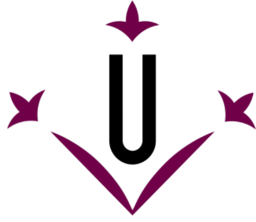

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# Warming and acidification-mediated survival to bacterial infection determine mortality and size patterns of early *Ostrea edulis* life stages

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ABSTRACT: The combined effects of temperature and seawater acidification were investigated across larval stages of the European flat oyster, *Ostrea edulis*, from veliger sizes released by gravid individuals to spat. Simultaneous experiments were also conducted to investigate the potential effects of reduced pH levels on bacterial growth that could provide a more comprehensive understanding on the effects of seawater acidification in larval mortality. Larvae (veliger, umbonate, and pediveliger) and spat were exposed to four temperature (18, 22, 26, and 30 °C) and four pH treatments (7.83-7.92 (ambient), low-reduced, medium-reduced, and high-reduced), with the latter conditions also used in bacterial experiments. Results showed increased larval mortalities at 30 °C (by *ca.* 49, and 44 % in veliger, and umbonate stages, respectively), although there was also a bottleneck in pediveligers from 22-30 °C and no effects on spat. In contrast, the survival of veligers increased with pH reductions by *ca.* 26 %, and was marginally increased in pediveligers in spite of large mortality at this stage. No shell malformations were observed at any larval stage or in spat and growth patterns tended to mirror those of survival. This coincided with lower bacterial growth, particularly for *Vibrio* spp., in the two lowest pH treatments, suggesting that seawater acidification may help to prevent pathogenicity in *O. edulis* larvae. Compared to available information on the vulnerability of other commercial bivalves to ocean acidification, our results suggest that this could be a more resilient species although further research is needed to investigate potential effects on gravid females and sperm.

KEYWORDS: Climate change · calcification · $CO_2$ · ocean acidification   bacterial growth · oyster larvae · Mediterranean

47 **INTRODUCTION**

48 Atmospheric $CO_2$ concentration [$CO_2$] has augmented from a pre-industrial level of
49 approximately 280 ppm to approximately 385 ppm, with further increases (700–1000 ppm)
50 anticipated by the end of the twenty-first century (IPCC 2013). Greenhouse effects of [$CO_2$] and
51 other greenhouse gases have triggered increased global average temperatures by *ca.* 0.2 °C
52 per decade over the past 30 years, with most of this added energy being absorbed by the
53 world's oceans (Hansen et al. 2006). In addition to the effects of temperature, marine
54 ecosystems may be particularly sensitive to increasing [$CO_2$] due to its rapid dissolution into
55 seawater causing alterations in the chemistry of inorganic carbon that reduce pH and the
56 calcium carbonate ($CaCO_3$) saturation (Doney et al. 2009). Effects of temperature rise and
57 ocean acidification may include a number of biological alterations such as decreased ocean
58 productivity (Behrenfeld et al. 2006), reduced growth and survival of calcifying organisms
59 (Hoegh-Guldberg et al. 2007), changes in species distributions, altered food web dynamics
60 (Vergés et al. 2014), and altered incidence of disease (Burge et al. 2014). Recent studies have
61 shown, however, that the importance of some of these effects may be considerably variable
62 across species and ontogenetic stages (Kurihara 2008, Hendriks et al. 2010), and emphasizes
63 the need of identifying which of them are the most resilient and the mechanisms involved.

64 To understand the potential effects of global change it is important to focus on the
65 population's bottlenecks which are usually early developmental and reproductive stages that
66 are developing their physiological capacities and have more specific environmental
67 requirements (Thorson 1950). Survival of adult and juvenile bivalves has been indicated a low
68 dependence on $pCO_2$ (Berge et al. 2006, Hendriks et al. 2010, Range et al. 2012), although
69 increasing temperature may also result on increasing mortality and metabolic rates (Basso et
70 al. 2015). In contrast, gametes, embryos, and larvae are generally more sensitive to both
71 temperature and elevated $pCO_2$ stress (Havenhand et al. 2008, Parker et al. 2009, 2010)
72 because they start the deposition of calcium carbonate ($CaCO_3$) shells and skeletons (Kurihara

73 et al. 2007). Yet, there is also a wide natural variability in pH ranges of seawater (7.5 to 8.5,
74 with even lower values being possible in semi-confined waters; Sverdrup et al. 1942), which
75 may partly account for observed differences in the responses of calcifying organisms to
76 acidification and complicates the generalization of patterns across species an ecosystems (see
77 review by Kurihara 2008). For instance, exposure to combined high temperature and $CO_2$ in
78 two species of oysters resulted on declined fertilization success, lower development of
79 embryos, and larval size, as well as an increase incidence of abnormal morphology, but
80 *Saccostrea glomerata* showed higher sensitivity compared to *Crassostrea gigas* (Parker et al.
81 2010). Similarly, Talmage and Gober (2011) found that increased temperature and $CO_2$,
82 negatively impact both juvenile and larval stages of different bivalve species, but *Mercenaria*
83 *mercenaria*, and *Argopecten irradians* showed stronger effects than *C. virginica*. In contrast,
84 the fertilization success in a suite of coastal marine invertebrates from SE Australia, including a
85 species of abalone (*Haliotis coccoradiata*), showed to be robust against near-future ocean
86 warming and acidification (Byrne et al. 2010).
87 Mortality of marine invertebrates has been indicated to exceed 90 % during early life stages
88 in their natural habitat and to be often mediated by infection and disease of larval stages
89 (Gillard 1959, Gosselin & Qian 1997, His et al. 1999). However, little is known on how global
90 change may alter bacterial abundance and disease on early developmental stages and the
91 overall response of the population. For instance, the reemergence of *Vibrio tubiashii*, a
92 bacterial pathogen of larval Pacific oysters (*C. gigas*), was linked to thermal shifts and changes
93 in seawater pH (Elston et al. 2008). Given that most bacteria have an optimal pH range that
94 maximizes colonial growth and determines community structure (Russell et al. 1979, Lauber et
95 al. 2009), deviations from this optimal range might result on variable rates of infection that
96 enhance or decrease the mortality of early life stages.
97 Currently, the most important bivalve aquacultural activities in the Mediterranean
98 (including the study region in the Ebro Delta) are those of the Mediterranean mussel (*Mytilus*

99 *galloprovincialis*), and the Pacific oyster (*C. gigas*) which appear to be vulnerable to global
100 change (Kurihara et al. 2008, Parker et al. 2010, Barros et al. 2013). The once important
101 production of *O. edulis* was drastically reduced during the 1970s and the 1980s due to
102 protozoan disease (Naciri-Graven et al. 1998), but little is known on its resilience capacity to
103 future global change scenarios, which could favor again its cultivation over more sensitive
104 species. In addition, studies aimed at investigating how seawater acidification interacts with
105 current outbreaks of *Vibrio parahaemolyticus* (Roque et al. 2009) and with the bulk bacterial
106 community are also necessary to understand mortality patterns and to secure the continuity of
107 commercial activities and the recruitment of populations.

108 In this context, the aims of this study were: (1) to investigate the effects of seawater
109 acidification (through elevated $pCO_2$) and temperature across larval (veliger, umbonate, and
110 pediveliger) and early benthic stages (spat) of the European oyster, *O. edulis*, after they were
111 released into the media (*ca.* 9 days; > 100 µm); and (2) to determine how global change
112 scenarios will affect the bacterial community associated to the intervalvar liquid of adult *O.*
113 *edulis* and the overall growth and survival of populations. We hypothesize that larvae released
114 into the media after early development may attain certain protection in size to variations in
115 seawater $pCO_2$, and that potential deleterious effects will decrease as larvae age. In regards of
116 the bacterial community, changes in the abundance (as a proxy of infection rates; see Lemire
117 et al. 2014) are expected, possibly depending on the optimal pH ranges for colonial growth for
118 each species.

