Combined effects of ocean warming and acidification on the larval stages of the European abalone Haliotis tuberculata

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Abstract

This study examined the physiological responses of the larval stages of *Haliotis tuberculata*, an economically important abalone, to combined temperature (17℃ and 19°C) and pH (ambient pH and -0.3 units, i.e., +200% increase in seawater acidity) in a full factorial experiment. Tissue organogenesis, shell formation, and shell length significantly declined due to low pH. High temperature significantly increased the proportion of fully shelled larvae at 24 hours post-fertilization (hpf), but increased the proportion of unshelled larvae at 72 hpf. Percentage of swimming larvae at 24 hpf, 72 hpf and 96 hpf significantly declined due to high temperature, but not because of low pH. Larval settlement increased under high temperature, but was not affected by low pH. Despite the fact that no interaction between temperature and pH was observed, the results provide additional evidence on the sensitivity of abalone larvae to both low pH and high temperature. This may have negative consequences for the persistence of abalone populations in natural and aquaculture environments in the near future.

Keywords: ocean acidification, global warming, climate change, marine mollusks, abalone larvae

Introduction

Increased CO_2 concentrations due to anthropogenic activities have led to increased oceanic CO_2 absorption, resulting in a phenomenon called ocean acidification (OA) that is concurrent with ocean warming (OW). Current projections suggest that by the year 2100, the average sea surface temperature will increase by 1°C– 3°C and the water pH will drop by 0.1 to 0.3 units (i.e., +30% to +200% increases in acidity; IPCC, 2014). OA has particularly deleterious impacts on marine calcifiers that produce calcium carbonate ($CaCO_3$) skeletons (Kroeker et al., 2013; Parker et al., 2013). These minerals are experiencing unprecedented decreases in their saturation states due to ongoing OA. This has a suppressive, narcotic effect and causes acidosis, which impairs many physiological processes (Feely et al., 2004; Portner, 2008; Doney et al., 2009). In contrast, the impacts of increased temperatures due to OW can stimulate physiological processes until they reach a threshold (Pörtner, 2010; Przeslawski et al., 2011; Byrne et al., 2011).

The combined impacts of OA and OW have been addressed with contrasting responses, such that the effects of OA and OW are either exacerbated (e.g., Di Santo 2015; D'Amario et al., 2020; Zittier et al. 2018; Rodolfo-Metalpa et al., 2011) or ameliorated (e.g., Kroeker et al. 2014; Davis et al., 2013; Knights et al., 2020; García et al., 2015; Jiang et al., 2018) in the presence of the other stressor (). Meta-analyses have suggested that the combined impacts of OA and OW on species physiology are especially devastating for the larval stages of many species (Przeslawski et al., 2015; Kroeker et al., 2013), presenting a major bottleneck for population persistence under changing oceanic conditions (Przeslawski et al., 2015). As a result, the responses of these early stages to combined OA and OW are now being paid greater attention (Przeslawski et al., 2015). Temperature and OA-related factors, such as pH, are among the paramount environmental factors that dictate the survival, morphology, physiology, and behavior of marine larvae, and in

68 particular, calcifying larvae (Przeslawski et al., 2015). Among all of the marine calcifiers studied
69 so far, mollusk larvae are the most vulnerable to the effects of OA (Przeslawski et al., 2015;
70 Kroeker et al., 2013).

71 The recent marine mollusks include over 43,000 recognized species That includes some of the
72 major $CaCO_3$ producers (Rosenberg, 2014) fulfilling crucial ecosystem functions, such as
73 creating habitat structures and food sources for benthic species (Parker et al., 2013). Therefore,
74 any negative impacts from environmental drivers can result in high ecological and economic
75 consequences (Narita et al., 2012). Abalones are important mollusks that have high ecological
76 and commercial values, and also provide food for human beings (Cook, 2016; Huchette and
77 Clavier, 2004). However, their natural populations have experienced severe declines due to
78 overexploitation (Micheli et al., 2008; Kashiwada and Taniguchi, 2007) and environmental
79 disturbances such as OW and bacterial diseases (Cook, 2016; Travers et al., 2009; Huchette and
80 Clavier, 2004; Morales-Bojórquez et al., 2008). On the other hand, abalone aquaculture is
81 expanding worldwide, understanding the effects of global change drivers on abalone physiology
82 is an important issue for the management of abalone populations in natural and aquaculture
83 environments (Morash and Alter, 2015).

84 Early life-history stages of abalones are negatively affected by OA and show a high percentage
85 of deformed larvae under low-pH conditions (Byrne et al., 2011; Crim et al., 2011; Guo et al.,
86 2015; Kimura et al., 2011; Zippay and Hofmann, 2010; Wessel et al., 2018; Swezey et al., 2020).
87 Other negative responses that have been reported in abalones in response to temperatures higher
88 than their physiological limit during development include abnormal appearance, reduced growth,
89 and trochophore mortality (e.g., Leighton, 1974; Pedroso, 2017). Although several studies have
90 shown larval abalones to be highly sensitive to OA (Santander-De Leon et al., 2018; Crim et al.,

2011; Tahil and Dy, 2016; Guo et al., 2015; Wessel et al., 2018), the combined effects of OA and OW are still not understood (Gao et al., 2020). Based on the limited data available, OA and OW may have deleterious impacts on abalone populations. Indeed, *Haliotis coccoradiata* embryos were dramatically affected by a combination of high temperatures (+2 to +4°C compared with control 20°C) and acidified conditions (-0.4 to -0.6 pH units), with only a small percentage surviving (Byrne et al., 2011).

Haliotis tubercula is a commercially important species in Europe, for which rearing over the whole life cycle is controlled in aquaculture (Huchette and Clavier, 2004; Courtois de Viçose et al., 2007). As for most marine molluscs, abalone species display a pelago-benthic life cycle with a larval planktonic stage followed by a critical metamorphosis into the benthic juvenile, making the specie highly sensitive to environmental changes (Byrne et al. 2011). The impacts of OA on all stages of the European abalone, *Haliotis tuberculata* (Linnaeus, 1758) have been recently studied (Wessel et al., 2018; Auzoux-Bordenave et al., 2020; Avignon et al., 2020). All the aforementioned studies reported adverse impacts of OA on *H. tuberculat*a, especially on the shell growth and calcification. However, *H. tuberculata* larvae were more impaired by OA than was any other life stage, showing significantly adverse effects in survival rate, morphology and development, growth rate and shell calcification (Wessel et al., 2018). While the impacts of OA on abalone larvae are worrying enough, high temperatures present another emerging threat for *H. tuberculata* populations (Travers et al., 2009; Huchette and Clavier, 2004). Furthermore, there is a lack of knowledge on how climate change will modify *H. tuberculata* larval settlement, behaviour and physiology in early life stages.

In this study, we investigated the combined effects of OA and OW on the early life stages of *H. tuberculata* in a full factorial experiment. Abalone larvae were exposed to four OA and OW

scenarios: ambient (pH 8.08.0 and 17°C); combined (pH 7.77.7 and 19°C); and individual effect scenarios (pH 8.08.0 and 19°C; pH 7.77.7 and 17°C) throughout the five days of larval development. We focused on three key larval stages: the trochophore stage, characterized by the set-up of the larval shell at 24 hours post-fertilization (hpf); the mature veliger stage (72 hpf); and the premetamorphic veliger stage (96 hpf), which is the last pelagic life stage before larval settlement (Jardillier et al., 2008; Auzoux-Bordenave et al., 2010). To better understand both the individual and combined effects of OA and OW, we investigated several biological parameters involved in growth, physiology and behavior throughout the larval development cycle of *H. tuberculata*.

Materials and methods

Abalone larvae production

The parental *H. tuberculata* stock was composed of wild broodstock (3 females and 7 males) collected from Saint Quay Portrieux (Brittany, France), and farmed abalones (4 females and 5 males) collected from an offshore sea-cage structure at the France *Haliotis* abalone farm (48°36'50 N, 4°36'3 W; Plouguerneau, Brittany, France). Mixing wild and farmed broodstocks prevent inbreeding and production of enough larvae for the experiment. In France *Haliotis* farm, after a 10-month period in nursery tank, abalone are placed in sea-cages and raised with similar environmental conditions as the wild abalone apart the protection from the predator attacks and high density until the age of 4 years. They are fed with algae collected on the shore, and submitted to the same temperature and pH as the wild broodstock.

The wild and farmed abalones were transferred from the sea and sea cages to the farm, respectively. There were given time to acclimatize to the farm's conditions over three months with natural running seawater maintained at 15°C and pumped twice a day from the sea, with *ad libitum* feeding to assure optimal reproduction maturity. Spawning was induced at the France *Haliotis* farm following usual procedures. Briefly, abalones were detached from the rearing aquarium and placed individually into 5-L buckets. Spawning was stimulated with ultraviolet light while gradually heating the filtered seawater from 18°C to 21°C over the course of 1 h. Abalones were allowed to spawn for a maximum of 5.5 h from the start of the experiment. Once spawned, the eggs were pooled and then divided into 12 batches. Spermatozoa from each of the 12 males (one male per batch of eggs) were added separately to avoid spermatic competition (Harney et al., 2018) at an optimal sperm concentration of approximately 100,000 spermatozoa per egg (Huchette et al., 2004). After 1 h, the fertilized eggs were pooled again and their density was estimated under a binocular microscope. The pooled fertilized eggs were then divided into 12 samples of 900,000 embryos per batch and transferred to the 12 hatching boxes.

