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Victoria Díaz-Castañeda, T. Erin Cox, Frédéric Gazeau, Susan Fitzner, Jérémy Delille, et al.. Ocean acidification affects calcareous tube growth in adults and reared offspring of serpulid polychaetes. *Journal of Experimental Biology*, 2019, 222 (13), pp.jeb196543. 10.1242/jeb.196543 . hal-02330604

HAL Id: hal-02330604

<https://hal.science/hal-02330604v1>

Submitted on 17 Jun 2023

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RESEARCH ARTICLE

Ocean acidification affects calcareous tube growth in adults and reared offspring of serpulid polychaetes

Victoria Díaz-Castañeda^{1,*}, T. Erin Cox^{2,3,*}, Frédéric Gazeau³, Susan Fitzer⁴, Jérémy Delille³, Samir Alliouane³ and Jean-Pierre Gattuso^{3,5}

ABSTRACT

The energetically costly transition from free-swimming larvae to a benthic life stage and maintenance of a calcareous structure can make calcifying marine invertebrates vulnerable to ocean acidification. The first goal of this study was to evaluate the impact of ocean acidification on calcified tube growth for two Serpulidae polychaete worms. *Spirorbis* sp. and *Spirobranchus triqueter* were collected at 11 m depth from the northwest Mediterranean Sea and maintained for 30 and 90 days at three mean pH_T levels (total scale): 8.1 (ambient), 7.7 and 7.4. Moderately decreased tube elongation rates were observed in both species at pH_T 7.7 while severe reductions occurred at pH_T 7.4. There was visual evidence of dissolution and tubes were more fragile at lower pH but fragility was not attributed to changes in fracture toughness. Instead, it appeared to be due to the presence of larger alveoli covered in a thinner calcareous layer. The second objective of this study was to test for effects on *S. triqueter* offspring development. Spawning was induced, and offspring were reared in the same pH conditions that the parents experienced. Trochophore size was reduced at the lowest pH level but settlement success was similar across pH conditions. Post-settlement tube growth was most affected. At 38 days post-settlement, juvenile tubes at pH_T 7.7 and 7.4 were half the size of those at pH_T 8.1. The results suggest future carbonate chemistry will negatively affect the initiation and persistence of both biofouling and epiphytic polychaete tube worms.

KEY WORDS: pH, *Spirorbis* sp., *Spirobranchus triqueter*, Transgenerational effects, Tube worms

INTRODUCTION

The uptake of anthropogenic carbon dioxide (CO₂) by the ocean generates changes in the carbonate chemistry of seawater. Dissolved inorganic carbon increases whereas the concentration of carbonate ions (CO₃²⁻) and pH decrease. This process is known as ocean acidification and it can have deleterious effects on some species, particularly calcifiers. The impact of ocean acidification on invertebrates has been studied in the past two decades (e.g. Byrne,

2011; Kroeker et al., 2013; reviewed by Kurihara, 2008). Many prior studies have shown that adult invertebrate calcifiers exposed to elevated CO₂ partial pressure (P_{CO_2}) exhibited physiological stress and reduced calcification rates (Gazeau et al., 2007; Miles et al., 2007; Pörtner et al., 2004).

Serpulidae is a large family of sedentary benthic polychaete worms that have a worldwide distribution and secrete and live within calcareous tubes. The family includes members from the subfamilies Spiborbinae, Filograninae and Serpulinae (Ten Hove and Kupriyanova, 2009). They live in varied marine habitats and are economically relevant as one of the most significant groups of marine fouling invertebrates. For example, many have undesirable effects such as clogging of seawater intake pipes and fouling of ship hulls (Bastida-Zavala et al., 2017; Hoagland and Turner, 1980). Many serpulids are epiphytes on marine plants or live attached to rocks and shells and a few species aggregate to form calcified reefs (Kupriyanova et al., 2001; Smith et al., 2005). They are also often major ecological components of marine communities; they occur in great numerical abundance or biomass, add structural complexity to the habitat, and link pelagic and benthic food webs by filter feeding (Kupriyanova et al., 2001).

The mineralogy and structure of serpulid polychaete tubes could make them vulnerable to ocean acidification (Smith et al., 2013). The calcareous tube is formed by secretions from a pair of exocrine glands in the ventral part of the peristomium; these glands are in contact with the surrounding seawater and discharge a mucopolysaccharide matrix (Neff, 1971) that induces the precipitation of calcium carbonate (CaCO₃) from seawater. The mixture of CaCO₃ and mucopolysaccharides is then deposited to the leading edge of the tube by the collar. The timing and details of the formation can be species specific but it tends to occur rapidly (within hours) and appears to be necessary for survival (Rouse and Pleijel, 2001). Furthermore, unlike many other groups in which calcification is impacted by ocean acidification such as corals, crustaceans and molluscs, most serpulid tubes lack an external organic layer which would isolate their calcified structures from the surrounding seawater and protect them from negative effects (Ries et al., 2009). Serpulid tubes vary in skeletal mineralogy from aragonite, a form of CaCO₃ that is highly soluble at low pH, to moderate to high-magnesium calcite, and many tubes are a mixture of the two forms (Smith et al., 2013; Vinn et al., 2008). The few studies that have investigated low pH effects on serpulid tube growth have found a range of responses from no effect at elevated temperature (Chan et al., 2013) to reduced growth rates and changes in mineralogy. Mineralogical changes often result in subsequent losses in fracture hardness and elasticity (Chan et al., 2012; Lane et al., 2013, 2015; Li et al., 2014).

Early life stages of invertebrates are often the most sensitive to environmental changes (Byrne, 2011; Kurihara, 2008). The transition from a pelagic to a benthic life history stage is irreversible and

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energetically costly, and it is when most of the mortality is thought to occur. Thus, effects in the early benthic stages could cause a major bottleneck in population dynamics (Gosselin and Qian, 1997). Indeed, several bioassays with invertebrate larvae reared under elevated P_{CO_2} have shown deformed larvae, reduced size at metamorphosis and smaller juveniles (Dupont et al., 2008; Kroeker et al., 2013; Kurihara, 2008).

The consequences of ocean acidification on the offspring of serpulid polychaetes are not yet fully understood. The success of larvae may depend on reproductive investment by parental generation, direct effects of lower pH conditions on larval and juvenile physiology, and their ability to adapt to rapid changes over generations. Lane et al. (2013) studied inter-generational adaptation in the serpulid polychaete *Hydroides elegans* and found that both paternal and maternal low pH exposure affects offspring growth rate, with evidence for transgenerational plasticity. Rodríguez-Romero et al. (2016) worked on six generations of the dorvilleid polychaete *Ophryotrocha labronica*, which does not produce calcium carbonate tubes. They found that in early generations (F1 and F2), fecundity was significantly lower at low P_{CO_2} but from F3 onwards there were no significant differences between the effects of P_{CO_2} treatments, indicating transgenerational adaptation. This highlighted the usefulness of transgenerational experiments for understanding the response to ocean acidification.

The objective of the present study was to test for the effects of ocean acidification on the serpulid polychaetes *Spirobranchus triqueter* and *Spirorbis* sp. These species have differing ecologies and belong to different serpulid phylogenetic clades (see Smith et al., 2013). *Spirobranchus triqueter* is a biofouling species, a broadcast spawner, and has a calcified operculum, whereas *Spirorbis* sp. broods eggs, lacks an operculum and lives epiphytically on seagrass and algae (Kupriyanova et al., 2001). These differing ecologies and evolutionary lineages may have consequences on their sensitivity to ocean acidification (Lucey et al., 2015; Smith et al., 2013). The tube growth of these polychaetes was measured after they were maintained under three pH_T (total scale) conditions (8.1, 7.7, 7.4) for 30–90 days. Additionally, we tested for effects on the offspring of *S. triqueter* under the same control and pH_T conditions. We hypothesized: (1) that calcareous tube growth is negatively impacted at low pH and (2) that early life stage development is also negatively affected from lowered parental investment and/or from direct effects of pH on development and growth.

