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ORIGINAL PAPER

High pCO_2 and elevated temperature reduce survival and alter development in early life stages of the tropical sea hare *Stylocheilus striatus*

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Abstract Elevated temperature (ocean warming) and reduced oceanic pH (ocean acidification) are products of increased atmospheric pCO_2, and have been shown in many marine taxa to alter morphology, impede development, and reduce fitness. Here, we investigated the effects of high pCO_2 and elevated temperature on developmental rate, hatching success, and veliger morphology of embryos of the tropical sea hare, *Stylocheilus striatus*. Exposure to high pCO_2 resulted in significant developmental delays, postponing hatching by nearly 24 h, whereas exposure to elevated temperature (in isolation or in combination with high pCO_2) resulted in accelerated development, with larvae reaching several developmental stages approximately 48 h in advance of controls. Hatching success was reduced by ~20 and 55% under high pCO_2 and warming, respectively, while simultaneous exposure to both conditions resulted in a nearly additive 70% reduction in hatching. In addition to these ontological and lethal effects, exposure of embryos to climate change stressors resulted in significant morphological effects. Larval shells were nearly 40% smaller under high pCO_2 and warming in isolation and up to 53% smaller under multi-stressor conditions. In general, elevated temperature had the largest impact on development, with temperature-effects nearly 3.5-times the magnitude of high pCO_2-effects. These results indicate that oceanic conditions congruent with climate change predictions for the end of the twenty-first century suppress successful development in *S. striatus* embryos, potentially reducing their viability as pelagic larvae.

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Introduction

Decreased oceanic pH (ocean acidification) is a product of increased atmospheric pCO_2, and has been shown in many taxa to lead to altered developmental timelines (Stumpp et al. 2011), inhibition of growth (Stumpp et al. 2011; Dorey et al. 2013), malformations in calcitic exoskeletons (Gazeau et al. 2013), and reduced larval fitness (Kroeker et al. 2010). However, the magnitude of these effects can vary significantly both across taxa (Kroeker et al. 2010; Harvey et al. 2013) and across life history stages within a species (Kurihara 2008; Harvey et al. 2013). Early life history stages are often the most susceptible to acute changes in the environment and frequently require a narrower range of optimal conditions to successfully complete their

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development (Kurihara 2008). Mortality in such stages can be extremely high (>90%) even under ambient natural conditions (Kurihara 2008) and thus sensitive early life history stages may represent important bottlenecks for population growth and persistence under a changing climate (Byrne and Przeslawski 2013).

In several marine taxa studied to date, exposure to acidification stress during early development is associated with developmental delays (Dupont and Thorndyke 2009; Busby et al. 2013) which, for species with planktonic larvae, is likely to have negative ecological consequences through increased risk of loss to predation and delayed settlement opportunity (Timmins-Schiffman et al. 2013). Indeed, early stage larvae of marine calcifiers are thought to be especially susceptible to ocean acidification for at least two reasons: first, developing larvae may have reduced capacity for compensatory acclimation relative to adults under acidification stress (Dupont et al. 2008; Kurihara 2008; Pörtner et al. 2011), and second, initiation of mineral deposition in larval forms is thought to begin with a thin layer of amorphous $CaCO_3$ which is the least robust form of $CaCO_3$ and highly prone to dissolution (Gazeau et al. 2013). Negative effects of ocean acidification on embryonic or larval calcification have been observed in multiple taxa including several species of gastropod molluscs (Gazeau et al. 2013; Manríquez et al. 2014; Noisette et al. 2014), but rarely in gastropods with encapsulated embryos (Ellis et al. 2009; Manríquez et al. 2014; Montory et al. 2009; Noisette et al. 2014) and fewer still investigating the effects of multiple stressors (Harvey et al. 2013; Kroeker et al. 2013).

Although embryonic shell development in free-swimming gastropod larvae is susceptible to sustained exposure to high pCO_2 (Kurihara 2008; Gazeau et al. 2013), such effects have not been well-studied in species with encapsulated embryos where intracapsular fluids may buffer against external environmental stressors such as reduced pH and lower carbonate ion availability (Ellis et al. 2009; Noisette et al. 2014). In several studies performed to date on acidification response of encapsulating gastropods (*Crepidulata fornicata*, *C. dilatata*, *Littorina obtusata*, *Concholepas concholepas, Bembicium nanum*, and *Dolabifera brazier*), encapsulation did not significantly reduce the effects of acidification on embryonic calcification and development (Ellis et al. 2009; Montory et al. 2009; Davis et al. 2013; Manríquez et al. 2014; Noisette et al. 2014). However, in other groups (e.g. polychaetes) negative effects of acidification have been shown to be ameliorated through parental investment in brooding or maintenance of embryos in protective egg casings (Lucey et al. 2015).

Recent meta-analyses have highlighted the complex responses of organisms to acidification stress in isolation (Hendriks et al. 2010; Kroeker et al. 2010; Gazeau et al. 2013); however, less focus has been directed to the impact of multiple other stressors acting in concert with acidification on calcifying organisms (Harvey et al. 2013; Kroeker et al. 2013; Breitburg et al. 2015). For example, increasing atmospheric pCO_2 has also resulted in ocean warming which has already had severe consequences for several taxa, resulting in shifted biogeographic range distributions and altered fitness (Stillman and Armstrong 2015). Further, there is mounting evidence that tropical species may be especially sensitive to warming (Stillman 2003; Tewksbury et al. 2008; van Heerwaarden et al. 2016) as a result of physiological trade-offs incurred in the evolution of high inherent tolerance limits at the expensive of thermal tolerance plasticity. Such trade-offs may result from thermal specialization of individual enzymes which are adapted to operate at higher temperatures but which lose functionality more rapidly as the environment warms (Hochachka and Somero 2002) or because warm-adaptation alters underlying metabolic processes (e.g. mitochondrial densities or oxygen demand) resulting in insufficient oxygen-supply capacity under increasing environmental temperatures (Pörtner et al. 2006). Whatever the mechanism, patterns of thermal trade-offs associated with warm-adaptation are pervasive and have been observed in many taxa (Deutsch et al. 2008; Tewksbury et al. 2008). However, little is known about how this potential thermal sensitivity might affect resilience to other stressors such as ocean acidification during simultaneous exposure. In a recent study on tropical littorinid and opisthobranch molluscs, simultaneous exposure to moderate warming reduced the negative impacts of acidification on development suggesting that responses of tropical species to mutliple stressors may be complex and, therefore, difficult to predict a priori (Davis et al. 2013).