Experimental design

To study the individual and combined impacts of OA and OW, we applied a full factorial design of two temperatures (17°C low and 19°C elevated) and two pH values (8.0 high and 7.7 low; with three replicate tanks per treatment (12 tanks in total; Fig. 1). We considered 17°C to be the ambiant ("low") temperature, as it was the temperature experienced by abalones during the summer reproduction period in Northern Brittany. According to Gac et al. (2020), temperature in the Northern Brittany reach maximal summer value of 17°C and present short-term variability of 0.1 to 0.4 °C mainly related to the tidal cycle and the day-time. Similarly, the high pH

158 corresponded to the naturally occurring pH during autumn in Northern Brittany (Qui-Minet et
159 al., 2018), that is, the high pH in this experiment was the ambient pH of the seawater pumped
160 into the France *Haliotis* site. The high temperature and low pH conditions were chosen based on
161 the global projections for the coming decades (IPCC, 2014). We chose the worst-case pH and
162 temperature scenarios; however, the high temperature treatment in this study is about 1°C lower
163 than the worst scenarios predicted for 2100. Abalone larvae were exposed to the experimental
164 conditions from 1 h post-fertilization until they reached the premetamorphic veliger stage. The
165 temperature was controlled using two central heating systems. For the 17°C treatment and pre-
166 heating of the 19°C treatment, we used an Aquahort Ltd Heat Pump (26 kW; THP26-3). To
167 reach 19°C, we supplemented this pump with a Charot Heat Pump (6 kW; 4911 type). Each
168 replicate was composed of three parts: I) a food-safe plastic head tank (60 L), in which water of
169 the appropriate temperature and CO_2 level was premixed and homogenized with a bubbling
170 system; II) a food-safe 22.5-L hatching box (30 cm x 50 cm x 15 cm), which was supplied with
171 reduced-flow running water from the head tank to avoid flushing the eggs out while maintaining
172 the desired pH and temperature. This box harbored the fertilized eggs to allow them to hatch
173 within the first 18 h. An evacuation pipe was connected to the rearing tank to allow automatic
174 larval transfer once the larvae could swim; this transfer was completed over four hours. III) A
175 350-L epoxy-coated larval tank, which was supplied by the head tank, to rear hatched larvae that
176 had left the hatching box. Natural 3-µm filtered seawater was allowed to run through the tanks at
177 all times (open circuit). To prevent larvae from leaving the tanks, a 40-µm net was placed at the
178 outflow. The flow rate from the head tanks to the rearing tanks was between 200–400 $mL \cdot min^{-1}$.
179 All tanks were exposed to an artificial photoperiod of 12 h light:12 h dark, which was provided
180 at a light intensity of 900–1000 lux at the water surface using 68-W natural spectrum light-

emitting diode (LED) lamps (Solar Natur, JBL). As pre-settlement abalone larvae are lecithotrophic (non-feeding), they were not fed during the experiment.

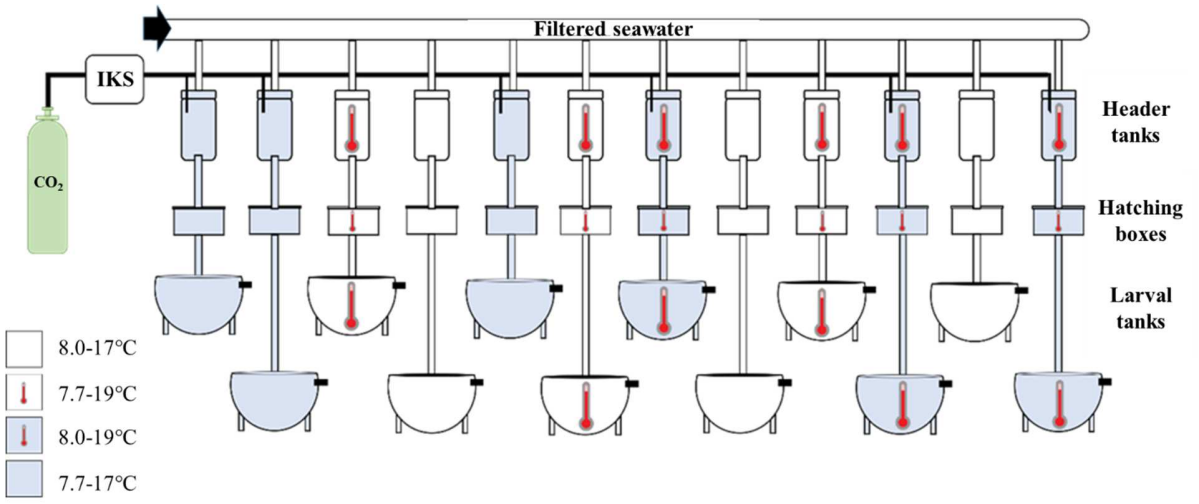


Fig. 1 Simplified design of the experimental system showing the flow of filtered seawater from the header tanks, the styrene hatching boxes, and the 350-L larval tanks (12 in total). Seawater exited the system via the black outflow at the top of larval tanks. IKS is a pH-regulated electrovalve system. The four treatments are indicated using two colors (white and blue) and a red thermometer for high temperatures.

Carbonate chemistry and pH control

Carbonate chemistry and pH control were monitored according to the methods of Avignon et al. (2020). Low-pH seawater was provided by bubbling CO_2 (Air Liquide, Paris, France) into the tanks through electrovalves regulated by a pH-stat system (Aquastar, IKS Computer System, Karlsbad, Germany) (one electrovalve and bubbling CO_2 system per header tank). The desired low-pH value was adjusted to be 0.3 units lower than the high pH, corresponding to natural pH

fluctuations along the Northern Brittany coast (the total scale (pH_T) range of 7.9–8.2; Qui-Minet et al., 2018). The values of the pH-stat system were adjusted from daily measurements of the electromotive force in the header tanks using a pH meter (Metrohm 826 pH mobile, Metrohm AG, Herisau, Switzerland) with a glass electrode (Metrohm Ecotrode Plus, Metrohm AG, Herisau, Switzerland). The electromotive force values were converted to pH units on the pH_T after calibration with Tris-HCl and 2-aminopyridine-HCl (AMP) buffers (Dickson et al., 2007).

Temperature and salinity were measured daily using a portable conductivity meter (ProfiLine Cond 3110, WTW, Oberbayern, Germany). Total alkalinity (A_T) was measured twice (n = 12 and n = 9) from 50-mL samples taken from each experimental tank. Seawater samples were filtered through 0.7-µm Whatman GF/F membranes, immediately poisoned with mercury chloride, and stored in a dark place at room temperature for later analysis.

A_T was determined from approximately 50 g of weighed samples using a potentiometric titration at 25°C with 0.1 M HCl and using a Titrino 847 plus Metrohm. The balance point was determined by the Gran method (Gran, 1952) according to Haraldsson et al. (1997). The accuracy of this method was ± 2 µmol·kg^{-1} and was verified by Certified Reference Material 182, provided by A. Dickson (Scripps Institute of Oceanography, University of South California, San Diego, United States). The seawater carbonate chemistry analysis included dissolved carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), dissolved inorganic carbon (DIC), pCO_2, aragonite saturation state (Ω_{ar}), and calcite saturation state (Ω_{calc}); these were determined by entering the values of pH_T, A_T, temperature, and salinity into CO2SYS software (Lewis and Wallace, 1998) using constants from Mehrbach et al. (1973) as refitted by Dickson and Millerro (1987).

219

220

221 *Hatching success*

222 Under pH 8.0 and 17°C, trochophore larvae typically hatch at 18–20 hpf (Jardillier et al., 2008).
223 To estimate the percentage of larvae that hatched in the 12 experimental hatching boxes, we
224 collected all of the unhatched larvae that were deposited at the bottom of the hatching boxes at
225 24 hpf, after the hatched larvae could swim and had evacuated to the 350-L rearing tank. Nine 1-
226 mL replicates were sampled from a 5-L bucket and fixed with 90% ethanol. Then, the total
227 number of unhatched larvae per tank was counted under a binocular microscope. To estimate the
228 percent hatching success of each tank, we used formula 1.

229 $$(1) = \frac{\text{number of swimming larvae at 24 hpf}}{(\text{number of swimming larvae at 24 hpf} + \text{number of unhatched larvae})} \times 100$$

230

231 *Morphometric assessment and percentage of swimming larvae*

232 The post-embryonic developmental stages of *H. tuberculata* were explained in detail by Jardillier
233 et al. (2008) and Auzoux-Bordenave et al. (2010). Our morphometric measurements were
234 focused at 24 and 72 hpf, which corresponded to the free-swimming trochophore and mature
235 veliger stages, respectively. Therefore, only the swimming larvae in the tank water column that
236 corresponded to these larval stages were sampled for further analyses.

237 The development timing of the larvae was investigated under a binocular microscope at 24, 72,
238 and 96 hpf to verify the larval stages before sampling according to Jardillier et al. (2008) and
239 Auzoux-Bordenave et al. (2010). At 24 and 72 hpf, the total number of larvae swimming in each

tank was estimated from six 10-mL replicates per tank (n = 18 per treatment). For morphometric, birefringence, and scanning electron microscopy (SEM) analysis, 10–15 L of seawater containing larvae was collected from each tank and filtered through a 40-µm sieve, then aliquoted into 15-mL tubes. Larvae were concentrated at the bottom of each sample by adding few drops of 70% ethanol; then, the samples were fixed and stored in 70% ethanol for polarized light microscopy and SEM analysis. At 96 hpf, all of the swimming larvae were filtered out of the 350-L tank using a 40-µm sieve and placed into buckets containing 5 L of seawater adjusted to the same temperature and pH as in their respective treatments. The percentage of swimming larvae at 96 hpf was calculated from eight 1-mL samples (n = 24 per treatment). The percentage of swimming larvae was calculated at 24 hpf, 72 hpf and 96 hpf as the total number of swimming larvae in the 350-L tank at each time point divided by the initial number of larvae that hatched.

Slide preparation for morphometric and birefringence analysis

To study morphometric characteristics and birefringence, 12 slides per larval stage (1 replicate per experimental tank) were prepared with the ethanol-fixed larvae (Wessel et al., 2018). Approximately 100 larvae were whole-mounted in about 500 µL of glycerol. All ethanol was removed before the samples were transferred into the glycerol. The slides were kept at room temperature for 5 to 10 min to allow any remaining ethanol to evaporate and let the larvae settle. Six spots of vacuum gel were deposited at the corners and middle edges of a square coverslip to prevent the larvae from being crushed. After placing the coverslip over the glycerol, the slides were gently sealed with clear nail polish. Each slide contained 150 larvae per treatment per larval stage. The first 50 larvae that were observed per slide, regardless of their orientation, shape, and development, were photographed for morphological analysis with an Olympus binocular microscope (Olympus, Hamburg, Germany) under phase contrast and under polarized light. The

same microscope was equipped with polarizing filters for the birefringence analysis. To avoid bias, a coding system was used to prevent the person taking pictures and analyzing the photos from learning which slides corresponded to the particular treatments. All images were taken with a digital camera (DS-Ri1, Nikon) at 20X magnification and a 40-ms exposure. Images were analyzed in ImageJ software (1.52a).

Morphometric analysis

Larval development, shell formation, and shell size were analyzed for larvae lying on the lateral side (n = 125–133 larvae per treatment). We used semi-quantitative categorizations to analyze larval development, whereby each larva was scored on a scale from 0 to 2 as one of three morphological categories (Table 1; Fig. 2).

Table 1 Semi-quantitative morphological categories of abalone larvae at 24 and 72 hpf. Categories were based on shell formation and soft tissue morphogenesis according to the developmental cycle of *Haliotis tuberculata* at 17 ± 0.5°C (Jardillier et al. 2008).