MATERIALS AND METHODS

Serpulid collection and calcein staining

Thirty *Posidonia oceanica* seagrass shoots (33 cm maximum leaf length) colonized by *Spirorbis* sp. were collected on 26 March 2014 at 11 m depth in a seagrass meadow within the Bay of Villefranche (NW Mediterranean Sea; 43°40.73'N, 07°19.39'E).

Spirobranchus triqueter (Linnaeus 1758) was collected 8 days later on 3 April 2014 on 2×1 m Perspex[®] sheets. Sheets were deployed on 6 March 2014 in the same meadow and at the same depth at which *Spirorbis* sp. was collected and, ~4 weeks later, had a single *S. triqueter* recruitment event colonizing its surface. At the time of collection, all tubes were of similar size (about 1.5 cm in length). The sheets were then cut into 20×10 cm plates.

Posidonia shoots with *Spirorbis* sp. and the plates harbouring *S. triqueter* were immediately brought to the laboratory and placed in a tank with buffered calcein (60 mg l⁻¹) for 48–72 h, in a temperature-controlled room (14°C). The purpose was to stain the tubes in order to have a mark for future growth measurements. The polychaetes were fed *ad libitum* with the microalgae *Isochrysis*

galbana during staining. The plates and seagrass shoots were then placed into the experimental setup (Fig. 1).

Experimental setup and pH control – *Spirorbis* sp. and *S. triqueter* parental generation

After staining with calcein, plates and seagrass shoots with attached serpulids were placed into nine transparent (Perspex) open-top 8.4 l cylinder tanks (Fig. 1). There were five shoots per tank. The nine tanks were randomly assigned to three pH treatments (three tanks per pH level) referred to as ambient, treatment 1 and treatment 2 with targeted pH_T levels of 8.1, 7.7 and 7.4, respectively. Tanks initially contained *Spirorbis* sp. on leaves and 8 days later, after collection, also contained *S. triqueter* on plates. Both species were kept in a flow-through system in a temperature-controlled room (14–15°C) under a 9.5 h:14.5 h light:dark photoperiod (to mimic day length at depth in March) provided by six 39 W Solar Nature Ultra light tubes (JBL) for up to 30 days for *Spirorbis* sp. and 90 days for *S. triqueter*. During this time, polychaetes were fed twice per week (75 ml, 60,000 cells ml⁻¹ of a culture of *I. galbana*). The flow through was interrupted for 1 h during feeding.

Seawater, pumped at 8 m depth, 100 m from the coast, was continuously supplied to three 200 l header tanks at a minimal rate of 50 l h⁻¹. From each header tank, seawater was delivered continuously by gravity to the three experimental tanks at each pH level. The flow into experimental tanks was adjusted to reach a renewal time of about 15 min, ensuring good water quality without large fluctuations of pH, nutrients, dissolved oxygen and temperature. A pump was located in each header tank to ensure mixing.

pH was controlled by bubbling pure CO₂ in the corresponding header tanks using a pH-stat system (IKS, Aquastar, Karlsbad, Germany). The pH electrodes of the pH-stat system were inter-calibrated every 2 days using a glass combination electrode (Metrohm Ecotrode Plus) calibrated on the total scale using Tris buffer with a salinity of 38 corresponding to the salinity of the seawater pumped from the bay.

Carbonate chemistry measurements

Every 2 or 3 days, during the experiments on both adult tube worms and *S. triqueter* offspring, samples were taken for determination of dissolved inorganic carbon (C_T) in each header tank and for total alkalinity (A_T) in the ambient header tank. Furthermore, during the experiment on offspring, approximately every 2–3 days, samples for C_T and A_T were taken prior to each seawater renewal (see below). Samples for C_T (60 ml) were immediately poisoned with 100 µl of a saturated solution of mercuric chloride (HgCl₂) to prevent biological activity and analysed within 2 days using an AIRICA (automated infra-red inorganic carbon analyser, Marianda, Kiel, Germany) coupled to a LI-COR infrared gas analyser (LI-6262), on triplicate 1.2 ml subsamples at 22°C. The instrument was calibrated before every set of measurements using certified reference material from A. Dickson (Scripps University, San Diego, CA, USA; batch 132). Samples for A_T (300 ml) were filtered on 47 mm diameter GF/F membranes, immediately poisoned with 100 µl of saturated HgCl₂ and analysed within 2 days. A_T was determined at 22°C on triplicate 50 ml subsamples by potentiometric titration on an 880 Titrand (Metrohm) coupled to a glass combination electrode (Ecotrode Plus, Metrohm), calibrated daily on the total scale using Tris buffers of salinity 38. A_T was calculated as described by Dickson et al. (2007). During the experiment, standards provided by A. Dickson (batch 132) were used to check precision and accuracy ($n=11$; 1.3 and 1.3 µmol kg⁻¹, respectively). The pH_T levels and temperature were also measured approximately every 2 days in each cylinder using a

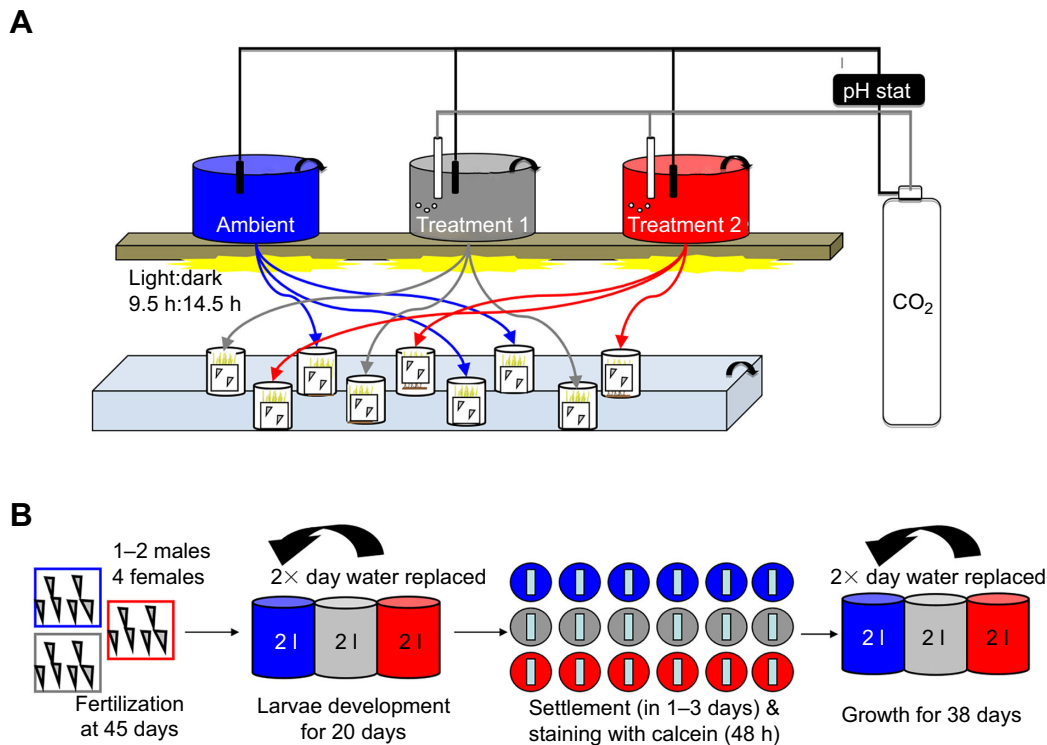


Fig. 1. Experimental design. (A) *Spirorbis* sp. on *Posidonia oceanica* shoots and *Spirobranchus triqueter* on tiles were collected from the field and exposed for 30 and 90 days, respectively, to three pH_T levels (total scale): ambient (pH_T 8.1), treatment 1 (pH_T 7.7) and treatment 2 (pH_T 7.4). Header tanks were used to feed three replicate cylinder tanks containing tube worms. A pH-stat system was used to bubble pure CO_2 into header tanks to maintain pH. (B) After 45 days, 1–2 males and 4 females of *S. triqueter* from each treatment were induced to spawn. Larvae were allowed to develop for 20 days in 2 l beakers and transferred to Petri dishes that contained a slide covered with biofilm for settlement. Worms settled within 1–3 days and were placed into a calcein bath for 48 h. Then, juveniles were placed into 2 l beakers and allowed to grow for 38 days. Offspring were exposed to experimental conditions for a total of 43 days. The water was changed in culture beakers twice per day.