119

120 **MATERIALS AND METHODS**

121 **Study species**

122 Gravid individuals of *O. edulis* were bought from an external aquaculture supplier
123 (Promociones Marsan S.L.) in Santa Pola, Alicante in early April 2014. Individuals were
124 maintained at 16 ºC with filtered seawater (1µm) renovated every 2 h, and were fed with a mix
125 of *Isochrysis aff. galbana*, *Tetraselmis chuii* and *Chaetoceros gracilis*. In species of brooding
126 bivalves such as *O. edulis*, internal fertilization usually prevents morphogenetic evaluation of
127 larval stages prior to their release to the external media at *ca.* 9 days age (shell size ranging
128 from *ca.* 130 to 160 µm). Hence, the effect of pH and temperature on the survival rate, and
129 size (shell length and width) of *O.edulis* was determined for the three other major free-living
130 oyster stages including late veliger, and early stages of umbonate larvae, pediveliger larvae,
131 and spat.
132
133 **Experimental treatments**

134 Two natural (18 and 22 °C) spring temperatures common during spawning and larval
135 periods (Carlucci et al. 2010) and two elevated temperatures (26 and 30 °C) usually occurring
136 at the Alfacs Bay (Ebro Delta, NW Mediterranean) during the summer period, were selected.
137 For each temperature, one ambient pH treatment and three lower pH concentrations were
138 considered to study the effects of pH reductions in *O. edulis* larval stages and spat. The
139 ambient pH concentration at the Alfacs Bay during the study period was found to range from
140 7.83 to 7.92, and based on this initial pH, reductions were set at *initial* pH concentrations of
141 7.6, 7.4, and 7.2.

142 Filtered seawater (sand and ozone filters) from the Alfacs Bay (salinity of 35) was available
143 at the IRTA-Sant Carles de la Ràpita bivalve hatchery through a water uptake system. The
144 seawater pH in the study area is subjected to strong temporal variability, with values ranging
145 from 7.8-7.9 up to 8.3 depending on phytoplankton blooms and freshwater supply into the bay

146 (Dr. M. Fernandez, personal communication). In particular, important freshwater discharges
147 occur from April to November associated to rice agriculture in the Ebro Delta (Garcés et al.
148 1999), which may have accounted for the lower pH values during the study.

149 The two highest temperature treatments (26 and 30 °C) were obtained by heating 15 L
150 tanks (one per temperature treatment) with aquarium thermostat heaters, whereas the two
151 lowest temperatures were obtained by using two aquarium chillers equipped with thermostat
152 control units (also one per temperature treatment). Ambient pH values of the bay were
153 consistently around 7.9 from early April (veliger experiment) to late July 2014 (spat
154 experiment) whereas temperature varied from 22 to 27 ºC and the total alkalinity (TA) from
155 2715 to 2775 µM $kg^{-1}$. The three lower pH concentrations used during the study were obtained
156 via manipulation of the ambient pH by direct bubbling of pure $CO_2$ into seawater (see Parker et
157 al. 2010). This was done by injecting $CO_2$ with a 20 mL syringe (connected to an aquarium
158 diffuser through a plastic tube in order to maximize $CO_2$ homogenization) into a 1 L container
159 with ambient seawater until the desired value which was then used to fill 120 mL sealed
160 containers with 110 mL of pH treated seawater. Experimental trials were first conducted to
161 assess deviations from *initial* pH levels (ambient, 7.6, 7.4, and 7.2) within the 120 mL sealed
162 containers without larvae at two temperatures (18 and 26 °C) 24 and 48 h after $CO_2$ injection
163 and showed non-significant variations ($<\pm0.1$ pH units; Student's *t* test, $P > 0.05$ for both 24h
164 and 48h replicates). Latter, the same procedure was used to manipulate pH conditions of
165 seawater within replicate containers with larvae ($N = 3$ for each temperature and pH
166 treatment) at each of the four experimental temperatures. pH values were always measured
167 initially and previously to water change two days later in order to account for possible
168 variations (mainly due to respiratory processes and temperature to a lesser extent), and the
169 average of the two initial and two final values used for $pCO_2$ calculations (see later). The
170 variability between initial and final values 48 h later (usually < 0.2 pH units in all treatments;
171 see Table 1) was used to define non-overlapping ranges of reduced pH (high-reduced,

172 medium-reduced, and high-reduced) to which individuals were exposed during each 4-day
173 experiment. In all instances, the glass pH electrode used for pH readings (Eutech Instruments)
174 was calibrated daily using NBS buffers.
175
176 **Effect of pH and temperature on the size of larvae and spat**

177 Larvae were maintained in the hatchery from veliger to the spat stages in order to ensure
178 rapid availability for experimental use. Larvae were maintained in a 500-L fiberglass tank at a
179 concentration of 2-5 larvae $mL^{-1}$, depending on larval stage. The tank was changed every 2
180 days and were thoroughly washed with bleach, rinsed with freshwater and then, filled again
181 with new water and the larvae (previously retained with appropriate mesh sizes that excluded
182 death vales of smaller size). The feed rates, stocking densities, and screen size used for
183 experimental larval selection (60 to 180 µm) and water changes were continually adjusted to
184 suit size and stage of development (see below). Larvae were collected for experimental
185 treatments at the beginning of each stage, except for the veliger, which was about 9 days' age.
186 Once larvae reached the pediveliger stage, they were transferred into a new tank containing
187 fine shell fragments in order to allow settlement and development of spat. Newly
188 metamorphosed spat (2–4 weeks after metamorphosis) were collected and used in
189 experimental treatments.

190 Larvae were transferred into each container treatment of pH and temperature at a
191 concentration of 5 larvae $mL^{-1}$ for the veliger stage, 3 larvae $mL^{-1}$ for the umbonate stage, and 2
192 larvae $mL^{-1}$ for pediveligers, following stocking densities in the mother tank. For spat, 15
193 individuals were transferred into each container as in Parker et al. (2010). Diets within
194 containers (and in the mother tank) consisted on *I. aff. galbana* during the first five days of
195 culture (which included the veliger experiment), and thereafter on a mix of *I. aff. galbana* and
196 *C. gracilis* or *C. calcitrans*, depending on availability (in a proportion of 60:40 and *ca.* 75-
197 $100 \cdot 10^3$ algal cells per mL every two days). Individuals within containers were fed at the

198 beginning of the experiment and after water changes 2 days later, in order to minimize larval
199 mortality due to disturbance and preserve experimental conditions. After 4 days, containers
200 were opened and 3 mL of neutral red colorant added in order to facilitate discrimination
201 between and living (*i.e*. filtering the colorant) and death individuals under the microscope
202 (Crippen & Perrier 1974, Jessopp 2007). After a few minutes, experiments were ended by the
203 addition of 10 mL of 5 % buffered formalin. Later, the survival rates (%) within each replicate
204 container was estimated by concentrating larvae or spat within a plate chamber and counting
205 live-dead occurrence under an inverted microscope (Leica DMI 3000 B), and stereomicroscope
206 (Leica M165 C), respectively. Lengths and width (µm) of the shell of 50 larvae (out of several
207 hundreds of individuals per container) and the 15 spat in each replicate container were
208 measured from random photographs using the Leica Application Suit program. In some
209 instances the number of living larvae was lower than 50, and the sample size was reduced.
210

211 **Effect of pH on bacterial growth**

212 This study was conducted after the larval experiments, in an attempt to account for some
213 unexpected effects of pH treatments in larval stages, whereas the influence of temperature
214 appeared to act at the physiological level as described in previous experiments with other
215 species (see the discussion section for further references). Hence, the potential effect of pH
216 reductions on the development of bacteria possibly introduced to the larval tank through the
217 water content within gills of breeding oysters and interfere with survival rates was investigated
218 for the same 4 treatment conditions than in larval stages. Preliminary samples (*N* = 3) were
219 collected from oyster gills by drilling a small hole in the shell and extracting the intervalvar
220 water with a syringe and kept within sterile ependorfs in order to confirm the presence of
221 bacteria. Simultaneously, ten adult oysters were kept within the same water volume than the
222 mother tank for larvae (see before) and water samples (*N* = 3) collected after 24 h collected
223 using sterile screw containers of 120 ml. Then, aliquots of the water with adult oysters were