Score	Shell formation	Soft tissue morphogenesis
2	Normal shell	Tissues normally developed
1	Shell partially and/or abnormally developed	Abnormal or partially developed
0	No shell	Undeveloped

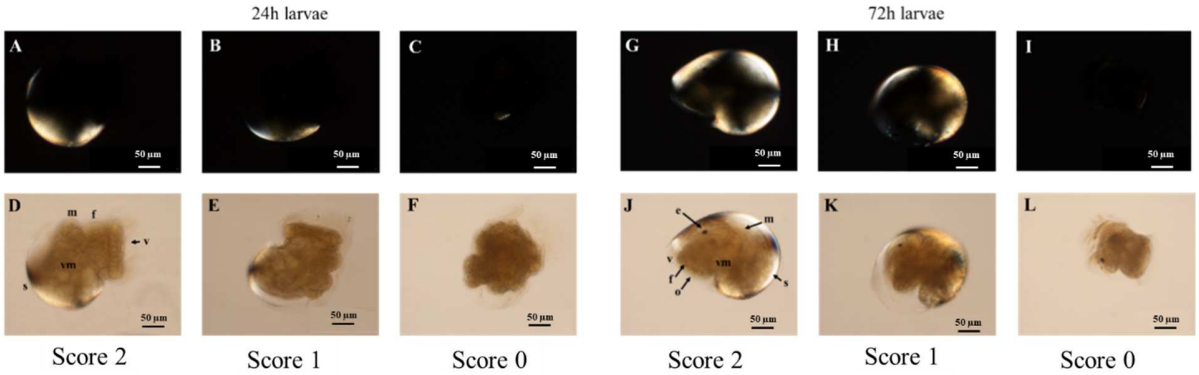


Fig. 2 Polarized microscope (A–C and G–I) and light microscope (D–F and J–L) images showing the morphological variables used for larval scoring at 24 hours post-fertilization (hpf; left) and 72 hpf (right). **A** and **G** Larvae with normal shell development (score = 2; the shell field covers the posterior area of the larval body, including the light half-circled area). **B** and **H** Partially and/or abnormally developed shell (score = 1). **C** and **I** Larvae with no shell development (score = 0). **D** Larvae at 24 hpf with normal tissue development (score = 2). **J** Larvae at 72 hpf with normal tissue development (score = 2) . **E** and **K** Partially and/or abnormally developed larvae (score = 1). **F** and **L** Underdeveloped larvae (score = 0). velum (v), mantle (m), shell (s), foot (f), visceral mass (vm), eyes (e), and operculum (o)

The maximum larval length (24 hpf) or total shell length (72 hpf) were measured using ImageJ software and used as indicators of larval size, with all individuals lying on the lateral side (Fig. 3). The mean length of larvae from each tank was calculated, and the average value for all tank replicates was presented as the mean larva size for each treatment (n = 3 tanks per treatment).

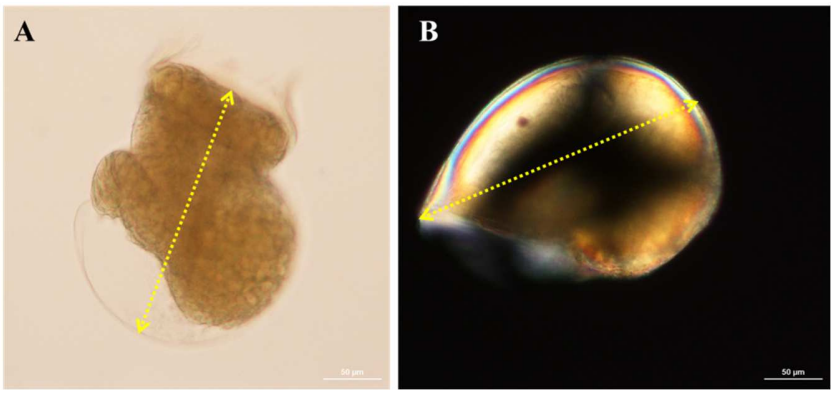


Fig. 3 Larval length measured at **A** 24 hours post-fertilization (hpf) and **B** 72 hpf. Arrow heads show the two ends of the measured lengths. Scales are 50 µm.

Birefringence analysis

We measured birefringence on a scale of 0–255 under cross-polarized light using an Olympus microscope, according to the method described by Wessel et al. (2018). Cross-polarized light that passes through $CaCO_3$ (an anisotropic material) is double-refracted, and shells with higher $CaCO_3$ content double-refract more light. Therefore, birefringence intensity can be used as a proxy for evaluating shell mineralization (Noisette et al., 2014). Birefringence was measured for at least 120 larvae per treatment at 24 and 72 hpf using ImageJ. The mean grayscale level (in pixels) was determined for each area of the larval shell showing birefringence, i.e., two zones for 24 hpf larvae, and three zones for 72 hpf larvae.

Scanning electron microscopy

Larvae stored in 70% ethanol were used for SEM analysis (four larvae per treatment). Samples were dehydrated in a series of increasingly concentrated ethanol solutions (90%, 95%, and 100%) and were critical point dried with liquid CO_2. Finally, the samples attached to the SEM

311 stubs were gold-coated and observed at 5 kV with a SIGMA 300 FE-SEM scanning electron
312 microscope (Plateau Technique de Microscopie Electronique, MNHN, Concarneau, France).

313

314 *Larval swimming behavior*

315 At 72 hpf, a 1-L sample was taken from each 350-L tank by carefully dipping a plastic bucket
316 into the middle of the tank and filling it to its maximum capacity. The bucket was covered with a
317 lid to minimize CO_2 exchange and ensure pH stability. The samples were kept in insulated boxes
318 to maintain their respective temperature treatments while they were transported (< 45 min) to the
319 Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) experiment station
320 (Argenton, France). Upon arrival, the buckets were placed in water baths adjusted to their
321 respective treatment temperatures to recover from their transport. After two hours, the samples
322 were examined for the behavioral analysis.

323 The pH treatments were randomly selected for behavioral analysis, alternating the elevated and
324 low temperatures. Before filtration, each bucket's temperature and pH were measured to ensure
325 that the treatment conditions had been maintained. Larvae were concentrated in a 40-µm sieve
326 and collected with a pipette. A total of 10–20 larvae per sample were distributed into 24-well
327 plates. The wells (16 mm in diameter) were filled with 500 µL of natural seawater with the same
328 temperature and pH as in their original treatment (n = 3 or 4 replicates per tank). All larvae were
329 allowed to habituate for 5 min prior to starting the test. Videos were recorded in the DanioVision
330 Observation Chamber® (Noldus Information Technology, the Netherlands) using a camera
331 (Basler, GigE) fitted with a 55-mm lens that was positioned 15 cm away from the microplate.
332 Each video was recorded for 30 seconds at 30 frames per second and 1920 × 1080 resolution. All

processed videos were analyzed under completely treatment-blind conditions using the software EthoVision XT 13.0 (Noldus Information Technology, Wageningen, the Netherlands). This software tracked active larval swimming for a maximum of 16 larvae simultaneously (not exceeded in our case) within a selected area. To reduce uncertainty, we pre-set a few criteria that were followed for each video. For example, if the larvae hit the walls of the well or if they collided, the behavioral variables after the collision were excluded from further analysis. Therefore, only larvae that were detected for at least 10 consecutive seconds without interruption were used in our analyses. If a larva did not move for 30 consecutive seconds, then no signal was detected by the program and a value of zero was indicated for the different behavioral variables of that larva.

The 2D_.distance moved was measured as the distance (cm) that each larva traveled from the center point of the well over 30 sec. The mean velocity ($cm \cdot ms^{-1}$) was calculated by dividing the distance moved by the unit of time (ms). The mean meander ($deg \cdot cm^{-1}$) was calculated as the mean change in a larva's direction of movement relative to its distance moved; this provided an indication of how convoluted the larva's trajectory was.

Larval metabolism

At 72 hpf, larval respiration rates were determined using closed incubations in acrylic respirometry chambers (Engineering and Design Plastics Ltd, Cambridge, UK). We carried out six incubations for each experimental tank: three incubations with larvae in 120-mL respirometry chambers, and three incubations with larvae-excluded seawater (control) in 80-mL respirometry chambers. We applied a correction to compensate for the differences in chamber volume. About

100 L of live larvae were siphoned from the experimental tanks and resuspended in 1 L of filtered seawater from the experimental tanks to reach a density of 100 larvae·mL^{-1}. The incubation chambers that contained larvae were filled with this same larval resuspension, whereas the control chambers were filled with filtered seawater from the experimental tanks. The chamber temperatures were maintained at the larval culture temperature during the incubations, either at room temperature (17°C) or at an high temperature (19°C) using a circulating water bath. The respiration rates were calculated from the measured differences in oxygen concentration during each incubation using a noninvasive fiber-optical system (FIBOX 3, PreSens, Regensburg, Germany) made up of an optical fiber and a reactive oxygen spot attached to the inner wall of each chamber. The reactive oxygen spots were calibrated using a 0% oxygen buffer that was prepared by dissolving 10 g of Na_2SO_3 into 1 L of seawater, and a 100% oxygen buffer that was prepared by bubbling air into 1 L of seawater for 20 min to achieve oxygen saturation. Oxygen consumption was measured over 1.5 to 2 hours and checked for linearity. Respiration rates were corrected for oxygen consumption in the controls and normalized to the number of larvae (µmol O_2.larva^{-1}·h^{-1}).

After each incubation (n = 9 per treatment), the exact number of larvae per chamber was determined from six 100-µL sample replicates per chamber that were distributed into a six-well plate.

For two of the three replicate incubations per experimental tank (n = 6 per treatment), the larvae were removed from the chambers, rinsed with distilled water to remove salt, and filtered through a pre-weighed Whatman GF/F 0.7-µm membrane. Filters containing larvae were dried in an oven at 60ºC for 48 h to determine their DW. The relative CaCO3 content (%) was calculated from the ash weight after burning at 550ºC for 5 h and the DW of each sample (g CaCO3·g^{-1} DW larvae).

