

# Impact of climate change on the ontogenetic development of 'solar-powered' sea slugs

Gisela Dionísio<sup>1,2,3,\*</sup>, Filipa Faleiro<sup>1</sup>, Meri Bilan<sup>1,4,5,6</sup>, Inês C. Rosa<sup>1,2</sup>,  
Marta Pimentel<sup>1</sup>, João Serôdio<sup>2</sup>, Ricardo Calado<sup>2</sup>, Rui Rosa<sup>1</sup>

<sup>1</sup>MARE – Marine and Environmental Sciences Centre, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Av. Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

<sup>2</sup>Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

<sup>3</sup>Naturalist, Science & Tourism, Rua da Ladeira, n°2, 9900-029 Horta, Faial, Azores, Portugal

<sup>4</sup>MARE – Marine and Environmental Sciences Centre, University of the Azores, 9900-862 Horta, Azores, Portugal

<sup>5</sup>IMAR - Institute of Marine Research, University of the Azores, 9900-862 Horta, Azores, Portugal

<sup>6</sup>University Department of Marine Studies, Livanjska 5, 21 000 Split, Croatia

**ABSTRACT:** Solar-powered animals are particularly susceptible to climate change because photosynthesis brings major risks to animals under environmental stress. Although some groups have been widely studied (e.g. corals), little information is available on how other less charismatic photosynthetic animals (e.g. sea slugs) will respond to future ocean conditions. This study is the first to evaluate the impact of future ocean conditions on the fitness of tropical photosynthetic sacoglossan sea slugs throughout different life stages. Adults of *Elysia clarki* were exposed (30 d) to conditions simulating present-day and predicted scenarios of ocean acidification ( $\Delta\text{pH} = 0.4$ ) and warming ( $+4^\circ\text{C}$ ). Egg masses were incubated under the same conditions as adult broodstock until 15 d after metamorphosis. Exposure to ocean acidification and warming scenarios led to a significant decrease in the number of spawned egg masses and in their membrane thickness. Moreover, a significant decrease in the volume of embryo capsules was accompanied by an increase in embryo volume. These findings suggest that sea slugs shifted their energy allocation towards embryo quality rather than to structures that confer protection from environmental challenges. Climate change-related stress significantly reduced the survival and length of veligers and increased the incidence of deformities, but did not affect chloroplast acquisition by juvenile slugs. The lower reproductive output of adults and the adverse impacts on early life stages recorded under future ocean conditions allows us to anticipate negative consequences for the recruitment of these sea slugs' populations in the oceans of tomorrow.

**KEY WORDS:** Acidification · Warming · Sacoglossan sea slugs · Ontogenetic development · Growth · Malformations · Chloroplast acquisition

Resale or republication not permitted without written consent of the publisher

## INTRODUCTION

Anthropogenic activities have contributed to an unprecedented increase in atmospheric carbon dioxide ( $\text{CO}_2$ ) concentration over the last decades. Indeed,  $\text{CO}_2$  partial pressure ( $\text{pCO}_2$ ) in the atmosphere increased 40% since 1750 (from 280 to 390 ppm; Hartmann et al. 2013), and a further rise up to

940 ppm is expected by the year 2100 (Pörtner et al. 2014). Approximately 25% of the emitted  $\text{CO}_2$  from anthropogenic activities has been dissolved in the ocean. A decrease of 0.1 units in the pH of surface waters was observed over the last decades, with projections indicating a further decrease between 0.14 and 0.42 units by the end of the 21st century (Pörtner et al. 2014). Another consequence of rising atmo-

spheric pCO<sub>2</sub> is an increase in global surface temperatures, with predictions pointing to an increase of up to 2.7°C by the end of the present century (Pörtner et al. 2014). The ocean is absorbing some of the heat from the atmosphere, with the functioning of marine ecosystems linked to naturally-occurring patterns, such as the El Niño/Southern Oscillation and the North Atlantic Oscillation (Hurrell & Deser 2009), and being strongly affected by climate variability.

Only in the last several years have researchers begun to understand how increasing ocean temperature and pCO<sub>2</sub> may affect marine life. A large body of literature has investigated the effects of increased ocean pCO<sub>2</sub> and temperature on the physiology (Anestis et al. 2007, Pörtner & Farrell 2008, Rosa & Seibel 2008, Todgham & Stillman 2013), development and growth (Przeslawski et al. 2005, Kurihara et al. 2008a, Byrne & Przeslawski 2013, Kroeker et al. 2013, Wolfe et al. 2013, Rosa et al. 2014a), calcification (Watson et al. 2009, Lischka et al. 2011, Gianguzza et al. 2013, Pimentel et al. 2014), and reproduction (Havenhand et al. 2008, Byrne et al. 2009) of marine taxa. Overall, most studies have shown that organisms can be negatively affected by ocean acidification and warming. Environmental changes during the reproductive conditioning of marine species can negatively affect fecundity and offspring survival (Muranaka & Lannan 1984, Rossiter 1996, Przeslawski & Webb 2009). Previous research has shown that climate change-related variables may have a negative impact on the number and quality of offspring of marine invertebrates, namely on egg production, incidence of larval malformations, survival and developmental rate (Beukema et al. 1998, Honkoop et al. 1998, Kurihara et al. 2008b, Parker et al. 2010, Hettinger et al. 2012, 2013, Davis et al. 2013, Rosa et al. 2014b).

