leading to a positive effect (faster growth compared to pH 8.2) of low pH at 20°C, a neutral effect at 24°C and a negative effect (slower growth) at 26°C.

The black sea urchin *Arbacia lixula* (Linnaeus, 1758) is currently one of the most abundant sea urchins in the Mediterranean (Benedetti-Cecchi et al., 1998; Palacín et al., 1998; Hereu et al., 2012) and tropical Eastern Atlantic (Hernández et al., 2013). It is recognized as a thermophilous species of tropical affinities (Stefanini, 1911; Mortensen, 1935; Tortonese, 1965) which probably spread through the Mediterranean in the Upper Pleistocene (Wangensteen et al., 2012) where it lives in suboptimal temperature conditions. Thus, it is a candidate species to be favoured by increased temperatures due to global change. *A. lixula* is an omnivore tending to carnivory (Wangensteen et al., 2011) which has a high potential to impact shallow rocky areas by originating or maintaining barren zones (Guidetti et al., 2003; Bonaviri et al., 2011). 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, 41°43’16” N, 2°56’24” 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.
2.2. *In vitro* fecundation and larval cultures

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

In the northwestern Mediterranean, the planktotrophic *A. lixula* larvae may be found in the water column between June and November and can be exposed to a wide range of temperatures (15 to 24°C; Fenaux, 1968; Pedrotti, 1993). Nevertheless, Pedrotti’s (1993) results suggest that the highest planktonic concentrations occur in October-November, when the temperature ranges from 16 to 19 °C. We compared four different scenarios: (i) Treatment I (16 °C, pH⟨T⟩ 8.1), corresponding to the lower range of the present temperature variability; (ii) Treatment II (17.5 °C, pH⟨T⟩ 8.1), an intermediate temperature; (iii) Treatment III (19 °C, pH⟨T⟩ 8.1), corresponding to the higher range of temperature presently experienced by the autumnal larvae; (iv) Treatment IV (19 °C, pH⟨T⟩ 7.7), corresponding to near-future ocean acidification scenario. Two replicates were 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 °C 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 (Nᵣ, number of larvae L⁻¹) was estimated as the mean of this four measures. Daily survival (SUR) was calculated as: SUR = (Nᵣ/N₀)*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 °C, both at pHT 8.1 and pHT 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 pHT 8.1 or 7.7, transferred to plastic plates with 3-mL wells and kept in FSW at 19 °C and pHT 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 pHT were measured twice a week. A_T was determined on filtered samples with a titration system (TitroLine alpha plus, SI Analytics). pHT (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 (pCO2, ΩCa and ΩAr) 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
Millero (1987). pH was maintained in each experimental bottle using a computerized feedback system (AquaMedic) that regulated pH by addition of pure gaseous CO₂ directly into the seawater (±0.02 pH units).

2.6. Statistical analyses

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

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

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

The survival curves for the larvae were considered to be derived from a hazard function following a 2-parameter Weibull distribution (Cox and Oakes, 1984). Thus, the
ratio of surviving larvae (SUR) at a given TOC, is given by \( \text{SUR} = \exp(-\lambda \cdot \text{TOC}^\beta) \), where \( \lambda \) is the scale parameter and \( \beta \) is the shape parameter. We calculated both parameters separately for every replicate using non-linear least-squares regressions (Bates and Watts, 1988), and pooled the replicates for each treatment, after verifying the absence of significant differences.

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

3. Results

3.1. Physicochemical variables

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

3.2. Larval growth and survival

The variation over time of larval size at different temperatures and pH is displayed in Fig. 2 and the ANCOVAs are listed in Table 2. No significant differences between replicates were found for any variable throughout all analyses, so replicates
have been pooled for clarity in the graphical representations. The larval size, measured as body length (BL) grew significantly faster with increasing temperatures (treatments I, II and III, Table 2a). The effect of a pH decrease from 8.1 to 7.7 at 19 ºC produced no appreciable difference in BL during the first eight days of culture, but originated significantly smaller larvae from then on (treatments III and IV, Table 2b).

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

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

3.3. Larval morphology

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 °C 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 °C, but was significantly different at 19 °C, 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 °C 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 °C 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 °C. 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.
3.4. Settlers count, size and survival

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

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

4. Discussion

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

Nevertheless, our results show that *A. lixula* larvae can be cultured and complete their development at temperatures between 16 and 19 °C, though the survival curve showed quite elevated mortality rates, especially during the first days of culture. The advantage of using Weibull distributions to describe the survival curve is their...
flexibility for modelling both increasing and decreasing hazard functions, depending on the value of the shape parameter $\beta$. All values obtained for $\beta$ in our study were smaller than 1 (Table 3), implying that the hazard functions decreased over time; that is, in the conditions of our experiments, the larval mortality was higher during the first days of the development and it diminished over time. Also, the parameter $\beta$ showed a clear trend to decrease with warming (Table 3), which suggests that the mortality remained more constant over time at low temperatures.