We examined the effects of multiple environmental stressors (elevated temperature and increased pCO_2) on development and survival in early life stages of the circumtropical, capsule-laying opisthobranch sea hare, *Stylocheilus striatus* (Family Aplysiidae). Gastropods, in particular sea hares, play an important role in benthic reef ecology as specialist grazers on toxic cyanobacteria that prevent the settlement of coral larvae (Kuffner and Paul 2004) and can cause phase shifts from coral to algal dominated reefs (Thacker et al. 2001). Early developmental stages of *S. striatus* have been described (Horwitz et al. 2017) and are known to be sensitive to anthropogenic noise pollution, suffering impaired development and increased mortality among larval hatchlings (Nedelec et al. 2014), but whether they are susceptible to other environmental stressors is unknown. We hypothesized that (1) despite encapsulation, elevated pCO_2 will reduce viability of *S. striatus* embryos, via potential reductions in development rate, net embryonic calcification, and hatching success; (2) that exposure

to elevated temperature will have negative developmental effects comparable to acidification treatments; and (3) that, during simultaneous exposure, elevated pCO_2 and elevated temperature will interact synergistically, exacerbating the negative developmental effects observed in *S. striatus* embryos reared under either condition in isolation.

Materials and methods

Due to timing constraints of the field season, this work was conducted in two experimental rounds, with different biological response variables assessed in each round enabling a more in-depth understanding of the effects of the multiple environmental stressors. In experimental round I (spring 2012), embryos of *S. striatus* were reared under treatment in closed-system conditions (i.e. sealed petri dishes) and time to developmental stage, larval survival, and larval shell morphology were recorded. In experimental round II (Fall 2015), embryos were reared under semi-closed conditions (modified 50 mL Falcon tubes) and early stage growth rates and embryonic viability were monitored over the first 72 h of development. For both experimental rounds, environmental temperatures at time of collection were similar (26.8 and 27 °C for rounds I and II, respectively) and within climatological norms reported previously (Leichter 2015). In both Experiment rounds, the ambient temperature treatments (27.0 and 28.2 °C, respectively) corresponded to the mean monthly seawater temperature on the back reef in Mo'orea during the experimental period (September and June) (Leichter 2015). The elevated temperature treatments (31 °C) were ~2 °C warmer than average recorded during the warmest months of the year (March–April) and within the range of highest recorded daily temperatures (Leichter 2015).

In experimental round I we investigated the following response variables: time to reach four discrete, pre-defined, developmental stages (referred to hereafter as embryonic developmental timing), proportion of larvae emerging from an egg ribbon (termed larval hatching success or survival), and shell size at hatching (height, width, and lateral surface area). In experimental round II we measured both the change in total embryo surface area over time (a proxy for embryonic growth rate) and the proportion of embryos at a given developmental stage 48 h post-fertilization (a proxy for early stage embryonic viability). With these definitions it was possible for an embryo to grow (i.e. increase in overall area/size), without developing (i.e. reaching a predefined developmental stage), and vice versa. Further details of each experimental round are described below.

Experimental round I: developmental timing, survival, and shell morphometrics

Broodstock collection and maintenance

In September and October 2012, adult *S. striatus* ($n = 56$) were collected from three sites at the fringing reef surrounding the northern and eastern coast of Mo'orea, (Society Archipelago, French Polynesia), and from populations residing in flow-through aquaria at the University of California Gump Field Research Station (Suppl. Fig. 1). The collection sites are characterized by low diurnal variability in temperature (±1 °C) and seasonal variation in temperature is on the order of ±2 °C with highest temperatures in April (~28.8 °C) and lowest in August/September (~26.5 °C) (Leichter 2015).

Specimens obtained from the field were collected from either the canopies of opportunistic alga (*Sargassum spp.*) colonizing damaged coral heads, or large cyanobacterial mats (*Lyngbya majuscula*) present along the substrate. Animals were transferred within 5 h of collection into an outdoor, flow-through aquarium (volume = 668 L) containing aerated, ambient seawater (temperature ± SD = 27 ± 1.5 °C, salinity ± SD = 35 ± 1). Sea hares were fed ad libitum with the cyanobacterium *L. majuscula* and were maintained for a minimum acclimation period of 2 weeks before any mating/oviposition was induced for experimental purposes.

Mating procedures

Twenty individuals were selected and paired randomly from the total pool of adult *S. striatus*. After selection, mating pairs ($n = 10$) were distributed into ten, 36 L aquaria. These aquaria used for *S. striatus* mating were housed within the primary holding tank and lateral openings were cut in each to allow for circulatory flow. To induce oviposition, mating pairs were starved and provided algal masses (*Sargassum spp.*) as a substrate to protect egg ribbons. Each individual aquarium used for *S. striatus* mating was scanned every few hours for oviposition of egg ribbons. After ~12 h, egg ribbons were removed from individual aquaria and examined under a microscope to determine developmental stage. Only those egg ribbons containing embryos which had not yet undergone first cellular cleavage were utilized in round I experiments.

Egg ribbon maintenance and treatment conditions

Each egg ribbon ($n = 11$, approximately 5–7 cm in length) was divided with a scalpel into two equal halves (hereafter termed strands). Strands from each ribbon were then transferred to two, sealed, 96 mL Petri dishes containing

either seawater enriched with CO_2 (acidified treatment, $pCO_2 \pm SD = 1114 \pm 199$ µatm, pH ± SD = 7.67 ± 0.06, see Seawater Acidification and Carbonate Chemistry, below), or untreated water (ambient control, $pCO_2 \pm SD = 421 \pm 105$ µatm, pH ± SD = 8.02 ± 0.06). Petri dishes were stored at ambient temperature ($\bar{x} \pm SD = 27 \pm 0.5$ °C) in a covered water bath to reduce light exposure and treatment water was changed daily. Every 24 h each strand was imaged using a dissecting microscope and optical light microscope camera (Leica MZ16; Leica DFC420). A minimum of three images (i.e. technical replicates) were taken of each strand daily. After the first larvae began hatching from a strand, strands were maintained at experimental conditions for an additional 24 h before images were taken of hatched embryos and of the remaining egg cases. Strands were frozen in treatment water at −20 °C and stored for less than 1 month prior to subsequent imaging (see estimation of viability below) for shell morphometric analysis.

After completion of the first developmental period (10 days), adult *S. striatus* were allowed an additional 5 days of recovery and re-acclimation before the aforementioned procedures were repeated, and new ribbons from ten mating pairs were produced (length ± SD = 6.3 ± 1 cm, $n = 10$ length) to test the effects of elevated temperature. Egg ribbons were divided into strands which were incubated in two pCO_2 treatments as described above, but in a 31 ± 1 °C water bath, and imaged daily until hatching. Care was taken to remove egg ribbons for only a short period each day (<15 min) in order to reduce exposure to ambient temperature conditions during imaging. Based on oxygen consumption data collected in round II Experiments, it is unlikely that oxygen dropped below non-lethal critical thresholds over the 24-h period between water changes (calculated oxygen concentration of 7.4 mg O_2 L^{-1} after 24 h versus O_2-P_{crit} = 2.25 mg O_2 L^{-1} reported for non-lethal effects in molluscan embryos) (Vaquer-Sunyer and Duarte 2008). However, given a respiratory quotient of 1.03 mol CO_2 per mole O_2 consumed, we estimate that pH could have dropped up to 0.2 units over the 24-h period in sealed dishes due to respiratory CO_2 production which would have brought pCO_2 conditions in experimental round I (pH 7.7 at start of incubation) closer to those applied in experimental round II (pH 7.5 maintained).