224 used to inoculate containers with ambient and reduced pH treatments (*N* = 3 each) and
225 incubated at 23 °C during 48 h. Samples were immediately processed by serially diluting all
226 them (1:10) in sterile saline water at 2.5 % NaCl, and then duplicate 0.1 ml aliquots plated on
227 Marine Agar (Scharlau, Spain) for total heterotrophs and Thisulphate Citrate Bile Salt for *Vibrio*
228 spp. (Scharlau, Spain) and incubated for 48 h at room temperature (22 °C+/-1 °C). After 48 h,
229 colony forming units (CFU) were counted and expressed in terms of total heterotrophs and
230 *Vibrio* spp. per ml.
231
232 **Relationship between pH and $pCO_2$ levels**
233 To determine the $pCO_2$ levels corresponding to pH measures, the TA was also quantified for
234 each pH treatment with a Hanna Checker alkalinity meter HI 755 (±5 ppm accuracy at 25 °C).
235 Following the determination, ppm values were transformed to $\mu M\ kg^{-1}$, and then, TA and
236 selected pH values (averaged initial and final values; NSB scale) were entered into a $CO_2$
237 system calculation program developed by Lewis et al. (1998), using the dissociation constants
238 of Mehrbach et al. (1973) refit by Dickson and Millero (1987), the $KSO_4$ constant of Dickson
239 recommended by the program, and the Lee total boron formulation. To account for possible
240 temperature effects, input (initial temperature of ambient seawater) and output temperatures
241 (that of experimental treatments) were also entered. Resulting $pCO_2$values were compared to
242 future $pCO_2$ scenarios predicted in the 5$^{th}$ Assessment Report of the IPCC (Intergovernmental
243 Panel on Climate Change; IPCC 2013).
244
245 **Data analyses**
246 A preliminary GLMz Model averaging by an Information Theoretic approach was conducted
247 (see Ibáñez et al. 2012 for a methodological description) with larval stage and spat survival and
248 the 4 pH values measured during each experiment (i.e., two initial and the 2 final pH values) as
249 explanatory variables in order to assess the effect of different pH values recorded during the

250 experiment (continuous variables and factor). Although pH values at the end of the experiment
251 were significantly associated to survival rate (Selection Probability = 0.97), these are affected
252 by remaining individuals (e.g., living individuals kept respiring and excreting $CO_2$); thus initial
253 pH values were used in further analyses. Based on this result, differences in survival (%) of *O.*
254 *edulis* larval stages and spat due to the effects of pH and temperature were investigated with a
255 3-way ANOVA with pH (4 levels: Ambient, low-reduced, medium-reduced, and high-reduced),
256 T °C (18, 22, 26, and 30 °C), and Stage (veliger, Umbonate, Pediveliger, and Spat).
257 Differences in shell size (length and width) across treatments of pH and T °C were
258 investigated with a 2-way ANOVA (pH and T) for each larval stage and spat. Given the large
259 mortality recorded for the Pediveliger stage (up to 100 % in some treatments), comparisons
260 with other stages could have confounded the results of the ANOVA and separate analyses
261 were considered as the most suitable approach.
262 For bacteria, differences in the abundance colony forming units of total heterotrophs per
263 ml across pH treatments were investigated with a one-way ANOVA. For vibrio, however, given
264 the large ranges of variation within some of the treatments, significant patterns were
265 investigated with a Levene's test for all treatments followed by pair-wise comparisons. All the
266 quantitative variables were log-transformed for the analyses, because homoscedasticity and
267 linearity were clearly improved. All factors were considered fixed effect factors.
268 ANOVA assumptions of normality (Chi-squared test) and heteroscedasticity (Cochran's test)
269 of data were not always achieved by transformation. The ANOVA F-statistic is, however, still
270 able to provide robust results, particularly where data are balanced and the working sample
271 size is large enough (Underwood 1997). For balanced designs, heterogeneity of variances has
272 been indicated to increase the probability of making a Type I error (Underwood 1997), and in
273 those instances, the risk was minimized by setting the level of significance to 0.01. All data
274 analyses were performed with STATISTICA 8.
275

276 **RESULTS**

277 **Patterns of pH variability**

278 Control containers with ambient seawater conditions showed an average decline of 0.19
279 pH units compared to ambient seawater pH without larvae and in equilibrium with
280 atmospheric $CO_2$ (7.89 and 7.70, respectively). In addition, there was a significant effect of
281 temperature (ANOVA, $F_{3, 48} = 7$, $P < 0.001$) across predetermined pH treatments ($F_{3, 48} = 200$, $P <$
282 $0.001$), but no interaction effects were detected (Fig. 1). Overall, pH values tended to decrease
283 with temperature, and SNK showed significant differences between 18 °C and the remaining
284 temperature treatments.

285

286 **Survival rates**

287 Results for the 3-way ANOVA showed significant differences among larval stages (S), pH,
288 temperature (T), as well as significant S x pH and S x T interactions (Table 2). Survival rates of
289 larvae were highest for the umbonate stage (67.51 ± 3.35 %), intermediate for the veliger
290 stage (43.08 ± 4.1 %), and lowest for the pediveliger stage (5.53 ± 1.4%). At the benthic spat
291 stage, individuals were little affected by pH and temperature treatments and showed very high
292 levels of survival (99.74 ± 0.17 %) (Fig 2. A-B).

293 Overall, survival rates showed a sharp decrease at the highest treatment temperature of 30
294 °C with maximums at 18 and 26 °C (Table 2). However, there were also important differences
295 in temperature tolerance across life-stages (*i.e.*, S x T interaction). The veliger stage showed a
296 peak of maximal survival at 26 °C; umbonates had similar values from 18 to 26 °C; pediveligers
297 declined beyond 18 °C; and no effects were detected for spat (see Fig. 2A). There was also a
298 significant increase in survival rates from ambient through lower pH treatments (mainly at the
299 high-reduced pH range; Table2), but effects were only significant for veliger and pediveliger
300 stages, which were more sensitive to experimental conditions and had the lowest rates of
301 survival (see Fig. 2B). No shell malformations, or decreased calcification rates (in terms of shell

302 thinning or brittleness) were observed for any of the larvae and spat stages at any pH
303 treatment, including low conditions at the high-reduced pH (see Fig. 3A-D).
304
305 **Size of larval and spat stages**

306 ***Veliger***: For temperature, significant effects were only observed in shell length, whereas pH
307 showed significant effects in both shell length and width (Table 3a). After the 4 experimental
308 days, lengths of individuals were the lowest at 18 °C (159.8 ± 0.85 µm) and 30 °C (160.1 ± 0.75
309 µm) and highest at 26 °C (164.1 ± 1.26 µm) (Fig. 4). Both length and width showed the lowest
310 values at ambient pH (158.4 ± 0.45 µm, and 177.5 ± 0.59 µm, respectively for length and
311 width) and the highest at the lowest pH treatment at the high-reduced pH (164.9 ± 1.1 µm,
312 and 188.6 ± 3.5 µm, also respectively for length and width) (Fig. 5).
313 ***Umbonate***: There were only significant effects of temperature in both shell length and
314 width after the four experimental days (Table 3b). Shell size was highest at 26 °C (203.8 ± 1.9
315 µm, and 230.9 ± 1.85 µm, respectively for length and width), lowest at 30 °C (194.09 ± 1.2 µm,
316 and 219.1 ± 1.1 µm respectively for length and width), and intermediate at 18 and 22 °C (199.5
317 ± 0.67 µm, and 227.5 ± 0.82 µm, respectively for length and width) (Fig. 4).
318 ***Pediveliger:*** Size effects due to temperature and pH conditions could not be investigated
319 due to low survival above 18 °C ($n$ = 17 at 22 °C, 4 at 26 °C, and 0 at 30 °C).
320 ***Spat:*** Shell size at this stage was 4,250.5 ± 47.2 µm in length per 4,564.5 ± 46.89 µm in
321 width, with no observable effects of temperature, pH, or their interaction.
322
323 **Effects of pH on bacterial growth**

324 There was *ca.* 47 times larger numbers on *Vibrio* spp. colony forming units per ml in
325 intervalvar oyster water than in the 500 L mother tank, together with *ca.* 2 times higher
326 numbers of total heterotrofs (see Fig. 6A).