'Solar-powered' sacoglossan sea slugs from the genus *Elysia* spp. are gastropod molluscs with high economic and ecological value (Dionísio et al. 2013). One of the most remarkable features of these organisms is their ability to retain photosynthetically active chloroplasts (kleptoplasts) 'stolen' from their algal food sources (Rumpho et al. 2000, Cruz et al. 2015). Additionally, these organisms are simultaneous hermaphrodites with internal cross-fertilization. *Elysia* spp. lay their egg masses and release free-living veliger larvae. Larvae can be planktotrophic or lecithotrophic depending on the environmental conditions. Larvae usually metamorphose into juveniles approximately 15 d after oviposition, using dietary preys as metamorphic inducers (Dionísio et al. 2013, 2017).

Climate change studies on photosymbiotic sea slugs are extremely scarce. To the best of our knowledge, there is only one short study that evaluates the effects of hypercapnia on *Elysia clarki* adults exposed for 24 h to reduced pH (Dionísio et al. 2015). This tropical species seems to be resistant to the acidified conditions expected for 2100, at least for short periods of time. No significant effect was observed on the fitness and photosymbiotic associations of this species, similarly to that observed in photosymbiotic acoel worms (Dupont et al. 2012). Understanding the capacity of organisms to acclimate to the expected changes in ocean pH and temperature is paramount for better prediction of the biological impacts of ocean climate change. This is particularly relevant for tropical species that are less capable for acclimation to climate change (Donelson et al. 2012).

To better understand the potential effects of climate-change-related variables on solar-powered sea slugs, the present study aims to evaluate, for the first time, how ocean acidification ( $\Delta\text{pH} = 0.4$ ) and warming (+4°C) affect the early ontogenetic development of the tropical photosynthetic sea slug *E. clarki*.

## MATERIALS AND METHODS

### Exposure of adults to different climate change scenarios

One hundred specimens of the tropical sea slug *Elysia clarki* ( $41.1 \pm 3.8$  mm of total length) were collected off the Florida Keys coastline and shipped to the Laboratório Marítimo da Guia by the Tropical Marine Centre, a marine aquarium wholesaler recognized for its efforts on the sustainable collection and trade of reef organisms and promotion of animal welfare.

Upon arrival, organisms were randomly placed in 4 recirculating systems, each one composed of 250 l aquaria. Each system was filled with 0.2  $\mu\text{m}$  filtered natural seawater and equipped with mechanical (100  $\mu\text{m}$  filter, Tropical Marine Centre, Portugal), chemical (REEF-Skim Pro 400, Tropical Marine Centre, Portugal) and biological (bioballs, Fernando Ribeiro, Portugal) filtration, as well as with UV irradiation (Vecton 600, Tropical Marine Centre, Portugal). Ammonia and nitrite levels were monitored weekly using colorimetric test kits (Aquamerck, Merck Millipore, Germany) and kept within recommended levels. Overhead tank illumination was provided by a dimmable LED artificial lighting apparatus (Aquabeam 1500 Ultima NP Ocean Blue,

Tropical Marine Centre, Portugal), consisting of 5 white XP-G LEDs (9000 K) and 5 XP-E blue LEDs (50 000 K) suitable for marine set-ups. Photosynthetically active radiation (PAR) was measured using a FluorPen FP100 light meter (Photo System Instruments, Czech Republic) and maintained at  $150 \pm 15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at water surface. Photoperiod was set to 14 h light:10 h dark. The macroalgae *Bryopsis plumosa* (previously acclimated for 2 d to the same conditions of the stocked sea slugs to which they were going to be supplied) was provided *ad libitum* as dietary prey. Food was never a limiting factor and was selected according species dietary regime (Curtis et al. 2007).

After an acclimation period of 2 wk at controlled conditions (26°C and pH 8.0, corresponding to the ambient temperature and pH conditions at the collection site), adults were divided into five 5 l tanks per treatment ( $n = 5$  individuals per tank,  $n = 25$  individuals per treatment). To achieve good results on broodstock reproduction (see Dionísio et al. 2013 for detailed information), the following issues were carefully addressed: (1) animals were stocked at optimal densities for this species, and (2) similar-sized animals were paired in each tank. Organisms were then exposed to a gradual increase of  $\text{pCO}_2$  and temperature during 5 d. After that period, organisms were exposed for 4 wk to 4 different treatments simulating present-day and predicted climate change scenarios of ocean acidification and warming: (i) Control treatment: normocapnia ( $\sim 400 \mu\text{atm}$ , pH 8.0) and average sea temperature (26°C); (ii) Acidification treatment: hypercapnia ( $\sim 1100 \mu\text{atm}$ , pH 7.6) and control temperature; (iii) Warming treatment: the respective warming scenario (+4°C, 30°C) and normocapnia; and (iv) Acidification + Warming treatment: the warming scenario and hypercapnia.