Larval settlement

Larval settlement, which involves attachment to the substrate to achieve metamorphosis, occurs about 4 days after fertilization in *H. tuberculata* at 17 ± 0.5°C (Jardillier et al., 2008). Therefore, the larval settlement rate (%) was evaluated using premetamorphic 96 hpf veliger larvae. Twenty-four glass aquaria (20 cm W × 35 cm L × 20 cm H) were filled with 12 L of seawater at the same temperature and pH as in the original tanks. Temperatures were maintained by using a water bath set at 19°C for half of the aquaria. The other 12 aquaria were left at the low temperature of 17°C. We used a closed system with no water renewal to prevent the larvae from escaping from the small aquaria. Therefore, the pH of the acidified treatment could not be adjusted during the 24-h settlement period. As a result, the pH_T increased from 7.69 ± 0.04 at the beginning of the test to 7.86 ± 0.03 at the end of the test. In each aquarium, we installed two 10 cm × 15 cm vertical polycarbonate plates, one covered with the green microalga *Ulvella lens*, and one without *U. lens*. *Ulvella lens* is one of the major inductive cues for the settlement of abalone larvae (De Viçose et al., 2010, 2012; Daume et al., 2004), and the absence of such cues can substantially reduce larval settlement rates (Searcy-Bernal et al., 1992; Slattery, 1992; Daume et al., 1999). After estimating the density of the larvae, a 1-mL volume was distributed using a pipette into two aquarium pseudo-replicates (n = 6 per treatment). The volume was adjusted to create a similar density of the larvae in each aquarium. After about 24 h, we used a binocular microscope to record the number of larvae that had settled on each plate. The water in each tank was filtered using a 40-µm filter to collect any larvae that were unsettled or that had attached to the sides and bottom of the tank. We applied low-pressure seawater to remove any larvae that had attached to the tank. The larvae that were collected from each tank were preserved in 70% ethanol for later counting. Finally, we calculated the percentage of settled

larvae per plate type (blank and *Ulvella*-covered) per treatment using the average of the two pseudo-replicates per tank (formula 3).

$$(3) = \frac{\text{number of settled larvae}}{\text{total larvae}} \times 100$$

One tank was lost for the pH-elevated and 17°C treatment, resulting in 2 instead of 3 replicates for that treatment.

Statistical analyses

All statistical analyses were performed in R (version 4.0.5) and RStudio software (RStudio, Boston, United States). Differences in hatching success, percentage of swimming larvae, shell length, behavioral parameters, larval respiration rate, and larval settlement on *Ulvella*-covered plates were assessed using a two-way analysis of variance (ANOVA) with pH, temperature, and the interaction between pH and temperature as fixed factors. For all of the aforementioned parameters, for each sampling time, the data were averaged per tank (n = 12 in total). Subsequently, our replicates are tanks (not individual larvae). The normality of the residuals was verified with a Shapiro–Wilk's test, and the homogeneity of variances was tested using Levene's test. To study the effects of pH and temperature on the settlement rate on blank plates, we applied a Wilcoxon rank sum test with continuity correction due to the presence of many zero values, and because the normality of the residuals could not be verified. To study the interaction between pH and temperature, we used a four-treatment-level Kruskal-Wallis rank sum test followed by a post hoc Wilcoxon rank sum test with continuity correction. The p and F values of two-way ANOVA are presented in the results, unless otherwise indicated.

To assess the frequency of the morphological and developmental parameters, we performed a four-treatment-level (low pH - low temperature; low pH - high temperature; high pH - low temperature; high pH - high temperature) chi-squared ($\chi 2$) test to evaluate the effects of pH and temperature on larval phenotypes at 24 and 72 hpf.

Results

Carbonate chemistry and physicochemical characteristics of seawater

The mean seawater temperature, salinity, and carbonate chemistry parameters are presented in Table 2. The salinity was 35.18 ± 0.04 psu in all experimental tanks, and remained stable over the course of the experiment. A_T measured in the experimental tanks was 2364 ± 17 $\mu Eq \cdot kg^{-1}$ and remained stable over the course of the experiment and between experimental aquaria. The mean pH_T values were 7.95 ± 0.04 and 7.68 ± 0.12 for the high and low-pH treatments, respectively. The average temperatures were 17.40 ± 0.55°C and 19.35 ± 0.53°C for the low and high temperature treatments, respectively (means ± SD).

Table 2 Mean parameters of seawater carbonate chemistry during the experiment. Total-scale seawater pH (pH_T), temperature, salinity, and total alkalinity (A_T) were used to calculate the partial pressure of CO_2 (pCO_2; µatm), dissolved inorganic carbon (DIC; $\mu mol \cdot kg^{-1}$ SW), HCO_3^-, CO_3^{2-}, aragonite saturation state (Ωar), and calcite saturation state (Ωcalc) using CO2SYS software. The pH_T and temperature values shown represent the average value for each treatment, measured daily over the 5 days of the experiment (n = 15 per treatment). Salinity was measured once a day over the 5 days of the experiment (n = 5 per treatment). Results are expressed as mean ± SD.

446

Treatment name	Treatment (pH-T°C)	Temperature (°C)	pH_T	pCO_2 (µatm)	DIC	HCO_3^- (µmol·kg^{-1})	CO_3^{2-} (µmol·kg^{-1})	Ωar	Ωcalc
High pH – low temperature	**8.0–17°C**	17.5 ± 0.6	7.99 ± 0.04	478 ± 49	2122 ± 16	1950 ± 24	156 ± 10	2.40 ± 0.15	3.72 ± 0.23
Low pH – high temperature	**7.7–19°C**	19.4 ± 0.6	7.66 ± 0.12	1161 ± 319	2245 ± 45	2118 ± 58	88 ± 24	1.36 ± 0.37	2.10 ± 0.56
High pH – high temperature	**8.0–19°C**	19.3 ± 0.5	7.91 ± 0.05	593 ± 74	2147 ± 23	1985 ± 35	142 ± 14	2.20 ± 0.22	3.39 ± 0.34
Low pH – low temperature	**7.7–17°C**	17.3 ± 0.5	7.71 ± 0.11	999 ± 263	2236 ± 39	2109 ± 51	91 ± 21	1.40 ± 0.32	2.173 ± 0.49

447

448 *Hatching success*

449 The larval hatching success did not differ by temperature, pH, or the interaction of the two ($p >$
450 0.05; Table S1).

451

452

453 *Percentage of swimming larvae*

At 24 hpf, 72 hpf and 96 hpf the percentage of swimming larvaewas significantly lower at 19°C than at 17°C (24h hpf : $F_{1,8} = 19.92$, p = 0.002, Fig. 4A; 72 hpf : $F_{1,8} = 5.52$, p = 0.046, Fig. 4C; 96 hpf : $F_{1,8} = 13.47$, p = 0.006, Fig. 4E). However, no pH effect (24h hpf : $F_{1,8} = 0.41$, p = 0.539, Fig. 4A;72 hpf : $F_{1,8} = 3.11$, p = 0.115, Fig. 4C; 96 hpf : $F_{1,8} = 0.04$, p = 0.845, Fig. 4E) and no interaction between temperature and pH (24h hpf : $F_{1,8} = 0.09$, p = 0.764, Fig. 4B; 72 hpf : $F_{1,8} = 0.29$, p = 0.602; Fig. 4D; 96 hpf : $F_{1,8} = 0.3$, p = 0.618; Fig. 4F) were found.