glass combination electrode (Ecotrode Plus) calibrated as described above. At the conclusion of study for *Spirorbis* sp. (at 40 days in the experiment for *S. triqueter* adults), we considered we had successfully shown the pH_T and temperature in experimental cylinders reflected the pH of the header tanks and the temperature of the room. Thereafter, pH and temperature measurements in the cylinders were interrupted and only C_T and A_T were measured ever 2 or 3 days in the header tanks for the remainder of the experiment with *S. triqueter*. The carbonate chemistry was assessed using C_T and A_T (flag 15) at a salinity of 37.5 with the corresponding room temperature in the R package seacarb (<https://CRAN.R-project.org/package=seacarb>).

Tube elongation – *Spirorbis* sp. and *S. triqueter* parental generation

Tube elongation rates were determined for *Spirorbis* on day 30 and for *S. triqueter* throughout the experiment at 36 and 90 days for the parental population. Polychaetes were photographed under a fluorescence Zeiss microscope at 515 nm, a wavelength under which calcein fluoresces yellow–green. Growth measurements were performed using ImageJ analysis software. New tube growth for *Spirorbis* sp. was estimated from the external arc of the coil to the stained line of the tube edge, while for *S. triqueter* it was estimated as the length from the staining line to the distal end of the tube.

Culturing of *S. triqueter* offspring and measurement of growth and development

Spawning

After *S. triqueter* had been maintained for 45 days, 4 females and 1–2 males were randomly selected from each treatment. Gametes

were obtained by breaking the posterior part of the tubes; this mechanical perturbation caused the release of eggs and sperm. Once they began spawning, they were immediately placed into a beaker (three in total, one for each pH level), at a seawater pH_T of 8.1, where fertilization took place. They were left for 30 min to allow embryos to develop. Offspring were cultured and maintained in the same temperature-controlled room as the parents.

Trochophore density and size

Embryos were then transferred to 2 l beakers (three in total, one for each pH level) containing filtered seawater at the respective pH level taken from the header tanks and closed with plastic wrap to avoid CO_2 exchange with the atmosphere. Fifteen hours after fertilization, the density of developed trochophores was estimated for each treatment in 1 ml samples, which were placed under a microscope and counted with a Sedgewick grid.

Trochophore larvae from each treatment were maintained in these 2 l beakers at the respective pH levels (Fig. 1). During development from embryo to competent larvae, seawater was changed twice a day and replaced with water from the respective header tanks. Approximately every 2 days for a month, seawater was collected from beakers to measure carbonate chemistry using the methods described above. Larvae were fed daily with 10 ml of a culture of *I. galbana* at a density of 50,000 cells ml^{-1} .

Two and 10 days after the appearance of trochophore larvae, 20 larvae from each treatment were placed into separate Petri dishes. The dishes were then placed for 3 min in a refrigerator to reduce mobility. Larval size was measured using a stereoscopic microscope with a micrometer previously calibrated using a scaled slide. Those

larvae that were not measured remained in culture until they reached metamorphic competence (three segments).

Settlement

After 20 days of larval culture, when the metatrochophore stage had been reached, a batch of 25–30 competent larvae from each of the three pH treatments were pipetted into 18 Petri dishes (six per treatment) filled with seawater from the respective header tanks. In each Petri dish, larvae were provided with a microscope slide covered in biofilm to induce settlement. Biofilm was obtained by placing the slides in running seawater for 8–10 days to induce settlement. After 48 h, settled larvae were counted and the percentage calculated.

Offspring tube elongation

Slides with settled *S. triqueter* were stained for 40 h in a calcein (100 mg l^{-1}) bath that had been buffered to a pH_T of 8.1. The 18 slides were then placed into 2 l glass beakers (three in total) filled with seawater at the respective pH level. At 15, 22 and 38 days after the appearance of the first settled larvae, the new tube length of juveniles was assessed using the same method as described for their parental generation.

Tube hardness and fracture toughness – *S. triqueter* parental generation

At the conclusion of the experiment, *S. triqueter* tubes from the parental generation were oven dried at 60°C for 48 h, stored in air-tight containers, and shipped to the University of Glasgow, UK. New growth of *S. triqueter* tubes (three per treatment, nine in total) was embedded in epoxy resin (EpoxyCure, Buehler) blocks and sliced transversely using a diamond-trim saw blade. Resin blocks were ultra-polished using aluminium oxide (0.3 and $1 \mu\text{m}$) and colloidal silica ($0.6 \mu\text{m}$). Fracture toughness was determined by Vickers hardness microindentation testing (Micro Vickers 401-MVA, Wilson Wolpert Co., Ltd). Indents were made on the calcite material surrounding the porous structures of the tubes midway between the pores and the resin ($n=6$ – 15 indents per tube). A load of 0.2 N was applied for 10 s , the lengths of the diagonals of the indent were measured to calculate the Vickers hardness and the length of the cracks developed from the corners of the indent was measured to determine fracture (K_{IC} ; Fitzer et al., 2015).

Statistical analysis

Replication was considered at the individual level with the exception of settlement success, when each slide was considered a replicate. Pooling of individuals was done to increase sample size for statistical analyses. Replicate containers were thus only considered as sources of variability. Subsequently, analyses were subjected to potential container effects. However, obvious differences in measurements between containers were not observed, all containers were a source of individual measurements, and sampling efforts (environment monitored, treated similarly, transferred between container type with development) were taken to minimize this effect. In instances where multiple measurement intervals were collected from the same pH treatment, data were treated separately by day. This data treatment was done because when data were pooled for statistical analyses in a two-factor test with pH treatment and time interval included as factors, data did not meet parametric requirements and could not be successfully transformed to meet parametric requirements. Therefore, all data were tested with separate one-way analysis of variance (ANOVA). Prior to testing, data were checked for normality and homogeneity of variance and transformed when necessary.

Measurements of tube elongation at 90 days in *S. triqueter* (parental generation) and measurements at 38 days in the offspring still did not meet parametric requirements and in these instances a Kruskal–Wallis test was used (see Table 3). Additionally, rates of tube elongation by *Spirorbis* sp. were square-root transformed prior to testing. Tukey's or Dunn's (when non-parametric) *post hoc* tests were used to examine for pairwise differences when significant main results were found. Unless otherwise noted, data are reported as $\text{means} \pm \text{s.e.m.}$ throughout.

RESULTS

Carbonate chemistry and environmental measurements

Spirorbis sp. and *S. triqueter* parental generation

Environmental conditions in the header tanks are shown in Table 1 and Table S1. Under ambient conditions, pH_T averaged 8.11 ± 0.04 (mean \pm s.d.) during culture of *Spirorbis* sp. and 8.13 ± 0.06 during culture of adult *S. triqueter*, while in the two low-pH conditions, it was, on average, 7.73 ± 0.05 and 7.72 ± 0.07 in treatment 1 and 7.40 ± 0.07 and 7.38 ± 0.14 in treatment 2 for the two species, respectively. Serpulid worms cultured at pH_T 8.1 and 7.7 were exposed to seawater that was always supersaturated with respect to both calcite and aragonite. In contrast, at the lowest pH condition, seawater was mostly undersaturated with respect to aragonite ($\Omega_a=0.4$ to 1.1) and close to saturation for calcite ($\Omega_c=0.6$ to 1.5). pH_T measured in the cylinders (from 31 March to 16 May) was very close to values determined in the header tanks (Fig. 2).