At the close of experimental round I, embryos were thus reared in four seawater treatments: 1) control (pH = 8.02, $T = 27$ °C), 2) high-temperature (pH = 8.02, $T = 31$ °C), 3) high pCO_2 (pH = 7.67, $T = 27$ °C), and 4) high pCO_2 and high-temperature (pH = 7.67, $T = 31$ °C). These values were selected based on current physicochemical variability and predicted warming trends for the next century in the tropical Pacific (Ganachaud et al. 2011; Hofmann et al. 2011). A full list of experimental seawater parameters for experimental round I is given in Table 1.

Timing of embryonic development

The times to reach four, pre-defined, developmental events were recorded from embryo images for each strand. These events were 1) embryonic movement [defined as the first time individual embryos were observed moving inside an egg capsule], 2) protoconch formation [clear division and color differentiation of an aragonitic protoconch], 3) eyespot formation, and 4) hatching. For each strand ($n = 44$ total strands; $n = 11$ per treatment), data on time to reach

Table 1 Measured and calculated abiotic experimental round I and II treatment conditions

Variable	Ambient pH mean ± SD	Low pH mean ± SD
Measured		
pH_T	8.02 ± 0.06/8.13 ± 0.09	7.67 ± 0.06/7.50 ± 0.09
Temp (°C)	Ambient 27.0 ± 0.5/28.2 ± 0.8 High 31.0 ± 0.5/30.8 ± 0.6	Ambient 27.0 ± 0.5/28.2 ± 0.8 High 31.0 ± 0.5/30.8 ± 0.6
Salinity (PSU)	36.0 ± 0.9/36.4 ± 0.1	35.7 ± 1.0/36.2 ± 1.0
A_T (µmol Kg^-)	2354.8 ± 98/2435.1 ± 24	2337.9 ± 105/2399.27 ± 17
Calculated		
pCO_2 (µatm)	420.89 ± 105/317.4 ± 74	1113.69 ± 199/1756.0 ± 203
DIC (µmol kg^{-1})	2033.7 ± 95/1994.0 ± 80	2200.1 ± 104/2308.0 ± 109
HCO_3^- (µmol kg^{-1})	1796.4 ± 122/1675.0 ± 114	2056.01 ± 124/2171.0 ± 131
CO_3^{2-} (µmol kg^{-1})	225.9 ± 14/311.1 ± 19	114.5 ± 9/94.12 ± 7
$\Omega_{Aragonite}$	3.635 ± 0.2/5.035 ± 0.3	1.857 ± 0.1/1.526 ± 0.08
$\Omega_{Calcite}$	5.464 ± 0.5/7.48 ± 0.7	2.79 ± 0.1/2.267 ± 0.1

Carbonate system parameters were calculated from salinity, temperature, total alkalinity (A_T), and pH (total scale) using the *R* package seacarb (Gattuso et al. 2015). All values are given as round I/round II

each developmental stage were standardized as the time post-oviposition.

Hatching success

Hatching success was defined as the number of embryos which had successfully completed development (hatched or nearly hatched, see below) at 10 days post-oviposition prior to storage at −20 °C (24 h post-initial-larval-emergence). Hatching success was estimated by subtracting the total number of unviable embryos within a strand from the total number of embryos in that strand and dividing by the total number of embryos within the strand ($n = 44$ total strands; $n = 11$ per treatment):

$$\text{Percentage Hatching Success} = \frac{N_{\text{total}} - N_{\text{unviable}}}{N_{\text{total}}} \times 100\%$$

The number of unviable embryos within a strand (N_{unviable}) was estimated by randomly selecting five, 0.50-mm sections of the egg ribbon, counting the total number of unviable embryos for each section, taking the average, and extrapolating that mean across the entire length of the strand. The total number of eggs in a strand (N_{total}) was calculated by counting every egg (irrespective of embryonic viability) in a 0.50-mm section of five different egg ribbons, (selected randomly, each at a different stage of development), taking the average, and then extrapolating that mean across the entire length of the egg ribbon. At 10 days post-oviposition, eggs remaining in the tube were considered unviable if their development lagged significantly behind the rest of the clutch (i.e. did not develop eyespots), if the embryo was unable to move and/or swim (generally due to visible deformities of the embryo) or if 2 + embryos were conjoined within a single capsule. An embryo was considered viable if the veliger had hatched out of the egg ribbon (empty egg case) or if the embryo was able to swim/crawl freely inside the egg ribbon at the time of imaging. The vast majority of unviable embryos had not developed past the eyespot stage and were visibly decomposing in the egg capsule.

Veliger shell morphometrics

Veliger shell morphology was measured in ten randomly selected individuals from each strand ($n = 440$ total veligers; $n = 110$ per treatment) at the time of hatching. Post removal from −20 °C storage, strands were thawed and visualized using a compound light microscope and optical light microscope camera (Leica DM2500; Leica DFC420). Three measures were obtained for each veliger using ImageJ64 (Schneider et al. 2012): lateral shell length, lateral shell width (spiral height), and 2-dimensional lateral shell area (Suppl. Fig. 2).

Seawater acidification and carbonate chemistry

Seawater was supplied from a depth of 12 m in Cook's Bay, Mo'orea, filtered through a sand filter (mesh size ~100 μm), and stored in a header tank. Parameters in header tanks were maintained at levels following a 2 × 2 temperature (27.0 ± 0.5 °C modern ambient, 31.0 ± 0.5 °C predicted future) cross pH (8.02 ± 0.06 present and 7.67 ± 0.06 predicted future) design. Elevated pCO_2 treatments were maintained by bubbling tanks with CO_2-enriched air. CO_2-enriched air was created using a solenoid-controlled gas regulation system (Model A352, Qubit Systems Inc.) that mixed pure CO_2 with ambient air to create gas mixtures with known pCO_2 (as detected by an Infrared Gas Analyzer [IRGA], Model S151, Qubit Systems Inc.). The flow of CO_2-enriched air into each header tank was adjusted using needle valves to correct deviations detected by pH measurements of tank seawater from the targeted values. The flow of seawater into each header tank was similarly adjusted using ball valves to correct deviations from targeted values and to ensure that total alkalinity (A_T) remained constant throughout the experiment. High pCO_2 and control water was then collected from header tanks and stored for no more than 48 h in sealed 1 L containers at 5 °C before being added to embryo-containing petri dishes for each experimental treatment.