327 For pH treatments, the number of *Vibrio* spp. colony forming units at ambient and 7.6 pH
328 units varied between 0 and 15,500, whereas at medium and high-reduced pHs the range was
329 only from 0 to 210 (see Fig. 6B), and drawn significant differences in Levene's test and
330 subsequent pair-wise comparisons (see Table 4). For total heterotrofs, ANOVA results also
331 evidenced significant differences between the low-reduced pH treatment and the rest.
332
333 **Relationship between pH and $pCO_2$ levels**
334 Results for the $CO_2$ system calculation program revealed that a significant increase in the
335 $pCO_2$ from current atmospheric levels would be necessary for obtaining experimental
336 conditions of pH. On the one hand, the reduction in ambient seawater pH due to the biological
337 activity of the larvae within the containers (*i.e.*, control treatments with no added $CO_2$)
338 corresponded to *ca.* 1.74-fold increase in air $pCO_2$. On the other, further acidification of
339 seawater in the low, medium, and high-reduced pH treatments increased current $pCO_2$
340 scenarios by *ca.* 2.7, 4, and 6-fold, respectively for each treatment (see Fig. 7). In spite of the
341 previously described relationship between pH and temperature, no temperature ($F_{3, 48} = 1.349$,
342 $P > 0.05$) or interaction effects ($F_{9, 48} = 0.89$, $P > 0.05$) were observed for calculated $pCO_2$ levels
343 (Fig. 7).
344
345 **DISCUSSION**
346 Our results show that seawater temperatures around 30 °C have negative effects on
347 survival rates of all larval stages of *O. edulis*, although there were also important mortalities at
348 lower temperatures (22-30 °C) in the pediveliger stage and no effects on spat. For pH
349 treatments, veligers displayed a significantly higher survival at lower pHs, and a similar trend
350 was also observed in pediveligers, although in marginal importance due to large mortality at
351 this stage. This coincided with lower numbers of colony-forming bacteria at medium and high-
352 reduced pH treatments, and no apparent shell malformations, shell thinning or brittleness

353 indicative of altered rates of calcium carbonate deposition. Although this pH range correspond

354 to $pCO_2$ levels far beyond those in scenarios predicted by the IPCC (IPCC 2013, our results

355 suggest that short-term $CO_2$ bubbling into seawater could minimize infection rates of larvae

356 reared under controlled aquaculture conditions. Our results confirm previous findings that

357 increasing temperature can have deleterious effects on the growth and survival of early stages

358 of marine invertebrates (His et al. 1989, Parker et al. 2009, 2010), but also provide new

359 possible scenarios to potential outcomes of seawater acidification in calcifying organisms

360 (Kurihara 2008), resulting from interactive effects with the bacterial community. Overall, our

361 results for the studied developmental stages suggest that this could be a more resilient species

362 to global change compared to other species of commercial bivalves such as the mussel *M.*

363 *galloprovincialis*, and the oysters *C. gigas* and *S. glomerata* (see Kurihara et al. 2008, Parker et

364 al. 2010, Barros et al. 2013). These patterns were especially robust for spat, –a critical stage for

365 aquaculture activities– that may be also affected by acidification in the other commercial

366 species (Kurihara 2008, Range et al. 2014).

367

368 **Survival rates of *Ostrea edulis* stages**

369 Larval stages were more sensitive than spat, which registered survival rates of *ca.* 100%, in

370 agreement with previous findings linking mortality to the development of physiological

371 functions (Thorson 1950). Among larval stages, the most sensitive period was that of

372 pediveliger (only 5.5% survived), possibly associated to high metabolic costs of metamorphosis

373 for benthic settlement (Widdows 1991, García-Esquivel et al. 2001). The veliger stage also

374 showed fairly low rates of survival (43 %), but was higher at the umbonate stage (67.5 %)

375 coupled with an increase in size of 20 % in 15 days. The lack of observable malformations and/

376 or decreases in shell size and thickness across treatments and *O. edulis* stages suggests that an

377 important component of the larval mortality was likely due to natural factors (Rumrill 1990),

378 and/ or confinement conditions within sealed containers. In contrast, in similar 4-day

379 experiments conducted with *Crasostrea gigas* and *S. glomerata* (Parker et al. 2010), authors
380 found large number of abnormalities in veligers (up to 100 % in some *S. glomerata*
381 treatments), and later stages also showed altered growth rates. Moreover, variability in the
382 results among species may be partially due to differential sensitivity to pH and temperature
383 during fertilization (Parker et al. 2010), which for our *O. edulis* larvae were ambient seawater
384 pH and conditions within gravid females.
385

386 **Effects of temperature on *Ostrea edulis* stages**

387 Temperature treatments were responsible for *ca.* 49, 44, and 22 % differences in mortality
388 for veliger, umbonate, and pediveliger stages, respectively. Similarly to a previous study
389 conducted to investigate temperature tolerance in larvae of *S. glomerata* (Dove and O'Connor
390 2007), we found that the optimum temperature for the survival of *O. edulis* veligers was 26 °C,
391 with higher survival compromise above this optimal level (*i.e.*, at 30 °C). This could also be the
392 case for fertilization, as indicated by laboratory experiments with *S. glomerata* (Parker et al.
393 2009). In contrast, negative temperature effects on umbonate larvae were only detected at 30
394 °C, whereas pediveliger suffered higher mortality beyond 18 °C, possibly because shallow
395 Mediterranean waters are typically stratified and benthic layers attain the lowest
396 temperatures during the spring-summer (Estrada 1996). Differences on larval size (both shell
397 length and width) followed the same patterns than survival rates at each stage, suggesting that
398 temperature treatments have an important effect on the growth rates during the experimental
399 period. In veligers, Parker et al. (2010) also found higher shell length of both *S. glomerata* and
400 *C. gigas* at 26 °C, confirming that this temperature is the optimal for this larval stage. Overall,
401 our results suggest that temperatures of around 30 °C consistently cause the highest
402 mortalities and lowest growth, although with important differences in the optimum
403 temperature levels across larval stages. Therefore, spring temperatures of around 20 °C in the
404 study region (Loureiro et al. 2009) added to potential rises predicted by the highest emission

405 RCP8.5 scenario for the end of the century (2.6 to 4.8 °C; IPCC 2013) may fall within the

406 deleterious temperature range for pediveliger and impact the remaining populations of *O.*

407 *edulis* in the Mediterranean sea.

408

409 **Effects of pH reductions on *Ostrea edulis* stages**

410 The effects of pH reductions were a significant increase in larval survival of *ca.* 26 % at the

411 veliger stage, along with a slight gain in size of about 3-4 %. For pediveligers, a slight increase

412 in survival of *ca.* 4 % was also observed, but given the high mortality observed at this stage this

413 result would require further experimental evidence. The variety of effects reported for

414 reductions in seawater pH seem to be strongly dependent on the experimental pH and

415 exposure period, as well as on species type (review by Kurihara 2008). This may be partly due

416 to the 7.5 to 8.5 range of pH variability in the open ocean -with reductions down to 7.0 or

417 lower in tide pools, bays, and estuaries due to dilution and production of $H_2S$ (Sverdrup et al.

418 1942)-, which may partly shape the tolerance of species to high levels of $pCO_2$. pH levels below

419 *ca.* 7.0 appear to cause consistent damage (e.g., decreased survival, growth rate, egg

420 production and hatching success), whereas above pH 7.0 different species show variable

421 degrees of sensitivity to seawater acidification (Kurihara 2008). On the one hand, control

422 seawater during our study ranged between 7.8 and 7.9, which has been reported to decrease

423 calcification, growth rates, and shell malformation in larvae of *C. gigas*, as well as in adult

424 stages of sea urchin and decapoda (Shirayama and Thornton 2005, Kurihara 2008). On the

425 other, our lowest pH treatment (*ca.* 2200 ppm of [$CO_2$]) was far beyond high end scenarios

426 predicted by the IPCC at 936 ppm (RCP8.5; IPCC 2013, and nonetheless, Calabrese and Davis

427 (1966) could not detect any deleterious effects in *C. virginica* and *M. mercenaria* larvae at pHs

428 higher than 6.75, even at longer durations of experimental exposure (10-12 days). In our

429 experiment, the duration was fixed at 4 days, following the methodology used by Parker et al.