Water temperature and pH were adjusted automatically by using a Profilux controlling system (GHL, Germany) connected to individual temperature and pH probes. Temperatures were automatically upregulated by heaters and downregulated using cooling systems (HC-1000A, Hailea, China). pH was monitored every 2 s and adjusted automatically via solenoid valves, being downregulated through the injection of a certified  $\text{CO}_2$  gas mixture (Air Liquid, Portugal) via air stones and upregulated by aerating the tanks with filtered air. Salinity was kept at  $35.0 \pm 1.0$  throughout the experiment. Seawater carbonate system speciation (Table 1) was calculated weekly based on total alkalinity (see Sarazin et al. 1999), pH, temperature and salinity measurements, using the CO2SYS software developed by Lewis & Wallace (1998) with dissociation constants from Mehrbach et al. (1973).

#### Exposure of egg masses to different climate change scenarios

A preliminary experiment was carried out in order to characterize the embryonic development of *E. clarki*. The different developmental stages (see Table 2) were established based on the appearance or disappearance of specific morphological structures, such as cilia, velum, shell, eyespots and propodium, among others (according to Thompson 1967, Trowbridge 2000).

Egg masses produced during adult exposure to different climate change scenarios were transferred to individual rearing boxes placed inside the parental tanks, thereby being maintained under the same conditions as the parental organisms. Development was monitored daily from the moment of egg mass

Table 1. Seawater carbonate chemistry during the exposure of *Elysia clarki* adults and early life stages to different temperature and pH conditions. Values for  $\text{pCO}_2$ ,  $\Omega_{\text{aragonite}}$  and  $\Omega_{\text{calcite}}$  were calculated from salinity, temperature, pH and total alkalinity (TA), using CO2SYS software. Values are given as means  $\pm$  SD

Treatment	Temperature (°C)	pH (total scale)	TA ( $\mu\text{mol kg}^{-1}$ )	$\text{pCO}_2$ ( $\mu\text{atm}$ )	$\Omega_{\text{aragonite}}$	$\Omega_{\text{calcite}}$
<b>Adults</b>						
Control	$26.0 \pm 0.1$	$8.0 \pm 0.1$	$2075.9 \pm 48.3$	$393.4 \pm 9.6$	$2.97 \pm 0.08$	$4.51 \pm 0.10$
Acidification	$26.0 \pm 0.1$	$7.6 \pm 0.1$	$2028.6 \pm 37.1$	$1144.7 \pm 21.3$	$1.30 \pm 0.02$	$1.96 \pm 0.03$
Warming	$30.0 \pm 0.1$	$8.0 \pm 0.1$	$2063.9 \pm 29.1$	$398.4 \pm 5.9$	$3.30 \pm 0.08$	$4.93 \pm 0.10$
Acidification + Warming	$30.0 \pm 0.1$	$7.6 \pm 0.1$	$2059.0 \pm 27.9$	$1181.6 \pm 16.3$	$1.51 \pm 0.02$	$2.23 \pm 0.03$
<b>Early stages</b>						
Control	$26.0 \pm 0.1$	$8.0 \pm 0.1$	$1952.5 \pm 53.3$	$381.6 \pm 9.2$	$4.13 \pm 0.10$	$2.73 \pm 0.06$
Acidification	$26.0 \pm 0.1$	$7.6 \pm 0.1$	$1837.9 \pm 36.3$	$1032.2 \pm 20.8$	$1.78 \pm 0.03$	$1.17 \pm 0.02$
Warming	$30.0 \pm 0.1$	$8.0 \pm 0.1$	$2004.5 \pm 21.3$	$386.3 \pm 4.3$	$4.75 \pm 0.05$	$3.18 \pm 0.03$
Acidification + Warming	$30.0 \pm 0.1$	$7.6 \pm 0.1$	$1856.9 \pm 27.3$	$1053.9 \pm 15.9$	$2.05 \pm 0.03$	$1.38 \pm 0.02$

Table 2. Developmental stages of *Elysia clarki* from embryo to juvenile.  
Days: minimum number of days after egg mass deposition at 26°C

Development stage	Description	Days
<b>Before hatching</b>		
Pre-veliger	Division stage, blastula and gastrula	0
Veliger	Development of shell, cilia and foot	3
	Development of nephrocyst and otocyst	5
	Development of eyespots	6
	Development of black/purple pigmentation, operculum and propodium	8
	Intracapsular metamorphosis	9
	Capsule discard	12
<b>After hatching</b>		
Juvenile	Metamorphic shell discard	13
	Acquisition of chloroplasts	14

deposition until 15 d post-hatching (after they had reached the juvenile stage). Juveniles were fed *ad libitum* with their preferred dietary prey, *Bryopsis plumosa*. The presence of food was assured before the juvenile stage was reached, in order to stimulate metamorphic competence.

#### Effects of different climate change scenarios on egg masses and embryos

The presence of egg masses was checked for daily during the 4 wk exposure period of adult sea slugs. Egg masses were counted, collected (by carefully detaching the egg masses from the substrate using a plastic nail), and photographed under a microscope (DM1000, Leica, Germany) equipped with a digital camera (DFC 450, Leica, Germany). The manipulation of egg masses was kept to a minimum to avoid any damage. Three to 4 egg masses from different tanks were then randomly selected per treatment and used as replicates. Different egg masses were used to evaluate different response variables.