Samples for determination of pH (pH_T) were collected from header tanks and measured immediately with an automatic titrator (T50, Mettler-Toledo) using 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS) buffer (Andrew G. Dickson) on the total scale and at a salinity of 35.0. Calibrations were done every third day based on the rationale that the pH cell was demonstrably stable as it drifted an average of 0.2 mV between 2 calibrations, which corresponded to a pH_T variation of <0.005 unit. Determination of pH using a pH cell yields uncertainty for pH measurements of <0.02 unit for seawater (Dickson 2010). Salinity was measured using a conductivity meter (YSI 63) and A_T was measured in duplicate. Analyses of A_T were performed on the day of seawater sampling using an open-cell potentiometric titration using an automatic titrator (T50, Mettler-Toledo). Measurements of A_T were conducted on duplicate 50 mL samples at room temperature (~23 °C) and A_T was calculated using a Gran function applied to pH values ranging from 3.5 to 3.0 as described previously (Dickson et al. 2007). Titrations of certified reference materials (CRM) provided by A. G. Dickson (strand 105) yielded A_T values within 4 mol kg^{-1} of the nominal value (SD = 4.1 mol kg^{-1}; $n = 12$). Parameters of the seawater carbonate system were calculated from salinity, temperature, A_T, and pH_T using the R package seacarb (Gattuso et al. 2015) (Table 1).

Experimental round II: early stage embryonic growth rate and viability

Broodstock collection and maintenance

In June 2015, adult *S. striatus* were collected from cyanobacterial mats (*L. majuscula*) on the northern shore of Mo'orea (Suppl. Fig.1) and transferred within 3 h of collection into an outdoor, flow-through aquarium (volume = 550 L) containing aerated seawater (temperature ± SD = 26.9 ± 1 °C, salinity ± SD = 35.96 ± 0.2). As in experimental round I, sea hares were fed ad libitum with the cyanobacterium *L. majuscula* and were maintained for a minimum acclimation period of 2 weeks before any mating/oviposition was induced for experimental purposes.

Mating procedures

Ten adults were selected and paired randomly from the total pool of adult *S. striatus*. After selection, mating pairs ($n = 5$) were distributed into five, 36 L aquaria as in round I. After ~12 h, egg ribbons were removed, and after examination under a microscope (as in round I) only those egg ribbons containing embryos which had not yet undergone first cellular cleavage were utilized in round II experiments.

Egg ribbon maintenance and treatment conditions

Total egg ribbon length was measured (length ± SD = 5.8 ± 1.2 cm, $n = 5$) before removing ~0.1 cm from each end. Because individual egg capsules could not be removed from the ribbon without disrupting the embryos within, the remaining ribbon was cut so as to divide it into two strand length treatments: long strands (~5 mm, "well-protected" embryos) and short strands (~1 mm, "exposed" embryos) in order to test for possible effects of protection afforded by the egg ribbon casing itself. To reduce edge effects of the total egg ribbon length, strands were cut such that both long- and short-strand treatments contained embryos from the end and middle of each ribbon. For each ribbon, groups of one long strand and five short strands were placed together in 12, replicate, 50 mL Falcon tubes corresponding to three replicates of the four seawater treatments (see treatment conditions below). The Falcon tubes were modified with 1 cm diameter holes in the lids and open bottoms both covered by 75 µm mesh. To maximize the exposure effects, all data on the short strand treatments were taken from the edge of the ribbon (i.e. embryos most exposed to external environment) and all measures from the long strands were taken from the middle-most 2 mm of the ribbon (i.e. most protected embryos).

Embryos were then reared using an extensive culture approach in which seawater within tubes was flushed every 12 h in order to avoid hypoxia and to maintain experimental parameters similar to experimental round I (see Culture Conditions and Seawater Acidification below). To confirm that embryos were not experiencing hypoxia during treatment, the concentration of dissolved oxygen in experimental tubes was measured once, fluorometrically, at the end of a 12-h interval using a PreSens Sensor Dish Reader with Oxodish® Optode Plate (PreSens Precision Sensing GmbH, Germany; SDR software v38). From these data, it was determined that dissolved oxygen values within experimental tubes never dropped below 94.7 ± 3.8% ($n = 8$) of aerated header-tank seawater. During experimental round II, embryos were reared in four seawater treatments: 1) control (pH = 8.13, $T = 28.2$ °C), 2) high-temperature (pH = 8.13, $T = 30.8$ °C), 3) high pCO_2 (pH = 7.50, $T = 28.2$ °C), and 4) high pCO_2 and high-temperature (pH = 7.50, $T = 30.8$ °C). A full list of experimental seawater parameters for experimental round II is given in Table 1.

Embryonic early growth rates

Embryo growth rates were calculated as change in total embryo area over time. Morphometric data on total embryo area were obtained from photographs taken every 24 h (over the first 72 h) of $n = 20$–21 randomly selected embryos per treatment ($n = 85$ total embyros). All morphometric measurements were generated using the image analysis software program Image J (Schneider et al. 2012). Embryonic growth rate was calculated as the slope of the best fit line of total embryo area versus time over the first 72 h of the experiment when this relationship was most linear (Fig. 1).

Embryo viability

Embryo viability was inferred from the number of embryos actively spinning at 48 h post-oviposition and was assessed from $n = 15$, 30 s video recordings of embryos taken from a randomly selected, representative strands in each replicate tank per treatment. For each video, a random subsample of $n = ~5$ individual embryos visible in the frame were selected and the proportion of these specimens that spent at least a portion of the time actively spinning within their egg cases was recorded. This yielded data on embryo viability for a total of $n = 85$ total embryos; $n =$ between 18 and 23 for each treatment.

Culture conditions and seawater acidification

In experimental round II, culture water was collected in Opunohu Bay, Mo'orea, at a depth of 8 m and filtered through a sand filter, before passing through UV-sterilization (Universal 12000 UV filter, Aqua Zonic) and a final 75-µm filtration. Filtered seawater was delivered to four 120 L

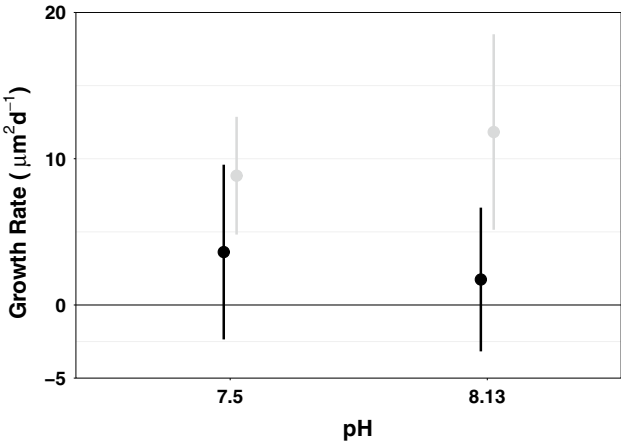

Fig. 1 Experimental round II: mean growth rates of *Stylocheilus striatus* embryos over the first 72 h of development under exposure to climatic stressors (elevated temperature, 30.8 °C, light gray; and/or High pCO_2, pH 7.5) and control conditions (pH 8.13 and 28.2 °C). Error bars represent one standard deviation from the mean

header tanks at a rate of 4 L hr^{-1}. Parameters in header tanks were maintained at levels similar to experimental round I following a 2 × 2 temperature (28.2 ± 0.8 °C modern ambient, 30.8 ± 0.6 °C predicted future) cross pH (8.13 ± 0.09 present and 7.50 ± 0.09) design. Natural diel variability in temperature and pH was simulated in each of the header tanks, with temperature/pH an average of 1 °C/0.1 unit lower during the evening (roughly 19:01 – 6:59). For each temperature by pH treatment, culture water was delivered from the header tanks via driplines (7.57 L hr^{-1} flow rate) to six replicate 1.7 L polypropylene aquaria which were lightly aerated and which each contained the mesh-bottom, embryo-bearing Falcon tubes.