Spirobranchus triqueter offspring

Environmental and carbonate chemistry parameters in header tanks and in the beakers in which offspring were reared (before water replacement) are shown in Tables 1 and 2. Under ambient conditions, pH_T averaged 8.10 ± 0.05 (mean \pm s.d.) in the beakers over the 28 day experimental duration. In the two low-pH conditions, pH_T increased by 0.1 – 0.2 units between water changes but the pH gradient between treatment levels was maintained (ambient > treatment 1 > treatment 2). For example, the mean pH_T in header tanks for treatment 1 and treatment 2 was 7.7 and 7.4 , respectively, whereas the mean \pm s.d. pH_T in beakers at the time of water change for treatment 1 was 7.82 ± 0.06 and for treatment 2 it was 7.57 ± 0.06 . Similarly, the aragonite and calcite saturation was proportionally increased from values measured in the header tanks at the time of water change.

Tube elongation – *Spirorbis* sp. and *S. triqueter* parental generation

Spirorbis sp. tube elongation (measured once at the end of the study) was affected by pH (Table 3, Fig. 3). Elongation rates were relatively reduced at the two lower pH levels and tubes grown at the lowest pH were thinner and broke easily.

Tube elongation by *S. triqueter* (parental generation) was also impacted by lowered pH conditions and rates differed over the duration of the experiment (Table 3, Fig. 3). Over 36 and 90 days, individuals maintained at ambient pH had the fastest rates of tube elongation: $0.23 \pm 0.02 \text{ mm day}^{-1}$ over 36 days, reduced to $0.17 \pm 0.01 \text{ mm day}^{-1}$ over 90 days. During the 36 days when serpulids in ambient conditions had the fastest rates of tube elongation, those exposed to the lower pH levels (treatments 1 and 2) had reduced elongation rates that significantly differed from rates measured under ambient pH, with those at pH_T 7.7 having greater rates than those at pH_T 7.4 (Table 3; ambient > treatment 1 > treatment 2). After a longer period of growth (90 days), individuals of *S. triqueter* kept at pH_T 7.7 and 7.4 had further reduced elongation rates (mean of

Table 1. Carbonate chemistry for *Spirorbis* sp. and *Spirobranchus triqueter* (adults and offspring) in header tanks under the three experimental conditions

| | Ambient | | | | | | | Treatment 1 | | | | | | | Treatment 2 | | | | | | |
|-------------------------------|----------|---|-----------------|------------------------------------|---|----------------|----------------|-----------------|------------------------------------|---|----------------|----------------|-----------------|------------------------------------|---|----------------|----------------|--|--|--|--|
| | T (°C) | A _T (μmol kg ⁻¹) | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c | | | | |
| <i>Spirorbis</i> sp. | 16.2±0.8 | 2551±6 | 8.11±0.04 | 367±37 | 2251±24 | 3.3±0.2 | 5.1±0.4 | 7.73±0.05 | 1011±128 | 2439±12 | 1.5±0.1 | 2.4±0.2 | 7.40±0.07 | 2268±401 | 2557±23 | 0.8±0.1 | 1.2±0.2 | | | | |
| <i>S. triqueter</i> | 15.9±1.2 | 2546±10 | 8.13±0.06 | 347±49 | 2242±20 | 3.3±0.2 | 5.1±0.3 | 7.72±0.07 | 1047±177 | 2440±26 | 1.5±0.2 | 2.3±0.3 | 7.38±0.14 | 2510±848 | 2565±46 | 0.8±0.2 | 1.2±0.3 | | | | |
| <i>S. triqueter</i> offspring | 14.9±0.4 | 2546±13 | 8.15±0.03 | 328±29 | 2235±17 | 3.4±0.2 | 5.2±0.3 | 7.73±0.10 | 1019±247 | 2438±39 | 1.5±0.3 | 2.3±0.5 | 7.41±0.18 | 2381±1038 | 2560±62 | 0.8±0.3 | 1.2±0.4 | | | | |

Carbonate chemistry was calculated from determined total alkalinity (A_T) and dissolved inorganic carbon (C_T) in each header tank with a salinity of 37.5 and measured temperature (T) within ambient, treatment 1 and treatment 2 header tanks over the experimental duration for *Spirorbis* sp. (30 days) and *Spirobranchus triqueter* adults (90 days) and offspring (43 days). pH_T, pH on the total scale; P_{CO₂}, partial pressure of carbon dioxide; Ω_a, saturation state with respect to aragonite; Ω_c, saturation state with respect to calcite. Data are means±s.d.

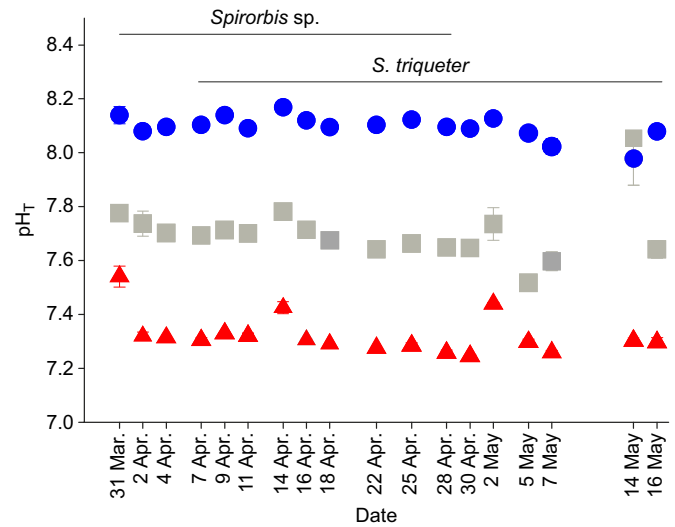


Fig. 2. pH_T in experimental cylinders containing *Spirorbis* sp. and *S. triqueter* (parental generation). pH_T was measured in March, April and May, for ambient (blue), treatment 1 (grey) and treatment 2 (red) conditions.

0.06±0.01 and 0.03±0.02 mm day⁻¹) that did not differ from each other (Table 3; ambient >treatment 1=treatment 2).

Spirobranchus triqueter trochophore density and size

Data were most congruent with the hypothesis that lowered pH conditions affect trochophore density and growth (Table 3, Fig. 4). Indeed, the mean density in treatment 2 (57±11.8 trochophores ml⁻¹) was substantially lower than the mean density under ambient conditions (101±12 trochophores ml⁻¹). Initial size estimates of larvae on day 2 were similar and all larvae increased in size from day 2 to day 10. However, at day 10 post-fertilization, the larvae at the lowest pH level were significantly smaller (Table 3; 376±7.9, 369.4±5.5 and 361.2±6.7 μm for ambient, treatment 1 and treatment 2, respectively).

Spirobranchus triqueter settlement

Most larvae attained metamorphic competence but at the lowest pH level (treatment 2), five abnormal larvae were observed (out of 25–30 larvae). All five had one side of the body more developed than the other, making the larvae swim in an unusual way. Settlement of metatrochophores took place first (21 days after fertilization) in treatment 2 (mean pH_T 7.4–7.6), then 24–48 h later (22–23 days after fertilization) for those maintained in ambient conditions and treatment 1. Settlement success of competent larvae ranged between 89% and 93% and no differences between treatments were detected (Table 3).