430 (2010) to study the effect of elevated $pCO_2$ and temperature on the fertilization and early

431 development of *C. gigas* and *S. glomerata*, although with contrasting results. In addition to
432 variability in species tolerance to reduced pH at a given developmental stage, variability in the
433 results may be due to veliger size at the start of the experiment (*ca.* 160 µm and 9 days old).
434 Usually, mortality rates are higher for small size classes than for larger individuals >1 mm
435 (Green et al. 2004), and the majority of works finding deleterious effects in early larval stages
436 were conducted during fertilization and embryogenesis (Kurihara & Shirayama 2004, Kurihara
437 et al. 2007, Parker et al. 2009, 2010). Also importantly, males of *O. edulis* release
438 spermatozeugmata (radially arrayed sperm cells attached by an extracellular matrix to a core
439 of acellular vesicles) into the water (Foighil 1989), and there is no information on how
440 variations of pH may affect the quality spermatozoa. In other species such as *C. gigas*, sperm
441 motility and velocity has been indicated to be decreased by seawater acidification (Barros et
442 al. 2013) so negative effects are possible. In spite of these gaps in knowledge, the
443 characteristics of the life history taken together with our results suggest that *O. edulis* could be
444 a resilient species to future scenarios of $pCO_2$ and seawater acidification.
445
446 **Effects of pH reductions on bacterial abundance and infection capacity**
447 Our results showed that bacteria entered the mother tank of the rearing system via the
448 parental intervalvar water during the initial transfer of larvae. Since bivalves are filter feeding
449 organisms, they tend to accumulate a great number of bacteria within their tissues, which
450 often lead to pathological condition (Bolinches et al. 1986, Roque et al. 2009). Bivalve larvae
451 also suffer from bacterial infection, which is considered as one of the main causes of mortality
452 in early stages (Guillard 1959, Gosselin and Qian 1997, His et al. 1999). Although direct
453 observation of bacteria during larval evaluation was not possible, samples contained multiple
454 bacterivorous flagellates (direct observation during the study), typically occurring after
455 bacterial infection and host mortality (Azam et al. 1983, Sanders et al. 1989), with higher
456 numbers occurring in the two highest pH treatments. Results from parallel experiments with

457 bacterial cultures from the same seawater used in larval experiments evidenced a higher
458 number of *Vibrio* spp. colony forming units at higher pH treatments (control and low-reduced
459 pH), whereas numbers of total heterotrofs were the highest at the low-reduced pH. Taken
460 together, these results suggest that larval mortality within sealed containers was decreased
461 (mostly at the veliger stage) due to enhanced $pCO_2$ and water acidification, which created a
462 less optimal environment for bacterial growth. In addition, given that no significant differences
463 in larval mortality were observed between the low-reduced pH and control treatments,
464 infection by *Vibrio* spp. appears to be the most likely pathogen responsible for the observed
465 patterns. In fact, pH values around 8 to 8.5 have been indicated as optimal pH ranges for
466 several *Vibrio* species, including *V. cholerae* and *V. parahaemolyticus* (Huq et al. 1984, Miles et
467 al. 1997), which commonly infect *O. edulis* and other species of commercial bivalves (Roque et
468 al. 2009). In contrast, *V. tubiashii,* another species of prevalent bivalve pathogen is indicated a
469 biphasic pH response with optimums at both pH 8.0 and 6.5, and weakened growth at pH 7.0
470 and 7.5 (Temperton et al. 2011). Therefore, lower pH values of 7.75 observed in regions of
471 strong upwelling on the continental shelf of western North America (Feely et al. 2008) alone,
472 cannot fully explain bacterial outbreak, and enhanced mortality of *C. gigas* larvae (Elston et al.
473 2008), although infection may had been facilitated by enhanced larval sensitivity at only
474 slightly acid pHs of around 7.8 (see Kurihara 2008), among other possible causes.
475
476 In summary, given the strong correlation observed between atmospheric and water
477 temperature in semi-confined coastal bodies with rearing bivalve activities such as the Alfacs
478 Bay (Solé et al. 2009), a potential rise in temperature of between 2.6 to 4.8 °C as predicted by
479 the RCP8.5 scenario for the end of the century (IPCC 2013) may be harmful for the species (due
480 to lower tolerance in pediveligers), unless reared to spat under controlled conditions. In
481 contrast, *O. edulis* larvae appear to be resilient to direct effects of seawater acidification in
482 physiological processes such as growth and shell deposition even far beyond the worst IPCC

483 scenario (IPCC 2013). Yet, further research is necessary to investigate the effects of increased
484 p$CO_2$ in gravid oysters and sperm. For bacterial infections, our results are amongst the first
485 contributions in the field, but it seems plausible that the influence of ocean acidification in
486 mediating potential pathogenic condition may be dependent on both the optimal pH ranges
487 for bacterial growth (also including beneficial bacteria) and the sensitivity of larvae and benthic
488 calcifying organisms to pH reductions, among other possible factors. In addition, potential
489 shifts in the abundance of bacterial communities are also likely to cascade down the food web
490 and alter the trophic dynamics of coastal systems. Yet, our results of mid-term (4 days)
491 exposure of *O. edulis* larvae to $CO_2$ bubbling could be of application for the aquaculture sector,
492 as an alternative/ complementary way to probiotic feeds (Avendaño and Riquelme 1999),
493 addressed to minimize the outbreaks of local *Vibrio* spp. within rearing tanks.
494
495
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503

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664

665 Fig. 1. Ranges of variability in pH treatments (low-reduced (LR), medium-reduced (MR), and
666 high-reduced (HR) pH) at each study temperature. Initial (2) and final (2) pH values at each
667 experiment (veliger, umbonate, pediveliger, and spat) were pooled to assess trends (*i.e.*, $n$ = 16
668 for each pH and T treatment). Error bars are SE.
669
670 Fig. 2. Percent survival of *Ostrea edulis* larval and benthic stages at: (A) the different
671 temperature (18 °C, 22 °C, 26 °C, and 30 °C), and (B) pH treatments (low, medium, and high-
672 reduced pH, and controls (C) with no $CO_2$). Error bars are SE.
673
674 Fig. 3. *Ostrea edulis* larval stages and spat at the high-reduced pH treatment showing no
675 malformation and/ or regions with low calcification. (A) veliger, (B) umbonate, (C) pediveliger,
676 and (D) spat.
677
678 Fig. 4. Size (length and width) of *Ostrea edulis* veliger and umbonate stages at each
679 temperature (18 °C, 22 °C, 26 °C, and 30 °C). Spat showed NS effects whereas pediveliger had a
680 very low $N$ and are not included. For each larval stage, letters indicate significant treatment
681 groupings in SNK. Error bars are SE.
682
683 Fig. 5. Size (length and width) of *Ostrea edulis* veligers at pH treatments (low, medium, and
684 high-reduced pH, and controls (C) with no $CO_2$). Umbonate and spat showed NS effects
685 whereas pediveliger had a very low $N$ and are not included. For each larval stage, letters
686 indicate significant treatment groupings in SNK. Error bars are SE.
687
688 Fig. 6. (A) Number of colony-forming bacteria (*Vibrio* spp. and total heterotrofs in marine agar)
689 per ml in oyster intervalvar water and mother tank water; and (B) number colony-forming of
690 *Vibrio* spp. and (C) total heterotrofs per ml at each experimental pH.

691

692 Fig. 7. $pCO_2$ levelscalculated for each pH and temperature treatment during the 4 combined

693 experiments (veliger, umbonate, pediveliger, and spat) using the$CO_2$ system calculation

694 program (Lewis et al.1998). The initial start shows the $pCO_2$ levels calculated for ambient

695 seawater without larvae or spat (362 ± 18.1). Error bars are SE.