In experimental round II, elevated pCO_2 treatments were maintained by controlled bubbling header tanks with pure CO_2 using an IKS Aquastar pH controller and solenoid-valve gas regulation system (CO_2 Art). Seawater pH_T was measured four times per day using a Professional Plus Multiparameter Instrument (YSI Quatro Dual, Model 1001 pH sensor). Measurements of pH_T were also conducted spectrophotometrically twice during the experimental run using *m*-Cresol Purple sodium salt dye using an Evolution 60S UV–Visible spectrophotometer against a Tris-buffered pH reference standard (Dickson Batch 13/Bottle 74) following modified best practice methods (Dickson et al. 2007). Seawater alkalinity was measured as described previously in experimental round I. All carbonate chemistry parameters are listed in Table 1.

Statistical analyses

Data from both experimental rounds I and II were analyzed using the statistical software program R (v 3.2.5; (R Development Core Team 2008)). For all datasets, outliers were identified and removed using the adjboxStats function of the "robustbase" package in R. Normality of data was tested using the shapiro test and test_normality functions of the "stats" and "LambertW" packages. Because all data showed non-normal distributions (binomial residual densities) which could not be corrected via simple transformations (e.g. log-normal, boxcox, or cubic-root transformation), non-parametric unrestricted permutation testing was used to investigate the effects of high pCO_2 and elevated temperature on response variables (Manly 2007). Pairwise permutation tests with *p* value adjustment for multiple comparisons were conducted using the pairwise.permutation. test function and used as post hoc tests for all significant permutation tests. In experimental round I, maternal origin was not tracked and, therefore, could not be included in statistical analyses of the response variables. In experimental round II, maternal origin was found to have no effect on embryonic growth rates (ANOVA, $P > 0.05$) and thus was not included as a factor in permutation tests. Results from all statistical analyses are given in Table 2.

Results

Description of egg masses

In captivity, *S. striatus* egg clutches were oviposited to both the fronds and stems of the algae *Sargassum spp.* and to the walls of the aquaria used for mating. *S. striatus* egg clutches are produced as a continuous, 1.0 mm wide egg ribbon averaging ~6 cm in length (Suppl. Fig. 4). Each mm of the ribbon contains approximately 145 round, 0.15 mm wide capsules, each holding between 1 and 4 eggs. Egg ribbons were initially pale yellow in color and slowly changed to pale amber. Incubated in ambient seawater conditions, an average of ~8500 veligers (estimated from experimental round I Hatching Success experiments) hatched per egg strand after a 7–9 day duration of development.

Experimental round I

Developmental timing

Mean time (± se) to first observed embryonic movement was 3.6 ± 0.1 and 4.2 ± 0.1 days for embryos reared under ambient and high pCO_2 conditions, respectively. This decreased to 1.9 ± 0.1 and 2.1 ± 0.02 days under elevated temperature and multistressor exposure, respectively. Mean time to eyespot development was 5.9 ± 0.1 and 6.2 ± 0.1 days for embryos reared under ambient and high pCO_2 conditions, respectively. This decreased to 4.0 ± 0.1 days under either elevated temperature or

Table 2 Results of statistical analyses

Response variable (round)	Factor	F value	DF[a] (residuals)	*P* value	Sample size (*n*)
Early growth rate (round II)	Elevated temperature	2.5	16	0.16	20
	High pCO_2	0.02	–	0.92	–
	Interactive effect	0.32	–	0.60	–
Days to movement (round I)	Elevated temperature	468.3	37	<0.0001	44
	High pCO_2	27.7	–	<0.0001	–
	Interactive effect	4.3	–	0.048	–
Days to protoconch (round I)	Elevated temperature	646.9	30	<0.0001	44
	High pCO_2	0.7	–	0.41	–
	Interactive effect	3.2	–	0.09	–
Days to eyespot (round I)	Elevated temperature	632.7	33	<0.0001	44
	High pCO_2	3.3	–	0.07	–
	Interactive effect	2.8	–	0.10	–
Days to hatch (round I)	Elevated temperature	285.6	33	<0.0001	44
	High pCO_2	4.8	–	0.03	–
	Interactive effect	15.4	–	0.0006	–
Hatching success (round I)	Elevated temperature	238.8	38	<0.0001	44
	High pCO_2	24.2	–	<0.0001	–
	Interactive effect	0.9	–	0.36	–
2D-Lateral shell area (round I)	Elevated temperature	470.1	411	<0.0001	440
	High pCO_2	477.5	–	<0.0001	–
	Interactive effect	76.9	–	<0.0001	–
Lateral shell width (round I)	Elevated temperature	2500.9	411	<0.0001	440
	High pCO_2	2333.7	–	<0.0001	–
	Interactive effect	76.5	–	<0.0001	–
Lateral shell length (round I)	Elevated temperature	1412.3	411	<0.0001	440
	High pCO_2	1259.2	–	<0.0001	–
	Interactive effect	75.1	–	<0.0001	–

All response variables were analyzed using unrestricted permutation of observations (Manly 2007) with high pCO_2 and elevated temperature as factors

[a] All treatment degrees of freedom were 1, given values are for residual degrees of freedom

multistressor exposure. Mean time to first larval emergence (i.e. hatching) was 7.7 ± 0.2 and 8.6 ± 0.2 days for embryos reared under ambient and high pCO_2 conditions, respectively. This decreased to 5.2 ± 0.2 days under either elevated temperature or multistressor exposure. Mean time to protoconch formation was 4.9 ± 0.1 and 5.1 ± 0.1 days for embryos reared under ambient and high pCO_2 conditions, respectively. This decreased to 3.0 ± 0.1 days and 2.9 ± 0.1 days under elevated temperature and multistressor exposure, respectively (Fig. 2).