The membrane thickness of each egg mass was determined based on 20 measurements taken along the membrane (Fig. 1). The length ( $L$ ) and width ( $W$ ) of at least 10 embryo capsules per egg mass were measured immediately after deposition (at the un-cleaved stage). The radius ( $R$ ) of at least 10 embryos per egg mass was also measured. All measurements were made using ImageJ software (v.1.8). Embryo capsule volume (ECV) was then determined assuming a prolate spheroid shape ( $ECV = 4/3 \pi W^2 L$ ), while embryo volume was calculated assuming a spheroid shape ( $EV = 4/3 R^3$ ).

#### Effects of different climate change scenarios on veligers

The development time was determined between the day of egg mass deposition and the day of hatching. At Day 8 after deposition, egg masses were observed and photographed under a microscope (DM1000, Leica, Germany) equipped with a digital camera (DFC 450, Leica, Germany). Three to 5 egg masses from different tanks were then randomly selected per treatment and used as replicates. Different egg masses were used to evaluate different response variables.

Survival was assessed by counting the number of live and dead veligers in each egg mass. The presence of deformities was inspected in each living veliger, namely: undeveloped or abnormal shell, abnormal or absent velum, undeveloped propodium, elongated body and complete body deformity. The percentage of larvae with deformities was then determined for each egg mass. At least 15 veligers per egg mass were measured for morphometric analysis. Shell length was measured across the aperture, along with the propodium diameter. According to Trowbridge (2000) and Bickell & Kempf (1983), propodium size can be used as an indicator of metamorphic competence for veligers because the full development of this structure, along with the growth of a dense cover of cilia in the central surface of the foot, enables the crawling behavior of juvenile slugs. All measurements were performed using the ImageJ software (v.1.48).



Fig. 1. *Elysia clarki* egg masses at the Pre-veliger stage. Embryo capsules and egg membrane thickness are shown. Scale bar = 100  $\mu$ m

### Effects of different climate change scenarios on juveniles

As for other opisthobranchs, *E. clarki* loses several morphological structures during metamorphosis, such as the velum, shell and operculum (Bickell & Kempf 1983). Therefore, the loss of these structures was inspected on a daily basis. In each treatment, at least 5 juveniles per egg mass were randomly selected from 4 different egg masses (which were obtained from different tanks). Juveniles were photographed under a microscope (DM1000, Leica) equipped with a digital camera (DFC 450, Leica). Images were then processed for total length measurements using ImageJ software (v.1.48). The acquisition of chloroplasts was also checked daily from hatching until 15 d post-hatching. After feeding on macroalgae, juvenile sea slugs acquire green pigmentation as a result of the incorporation of intact chloroplasts into specific cells lining the digestive gland.

### Statistics

Two-way ANOVA were conducted to evaluate the impact of ocean acidification (2 levels: 8.0 and 7.6) and warming (2 levels: 26 and 30°C) and their potential interactive effect on egg masses (number, membrane thickness, capsule and embryo volumes),

veligers (development time, survival, shell length, propodium diameter and incidence of deformities), and juveniles (total length). Only the mean measures of each egg mass were used as replicates. Percentage data (i.e. survival and deformities) were square-root transformed. The assumptions of normality and homogeneity of variances were verified using the Kolmogorov-Smirnov and Levene's tests, respectively. Subsequently, post-hoc tests (Tukey HSD, Fisher LSD or Unequal N HSD) were performed. All statistical analyses were done with results considered significant at  $p \leq 0.05$ , using STATISTICA v.12.6 software (StatSoft).

## RESULTS

### Effects of different climate change scenarios on egg masses and embryos

The number of egg masses laid by adults *Elysia clarki* (Fig. 2A) decreased significantly (68%) when exposed to future acidified conditions ( $F_{1,16} = 25.5$ ,  $p < 0.01$ ) but it was not significantly affected by warming ( $F_{1,16} = 3.7$ ,  $p = 0.07$ ) or its interactive effect with pH ( $F_{1,16} = 2.4$ ,  $p = 0.14$ ). Egg mass membrane (Fig. 2B) was significantly thinner under reduced pH ( $F_{1,8} = 8.79$ ,  $p = 0.02$ ), but membrane thickness was not significantly affected by temperature ( $F_{1,8} = 3.36$ ,