Tube elongation – *S. triqueter* offspring

One hour after settlement, larvae began to secrete a transparent primary tube, and 24 h later they were cemented permanently in place, building the secondary calcified white tube. Initially, the calcified tubes in the lowest pH treatment were significantly smaller (ambient: 1.67±0.03 mm, treatment 1: 1.67±0.02 mm and treatment 2: 1.64±0.02 mm), but a week later (22 days), juveniles in treatment 1 and 2 showed tube elongation rates similar to those in ambient conditions (Table 3, Fig. 4). Then, 38 days post-settlement, *S. triqueter* juveniles in ambient conditions had rates almost double those of previous days while juveniles at the lower pH levels (treatment 1 and 2) continued to elongate tubes at a similar rate, slightly above the rate recorded at 22 days.

Table 2. Carbonate chemistry for *Spirobranchus triqueter* offspring in beakers under the three experimental conditions

| Date | Day | T (°C) | Ambient | | | | | Treatment 1 | | | | | Treatment 2 | | | | | |
|-------------------|-----|-----------------|---|------------------|------------------------------------|---|----------------|----------------|------------------|------------------------------------|---|----------------|----------------|------------------|------------------------------------|---|----------------|----------------|
| | | | A _T (μmol kg ⁻¹) | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c | pH _T | P _{CO₂} (μatm) | C _T (μmol kg ⁻¹) | Ω _a | Ω _c |
| 23 May | 2 | 18.1 | 2546 | 8.06 | 416 | 2258 | 3.2 | 4.9 | — | — | — | — | — | — | — | — | — | |
| 24 May | 3 | 14.7 | 2510 | 8.03 | 454 | 2275 | 2.6 | 4.0 | — | — | — | — | — | — | — | — | — | |
| 25 May | 4 | 15.4 | 2521 | 8.06 | 422 | 2264 | 2.8 | 4.4 | 7.74 | 966 | 2411 | 1.5 | 2.3 | — | — | — | — | |
| 26 May | 5 | 14.2 | 2537 | 8.14 | 339 | 2242 | 3.2 | 4.9 | 7.83 | 779 | 2399 | 1.7 | 2.7 | — | — | — | — | |
| 28 May | 8 | 14.4 | 2535 | 8.14 | 340 | 2239 | 3.2 | 5.0 | 7.85 | 738 | 2387 | 1.8 | 2.8 | 7.56 | 1529 | 2497 | 1.0 | 1.5 |
| 31 May | 11 | 15.2 | 2543 | 8.15 | 325 | 2228 | 3.4 | 5.3 | 7.75 | 958 | 2431 | 1.5 | 2.4 | 7.59 | 1414 | 2489 | 1.1 | 1.7 |
| 5 June | 16 | 15.5 | 2546 | — | — | — | — | — | — | — | — | — | — | 7.53 | 1647 | 2512 | 1.0 | 1.5 |
| 6 June | 17 | 14.6 | 2542 | — | — | — | — | — | 7.78 | 866 | 2418 | 1.6 | 2.5 | — | — | — | — | — |
| 7 June | 18 | 14.8 | 2544 | — | — | — | — | — | 7.89 | 667 | 2375 | 2.0 | 3.1 | 7.54 | 1614 | 2511 | 1.0 | 1.5 |
| 10 June | 21* | 15.0 | 2549 | — | — | — | — | — | 7.91 | 638 | 2370 | 2.1 | 3.3 | 7.54 | 1607 | 2514 | 1.0 | 1.5 |
| 13 June | 24* | 15.0 | 2537 | 8.16 | 320 | 2222 | 3.4 | 5.3 | 7.85 | 735 | 2384 | 1.9 | 2.9 | 7.68 | 1124 | 2451 | 1.3 | 2.1 |
| 17 June | 28* | 15.0 | 2539 | 8.08 | 395 | 2269 | 3.0 | 4.6 | — | — | — | — | — | — | — | — | — | — |
| Means±s.d. | | 15.1±1.2 | 2540±12 | 8.10±0.05 | 380±47 | 2250±18 | 3.1±0.3 | 4.8±0.4 | 7.82±0.06 | 793±125 | 2397±22 | 1.8±0.2 | 2.8±0.3 | 7.57±0.06 | 1489±198 | 2496±24 | 1.1±0.1 | 1.6±0.2 |

Carbonate chemistry was calculated from determined A_T and C_T with a salinity of 37.5 and measured temperature (T) within ambient, treatment 1 and treatment 2 culture beakers just prior to water changes over the 28 day experimental duration for *S. triqueter* offspring. T_A, total alkalinity; pH_T, pH on the total scale; P_{CO₂}, partial pressure of carbon dioxide; Ω_a, saturation state with respect to aragonite; Ω_c, saturation state with respect to calcite. Data were collected in May–June 2014. Means±s.d. are in bold.

*Measurements for juveniles (1–2 days post-settlement).

Fracture toughness and hardness – *S. triqueter* parental generation

Fracture toughness (K_{IC}) was greatest for tubes maintained at the intermediate pH level (treatment 1), but was similar for worms measured under all three conditions (Table 3, Fig. 5B). Likewise, tubes maintained at the mid-pH level (treatment 1) were significantly harder than those in the low pH condition (treatment 2) (Table 3, Fig. 5A). Alveoli size visually differed among treatments (Fig. 6). Alveoli were present inside the two lateral tube flanges. Alveoli form in tube growth and house the notopodial chaetae (Hedley, 1958). As the tube elongates, the chaetae move forward and prevent calcareous deposition in a new location. In cross-section, the tube section formed at the lowest pH (treatment 2) had the largest alveoli with a thinner calcareous layer corresponding to the significantly reduced hardness of the tube. However, the tubes grown in treatment 1 had very small alveoli, noticeably smaller than those in tubes at ambient pH_T.

DISCUSSION

Our results support the hypothesis that ocean acidification will affect the calcification of serpulid tubes. The lowered pH and the subsequent lowered saturation states of calcite and aragonite reduced tube growth and appeared to alter tube integrity in both species.

Tube growth

According to Waldbusser et al. (2015), the effects that ocean acidification has on shell formation of bivalve larvae are modulated by a combination of dissolution of the tube and physiological changes to the internal acid–base balance, affecting the rate of new CaCO₃ deposition. Dissolution and reduced growth under acidified conditions have also been reported in other serpulid species. A well-studied tropical serpulid, *Hydroides elegans*, raised under lowered pH (7.8–7.4 NBS) produced smaller, thinner, less dense and weaker tubes (Chan et al., 2012; Li et al., 2014, 2016). In one report, most of the *H. elegans* larvae raised at pH conditions below 7.7 (NBS scale) were unable to produce calcified tubes at the time of metamorphosis and juvenile calcification was also negatively affected (Lane et al., 2013). In the Baltic Sea, tube growth of *Spirorbis spirorbis*, an epiphytic species on algae, was decreased by 25% and 40% at 1200 and 3150 μatm CO₂, respectively (Saderne and Wahl, 2013). Adult and juvenile tubes exhibited dissolution that in some cases exposed the worms and their embryo bags (Saderne and Wahl, 2013).