Elevated temperature and high pCO_2 treatments had significant individual and combinatorial effects on the ontological timing of embryonic development in *S. striatus* (Fig. 2; Table 2). For all developmental stages, exposure to elevated temperature, either alone or in combination with high pCO_2, hastened development, resulting in early emergence of key embryonic features ~48 h in advance of control groups. These effects were primarily temperature driven and simultaneous exposure to acidification did not significantly alter developmental rates for any embryonic stage (Manly's Paired Permutation Analysis, Table 2). Exposure to high pCO_2 had variable effects on development, increasing time to embryo movement and hatching ($\Delta t \pm SD = +14 \pm 5$ and $+20 \pm 16$ h, respectively), but not significantly altering time to protoconch or eyespot formation (Manly's Paired Permutation Analysis, Table 2). There was a significant interaction between high pCO_2 and elevated temperature treatments in two of the development variables: the time to first embryonic movement and time to hatching (Table 2).

Hatching success

Mean percentage (± se) of successful hatching was 93.7 ± 1.0 and 74.7 ± 4.2% for embryos reared under ambient and high pCO_2 conditions, respectively. This

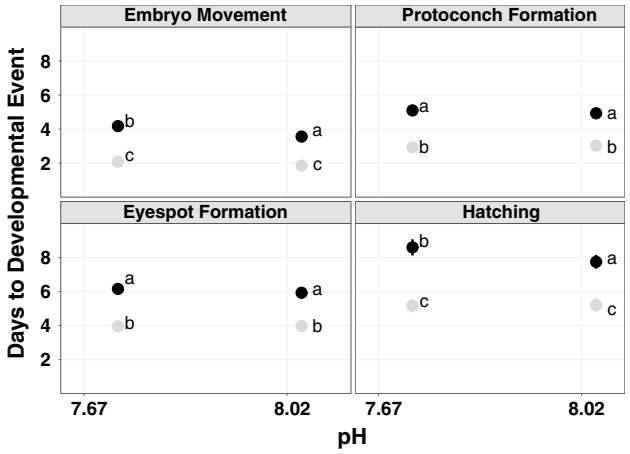

Fig. 2 Experimental round I: developmental rates of *Stylocheilus striatus* embryos incubated under climatic stressors (elevated temperature, 31 °C, *light gray*; and/or high pCO_2, pH 7.67) and under control conditions (pH 8.02 and 27 °C). Values represent the mean number of days post-oviposition to reach each developmental stage (embryonic movement, protoconch formation, eyespot formation, hatching). Error bars represent 95% confidence intervals around the mean. *Letters* highlight significant differences between means

decreased to 39.3 ± 2.4 and 25.7 ± 3.9% under elevated temperature and multistressor exposure, respectively.

Both exposure to high pCO_2 and elevated temperature decreased *S. striatus* hatching success with temperature exhibiting the larger negative effect (Fig. 4). Embryos exposed to both conditions simultaneously exhibited similar hatching success to those reared under high temperature alone and there was no significant interaction between elevated temperature and high pCO_2 treatments on hatching success in *S. striatus* (Table 2).

Veliger shell morphology

Mean shell area (± se) was 6596.2 ± 68.8 and 4202.4 ± 78.4 μm^2 for embryos reared under ambient and high pCO_2 conditions, respectively. This decreased to 4164.6 ± 67.4 and 3157.6 ± 124.3 μm^2 under elevated temperature and multistressor exposure, respectively.

All measured morphological characteristics of the veliger shells were significantly affected by high pCO_2 and elevated temperature treatments, and there was a general trend towards decreasing shell size with increasing departure of environmental conditions from ambient norms during incubation. Lateral shell length and width (spiral height; Fig. 5), as well as 2-dimensional shell area (Fig. 6) were all significantly reduced under both high pCO_2 and elevated temperature treatments, alone and in combination. Embryos exposed to both stressors simultaneously exhibited a synergistically greater reduction in shell morphometrics and area than under exposure to either stressor alone. In general, exposure to high pCO_2 during development also led to greater variance in shell morphometric parameters between individuals post-hatching (Fig. 5, 6).

Experimental round II

There was no significant difference in embryonic growth rates between short (i.e. exposed) and long (i.e. well-protected) strands in any treatment condition (Welch Two Sample T-Test, t(547.32) = -0.5,P = 0.6, Suppl. Fig. 3) and, therefore, these data were combined for all subsequent analyses described below.

Early growth rates (first 72 h)

Mean early growth rate (± se) was 1.7 ± 2.8 and 3.6 ± 3.4 μm^2 d^{-1} for embryos reared under ambient and high pCO_2 conditions, respectively. This increased to 11.8 ± 3.9 and 8.8 ± 2.3 μm^2 d^{-1} under elevated temperature and multistressor exposure, respectively.

There was no significant effect of any abiotic stressor on embryonic growth rates (calculated as the slope of the best fit line of embryo area versus time) during the first 72 h of development in *S. striatus* (Fig. 1; Manly's Paired Permutation Analysis, Table 2).

Early embryo viability (percentage spinning)

Mean percentage (± se) of embryos spinning at 48 h post-oviposition was 64.3 ± 5.3 and 72.2 ± 6.1% for embryos reared under ambient and high pCO_2 conditions, respectively. This increased to 72.7 ± 6.0 and 86.4 ± 4.4% under elevated temperature and multistressor exposure, respectively.

At 48 h post-oviposition, only embryos reared under the multistressor treatment showed an increased proportion of individuals moving (Fig. 3, Manly's Paired Permutation Analysis, Table 2). There was no interactive effect of temperature and acidification on proportion of embryos spinning by 48 h post-fertilization.

Discussion

This study examined embryonic development of the tropical sea hare *S. striatus* under present day and elevated pCO_2 and warmer conditions, alone and in combination. Exposure to high pCO_2 had a primarily negative effect on development, resulting in delayed development times (Fig. 2), smaller size at hatching (Figs. 5, 6), and reduced hatching success (Fig. 4). Exposure to elevated temperature yielded more varied results. Elevated temperature had no significant effect on early growth rates over the first 72 h of

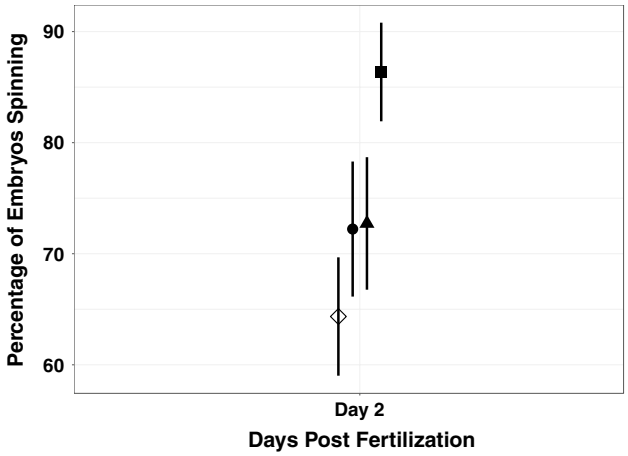

Fig. 3 Experimental round II: percentage of embryos visibly moving within egg capsules (egg viability) at 48 h post-fertilization incubated under control conditions (28.2 °C, pH 8.13, *diamond*), elevated temperature (30.8 °C, *filled triangle*), high pCO_2 (pH 7.50, *filled circle*) and multistressor conditions (30.8 °C, pH 7.50, *filled square*). *Values* represent the mean percentage of embryos that were spinning in a given egg ribbon section and error bars represent 95%-confidence intervals around the mean