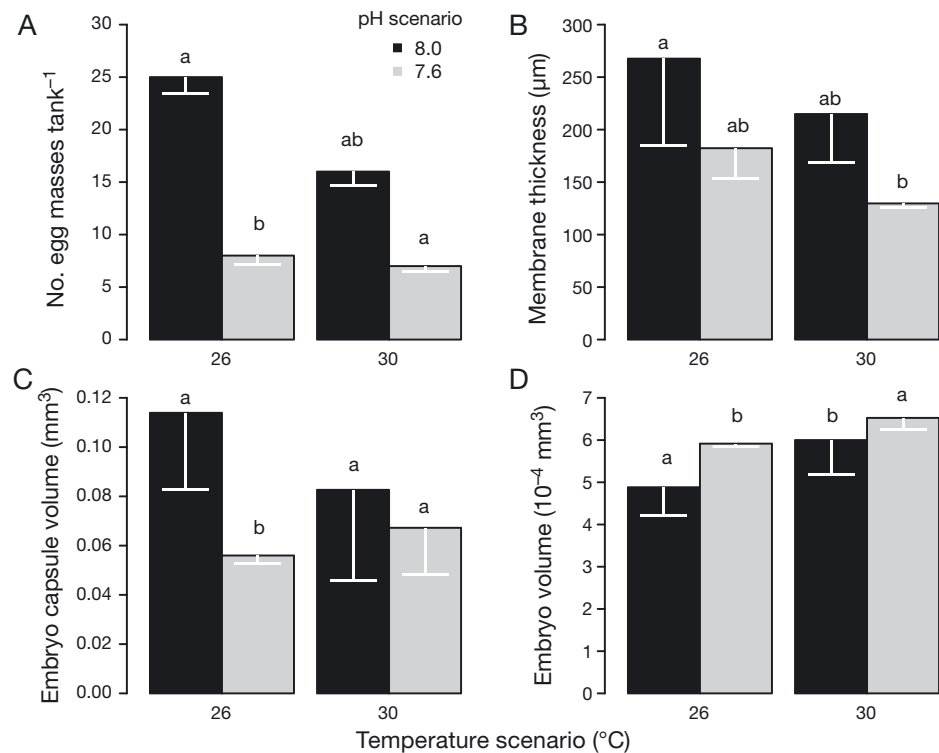


Fig. 2. Effects of ocean acidification and warming on the number and size of *Elysia clarki* egg masses and embryos. (A) Number of egg masses; (B) membrane thickness of egg masses; (C) embryo capsule volume; and (D) embryo volume, under different climate change scenarios: Control (26°C, pH 8.0); Acidification (26°C, pH 7.6); Warming (30°C, pH 8.0); and Acidification + Warming (30°C, pH 7.6) treatments. Values are given as means  $\pm$  SD. Different letters represent significant differences between treatments ( $p < 0.05$ )

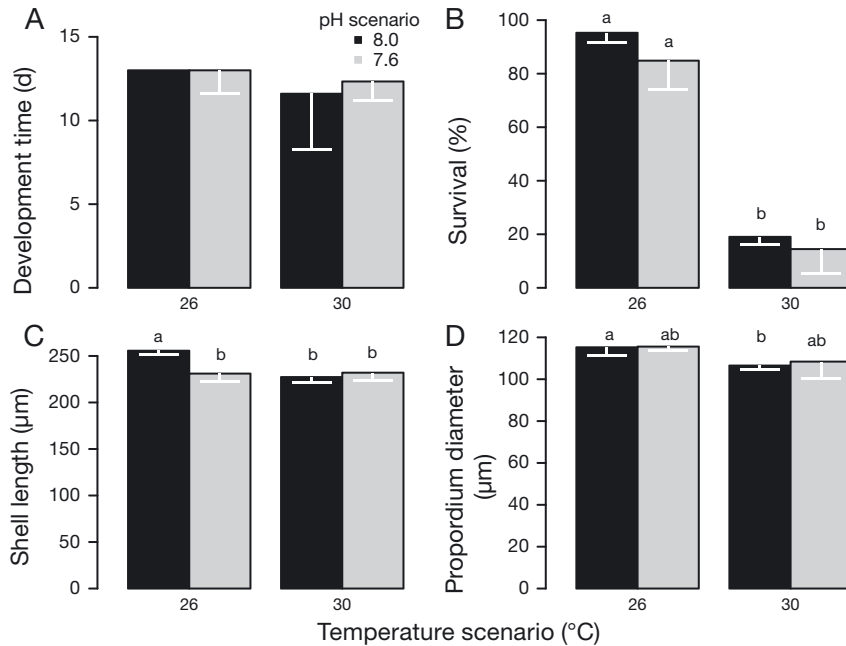


Fig. 3. Effects of ocean acidification and warming on the development of *Elysia clarki* veligers (8 d after egg laying). (A) Development time (from egg laying till hatching); (B) survival; (C) shell length; and (D) propodium diameter, under different climate change scenarios: Control (26°C, pH 8.0); Acidification (26°C, pH 7.6); Warming (30°C, pH 8.0); and Acidification + Warming (30°C, pH 7.6) treatments. Values are given as means  $\pm$  SD. Different letters represent significant differences between treatments ( $p < 0.05$ )

$p = 0.10$ ) or by its interactive effect with pH ( $F_{1,8} = 0.0$ ,  $p = 1.00$ ). Embryo capsule volume (Fig. 2C) notably decreased with acidification ( $F_{1,8} = 6.5$ ,  $p = 0.04$ ) but was not affected by warming ( $F_{1,8} = 0.7$ ,  $p = 0.04$ ) or by its interactive effect with acidification ( $F_{1,8} = 2.2$ ,  $p = 0.18$ ). Embryo volume (Fig. 2D) increased significantly with both decreasing pH ( $F_{1,8} = 6.1$ ,  $p = 0.04$ ) and increasing temperature ( $F_{1,8} = 0.7$ ,  $p = 0.44$ ), but without significant interactive effects ( $F_{1,8} = 0.7$ ,  $p = 0.44$ ). Embryos were bright white in colour at oviposition and assumed a yellowish colour with subsequent development. This pattern did not change among experimental treatments.