Mineral composition

The fact that most serpulid tubes lack an outer organic protective layer and are composed of aragonite, calcite and, for some, high-magnesium calcite makes them sensitive to acidified conditions (Chan et al., 2012; Smith et al., 2013; Vinn, 2013; Vinn and Kupriyanova, 2011). Mineral composition of serpulid tubes shows a strong phylogenetic signal, but it can be somewhat plastic (Smith et al., 2013). *Spirorbis spirorbis* (as *S. borealis*) have been reported to be 10–14% aragonite with the rest being moderate- to high-magnesium calcite depending on environmental factors such as temperature (Bornhold and Milliman, 1973). This bimineralic composition is typical for *Spirorbis* spp. and both mineral types are highly soluble. Therefore, this serpulid group is suspected to be highly sensitive to dissolution (Smith et al., 2013); results in the present study support this hypothesis. *Spirobranchus triqueter* tubes have been reported to be composed of 98.4% moderate to high calcite and 1.6% aragonite (Vinn et al., 2008). In contrast, polychaete species belonging to the genus *Hydroides* are reported to be almost entirely aragonitic (Smith et al., 2013). However, biomineralogical

Table 3. Differences in *Spirorbis* sp. and *S. triqueter* tube elongation, development and tube fracture hardness and toughness under the three experimental conditions

| Parameter | <i>n</i> | d.f. | MS | <i>F</i> (or <i>H</i>) | <i>P</i> | Pairwise <i>post hoc</i> |
|--|----------|------|---------------|-------------------------|------------------|----------------------------|
| Tube elongation rate | | | | | | |
| <i>Spirorbis</i> sp. (square-root transformed) | 14–15 | 2 | 0.315 | 16.30 | <0.001 | Ambient>1*>2** |
| <i>S. triqueter</i> parental generation | | | | | | |
| 36 days | 13 | 2 | 0.084 | 92.55 | <0.001 | Ambient>1***>2** |
| 90 days (Kruskal–Wallis) | 23–28 | 2 | | 28.45 | <0.001 | Ambient>1=2* |
| <i>S. triqueter</i> offspring | | | | | | |
| Trochophore size | | | | | | |
| 2 days post-fertilization | 10 | 2 | 9.733 | 2.39 | 0.111 | N/A |
| 10 days post-fertilization | 10 | 2 | 564.133 | 12.35 | <0.001 | Ambient=1>2** |
| Settlement success | 6 | 2 | 41.8 | 0.93 | 0.415 | N/A |
| Juvenile tube growth | | | | | | |
| 15 days post-settlement | 11–13 | 2 | 0.0000207 | 9.83 | <0.001 | Ambient=1>2** |
| 22 days post-settlement | 11–13 | 2 | 0.00000451 | 4.13 | 0.025 | Ambient=1, Ambient=2, 1>2* |
| 38 days post-settlement (Kruskal–Wallis) | 18 | 2 | | 32.82 | <0.001 | Ambient>1=2* |
| Hardness | 3 | 2 | 35,203 | 16.47 | <0.001 | Ambient<1 and >2, 2>1** |
| Fracture toughness (<i>K_{IC}</i>) | 3 | 2 | 5,982,627,612 | 0.50 | 0.606 | N/A |

Results of one-way ANOVA or Kruskal–Wallis (as specified). Significant differences are in bold and the results of pairwise *post hoc* tests are shown (ambient, treatment 1, treatment 2): * $P<0.05$, ** $P<0.01$.

plasticity (increase in the ratio of magnesium to calcite) in response to CO₂-induced ocean acidification was observed in the biofouling species *Hydroides crucigera* (Ries, 2011). Ontogenetic mineral changes have also been noted; juvenile *H. elegans* tubes contained more amorphous calcium carbonate and were predominantly aragonitic whereas adult tubes were bimineralic with considerably more calcite (Chan et al., 2012). Probably, early stages of *S. triqueter* are less aragonitic than *H. elegans* and this gives them more resistance to changes in seawater chemistry, as we did not observe polychaetes unable to build tubes. However, lowered pH conditions

had the greatest impact post-settlement on *S. triqueter* juvenile tube elongation. Thus, an ontogenetic change in mineral composition is a possibility in this species but more studies are needed on the topic. Also, the present study may underestimate effects on *S. triqueter*, because the calcified opercula, known to be almost entirely aragonitic in this group (see Smith et al., 2013), were not investigated.

Tube hardness and elasticity

Alterations in tube mineralogy can result in deterioration of tube hardness and elasticity which are two factors that can have important

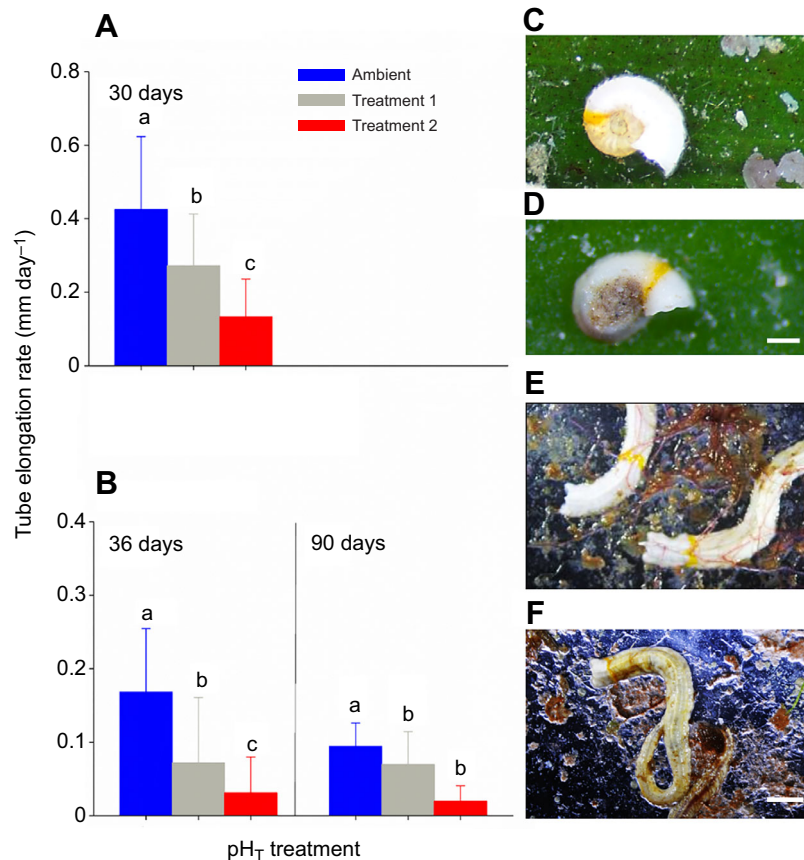


Fig. 3. Tube elongation rate. (A,B) Tube elongation rate (mean±s.e.m.) for *Spirorbis* sp. at 30 days (A) and *S. triqueter* (parental generation, B) at 36 and 90 days (tested separately) of experimental exposure to ambient, treatment 1 and treatment 2 conditions. Sample sizes were as follows: *S. triqueter* at 36 days, all treatments, $n=13$; *Spirorbis* sp., $n=15, 14, 15$ for ambient, treatment 1 and 2 conditions, respectively; and *S. triqueter* at 90 days: $n=23, 28, 25$ for ambient, treatment 1 and 2 conditions, respectively. Letters above bars represent statistical results from pairwise comparisons when main effects were found in one-way ANOVA or Kruskal–Wallis test (see Table 3). (C–F) Visual differences in new growth, after the yellow calcein mark, between treatments for *Spirorbis* sp. (C, ambient; D, treatment 2) at 30 days and *S. triqueter* at 90 days (E, treatment 1; F, treatment 2). Scale bars: C and D, 1 mm; E and F, 9 mm.