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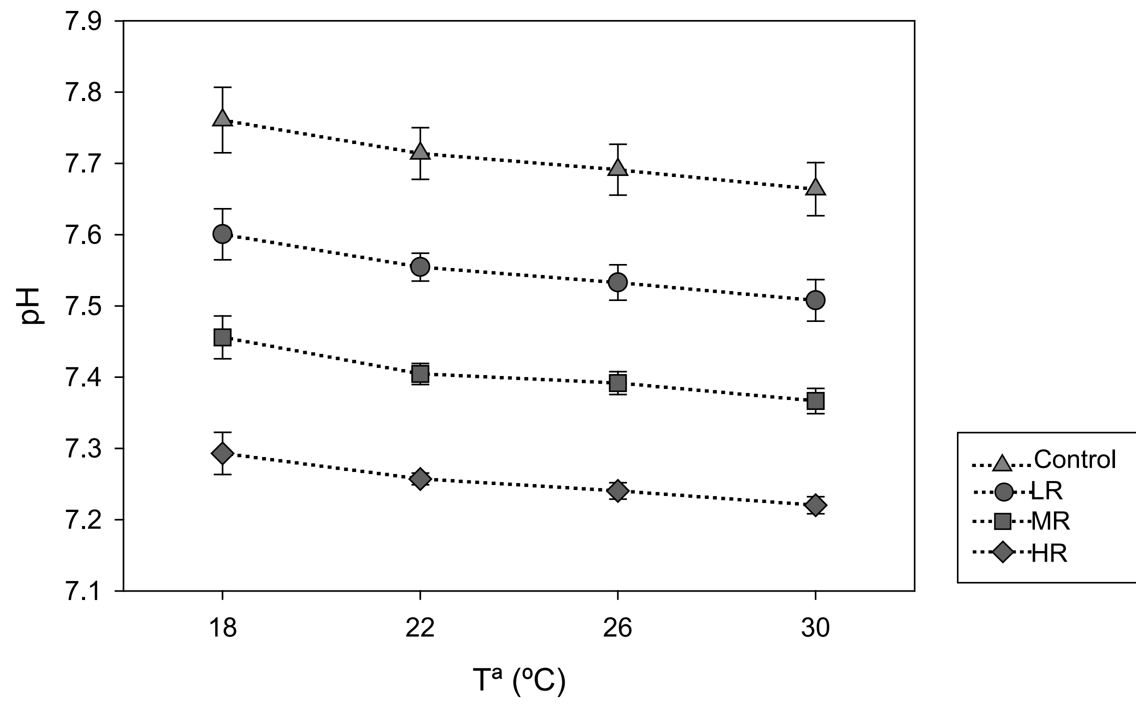


708 Fig. 1.

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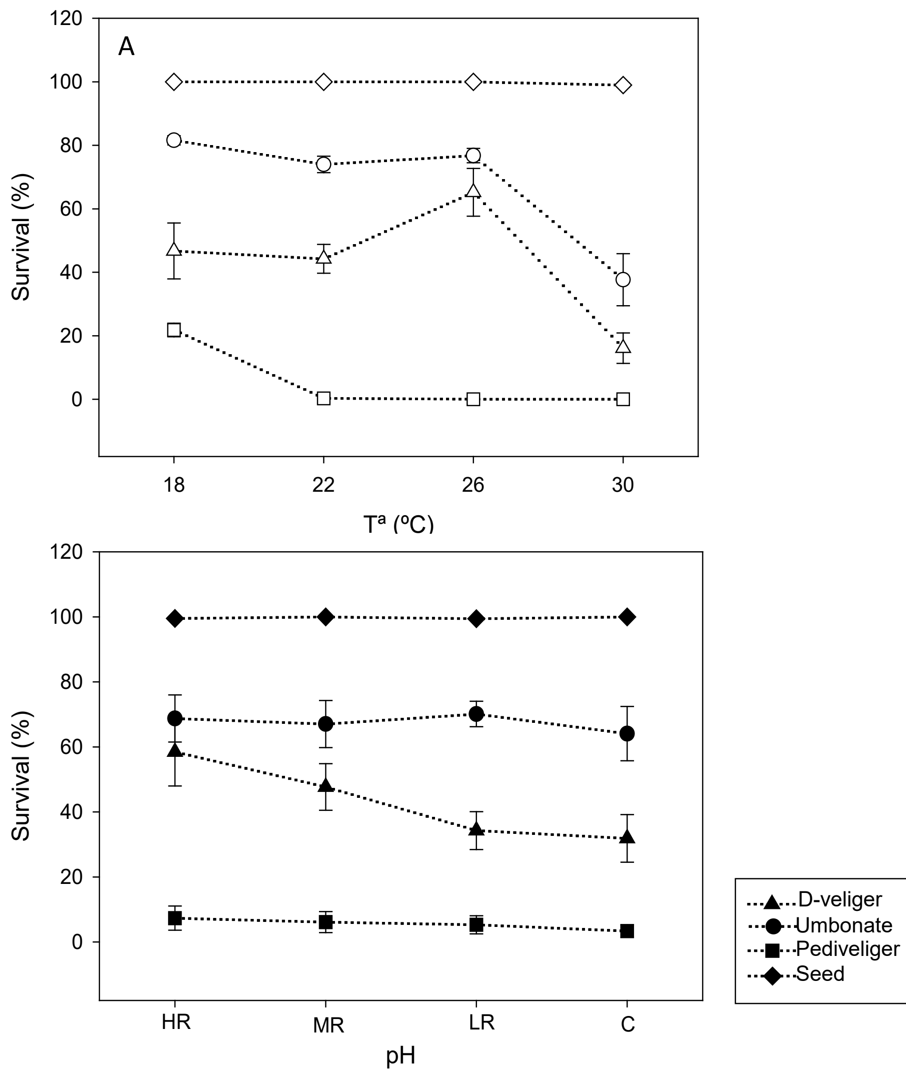


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728 Fig. 2.

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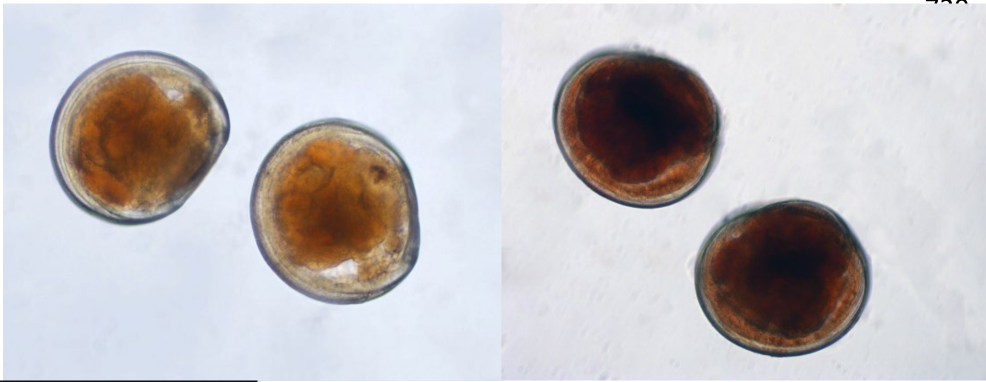

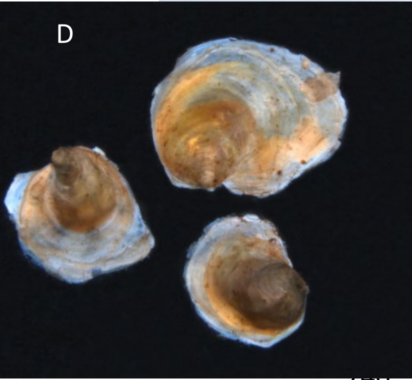


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742 Fig. 3.

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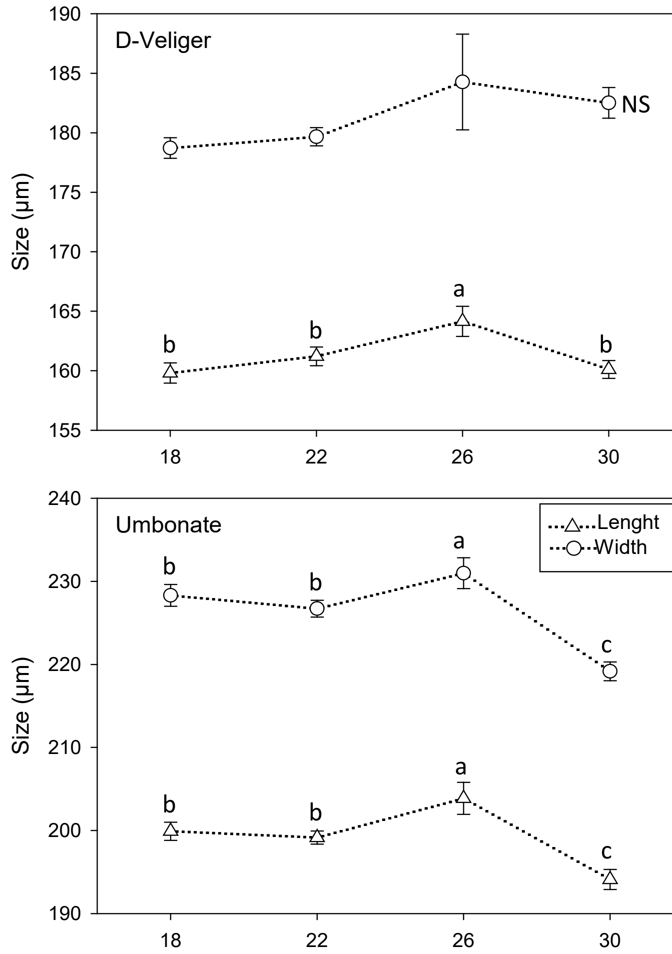