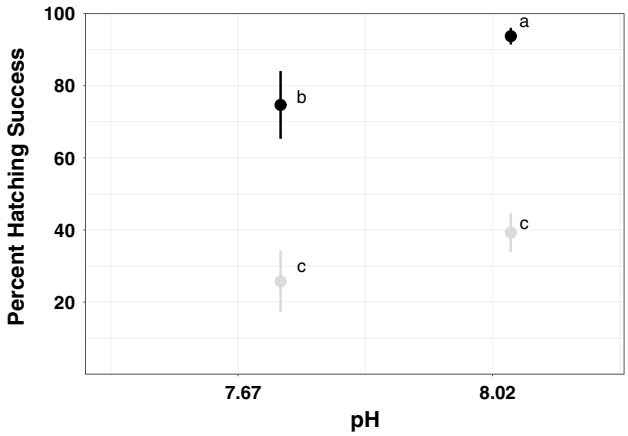

Fig. 4 Experimental round I: percentage hatching success of embryos incubated under climatic stressors (elevated temperature, 31 °C, *light gray*; and/or high pCO_2, pH 7.67) and control conditions (pH 8.02 and 27 °C). *Values* represent the mean percentage of embryos that successfully hatched from an egg ribbon and *error bars* represent 95% confidence intervals around the mean. *Letters* indicate significant differences between means

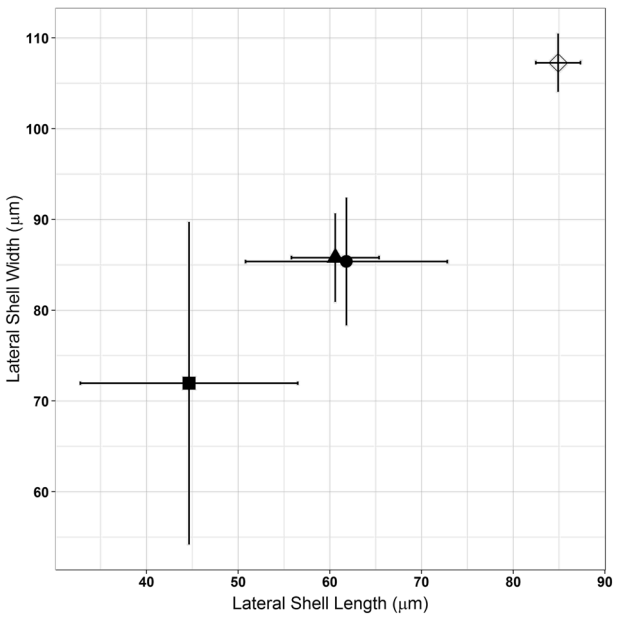

Fig. 5 Experimental round I: veliger-stage larval shell morphometrics. Plotted are lateral shell length and width (spiral height) of veliger larvae of *Stylocheilus striatus*, post-hatching, for larvae reared under control conditions (27 °C, pH 8.02, *diamond*), elevated temperature (31 °C, *filled triangle*), high pCO_2 (pH 7.67, *filled circle*) and multistressor conditions (31 °C, pH 7.67, *filled square*). *Error bars* represent 95% confidence intervals around the mean

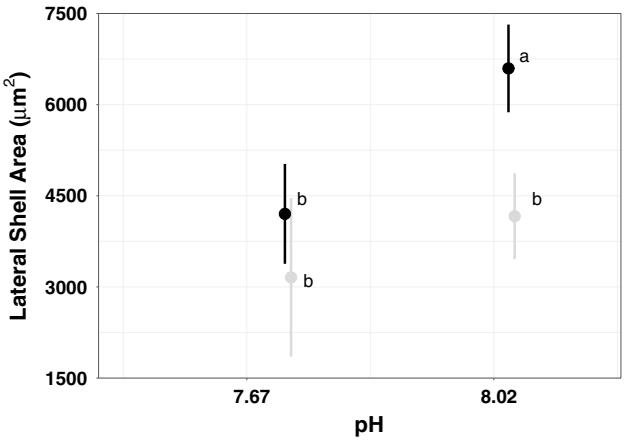

Fig. 6 Experimental round I: lateral shell area of embryos incubated under climatic stressors (elevated temperature, 31 °C, *light gray*; and/or high pCO_2, pH 7.67) and control conditions (pH 8.02 and 27 °C). *Values* represent the mean lateral shell area of a treatment group and *error bars* represent 95% confidence intervals around the mean. *Letters* indicate significant differences between means

development (Fig. 1), but resulted in precocious development of key embryonic features and reduced overall development time (Fig. 2). However, this came at the expense of decreased hatching success (Fig. 4) and reduced shell size (Figs. 5, 6) under elevated temperature. Taken together, these results suggest that while elevated temperature may reduce the time an individual larva remains within an egg capsule (a potentially positive effect), it tends to result in decreased larval size at hatching (associated with reduced fitness for individuals) and reduced emergence rates relative to control conditions (reduced fitness at the population level) both of which may outweigh the benefits of accelerated development.

For all response variables except early growth rate (in which there was no effect of any abiotic stressor) and shell calcification (in which effect sizes were equally strong for all exposure treatments), temperature had a stronger effect on larval development in *S. striatus* than acidification. Effect sizes of exposure to elevated temperature were on average, 3.5-times larger (and up to ~9-times larger) than responses to high pCO_2 (Fig. 7). Similar primacy of temperature as an abiotic stressor has been observed in many species (Hochachka and Somero 2002), including the biological responses of respiration and cardiac performance as well as calcification and survival in crustaceans (Harvey et al. 2013; Paganini et al. 2014), calcification, growth, and survival in echinoderms (Nguyen et al. 2012; Harvey et al. 2013; Wangensteen et al. 2013), and growth and survival in molluscs (Harvey et al. 2013; Zhang et al. 2015).

In general effect sizes increased with increasing exposure time rather than exposure severity. For example, although exposure pH was 0.2 units lower in round II experiments (early-stage growth rates and viability) relative to round I experiments (developmental timing, hatching success, and shell size at hatching) effects of acidification were ~4-times greater in the latter (Fig. 7). This suggests that cumulative costs of exposure to reduced pH may outweigh the effects of a more severe, acute, exposure over the first 72 h of growth in *S. striatus* embryos. It is also likely that effects of exposure to pH 7.5 would have been accentuated had round II experiments been carried out through hatching as in round I. Exposure temperatures were more similar between experimental rounds and showed the same pattern with stronger effects resulting from longer exposures.