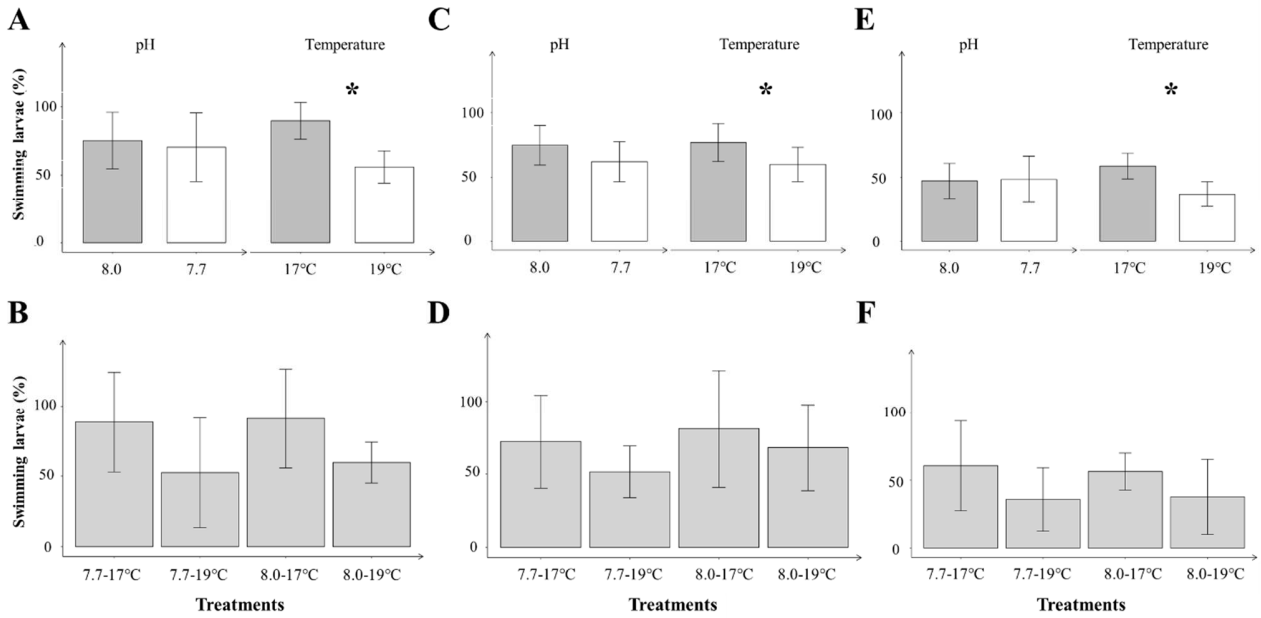


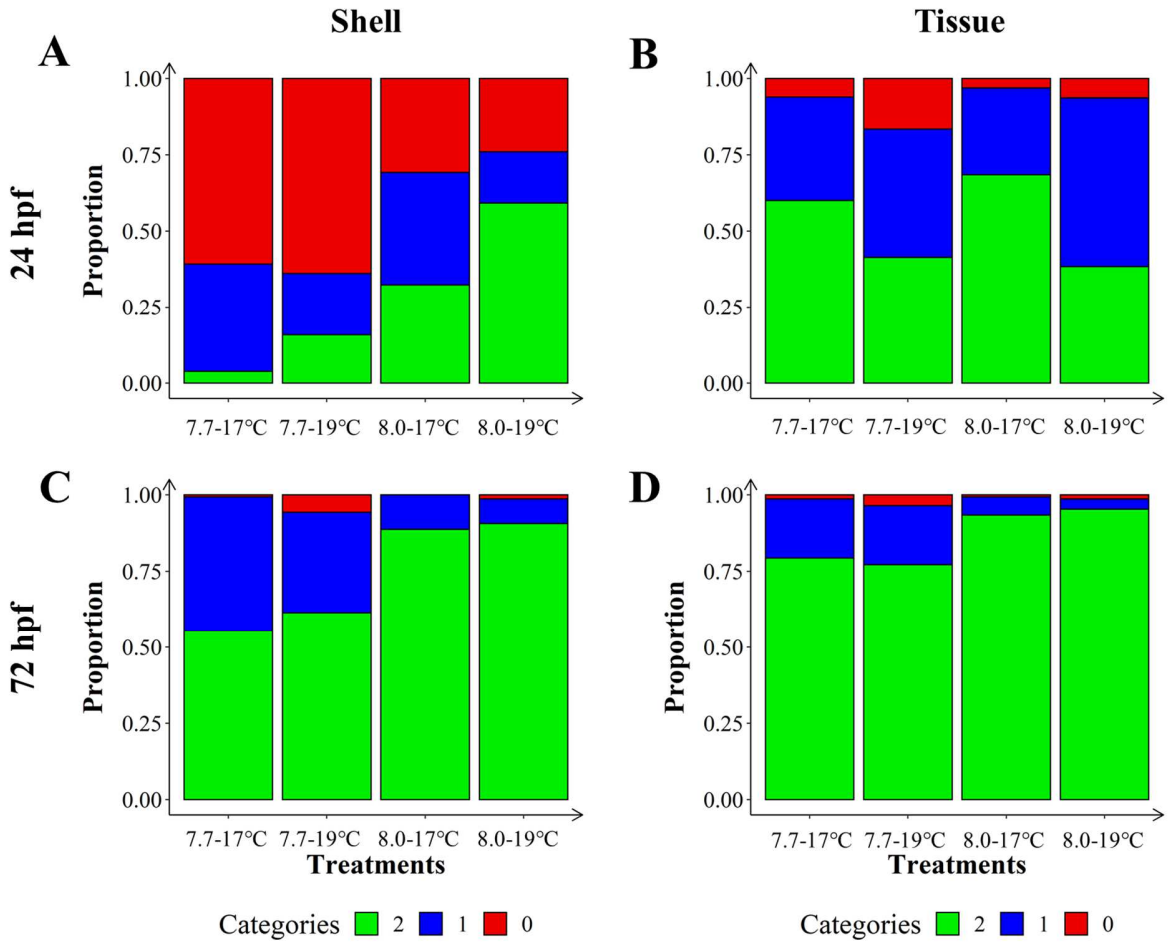
Fig. 4 Effects of pH and temperature on *Haliotis tuberculata* percentage of swimming larvae at **A)** 24 hours post-fertilization (hpf), **C)** 72 hpf, and **E)** 96 hpf (n = 6 tanks per treatment). Combined treatment effects of pH and temperature on *H. tuberculata* percent swimming larvae at **B)** 24 hpf, **D)** 72 hpf, and **F)** 96 hpf (n = 3 tanks per treatment). bar values represent means and error bars represent the standard deviation of the mean. Asterisks denote significant differences between temperature treatments by two-way ANOVA.

469

470 *Morphometric characteristics*

471 <u>*24 hpf larvae:*</u> Shell formation was significantly affected by temperature (X^2 (2) = 54.59, p <
472 0.001), pH (X^2 (2) = 94.82, p < 0.001), and the four treatment levels (low pH - low temperature,
473 low pH - high temperature, high pH - low temperature, high pH - high temperature); X^2 (6) =
474 130.71, p < 0.001) (Fig. 5A; Table S2). Larvae exposed to pH 7.7 were 4.5 times less likely to
475 have normal shell formation compared with larvae exposed to pH 8.0 (9.9% at pH 7.7 versus
476 45% at pH 8.0; Table S2). Larvae exposed to 19°C temperatures were 3 times as likely to have
477 normal shell formation compared with larvae exposed to 17°C temperatures (45% at 19°C versus
478 15% at 17°C; Table S2). The larvae exposed to the low pH and low temperature treatment had
479 the lowest proportion of normal shell formation, whereas larvae exposed to the high pH and high
480 temperature treatment had the highest proportion of normal shell formation (4% vs 59%,
481 respectively) (Fig. 5A; Table S2).

482 Similarly, tissue organogenesis at 24 hpf was significantly affected by temperature (X^2 (2) =
483 24.75, p < 0.001), pH (X^2 (2) = 7.83, p = 0.020), and the four treatment levels (low pH - low
484 temperature, low pH - high temperature, high pH - low temperature, high pH - high temperature;
485 (X^2 (6) = 45.46, p < 0.001) (Fig. 5B; Table S2). Larvae exposed to the high temperature had a
486 lower proportion of normally developed tissues than did larvae exposed to the low temperature
487 (39% at 19°C versus 61% at 17°C; Table S2), whereas larvae exposed to both low-pH and high
488 pH conditions had similar tissue organogenesis (51% at pH 7.7 versus 54% pH 8.0).



489

490 **Fig. 5** Proportional distribution of larvae at 24 hours post-fertilization (hpf) (**A** and **B**) and at 72
491 hpf (**C** and **D**) according to their morphological categories. Colored bars indicate the degree of
492 malformation for each variable. A and C: shell formation score (2 = normal shell; 1 = partially
493 and/or abnormally developed shell; 0 = no shell). B and D: tissue organogenesis score (2 =
494 normally developed tissues; 1 = partially or abnormally developed tissues; 0 = undeveloped
495 tissues). The frequencies of each category are shown (n = 518 larvae in total).

496

497 *72h larvae:* Shell formation was significantly affected by temperature (X^2 (2) = 10.61, p =
498 0.005), pH (X^2 (2) = 71.19, p < 0.001), and the four treatment levels (low pH - low temperature,

low pH - high temperature, high pH - low temperature, high pH - high temperature; X^2 (6) = 84.76, $p < 0.001$) (Fig. 5C; Table S2). Larvae exposed to pH 7.7 had a significantly lower proportion of specimens with normal shell development compared with larvae exposed to the high pH of 7.9 (60% at pH 7.7 versus 90% at pH 8.0) (Fig. 5C; Table S2). The larvae in the high pH and high temperature treatment showed the highest proportion of specimens with normal shell development (92%), followed by those in the high pH and low temperature treatment (88%). Larvae exposed to the low-pH and low temperature treatment had the lowest proportion of specimens with normal shell development (56%; Fig. 5C; Table S2).

Tissue organogenesis at 72 hpf was significantly affected by pH (X^2 (2) = 36.15, $p < 0.001$) and by the four treatment levels (low pH - low temperature, low pH - high temperature, high pH - low temperature, high pH - high temperature; X^2 (6) = 39.54, $p < 0.001$) (Fig. 5D; Table S1), but was not significantly affected by temperature (X^2 (2) = 2.70, $p = 0.26$). Larvae exposed to the low-pH had a significantly lower proportion of specimens with normally developed tissues (78%) compared with larvae exposed to the high pH (95%; Table S2).

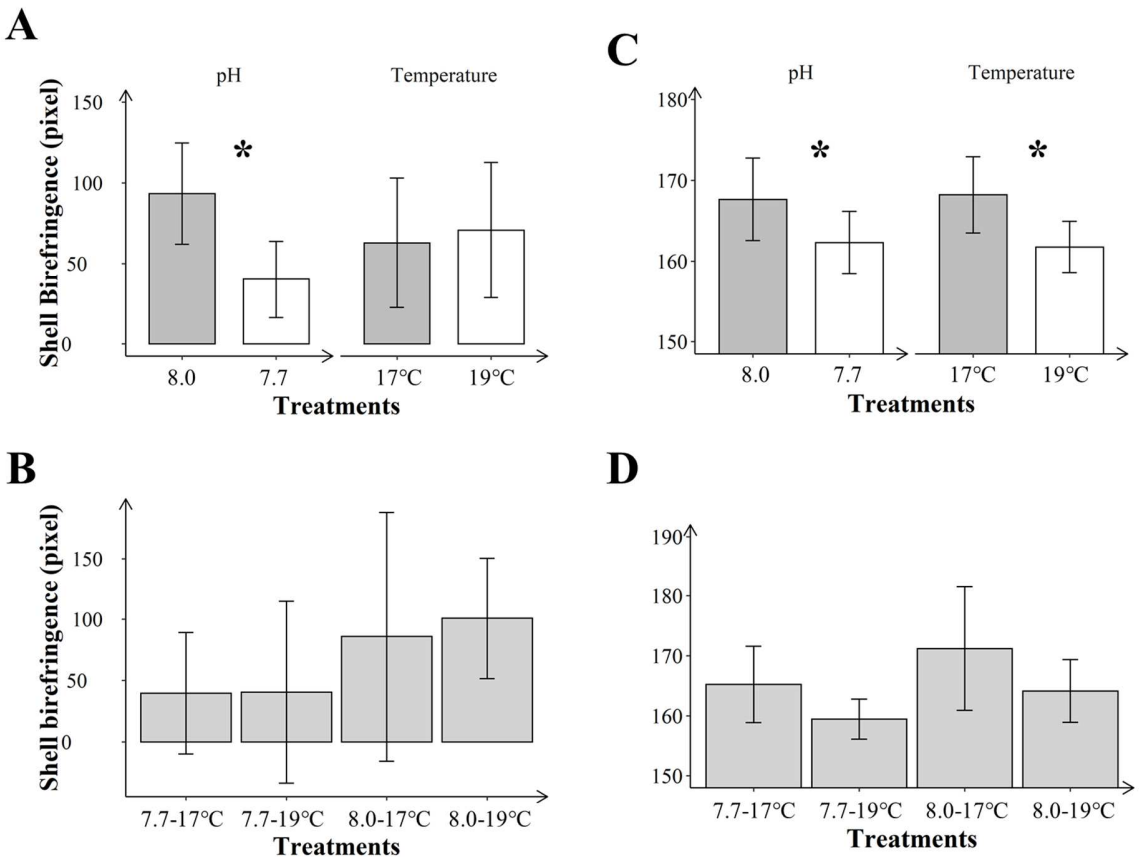
Shell length

The shell length of the 24-hpf larvae could not be measured in the low-pH treatments because there were very few larvae with normally developed shells (Fig. 5A). The shell length of the 72-hpf larvae was significantly shorter in the low-pH treatments compared with the high pH treatments ($F_{1,8}$ = 11.90, $p = 0.008$; pH 8.0_{mean} = 265.01 ± 6.69 µm; pH 7.7_{mean} = 258.57 ± 10.61 µm). However, the temperature had no effect on larval shell length at 72 hpf ($F_{1,8}$ = 4.66, $p = 0.063$; $17°C_{mean}$ = 264.83 ± 8.49 µm; $19°C_{mean}$ = 260.28 ± 8.89 µm). No interaction between

temperature and pH was observed ($F_{1,8}$ = 1.60, p = 0.24; 8.0–17°C$_{mean}$ = 265.92 ± 6.92 μm; 8.0–19°C$_{mean}$ = 264.09 ± 6.34 μm; 7.7–17°C$_{mean}$ = 263.00 ± 10.38 μm; 7.7–19°C$_{mean}$ = 254.44 ± 9.09 μm).