760 Fig. 4.

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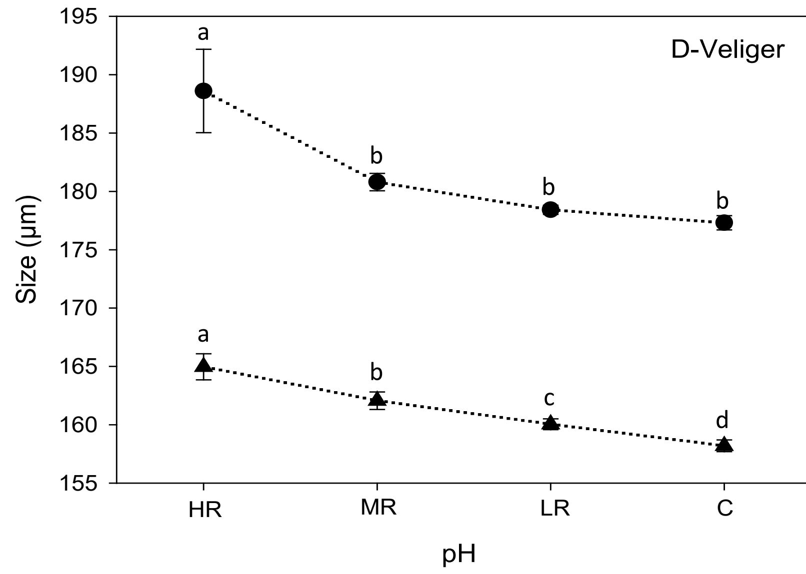


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773 Fig. 5.

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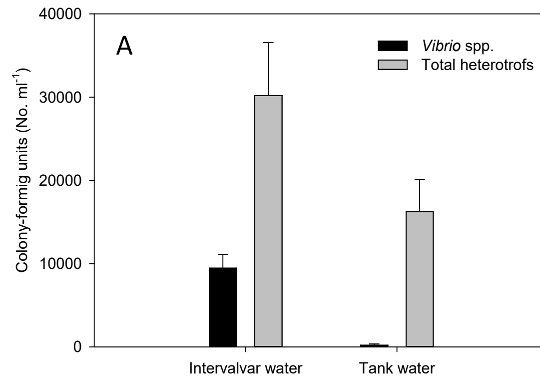


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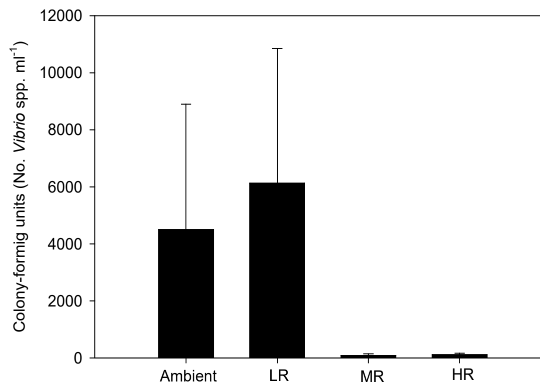


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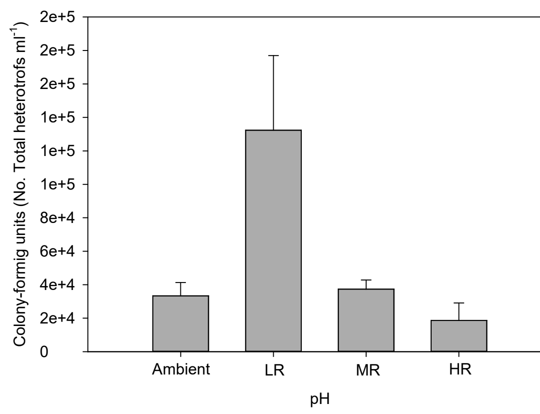


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794 Fig. 6.

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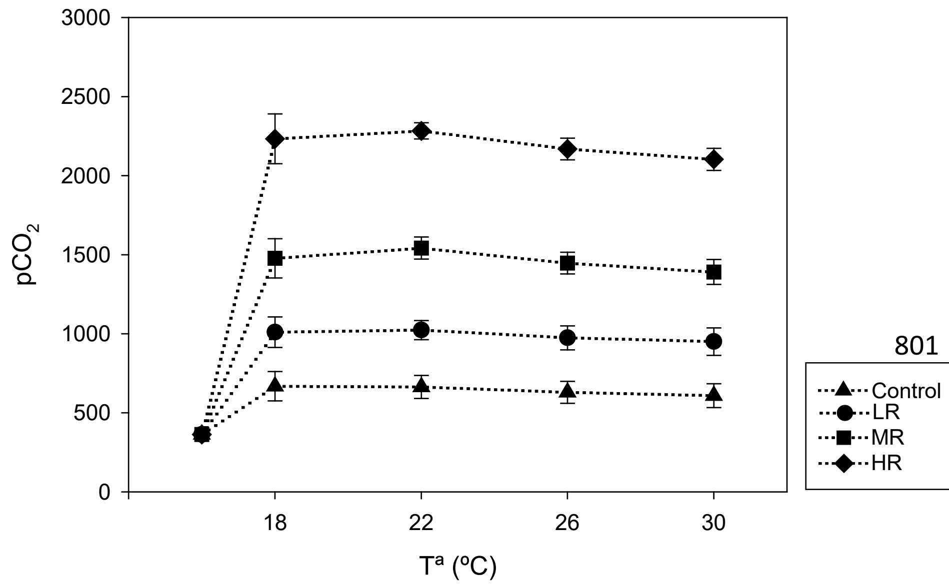


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806 Fig. 7.

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808 Table 1. Temporal variation in seawater pH within pots from initial values to water change two
809 days later. Values are averages of the two replicate measures (initial and final) across the four
810 experiments (D-veliger, umbonate, pediveliger, and seed). Errors are SE.

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| **Ta (ºC)** | **pH** | **$pH_i$** | **$pH_f$** |
|---|---|---|---|
| 18 | Control | 7.90 ± 0 | 7.72 ± 1.1E$^{-3}$ |
| | 7.6 | 7.60 ± 0 | 7.59 ± 5.1E$^{-16}$ |
| | 7.4 | 7.40 ± 0 | 7.5 ± 8.3E$^{-4}$ |
| | 7.2 | 7.20 ± 0 | 7.37 ± 4E$^{-4}$ |
| 20 | Control | 7.90 ± 0 | 7.62 ± 1.5E$^{-3}$ |
| | 7.6 | 7.60 ± 0 | 7.51 ± 1.5$^{E-3}$ |
| | 7.4 | 7.40 ± 0 | 7.40 ± 7E$^{-4}$ |
| | 7.2 | 7.20 ± 0 | 7.31 ± 4E$^{-4}$ |
| 26 | Control | 7.90 ± 0 | 7.58 ± 2.9E$^{-3}$ |
| | 7.6 | 7.60 ± 0 | 7.46 ± 1.1E$^{-3}$ |
| | 7.4 | 7.40 ± 0 | 7.37 ± 4E$^{-4}$ |
| | 7.2 | 7.20 ± 0 | 7.27 ± 1.7E$^{-3}$ |
| 30 | Control | 7.90 ± 0 | 7.52 ± 2.7E$^{-3}$ |
| | 7.6 | 7.60 ± 0 | 7.41 ± 1.5E$^{-3}$ |
| | 7.4 | 7.40 ± 0 | 7.32 ± 8E$^{-4}$ |
| | 7.2 | 7.20 ± 0 | 7.23 ± 1.8E$^{-3}$ |

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814 Table 2. 3-way ANOVA results testing for temperature and pH effects in survival rates (%)
815 across larval stages of *Ostrea edulis*: Veliger (V), Umbonate (U), Pediveliger (P), and Spat (S).
816 Significant results are indicated in **bold**. In SNK, groupings are shown for significant
817 interactions. Ph treatments are abbreviated as low-reduced (LR), medium-reduced (MR), high-
818 reduced (HR), and Controls (C) with ambient pH water and larvae or spat.