#### Effects of different climate change scenarios on veligers

*E. clarki* embryogenesis (Fig. 3A) lasted  $13 \pm 0$  d under control conditions. At higher temperatures, the duration of intracapsular development slightly decreased to 11 to 12 d, but this effect was not statistically significant ( $F_{1,11} = 0.8$ ,  $p = 0.37$ ). The survival of veligers (Fig. 3B) was  $95.2 \pm 1.5\%$  under control conditions and significantly decreased to 85% with decreased pH ( $F_{1,19} = 6.5$ ,  $p = 0.02$ ) and to 19% with increased temperature ( $F_{1,19} = 632.4$ ,  $p < 0.01$ ). No significant interaction was found between both factors ( $F_{1,19} = 1.0$ ,  $p = 0.33$ ).

Shell size was affected by future ocean conditions (Fig. 3C), with both pH ( $F_{1,8} = 6.0$ ,  $p = 0.04$ ) and temperature ( $F_{1,8} = 11.3$ ,  $p = 0.01$ ) correlating with signif-

icantly decreased shell length independently, as well as synergistically ( $F_{1,8} = 13.0$ ,  $p = 0.01$ ). In contrast, propodium diameter (Fig. 3D) was not affected by the interactive acidification and warming conditions ( $F_{1,8} = 0.08$ ,  $p = 0.78$ ) or independently by acidification ( $F_{1,8} = 0.17$ ,  $p = 0.69$ ), although it was negatively affected by the increased temperature ( $F_{1,8} = 8.78$ ,  $p = 0.02$ ).

The most common deformities observed during *E. clarki* development (Fig. 4) included elongated malformed body, abnormal velum (elongated), and malformed propodium and shell (irregular shape). The incidence of deformities (Fig. 5) was significantly affected by warming ( $F_{1,13} = 26.1$ ,  $p < 0.01$ ) and acidification ( $F_{1,13} = 27.4$ ,  $p < 0.01$ ). The percentage of deformities increased from  $1.5 \pm 2.1\%$  under control conditions to  $27.8 \pm 14.0\%$  under acidification and to  $28.5 \pm 21.0\%$  under warming, reaching  $61.0 \pm 7.8\%$  under the future ocean scenario with a synergistic effect of acidification and warming.

#### Effects of different climate change scenarios on juveniles

Juveniles (Fig. 6) reared at acidified conditions were 21% smaller than those at control conditions ( $F_{1,8} = 38.4$ ,  $p < 0.01$ ; Fig. 6), but their length was not significantly affected by warming ( $F_{1,8} = 3.7$ ,  $p = 0.09$ ) or its interactive effects with acidification ( $F_{1,8} = 0.8$ ,  $p = 0.40$ ). The loss of the operculum usually occurred simultaneously or immediately after the loss of the