Shell calcification

The mean birefringence (number of grayscale pixels) at 24 hpf was significantly lower at pH 7.7 than at the high pH of 7.9 ($F_{1, 8}$ = 8.55, p = 0.019; Fig. 6A). However, birefringence (and correspondingly, shell calcification) did not differ significantly by temperature ($F_{1, 8}$ = 1.33, p = 0.281; Fig. 6A) or by the interaction between temperature and pH ($F_{1, 8}$= 0.01, p = 0.920; Fig. 6B). The mean birefringence at 72 hpf was significantly lower in the pH 7.7 compared with pH 8.0 ($F_{1, 8}$ = 12.21, p = 0.008) and 19°C compared with 17°C ($F_{1, 8}$ = 17.83, p = 0.003) treatments, indicating reduced shell calcification in low pH treatment and high temperature treatment compared with the ambient pH and temperature respectively (Fig. 6C). However, the interaction between temperature and pH did not significantly affect shell birefringence at 72 hpf ($F_{1, 8}$ = 0.08, p = 0.784; Fig. 6D).



536

537 **Fig. 6** Individual effects of pH and temperature on mean shell birefringence of larvae at **A)** 24
538 hpf and **C)** 72 hpf (n = 6 tanks per treatment). Combined effects of pH and temperature on mean
539 shell birefringence of larvae at **B)** 24 hpf and **D)** 72 hpf (n = 3 tanks per treatment). bar values
540 represent means and error bars represent the standard deviation of the mean. Asterisks indicate
541 significant differences between either temperature or pH treatments, as determined by two-way
542 ANOVA.

543

544

545

546

547 *Scanning electron microscopy*

548 In the high pH treatments (7.9), the shell surface of larvae observed at 24 hpf showed a normal
549 granular texture with an alveolar pattern (Fig. 7A, B, E, F). However, the larvae reared in the
550 low-pH treatments (7.6) showed signs of shell deformation and microfractures (Fig. 7C, D).
551 Details of the shell surface boxed in Figures 7C and 7D revealed irregularities and mineralization
552 defects that were probably due to shell dissolution. pH 8.0The shell surface of larvae observed at
553 72 hpf were of similar shape and pattern in both the low (7.7) and high (8.0) pH treatments (Fig.
554 8A–D). At higher magnification, the shell surfaces of high pH larvae revealed a homogeneous
555 granular texture and alveolar pattern (Fig. 8E, F). In the low-pH treatments, the details of the
556 shell surface boxed in 8C and 8D revealed surface heterogeneities and small holes within the
557 alveolar network suggesting mineral dissolution. (Fig. 8G, H)

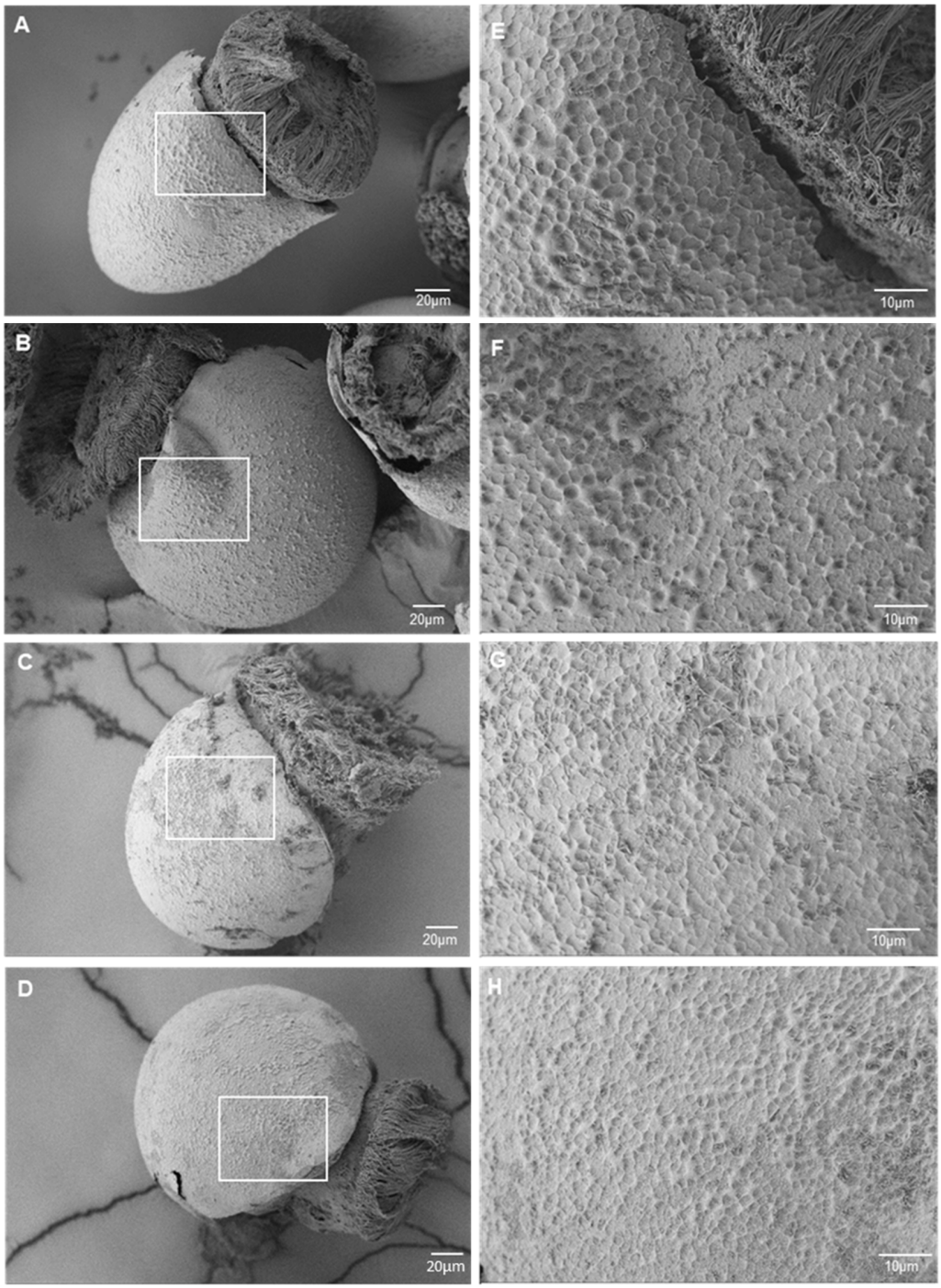
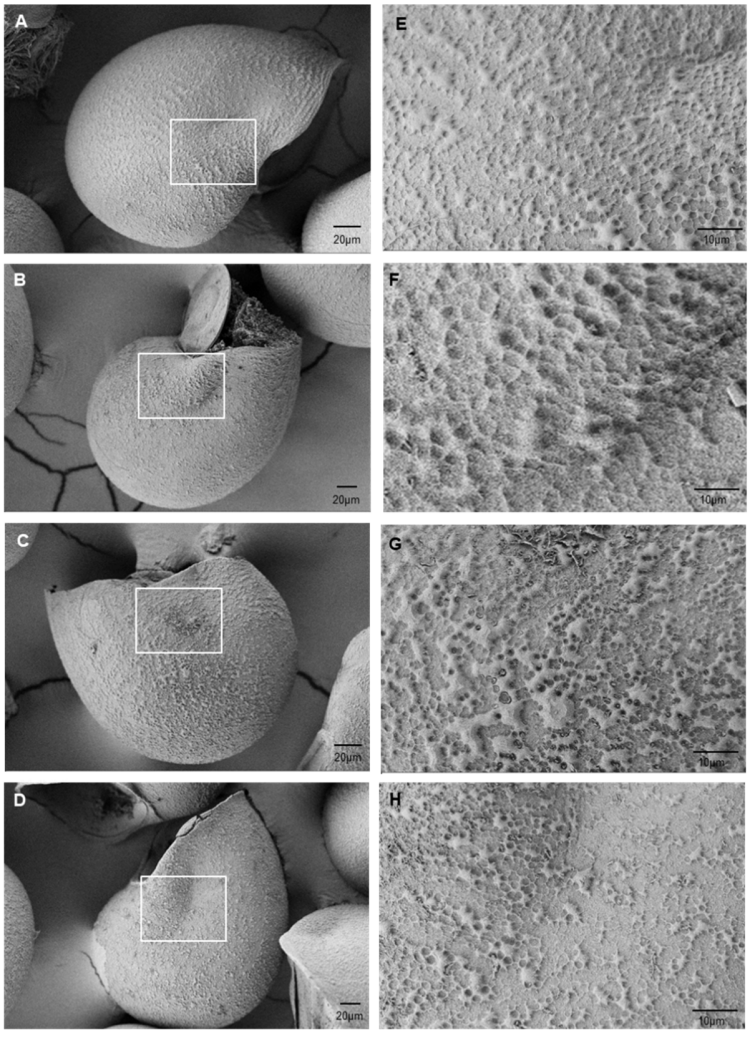


Fig. 7 Scanning electron microscopy (SEM) images of *Haliotis tuberculata* larvae at 24 hpf in the four experimental treatments **A)** pH 8.0–17°C; **B)** pH 8.0–19°C; **C)** pH 7.7–17°C; and **D)** pH

561 7.7–19°C. Lateral views of the larvae show the protoconch shell and the velum. **E–H** show
562 magnified views of the corresponding white squares in A–D.

563

564 The shell surface of larvae observed at 72 hpf were of similar shape and pattern in both the low
565 (7.7) and high (8.0) pH treatments (Fig. 8A–D). The magnified areas of the shells in all
566 treatments revealed a typical granular texture and alveolar pattern (Fig. 8E–H). However, the
567 surface heterogeneities and small holes within the alveolar network in the low-pH treatments
568 (Fig. 8G, H) suggest mineral dissolution.



569

570 **Fig. 8** Scanning electron microscopy (SEM) images of *Haliotis tuberculata* larvae at 72 hpf in
571 the four experimental treatments **A)** pH 8.0–17°C; **B)** pH 8.0–19°C; **C)** pH 7.7–17°C; and **D)** pH
572 7.7–19°C. Lateral views of the larvae show the well-developed protoconch covering the larval
573 body. In **A**, the protoconch is well developed and covers the larval body. In **B**, the operculum
574 closes the shell aperture after complete retraction of the veliger. **E–H** show magnified views of
575 the corresponding white squares in A–D.

576

577

578 *Larval behavior*

579 None of the larval behavioral parameters (Distance moved (total cm^{-1}), mean velocity ($cm \cdot ms^{-1}$),
580 mean meander ($deg \cdot cm^{-1}$) differed significantly due to pH, temperature, or the interaction
581 between pH and temperature (Table S3).