For some phenotypic characters (days to first embryonic movement, overall time to hatching, and shell morphometrics—length, width, and 2-dimensional lateral shell area), there was a strong interactive effect of temperature and high pCO_2 (Figs. 4, 5, 6) highlighting the importance of examining simultaneous exposure to multiple environmental stressors for understanding ecological responses of calcifying organisms under predicted future ocean conditions. This multistressor interactive effect was most pronounced for larval shell area and *S. striatus* exposed to both stressors simultaneously exhibited shells that were ~30% smaller than those formed under either abiotic condition in isolation (Figs. 5, 6). Reduced larval size has been linked to reduced settlement success and increased mortality (through inefficient food intake or increased vulnerability to predation) in several species (Anger 1987; Anderson 1988; Houde 1989; Garrido et al. 2015). We observed the lowest hatching success among the smallest size class of *S. striatus* embryos (i.e. those reared under multistressor conditions, Fig. 4) suggesting that this synergistic reduction of size under multiple abiotic stressors is likely to have significant ecological consequences for *S. striatus* under future ocean conditions.

Testing the hypothesis regarding increased susceptibility of tropical species such as *S. striatus* to the combined effects of warming and acidification is at present difficult due to the lack of comparative data from other molluscan (and especially gastropod) taxa. However, comparison of the combined effects of high pCO_2 and warming on larval survival between *S. striatus* and two temperate bivalves (*Mercenaria mercenaria* and *Argopectens irradians*) suggests that *S. striatus* is not particularly sensitive to these climate stressors (Talmage and Gobler 2011). For example, exposure of *S. striatus* larvae to temperature and pH conditions expected to occur by the year 2100 reduced survival by 23.8% (per 0.2 pH units) (Fig. 4) which is comparable to the 20.6 and 23.9% reductions exhibited in two temperate bivalve larvae studied previously (Talmage and Gobler 2011). This would seem to suggest that although tropical

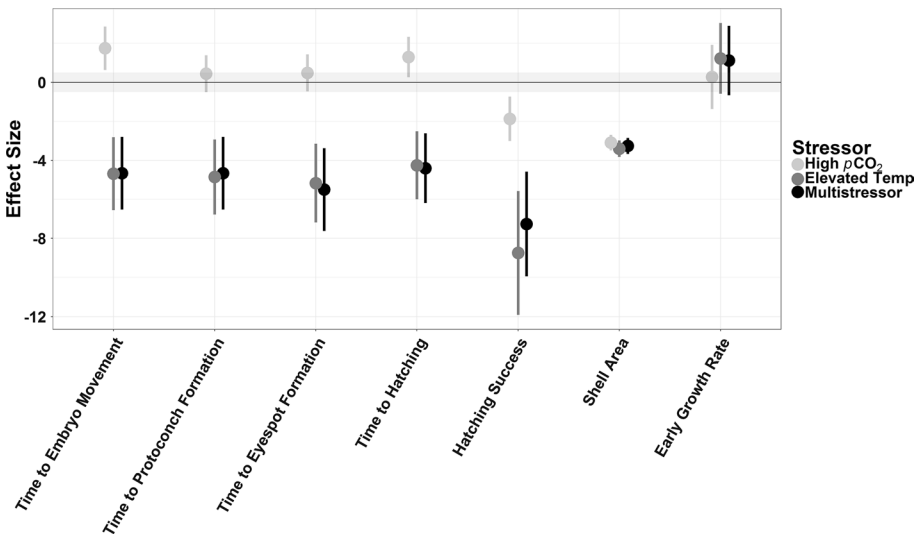

Fig. 7 Calculated Cohen's D effect sizes of examined environmental stressors (high pCO_2, *light gray*; elevated temperature, *dark gray*; and multistressor, *black*) on developmental and morphological features of *S. striatus* embryos. *Gray region* denotes "small to negligible" effects threshold (Cohen 1992). *Error bars* represent 95% confidence intervals around the mean. All data except "Early Growth Rate" are from experimental round I conducted at 31 °C and pH 7.67. Early growth rate data are from experimental round II conducted at 31 °C and pH 7.50

species such as *S. striatus* may be especially vulnerable to future warming, their ability to cope with additional simultaneous abiotic stressors is not significantly compromised as a result. One possible explanation for this resilience is that heat and acidification stress-response pathways may be shared in *S. striatus* thereby conferring increased resistance to both stressors when encountered simultaneously (an example of cross-tolerance) (Todgham and Stillman 2013). Further investigation, however, is needed to determine the mechanistic underpinnings of the responses to elevated temperature and high pCO_2 observed in this study.

As reported for the gastropod *Crepidula fornicata* (Noisette et al. 2014), encapsulation did not seem to confer any significant benefit to embryonic *S. striatus* during exposure to high pCO_2. Embryos which developed fully within the protective jelly-coating of the egg ribbon were equally as affected by environmental conditions as those that developed at the extreme edges (Suppl. Fig. 3). Although the buffering capacity of intracapsular fluids has been proposed as a mechanism for protecting embryonic molluscs from environmental pH fluctuations (Ellis et al. 2009; Manríquez et al. 2014), the ecological effects of encapsulation in *S. striatus* seem to be minor under predicted oceanic pH reduction. Exposure to increased pCO_2 significantly affected larval calcification rates, resulting in smaller shells at hatching (Fig. 5). This implies that, unlike other groups such as annelids, parental investment in egg-protection mechanisms in *S. striatus* does not significantly improve either larval development or survival under acidification exposure. However, a recent study conducted on the closely related sea hare *Aplysia punctata* found that calcification in adults was particularly resistant to reduced pH and animals were able to maintain calcification rates equivalent to those under ambient pH despite a 30% reduction in overall metabolic rate (Carey et al. 2016). Assuming a similar robustness of calcification in adult *S. striatus,* these results suggest that deleterious effect of exposure to reduced pH in gastropods may be accentuated in early life stages after which impacts to calcification may be trivial.

Tolerance limits of early life history stages may limit species' distributions and act as bottlenecks for population persistence under future climate change conditions (Harley et al. 2006; Fabry et al. 2008; Brierley and Kingsford 2009). In addition, because of their increased sensitivity to environmental change, early life history stages of these species are often implicated as key periods for selection and embryonic tolerances are likely to play a crucial role in defining suitable habitats and determining species persistence. The results of this study clearly demonstrate that future oceanic conditions congruent with current IPCC climate change projections (IPCC 2014) have detrimental effects on the developmental success of encapsulated gastropod embryos. While exposure to high pCO_2 did result in significant developmental delays and reduced shell size at hatching, overall, elevated temperature had the strongest negative effects on development in *S. striatus* leading to significant reductions in larval size and viability. These results suggest that rising temperature, rather than increasing acidity, may be the primary abitotic stressor of physiological response in early life stages in *S. striatus*.

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Compliance with ethical standards

Conflict of interest All applicable international and institutional guidelines for the care and permissions for the use of animals were followed during the conduct of this research. The authors declare no conflicts of interest in regards to the study presented here.

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