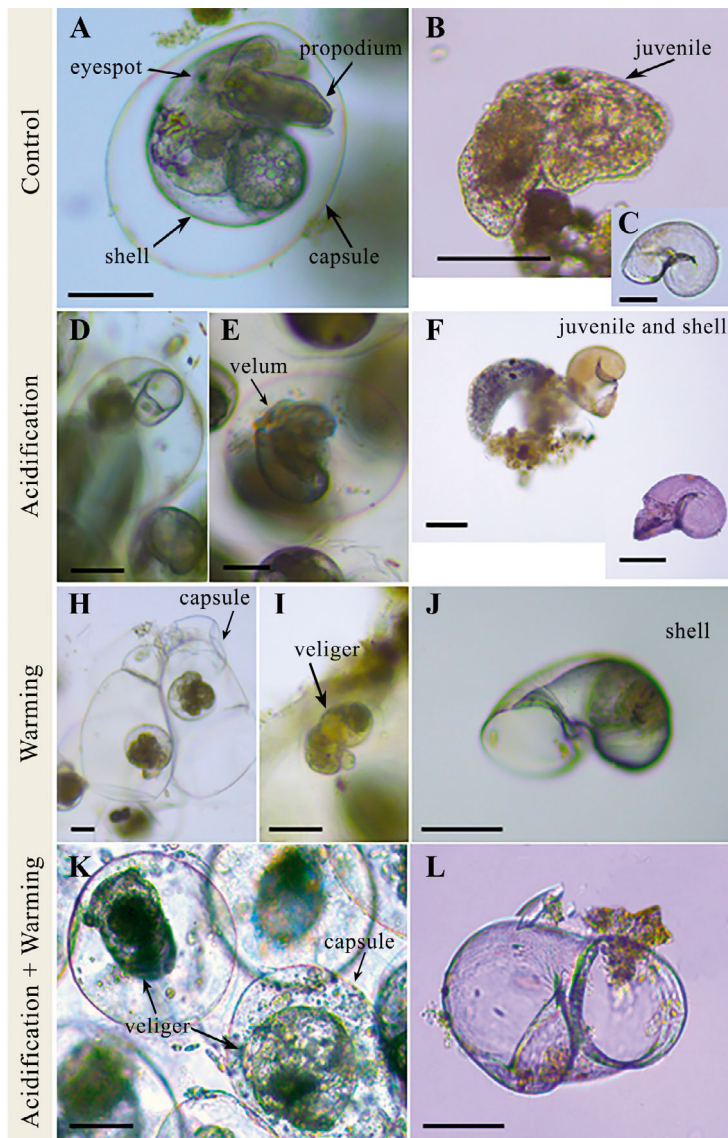


Fig. 4. Most common deformities observed during *Elysia clarki* development, under different climate change scenarios: Control (26°C, pH 8.0); Acidification (26°C, pH 7.6); Warming (30°C, pH 8.0); and Acidification + Warming (30°C, pH 7.6) treatments. (A) Normal intracapsular metamorphosis; (B) newly metamorphosed juvenile actively exploring algal surfaces; (C) shell after hatching; (D) abnormal development (body and shell deformities); (E) abnormal shell, velum and propodium; (F) juvenile after hatching and respective damaged shell; (G) shell loss; (H) abnormal capsules; (I) abnormal veliger; (J) abnormal shell; (K) abnormal development (body and shell deformities); (L) abnormal shell. Scale bar (A,D–L) = 100  $\mu$ m; (B,C) = 200  $\mu$ m

shell, regardless of the treatment. Newly metamorphosed juveniles were all aposymbiotic, but, within a few hours after metamorphosis, juveniles actively explored algal surfaces and began to feed. In all experimental treatments, chloroplast acquisition was observed and maintained until the end of the experiment (during at least 15 d).

## DISCUSSION

The present study found that the environmental conditions to which breeding adults of the photosynthetic sacoglossan sea slug *Elysia clarki* were exposed during reproduction shaped the condition of their progeny. Overall, the major biological outcomes of exposing *E. clarki* to future ocean conditions are negative and will likely reduce the recruitment of such tropical photosynthetic sea slugs. More specifically, acidification and warming had a negative impact on egg mass release, as well as on survival, growth and incidence of deformities during the early life stages of development.

Increased pH was the factor that most affected the number of egg masses released by adults, the membrane thickness of egg masses and the embryo capsule volume. Because both spawning and capsule production are energetically costly (Pechenik 1979), the results suggest that parental organisms exposed to future ocean conditions decreased the energy allocated to improve egg mass quantity and quality. This is possibly explained by modifications in energy allocation from processes such as growth and reproduction to primary processes such as respiration and repair of physiological damage under stressful environmental conditions (Wieser 1994). Moreover, adults under acidified and warming conditions seem to have allocated additional resources to improve embryo quality; embryos were 21 to 33% larger in volume under high pCO<sub>2</sub> and temperature scenarios. Increased egg size has been previously described as a common response of adults exposed to environmental stresses, including temperature and acidification (Allen et al. 2008, Moran & McAlister 2009, Parker et al. 2012). This may represent an adaptive strategy to confer a competitive advantage that may persist throughout development because larger egg size is broadly correlated with higher energy content, reduced dependence on exogenous food, and increased growth and survival (Moran & McAlister 2009). However, this strategy generally carries high costs to fecundity (maternal organisms adaptively adjust offspring size and phenotype across life-history stages; see detailed information in Allen et al. 2008). As already observed

shell, regardless of the treatment. Newly metamorphosed juveniles were all aposymbiotic, but, within a few hours after metamorphosis, juveniles actively explored algal surfaces and began to feed. In all experimental treatments, chloroplast acquisition was observed and maintained until the end of the experiment (during at least 15 d).

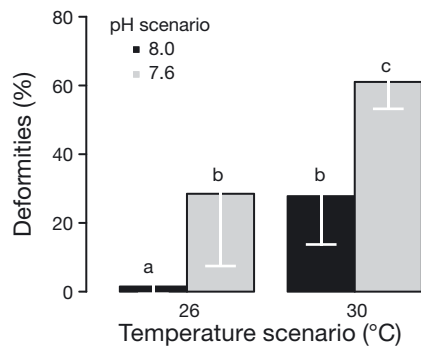


Fig. 5. Effects of ocean acidification and warming on the incidence of deformities on *Elysia clarki* veligers (8 d after egg laying), under different climate change scenarios: Control (26°C, pH 8.0); Acidification (26°C, pH 7.6); Warming (30°C, pH 8.0); and Acidification + Warming (30°C, pH 7.6) treatments. Values are given as means  $\pm$  SD. Different letters represent significant differences between treatments ( $p < 0.05$ )