582

583

584

585 *Respiration rate*

586 The respiration rate of larvae did not differ significantly between pH ($F_{1, 8} = 1.54$, $p = 0.250$; two-
587 way ANOVA), or temperature ($F_{1, 8} = 2.57$, $p = 0.15$; two-way ANOVA). No interaction was
588 found between pH and temperature for larval respiration rate ($F_{1, 8} = 5.66$, $p = 0.045$).

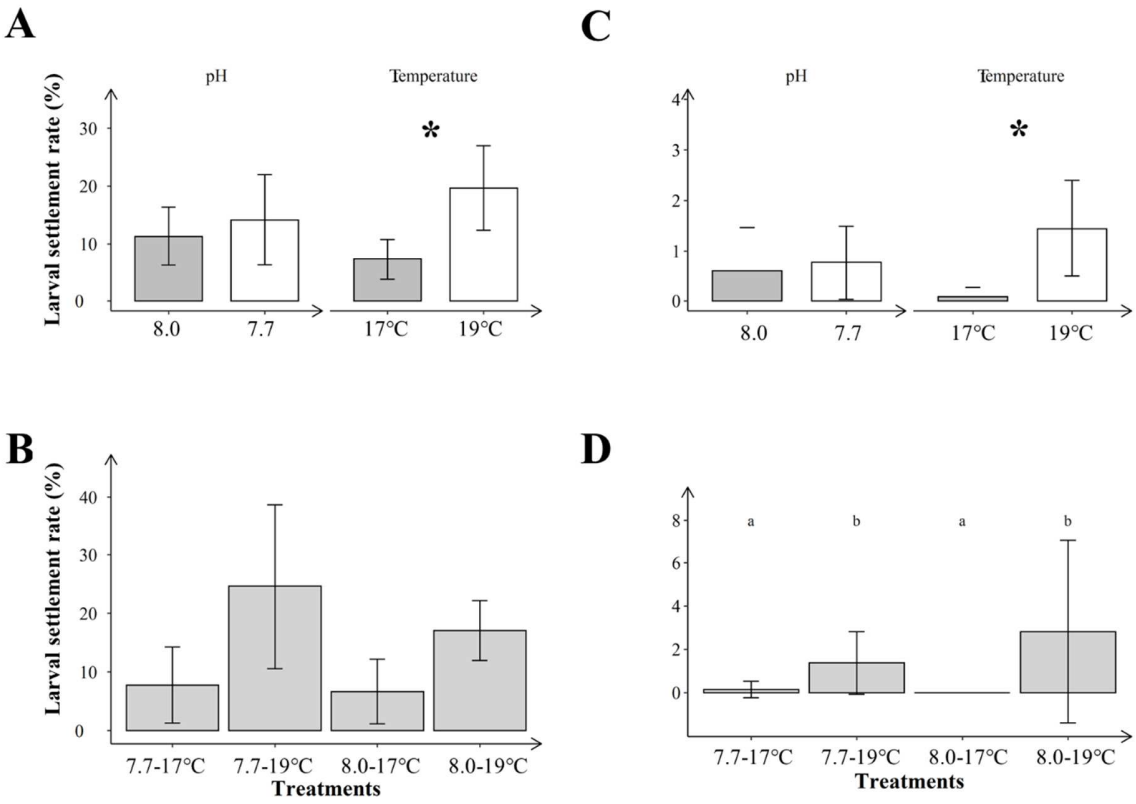
589

590

591 *Larval settlement*

592 The larval settlement on *Ulvella*-covered plates was significantly higher in the high temperature
593 (19°C) treatments than in the low temperature (17°C) treatments ($F_{1,7}$ = 14.02, p = 0.007; Fig.
594 9A). No significant differences were observed between pH treatments ($F_{1,7}$ = 1.40, p = 0.275;
595 Fig. 9A) or from the interaction between pH and temperature ($F_{1,7}$ = 0.47, p = 0.52; Fig. 9C).

596 On blank plates, we found significant effects from temperature (W = 0, p = 0.005; Wilcoxon rank
597 sum test with continuity correction; Fig. 9C) and from the four treatment comparisons (H (3) =
598 8.70, p = 0.033; Kruskal–Wallis) (Fig. 9D). Larval settlement on the blank plates was
599 significantly higher in the high temperature (19°C) treatments than in the low temperature (17°C)
600 treatments (Fig. 9B). No significant differences were found between the low-pH and high pH
601 treatments (W = 14, p = 0.923; Wilcoxon rank sum test with continuity correction; Fig. 9C).



602

603 **Fig. 9** Effects of pH and temperature on larval settlement on **A)** plates covered with the green
604 microalga *Ulvella lens* (n = 6 tanks per treatment; two-way ANOVA) and **B)** blank plates (n = 6
605 tanks per treatment; Wilcoxon rank sum test with continuity correction). Combined effects of pH
606 and temperature on larval settlement on **C)** *Ulvella*-covered plates and **D)** blank plates at 96
607 hours post-fertilization (hpf) (n = 3 tanks per treatment). bar values represent means and error
608 bars represent the standard deviation of the mean. Different lowercase letters indicate significant
609 differences among treatments. Asterisks indicate significant differences between either
610 temperature or pH treatments.

611

612

Discussion

In this study, we exposed *H. tuberculata* larvae to the individual and combined effects of OA and OW. We found that a 0.3-unit decrease in pH induced decreases in or no effects on larval responses, whereas a 2°C increase in temperature caused broader effects, resulting in either increased/enhanced, decreases/diminished larval responses or had no impacts. However, no significant interactive effects of temperature and pH were observed for the variables measured. Despite that, some treatment effects were recorded for the parameters that were not analyzed using ANOVA (Table 3). Among the biological parameters investigated in this study, shell formation, length, and calcification appeared to be the most sensitive to the effects of temperature and pH. The results of this study lend further support to the results from recent meta-analyses suggesting that the interactive effects of OA and OW on calcifying species, including mollusks, are not as ubiquitous as OA's and OW's individual effects (Kroeker et al., 2013; Przeslawski et al., 2015).

The hatching success of trochophore larvae of *H. tuberculata* was not significantly affected by temperature, pH, or the interaction of the two. These results are in line with those of Pedroso (2017), who found no impact on the hatching success of *H. asinina* in response to raising the ambient temperature of 29°C by 2°C. The lack of significant effects by pH or by the interaction of pH and temperature was likewise similar to previous findings. Guo et al. (2015) found no effect of pH_{NBS} 7.94 on the hatching success s of *H. diversicolor* and *H. discus*, whereas both species experienced reduced hatching success s when the pH value was decreased to 7.71 or lower, compared with the high pH of 8.15. In two separate studies on the hatching success of *H. discus hannai*, Kimura et al. (2011) and Li et al. (2013) found no impact of exposure to pH_{NBS} 7.6 and higher, compared with pH of 8.02. Likewise, Tahil and Dy (2016) found that pH_{NBS} 7.78

636 did not affect the *H. asinina* hatching success compared to the pH of 7.97; however, pH
637 treatments of 7.60 and 7.40 negatively affected the hatching success. Our results show that *H.*
638 *tuberculata* larvae can hatch at the lower pH values and high temperatures expected in the
639 coming decades. Such tolerance to OA and OW in the early larval stages has already been
640 reported for other marine species (Przeslawski et al., 2015), and might originate from protective
641 factors in the egg (Hamdoun and Epel, 2007).

642 An increased number of larvae with tissue abnormalities was observed under high temperature
643 treatments. This was accompanied by significant declines in the percentage of swimming larvae
644 in the high temperature treatments compared to the low temperature treatments at 24, 72 and 96
645 hpf. Low pH, also, reduced the normal tissue development of the larval stages. A study on *H.*
646 *asinina* found < 1% of normal trochophores at the reduced pH of 7.60, compared with the
647 ambient pH of 7.97 (Tahil and Dy, 2016). Parker et al. (2011) also reported slower larval
648 development in the Sydney rock oyster, *Saccostrea glomerata*, when the ambient pH (pH_{NBS} 8.2)
649 was reduced by 0.3 pH units. A study by Wessel et al. (2018) on *H. tuberculata* showed a
650 significant increase in the number of 30-hpf larvae with abnormal and delayed development at
651 pH_T 8.0compared with larvae at pH_T 7.7 and 7.6. These findings suggest that a difference of only
652 a few hours of exposure to stressors can influence their effects during larval development.

653 Larval shell formation was also impaired under low pH, similar to previous studies on *H.*
654 *coccoradiata* (pH_{NBS} = 7.8 and 7.6 compared with the ambient pH of 8.2; Byrne et al., 2011); *H.*
655 *kamtschatkana* (reported as pCO_2 values of 800 µatm and 1800 µatm compared with a control of
656 400 µatm; Crim et al., 2011); *H. asinina* (pH_{NBS} = 7.85 and 7.65 compared with the ambient pH
657 of 8.15; Santander-De Leon et al., 2018); and *H. tuberculata* (pH_T = 7.68 and 7.58 compared
658 with the ambient pH of 8.00; Wessel et al., 2018). Shell calcification of *H. tuberculata* larvae

659 was also reduced under low pH, as shown by the decrease in birefringence intensity measured at
660 24 and 72 hpf. Additional SEM observations revealed differences in larval shell texture and the
661 presence of numerous small holes on the outer shell surface, suggesting that $CaCO_3$ dissolution
662 may be responsible for the reduced calcification seen in larvae raised at a lower pH. The lack of
663 calcification observed under cross-polarization microscopy and SEM is consistent with previous
664 results from Wessel et al. (2018), and provides further evidence that abalone larvae deposit less
665 $CaCO_3$ and produce more fragile and thinner shells when exposed to OA. Our results confirm, as
666 reported for numerous mollusk species, that the growing calcified shells of larval and juvenile
667 abalones are highly sensitive to OA (Gazeau et al., 2013; Przeslawski et al., 2015). Naturally
668 occurring *H. tuberculata* populations may be at greater risk from future pH reduction, as early
669 shell alterations may impair larval development and recruitment.

670 In this study, the impaired process of shell formation induced by low pH was mitigated by high
671 temperature, resulting in a higher proportion of 24- and 72-hpf larvae with normal shell
672 development as shown in Fig. 5A and 5C. Observations of temperature reducing the negative
673 impacts of OA on shell calcification and development have been previously reported for larval
674 and juvenile mollusks (Davis et al., 2013; Ko et al., 2014).