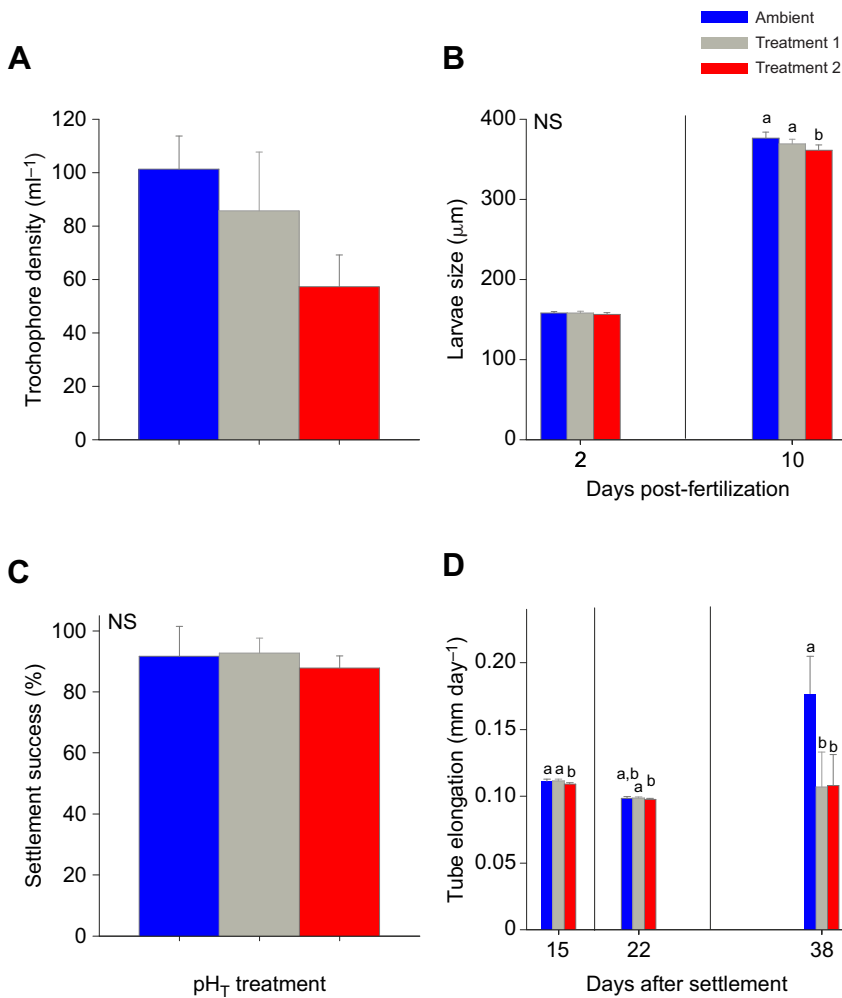


Fig. 4. *Spirobranchus triqueter* early life history stage development and juvenile tube elongation rates when cultured under the three treatment conditions.

(A–C) Trochophore density (A), trochophore size (B) and settlement success (C) under ambient, treatment 1 and treatment 2 conditions. (D) Juvenile tube elongation (means \pm s.e.m.) under ambient, treatment 1 and treatment 2 conditions. Sample sizes were as follows: trochophore density and size under all treatment conditions, $n=10$; settlement success under all treatment conditions but not statistically tested, $n=6$; ambient, treatment 1 and treatment 2 conditions at 15 and 22 days, $n=11, 12, 13$, respectively; and all treatment conditions at 38 days, $n=18$. Lines within graphs indicate distinct measurement intervals, which were tested separately. Letters above bars represent statistical results from pairwise comparisons when main effects were found in one-way ANOVA or Kruskal–Wallis test (NS, no significant differences found; see Table 3).

ecological consequences on survival (predation, withstanding wave force; Chan et al., 2012; Li et al., 2014). However, for *S. triqueter*, in the present study, the change in alveolus size, reduced hardness and unchanged fracture toughness (K_{IC}) presumably accounts for the fragility of the tube at pH_T 7.4. *Spirobranchus triqueter* may have some ability to compensate for conditions at pH_T 7.7, as evidenced by the greater hardness and smaller alveoli. Perhaps, *S. triqueter* allocates energy to maintain structural tube integrity at the expense of growth. The price of compensating for lowered pH has an associated energy cost. Such changes in the energy budget have been observed in several calcifiers such as molluscs and foraminifera (Pan et al., 2015; Wood et al., 2008). When additional energy is channelled to calcification in order to repair dissolved zones of the tube or shell, it cannot be used for other processes, restricting growth and reproductive output. Furthermore, the ability to change tube morphology in response to environmental conditions has been observed. For example, *H. elegans* subjected to both elevated temperature and lowered pH conditions produced longitudinal keels that increased the volume of tubes and mitigated the weakness observed under acidified conditions at lower temperature (Li et al., 2016).

Larval development

Few studies have noted ocean acidification effects on serpulid larval size or metamorphosis (Lane et al., 2013, 2015). We observed reduced density and size but no impact on metamorphosis when

larvae were cultured at pH_T 7.4 (Fig. 4). Some authors (Kurihara, 2008; Parker et al., 2010) have reported that effects at adult stages may have ‘carry-over’ consequences from one life history stage to the next. In the present study and in many studies on the topic to date (e.g. Chan et al., 2012; Li et al., 2014, 2016), fertilization of tube worm gametes has occurred at a pH_T of 8.1. It has been reported that pH, salinity and temperature do not directly alter fertilization in tube worms such as *H. elegans* (Li et al., 2014). However, without an acclimation period or some examination to rule out carry-over effects, fertilization under ambient conditions prior to treatment conditions may mitigate or, alternatively, cause cellular stress and exacerbate the impact of low pH on early development. Future efforts should ensure that all life stages are kept at treatment conditions to better determine ocean acidification effects. Nevertheless, decreased size in early developmental stages of marine organisms has been shown to affect juvenile fitness by reducing competitive ability and increasing post-settlement mortality (Anil et al., 2001).

The negative effect observed during early development of *S. triqueter* (Fig. 4) could be explained by the lower pH_T (7.4) used and exposure of both parents to lowered pH conditions, as differential sex effects have been noted (see Lane et al., 2015). The more extreme ocean acidification scenario could cause great stress on the parental generation or more drastic direct effects on acid–base balance or ion regulation in trochophore larvae. For example, from a 5 day transplant experiment, the polychaete *Sabella spallanzanii*

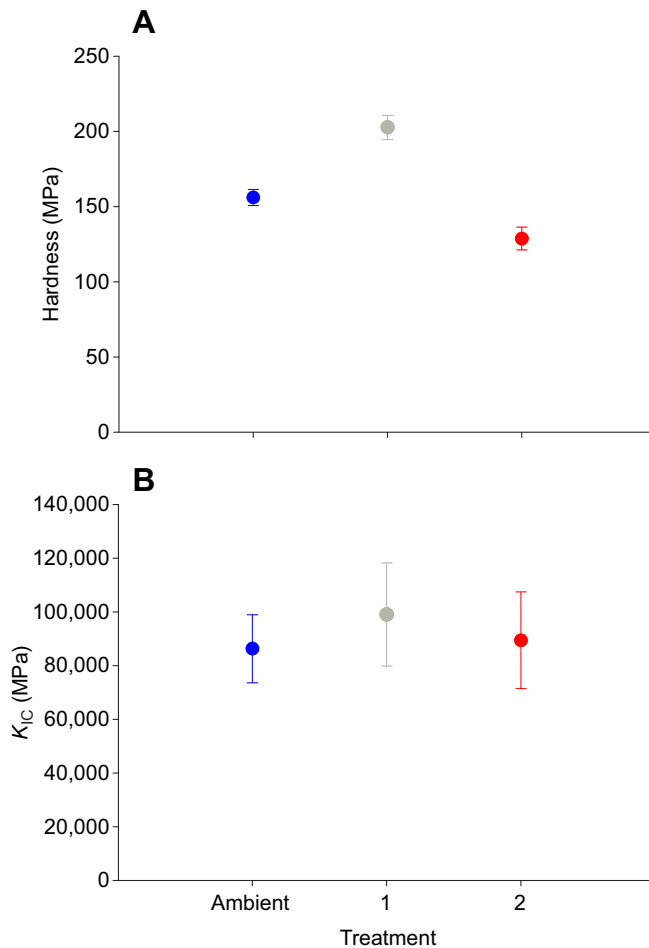


Fig. 5. Fracture hardness and toughness of new growth of *S. triqueter* (parental generation) after 90 days of exposure to the three conditions. (A) Hardness. (B) Fracture toughness (K_{IC}). Data are means \pm s.e.m., $n=3$ (see Table 3 for statistical results).

was estimated to only be able to survive in the short term under low pH conditions because ocean acidification causes a significant decrease in carbonic anhydrase concentration and an increase in energy metabolism (Turner et al., 2015). The mechanism resulting in smaller trochophores at 10 days (not 2 days) post-fertilization is unclear (Fig. 4), but there are several accounts of reduced larval size in invertebrates under acidified conditions (Kroeker et al., 2010). Echinoderm larvae of the sea urchins *Hemicentrotus pulcherrimus* and *Paracentrotus lividus* subjected to elevated CO_2 presented delayed development and smaller size (Byrne et al., 2013; Dupont et al., 2008). In the present study, however, lowered pH conditions did not delay settlement.