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| Survival (%) | df | MS | F | p |
|---|---|---|---|---|
| Stage (S) | 3 | 75890.1 | 483.07 | **0.0000** |
| Ta (T) | 3 | 5852.8 | 37.25 | **0.0000** |
| pH (P) | 3 | 676.0 | 4.30 | **0.0062** |
| S x T | 9 | 1789.3 | 11.38 | **0.0000** |
| S x P | 9 | 427.9 | 2.72 | **0.0061** |
| T x P | 9 | 312.2 | 1.98 | 0.0458 |
| S x T x P | 27 | 226.7 | 1.44 | 0.0911 |
| Error | 128 | 157.1 | | |

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*C* = **0.55** 825

**SNK (S x T):** S(30)=S(26)=S(22)=S(18)>U(18)≥U(26)=U(22)=V(26)>V(18)=V(22)=U(30)>V(30)=P(18)>P(22)=P(26)=P(30) 826

**SNK (S x P):** S(HR)=S(MR)=S(LR)=S(C)>U(LR)=U(HR)=U(MR)=U(C)=V(HR)>V(MR)>V(LR)>V(C)>P(HR)≥P(MR)=P(LR)≥P(C) 827

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830 Table 3. 2-way ANOVA results testing for effects of temperature and pH in the size (length and
831 width) at each larval stage (except pediveliger) and spat. Significant results are indicated in
832 **bold**. SNK groupings for significant effects are indicated in Fig. 3-4.

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| ANOVA | Length (μm) | | | | Width (μm) | | | |
|---|---|---|---|---|---|---|---|---|
| a) D-veliger | df | MS | *F* | *P* | df | MS | *F* | *P* |
| Ta (T) | 3 | 47 | 12.1 | **0.0000** | 3 | 78 | 2.8 | 0.0538 |
| pH (P) | 3 | 101 | 25.9 | **0.0000** | 3 | 311 | 11.2 | **0.0000** |
| T x P | 9 | 4 | 1.0 | 0.4698 | 9 | 79 | 2.8 | 0.0140 |
| Error | 32 | 4 | | | 32 | 28 | | |
| | *C*= 0.45 (NS) | | | | *C*= **0.84** | | | |
| b) Umbonate | df | MS | *F* | *P* | df | MS | *F* | *P* |
| Ta (T) | 3 | 193 | 9.96 | **0.0000** | 3 | 308 | 18.70 | **0.0000** |
| pH (P) | 3 | 18 | 0.92 | 0.4399 | 3 | 12 | 0.74 | 0.5376 |
| T x P | 9 | 28 | 1.44 | 0.2147 | 9 | 47 | 2.84 | **0.0140** |
| Error | 32 | 19 | | | 32 | 16 | | |
| | *C*= 0.53 (NS) | | | | *C*= 0.46 (NS) | | | |
| c) Seed | df | MS | *F* | *P* | df | MS | *F* | *P* |
| Ta (T) | 3 | 227721 | 2.248 | 0.1017 | 3 | 264347 | 2.782 | 0.0568 |
| pH (P) | 3 | 30845 | 0.304 | 0.8219 | 3 | 80579 | 0.848 | 0.4778 |
| T x P | 9 | 113877 | 1.124 | 0.3748 | 9 | 98374 | 1.035 | 0.4344 |
| Error | 32 | 101303 | | | 32 | 95012 | | |
| | *C*= **0.67** | | | | *C*= 0.47 (NS) | | | |

848 Table 4. Results of Levene's tests for ranges of variation in the abundance of *Vibrio* spp. across
849 all pH treatments and pair-wise comparisons; and ANOVA results on the abundance of total
850 heterotrofs across pH treatments. Significant results are indicated in **bold**. For SNK see Fig. 7.
851 pH treatments abbreviations as in Table 2.

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| **Vibrio** | | | | |
|---|---|---|---|---|
| Levene tests | MS Effect | MS Error | *F* | *P* |
| All pH treatments | 35899164 | 3724132 | 9.639 | **0.0049** |
| Ambient vs. LR | 216600 | 7446383 | 0.029 | 0.8728 |
| Ambient vs. MR | 50266852 | 3232546 | 15.55 | **0.0169** |
| Ambient vs. HR | 50498674 | 3232669 | 15.62 | **0.0167** |
| LR vs. MR | 57082785 | 4215596 | 13.54 | **0.0212** |
| LR vs. HR | 57329807 | 4215719 | 13.59 | **0.0210** |
| MR vs. HR | 266,66 | 1881.48 | 0.141 | 0.7256 |
| **Total heterotrofs** | | | | |
| ANOVA | df | MS | *F* | *P* |
| pH | 3 | 8.081E+09 | 4.913 | **0.0319** |
| | 8 | 1.644E+09 | | |
| Transformation: √x | | | | |
| Cochran's C: 0.088 (NS) | | | | |

855 Table 5. $pCO_2$ levels calculated for each pH and temperature treatment during the 4 combined
856 experiments (D-veliger, umbonate, pediveliger, and spat) using the$CO_2$ system calculation
857 program (Lewis & Wallace 1998). Indicated pH values were averaged across the 4 experiments.
858 For TA, values were measured previously to inclusion of containers within temperature tanks,
859 and also averaged across the 4 experiments. The presence/ absence of larvae or spat in the
860 meassurements is indicated. Error bars are SE. pH treatments abreviations as in Table 2.

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| Indiv. | TºC | pH treatment | pH average | TA (µM $kg^{-1}$) | $pCO_2$ (ppm) |
|---|---|---|---|---|---|
| No | 22-27 | Ambient | 7.90 ± 0.02 | 2735 ± 14.1 | 362 ± 18.1 |
| Yes | 18 | Control | 7.76 ± 0.05 | 2742.5 ± 11 | 667.1 ± 92.1 |
| Yes | 18 | LR | 7.60 ± 0.04 | 2740 ± 15.5 | 1009 ± 96.3 |
| Yes | 18 | MR | 7.46 ± 0.03 | 2740 ± 15.5 | 1476.9 ± 124 |
| Yes | 18 | HR | 7.29 ± 0.03 | 2730 ± 8.6 | 2232 ± 157.3 |
| Yes | 22 | Control | 7.71 ± 0.04 | 2742.5 ± 11 | 662.9 ± 72.7 |
| Yes | 22 | LR | 7.55 ± 0.02 | 2740 ± 15.5 | 1022 ± 60.3 |
| Yes | 22 | MR | 7.40 ± 0.01 | 2740 ± 15.5 | 1541.7 ± 70 |
| Yes | 22 | HR | 7.26 ± 0.01 | 2730 ± 8.6 | 2282.5 ± 51 |
| Yes | 26 | Control | 7.69 ± 0.04 | 2742.5 ± 11 | 629 ± 69 |
| Yes | 26 | LR | 7.53 ± 0.02 | 2740 ± 15.5 | 972.9 ± 75.8 |
| Yes | 26 | MR | 7.39 ± 0.02 | 2740 ± 15.5 | 1446.7 ± 68 |
| Yes | 26 | HR | 7.24 ± 0.01 | 2730 ± 8.6 | 2168 ± 68.6 |
| Yes | 30 | Control | 7.66 ± 0.04 | 2742.5 ± 11 | 607.6 ± 75.3 |
| Yes | 30 | LR | 7.51 ± 0.03 | 2740 ± 15.5 | 949.2 ± 86.6 |
| Yes | 30 | MR | 7.37 ± 0.02 | 2740 ± 15.5 | 1390 ± 79.1 |
| Yes | 30 | HR | 7.22 ± 0.01 | 2730 ± 8.6 | 2103 ± 69.8 |