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

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

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

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

Another recent work studied the culture and settlement of *A. lixula*. Privitera et
al. (2011) reported that larvae from Genoa populations cultured at 18 °C suffered 100%
mortality at 7 days, while the same larvae reared at 22 °C survived and reached the
competent stage at approximately 20 days. Our results show that *A. lixula* larvae from
northwestern Mediterranean are indeed able to develop at lower temperatures, down to
16 °C, 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 °C at two different pH values. They reported an interesting interaction between pH and temperature. Thus, slightly acidic pH accelerated growth at 20 °C, while it has a neutral effect at 24 °C and a negative effect at 26 °C. Our results showing enhanced survival rates using pH 7.7 at 19 °C are in accordance with a positive effect of slight acidification for *A. lixula* at temperatures around 20 °C, 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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References


**Legend to figures**

**Fig. 1.** Measured distances for the morphological study of *Arbacia lixula* pluteus larvae. BL: body length. BW: Body width, BRL & BRR: Body rods lengths (left and right); POL & POR: Post-oral rods lengths (left and right).

**Fig. 2.** Effect of temperature and pH on individual growth (body length) of *Arbacia lixula* larvae. Since no differences were found between replicate cultures, replicates have been pooled for clarity.

**Fig. 3.** Survival curves for *Arbacia lixula* larvae cultured at different temperatures and pH in function of time of culture (A) or body length (B). The interpolation curves in A were calculated assuming hazard functions following a Weibull distribution. Since no differences were found between replicate cultures, replicates have been pooled for clarity.

**Fig. 4.** Maximum total length (A), maximum body rod length (B), body width (C) and asymmetry index (D) plotted against body length of *Arbacia lixula* larvae grown at different conditions of temperature and pH. Since no differences were found between replicate cultures, replicates have been pooled for clarity.

**Fig. 5.** Typical morphology and size of *Arbacia lixula* larvae grown under different conditions, after eight (upper row) or fourteen (lower row) days of culture. The four treatments tested are shown.
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.
Table 1

Physicochemical variables measured in the four experimental treatments (mean ± SD). Partial pressure of carbon dioxide (pCO₂), total dissolved inorganic carbon (C_T) and calcium carbonate saturation state for calcite and aragonite (Ω_Ca, Ω_Ar) were calculated from temperature, pH_T and total alkalinity (A_T).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T (°C)</th>
<th>pH_T</th>
<th>A_T (μmol/kg)</th>
<th>pCO₂ (μatm)</th>
<th>C_T (μmol/kg)</th>
<th>Ω_Ca</th>
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<tbody>
<tr>
<td>I. 16 °C pH 8.1</td>
<td>16.3±0.4</td>
<td>8.09±0.05</td>
<td>2638±39</td>
<td>547±79</td>
<td>2403±56</td>
<td>4.13±0.38</td>
<td>2.67±0.24</td>
</tr>
<tr>
<td>II. 17.5 °C pH 8.1</td>
<td>17.5±0.3</td>
<td>8.08±0.03</td>
<td>2637±78</td>
<td>548±53</td>
<td>2384±77</td>
<td>4.45±0.29</td>
<td>2.88±0.19</td>
</tr>
<tr>
<td>III. 19 °C pH 8.1</td>
<td>18.8±0.3</td>
<td>8.09±0.04</td>
<td>2630±44</td>
<td>548±60</td>
<td>2379±53</td>
<td>4.42±0.27</td>
<td>2.87±0.17</td>
</tr>
<tr>
<td>IV. 19 °C pH 7.7</td>
<td>18.8±0.3</td>
<td>7.69±0.04</td>
<td>2658±61</td>
<td>1575±153</td>
<td>2590±56</td>
<td>1.95±0.15</td>
<td>1.27±0.10</td>
</tr>
</tbody>
</table>
Table 2