Additionally, invertebrate larvae with short life cycles have the potential for short-term evolution in response to ocean acidification; this capacity of adaptation can reduce biological impacts of climate change (Hoffmann and Sgrò, 2011). Polychaete worms transplanted to CO_2 vents in Ischia (Italy) have shown acclimation, adaptation and plasticity to elevated P_{CO_2} environments (Calosi et al., 2013). No acclimation was observed for *S. triqueter* in the present study (Figs 3 and 4); experiments with several generations may be needed in order to evaluate the adaptive capability of this species. If there was transgenerational plasticity, it was not apparent with our study design. For instance, Lane et al. (2015) concluded *H. elegans* exhibited transgenerational plasticity only when the first-generation

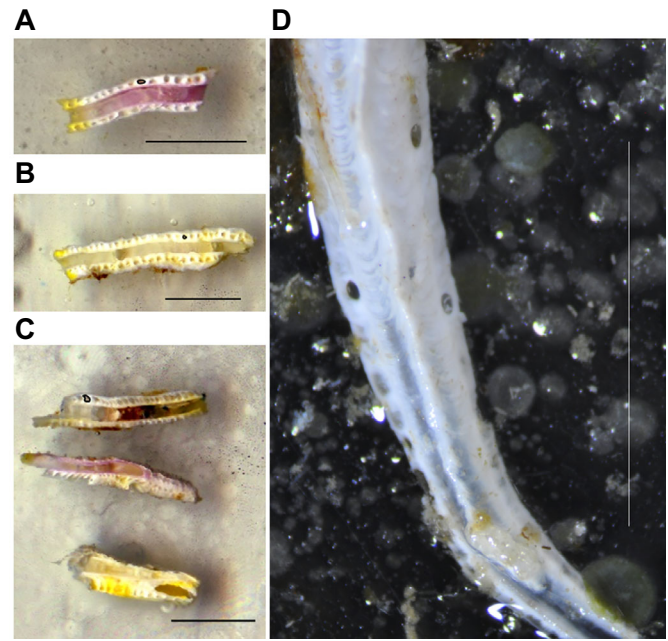


Fig. 6. Fragility of *S. triqueter* (parental generation) tubes after 90 days of exposure. (A–C) Tubes from the ambient (A), treatment 1 (B) and treatment 2 (C) conditions, sectioned and prepared for measures of fracture toughness and hardness. An alveolus in each treatment is outlined in black to indicate the difference in size. Note the small alveoli after exposure to treatment 1 and the large alveoli covered in a thin calcareous layer after 90 days under treatment 2. (D) A close-up ($\sim 4\times$) image of an intact tube at pH 7.4 (treatment 2), showing the exposed alveoli and the thin calcareous layer. Scale bar is adjusted in all images for direct comparison.

offspring were cultured in ambient pH (NBS, 8.1; i.e. mismatch between parental and offspring exposure).

Ecological relevance

Reduced tube growth and the lower number of trochophores produced under the lower pH conditions (Fig. 4) are likely to affect serpulid recruitment. Examinations of serpulid establishment in natural settings support this conclusion but also highlight the importance of including ecological interactions and environmental variability when predicting population responses. Observational studies along CO_2 vents have found a reduction in the number of serpulid individuals nearer to the vent source, citing both direct effects on calcification and decreased competitive ability with more-tolerant species as an explanation for this (Cigliano et al., 2010; Donnarumma et al., 2014; Martin et al., 2008). Species of *Spirorbis* that settle on photosynthesizing organisms can be somewhat buffered from ocean acidification effects by the chemically mediated environment provided by their host (Cox et al., 2017; Hendriks et al., 2014; Saderne and Wahl, 2013). However, to be buffered, the benefits during the day need to be great enough to offset the negative effects of respiration at night. In addition, the tube worm response can be driven by temperature (Campbell and Fourqurean, 2014; Ni et al., 2017). For example, *S. spirorbis*, in the laboratory, had reduced recruitment on a furoid alga at extreme P_{CO_2} (3150 μatm) but not at moderate levels (1200 μatm ; Saderne and Wahl, 2013). An *in situ* benthocosm study, using the same species, concluded that early life stages are promoted by moderately warm temperatures. However, high temperatures exacerbate tube dissolution and do not alter growth when water is undersaturated with respect to $CaCO_3$ (Ni et al., 2017). In the northwest Mediterranean Sea, *Spirorbis* sp. recruited less on tiles placed

inside an *in situ* acidified enclosure than on tiles placed in the control enclosure at ambient pH. Also, the density of epiphytic *Spirorbis* sp. on *P. oceanica* seagrass leaves appeared to be unaffected in both enclosures, leading to the conclusion that the chemically mediated boundary layer around the leaf provided an ocean acidification refuge (Cox et al., 2017). In contrast, *Spirorbis* sp. had fewer individuals on leaves of the subtropical seagrass *Thalassia testudinum* exposed to lower pH and the effect was greater during winter months (Campbell and Fourqurean, 2014). Moreover, food availability is a critical factor in larval development and juvenile growth (Olson and Olson, 1989). In the present study, worms were fed adequately and that could have allowed for some resistance to more negative effects.

Future implications under global climate change

Ocean warming will shape the response to ocean acidification. It is predicted that tropical tube worms will shift distribution towards the poles with continued global climate change (Faroni-Perez, 2017). In the predictive model, which considered both warming and acidification, elevated temperature was the most influential driver of distributions, although Faroni-Perez (2017) points to the need for more information on the effects of ocean acidification on tube worms to inform models. This shift in distribution should cause alarm because serpulid reefs allow for greater biodiversity and their loss can have implications on temperate shallow-water ecosystem function. The composition of biofouling communities will also probably change significantly with a decrease in invertebrate calcifiers, particularly Serpulidae.

Acknowledgements

We are grateful to S. Schenone and L. Urbini for their assistance with the experiment. We also thank P. Mahacek and A. Le Fur for their assistance in construction of settlement tiles and tanks. We thank C. Rouvière for assisting with the imaging of calcein markers and S. Marro for providing the algal cultures. D. Luquet, G. de Liège and D. Robin kindly assisted in diving for organism collections.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V.D.-C., F.G., J.G.; Methodology: V.D.-C., T.E.C., F.G.; Formal analysis: T.E.C., S.F., S.A.; Investigation: V.D.-C., T.E.C., F.G., S.F., J.D., S.A.; Writing - original draft: T.E.C., J.G.; Writing - review & editing: V.D.-C., T.E.C., F.G., S.F., J.D., S.A.; Supervision: F.G., J.G.; Project administration: F.G., J.G.; Funding acquisition: F.G., J.G.

Funding

This work was funded by Foundation BNP Paribas through the eFOCE (European Free Ocean Carbon dioxide Enrichment) project, and the European Commission through the project Mediterranean Sea Acidification in a changing climate (MedSea; grant agreement 265103) and the MISTRALS-MERMEX (INSU, CNRS) programme.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.196543.supplemental>

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