675 Shell length of *H. tuberculata* larvae was significantly reduced by low-pH conditions, but no
676 temperature or interaction effects were observed. Crim et al. (2011) reported a 5% reduction in
677 larval shell size in the northern abalone *H. kamtschatkana* after an 8-day exposure to acidified
678 conditions (reported as CO_2 levels 400 µatm vs 800 µatm), which is consistent with our results
679 (4% reduction at 24 hpf and 6% reduction at 72 hpf). Similarly, the larvae of *H. asinina* (pH_{NBS} =
680 7.85 and 7.65 compared with the ambient pH of 8.15; Santander-De Leon et al., 2018); *H.*
681 *diversicolor*; and *H. discus hannai* (pH_{NBS} = 7.94 and lower compared with the high pH of 8.15;

682 Guo et al., 2015) experienced reductions in shell length at low-pH compared with high pH
683 conditions. In a study by Onitsuka et al. (2018), larvae of *H. discus hannai* at pH_{NBS} 7.79 did not
684 show any significant reduction in shell length compared with larvae at the high pH_{NBS} of 7.99.

685 In our study, the lack of significant effects on shell size from temperature or from the interaction
686 between temperature and pH is consistent with results obtained for other marine mollusks. For
687 example, Thiyagarajan and Ko (2012) reported no impact of high temperature (30°C compared
688 with 24°C), or temperature–pH interaction on the shell size of the Portuguese oyster *Crassostrea*
689 *angulata*. On the other hand, Talmage and Gobler (2011) recorded decreased larval size in two
690 bivalves (*Mercenaria mercenaria* and *Argopecten irradians*) resulting from both a reduced
691 pH_{NBS} of 7.8 (compared with the ambient pH of 8.08) and an high temperature of 28°C
692 (compared with the ambient temperature of 24°C). These contrasting effects confirm a high
693 variability in the responses of marine mollusk larvae to both isolated and combined stressors.

694 The absence of direct effects of pH and temperature on larval physiology and behavior does not
695 rule out the existence of their indirect effects through cellular and molecular mechanisms that
696 were not measured in the present study. Indeed, larval developmental stages are characterized by
697 huge changes (in physiology and sensitivity) over a short period of time (5 days) and the
698 measurements of physiological and behavioral variables do not account for the whole
699 developmental processes that may be impacted by the stressors. Complementary studies at the
700 molecular level (i.e., gene expression, protein and enzyme synthesis, etc.) will be needed to fully
701 understand the fine developmental changes induced by the climate change drivers (Portner et al.
702 2010).

703

Larval settlement was greater at high temperatures than at the low temperature on both *Ulvella*-covered and blank plates. The shorter larval duration that corresponded with high temperature in this study is consistent with previous studies of related *Haliotis* species, such as *H. sorenseni*, *H. rufescens*, *H. corrugata,* and *H. fulgens* (Leighton, 1972, 1974). This suggests that high temperatures resulting from global warming reduce the duration of the planktonic larval stage and accelerate the settlement rate (Byrne et al., 2011). This may be an advantage (Byrne et al., 2011), as a longer planktonic stage may reduce the chances of survival due to increased risk of predation and exposure to other environmental stresses (Parker et al., 2013). We did not record any significant effects of pH on *H. tuberculata* settlement, which is in line with the findings of another study on *H. kamtschatkana* (Crim et al., 2011). However, the literature contains contradictory results, with a tendency toward a lower settlement rate of the New Zealand abalone, *H. iris*, in low-pH conditions (Espinel-Velasco et al., 2020) as well as for the donkey's ear abalone, *H. asinina*, in three low-pH treatments (Tahil and Dy, 2015).

Our results showed that in every treatment, the settlement rate of *H. tuberculata* larvae on *Ulvella*-covered plates was higher than the settlement rate on blank plates by a factor of at least 15. This supports the notion that settlement cues increase marine invertebrate larval settlement (Hadfield and Paul, 2001; Pawlik, 1992; Rodriguez et al., 1993). However, lowered pH may cause indirect effects, due to the effect of low pH on the settlement substrate. For example, O'Leary et al. (2017) reported that the settlement rate of *H. rufescens* under both present-day and low-pH treatments increased from 11% in tanks with no crustose coralline algae, to ~70% in the presence of crustose coralline algae. This raises another concern regarding the indirect impacts of OA and OW on abalone larval settlement. Coralline algae are common settlement cues for abalone larvae (De Viçose et al., 2010, 2012; Williams et al., 2008; Roberts et al., 2004; Roberts

and Nicholson, 1997), and they are decreasing due to OA and OW (Kuffner et al., 2008; Martin and Gattuso, 2009; Diaz-Pulido et al., 2012; Hofmann et al., 2012). Therefore, even in the absence of the direct impacts of OA and OW on abalone larvae, such environmental changes may indirectly reduce larval settlement by reducing their settlement cues. On the other hand, if *Ulvella* species, like many non-calcifying macroalgae, can benefit from high temperatures and reduced pH (Koch et al., 2013; Hofmann et al., 2012), then abalone larvae may be able to maintain their normal settlement rates. This presents an area requiring further examination.

None of the other behavioral responses (Distance moved (total cm^{-1}), mean velocity ($cm \cdot ms^{-1}$), mean meander ($deg \cdot cm^{-1}$),) differed as an effect of pH, temperature, or the interaction between the two. These outcomes parallel the responses of 2-day-old larvae of the Pacific oyster, *Crassostrea gigas*, when exposed to high pH and low (-0.3 units) pH conditions (Valentini, 2019). Although the method used in this research to study larval behavior is very common, it is noted that applying such a two-dimensional method may not allow the abalone larvae to express natural swimming behavior. Therefore, developing three dimensional methods are desired for future studies.

The lack of significant changes in larval behavior may partly justify the lack of changes observed in larval respiration. Larval respiration of *H. tuberculata* did not differ due to the individual or combined factors of pH and temperature. Although very low pH and highly high temperatures (i.e., out of the projected ranges for the year 2100) are known to amplify respiration (Campanati et al., 2018; Waldbusser et al., 2015; Liu and He, 2012; Padilla-Gamiño et al., 2013), some studies have confirmed that respiration was not impacted by a -0.3 unit pH reduction and/or a +3°C temperature increase above the ambient conditions (Frieder et al., 2017). For example, the respiration rate of the larvae of *Mytilus californianus* did not change when the ambient pH was

reduced from 8.3 to 7.8 (Waldbusser et al., 2015). Similarly, Campanati et al. (2018) found no impact on the oxygen consumption of *Reishia clavigera* larvae when the ambient pH was reduced from 8.1 to 7.6. High pCO_2 in seawater likely increases the maintenance costs of acid–base homeostasis, the intracellular ion balance required for protein folding and pH-sensitive physiological processes (Rivest and Hofmann, 2014; Lefevre, 2016). Therefore, our observation in this study of a lack of increase in *H. tuberculata* metabolic rates under acidified conditions may suggest that the larvae were able to use existing pools of ion pumps to conserve their acid–base homeostasis (Portner and Reipschlager, 1996; Guppy and Withers, 1999).

Table 3 Full results summary showing the effects of temperature, pH, and their interaction on the various parameters observed during this study. Arrow direction indicates whether these factors decreased/diminished (↓), increased/enhanced (↑), or had no significant effect (ns) on the measured parameters of *H. tuberculata* at 24, 72, and 96 hours post-fertilization (hpf). Empty cells indicate that the corresponding results were not applicable because they were not measured. Where nonparametric tests were used (i.e., for shell formation and tissue formation), treatment impacts are shown instead of interaction effects.

Parameter	**Temperature effect**			**pH effect**			**Interaction effect**		
	24 hpf	**72 hpf**	**96 hpf**	**24 hpf**	**72 hpf**	**96 hpf**	**24 hpf**	**72 hpf**	**96 hpf**
Hatching success	ns			ns			ns		
Percentage of swimming larvae	↓	↓	↓	ns	ns	ns	ns	ns	ns

Shell formation	↑	↓		↓	↓		8.0 - 17°C = ambient 7.7 - 19°C =↓ 8.0 - 19°C = ↑ 7.7 - 17°C =↓	8.0- 17°C = ambient 7.7 - 19°C =↓ 8.0 - 19°C = ns 7.7 - 17°C =↓	
Tissue formation	↓	ns		↓	↓		8.0 - 17°C = ambient 7.7 - 19°C = ↓ 8.0 - 19°C = ↓ 7.7 - 17°C = ns	8.0 - 17°C = ambient 7.7 - 19°C = ↓ 8.0 - 19°C = ns 7.7 - 17°C = ↓	
Shell length		ns			↓			ns	
Shell calcification	ns	↓		↓	↓		ns	ns	
Respiration rate		ns			ns			ns	
Larval swimming behaviors		ns			ns			ns	
Larval settlement			↑			ns			ns

766

767 In conclusion, the majority of the responses of *H. tuberculata* larvae were affected by low pH
768 and high temperature, but not by their interactions as summarized in Table 3. We did not detect
769 any significant interaction between the high temperature and low-pH stressors. However, such
770 interactions may exist, but we were not able to detect them using non-parametric tests. . For
771 example, although high temperature accelerated shell formation at 24 hpf, this was partially
772 mitigated by low pH. High temperature, however, turned into a threat by increasing the larval
773 tissue malformation at 24 hpf. High temperature decreased the percentage of swimming larvae at

all stages. It also declined shell calcification at 72 hpf despite the fact that it did not affect that of larvae at 24 hpf. By the end of the larval stage at 96 hpf, high temperature accelerated the larval settlement rate, which might reduce its detrimental effects on larval survival. Low pH, however, did not have any impact on the larval settlement. Low pH impaired all shell formation related parameters in all stages. However, like high temperature, it did not affect processes such as hatching success, respiration rate and swimming behaviors. Even though the projected rates of pH decrease and temperature increase over the coming decades may not seem to be an obstacle for *H. tuberculata* larval production, it seems that under OA and OW, the majority of this species' responses are susceptible to changes in either temperature or pH. Thus, *H. tuberculata* larvae will have to cope with both stressors. In nature, this may happen through adaptation. In abalone farms, this can occur using various strategies. For example, first, by monitoring of the daily changes in seawater pH, it can be arranged to and tadjustment of avoid pumping low pH seawater into the farms whenduring the hours when the CO2 concentrations in the seawater is at its peak ion (Murie and Bourdeau 2020). If farms have access to large-volume storage tanks, adding kelp species to the tanks may reduce CO2 concentrations as kelp canopies have the ability to alter local seawater chemistry (Murie and Bourdeau 2020). Inclusion of an integrated multi-trophic aquaculture system based on algae might be helpful (Bolton et al. 2009) Addition of chemicals to increase seawater alkalinity and/or pH might be another solution for the hatcheries with a recirculating system. The selection of tolerant broodstocks to OA and GW can be also undertaken. However, due to the limitations of each of the aforementioned methods, the combinations of them may lead to more sustainable abalone productions.

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