Analysis of covariance testing the effects of temperature (a) and pH (b) on *Arbacia lixula* larval growth. BL: body length, TOC: time of culture, T: temperature.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>( F )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN(TOC)</td>
<td>1</td>
<td>1219.23</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>299.62</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LN(TOC) x T</td>
<td>2</td>
<td>115.90</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Replicate(T)</td>
<td>3</td>
<td>0.06</td>
<td>0.98</td>
</tr>
<tr>
<td>Residuals</td>
<td>230</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>( F )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN(TOC)</td>
<td>1</td>
<td>1475.94</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>9.17</td>
<td>0.0031</td>
</tr>
<tr>
<td>LN(TOC) x pH</td>
<td>1</td>
<td>6.68</td>
<td>0.011</td>
</tr>
<tr>
<td>Replicate(pH)</td>
<td>2</td>
<td>2.00</td>
<td>0.14</td>
</tr>
<tr>
<td>Residuals</td>
<td>153</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Analysis of covariance for *Arbacia lixula* larvae survival data. SUR: Survival rate, TOC: Time of culture, T: Temperature, BL: Body length.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. SUR ~ Ln(TOC) + T + Ln(TOC) x T + Replicate(T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln(TOC)</td>
<td>1</td>
<td>849.33</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>20.71</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Ln(TOC) x T</td>
<td>2</td>
<td>1.11</td>
<td>0.33</td>
</tr>
<tr>
<td>Replicate(T)</td>
<td>3</td>
<td>0.93</td>
<td>0.43</td>
</tr>
<tr>
<td>Residuals</td>
<td>102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. SUR ~ Ln(TOC) + pH + Ln(TOC) x pH + Replicate(pH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln(TOC)</td>
<td>1</td>
<td>420.62</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>4.69</td>
<td>0.033</td>
</tr>
<tr>
<td>Ln(TOC) x pH</td>
<td>1</td>
<td>15.98</td>
<td>0.00014</td>
</tr>
<tr>
<td>Replicate(pH)</td>
<td>2</td>
<td>1.16</td>
<td>0.32</td>
</tr>
<tr>
<td>Residuals</td>
<td>82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. SUR vs Ln(BL) + T + Ln(BL) x T + Replicate(T)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln(BL)</td>
<td>1</td>
<td>283.90</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T</td>
<td>2</td>
<td>192.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Ln(BL) x T</td>
<td>2</td>
<td>13.70</td>
<td>0.0003</td>
</tr>
<tr>
<td>Replicate(T)</td>
<td>3</td>
<td>0.52</td>
<td>0.47</td>
</tr>
<tr>
<td>Residuals</td>
<td>230</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. SUR vs Ln(BL) + pH + Ln(BL) x pH + Replicate(pH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln(BL)</td>
<td>1</td>
<td>490.20</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>pH</td>
<td>1</td>
<td>1.37</td>
<td>0.24</td>
</tr>
<tr>
<td>Ln(BL) x pH</td>
<td>1</td>
<td>14.45</td>
<td>0.0002</td>
</tr>
<tr>
<td>Replicate(pH)</td>
<td>2</td>
<td>2.17</td>
<td>0.14</td>
</tr>
<tr>
<td>Residuals</td>
<td>153</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\lambda$ (day$^{-\beta}$)</th>
<th>$\beta$</th>
<th>SSR</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: 16.0°C pH 8.1</td>
<td>0.304 ± 0.034</td>
<td>0.642 ± 0.050</td>
<td>0.223</td>
<td>0.87</td>
</tr>
<tr>
<td>II: 17.5°C pH 8.1</td>
<td>0.313 ± 0.025</td>
<td>0.572 ± 0.035</td>
<td>0.050</td>
<td>0.95</td>
</tr>
<tr>
<td>III: 19.0°C pH 8.1</td>
<td>0.301 ± 0.026</td>
<td>0.531 ± 0.035</td>
<td>0.149</td>
<td>0.89</td>
</tr>
<tr>
<td>IV: 19.0°C pH 7.7</td>
<td>0.434 ± 0.039</td>
<td>0.338 ± 0.035</td>
<td>0.200</td>
<td>0.72</td>
</tr>
</tbody>
</table>
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.

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

The figure shows the relationship between body length (µm) and time of culture (days) at different temperatures and pH levels:

- I: 16.0°C pH 8.1
- II: 17.5°C pH 8.1
- III: 19.0°C pH 8.1
- IV: 19.0°C pH 7.7

The graph displays a clear trend of increasing body length over time for each condition, indicating a positive correlation between culture time and body length at these specific conditions.
Fig. 3.

Larval survival (%) vs. Time of culture (days)

Larval survival (%) vs. Ln[Body length (μm)]
Fig. 4.
Fig. 5.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>pH</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.0 °C</td>
<td>8.1</td>
<td>8</td>
</tr>
<tr>
<td>17.5 °C</td>
<td>8.1</td>
<td>8</td>
</tr>
<tr>
<td>19.0 °C</td>
<td>8.1</td>
<td>14</td>
</tr>
<tr>
<td>19.0 °C</td>
<td>7.7</td>
<td>14</td>
</tr>
</tbody>
</table>

200 μm
Fig. 6.

A box plot showing the distribution of settler diameter (μm) at different pH levels. The plot compares pH 8.1 and pH 7.7.
Fig. 7.

![Graph showing settler survival (%) for different pH ranges. The y-axis is labeled as "Settlers survival (%)" and the x-axis shows different pH ranges: pH 8.1 to 8.1, pH 7.7 to 8.1, pH 8.1 to 7.7, pH 7.7 to 7.7. The bars indicate the mean survival with error bars showing variability.]