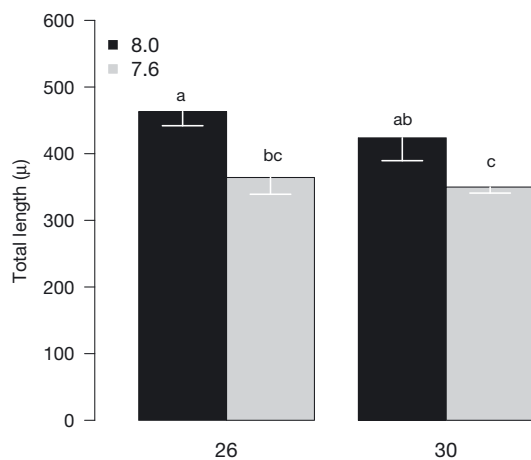


Fig. 6. Effects of ocean acidification and warming on the length of recently metamorphosed juveniles of *Elysia clarki*, under different climate change scenarios: Control (26°C, pH 8.0); Acidification (26°C, pH 7.6); Warming (30°C, pH 8.0); and Acidification + Warming (30°C, pH 7.6) treatments. Values are given as means  $\pm$  SD. Different letters represent significant differences between treatments ( $p < 0.05$ )

for other marine molluscs (Rawlings 1994), photosynthetic sea slugs under future climate change scenarios seem to prioritize energy allocation towards embryo quality, rather than to the structures that may confer them protection from environmental stresses and predation.

Increased temperature and  $p\text{CO}_2$  did not have a significant effect on the duration of embryo development. Higher temperatures are known to accelerate development and consequently decrease the development time (Przeslawski et al. 2005), but in this study, the slight decrease observed in the devel-

opment time of embryos (from 13 to 11–12 d) was not statistically significant. Moreover, no significant effect of high  $p\text{CO}_2$  was observed on the duration of intracapsular development, which goes against previous findings reporting slower development rates of gastropods under hypercapnic conditions (Byrne & Przeslawski 2013, Li et al. 2013).

Both acidification and warming had a negative impact on the survival of *E. clarki* veligers. High temperature was, however, the major driver affecting larval survival, leading to a decrease of >81 percentage points.

Intracapsular oxygen availability is one of the main factors affecting embryo development of marine gastropod species, particularly those aggregating the offspring in capsules, because of the low oxygen diffusion rates that the capsule wall allows (Cancino et al. 2011). Increased embryo metabolism and decreased intracapsular oxygen levels under high temperature may affect survival and normal embryonic development (Strathmann & Strathmann 1995, Deschaseaux et al. 2010, Cancino et al. 2011). This is of great concern because the mortality of juvenile gastropods is already estimated to exceed 90% under natural conditions (Gosselin & Qian 1997). A further increase in larval mortality associated with future ocean conditions will unquestionably affect recruitment and negatively affect adult populations (Parker et al. 2012).

Besides the impact on larval survival, future ocean conditions also affected the size of *E. clarki* veligers and juveniles. Veligers exposed to both acidification and warming showed a reduced shell length as compared to the control treatment. The negative impact of high  $p\text{CO}_2$  on shell size has already been documented for other molluscs (Watson et al. 2009, Lischka et al. 2011, Noisette et al. 2014). Moreover, shell length did not increase with temperature as would be expected due to faster growth. Instead, shell length decreased, indicating a potential reallocation of energy from shell formation to the support of stress-related physiological maintenance. Such a potential shift in energy allocation associated with environmental stress has already been observed in other studies (Mackenzie et al. 2014). Besides shell length, the propodium diameter of veligers also decreased under environmental stress, particularly warming. The development of the propodium is a sign of larval competence and acquisition of crawling behaviour (Kriegstein et al. 1974). Reduced propodium size at elevated temperature may affect crawling and impair settlement (Kriegstein et al. 1974), and ultimately decrease juvenile survival. Furthermore, juvenile size was also affected by acidification. Juveniles



were >20% smaller under hypercapnia, which is in agreement with previous studies that suggest seawater pH to be a growth-limiting factor for gastropods (Glass & Darby 2009, Naylor et al. 2014). In contrast, the lack of a significant thermal effect on juvenile size was not expected, particularly because growth is expected to be higher with increasing temperature.

Besides growth, future ocean conditions also affected the normal development of *E. clarki* early stages. Under high temperature and pCO<sub>2</sub> conditions, the incidence of deformities affected >60% of the veligers and was 40-fold higher than in control conditions. The occurrence of abnormal shells is a common response in mollusc larvae exposed to high pCO<sub>2</sub>, due to a reduced availability of carbonate ions (Noisette et al. 2014). An increased incidence of deformities in larvae exposed to warming and acidification is expected to negatively affect their chances of survival in the future.

Despite all the negative effects that ocean acidification and warming had on *E. clarki* development, there is one important feature that was not affected (at least on such a short experiment): the capacity to sequester functional chloroplasts 'stolen' from their algal food source. Other studies also found that the temperate sea slug *Elysia viridis* (Dionísio et al. 2015) and the photosynthetic acoel worm *Symsagittifera roscoffensis* (Dupont et al. 2012) were able to acquire and maintain photosymbionts in short-term ocean acidification experiments. However, long-term experiments are now required to understand if and how climate change may impact the mechanisms that allow *E. clarki* and other sacoglossan sea slugs to maintain 'stolen' plastids fully functional inside meta-zoan cells.

To the best of our knowledge, this is the first time that early life stages of photosynthetic sea slugs have been investigated in relation to environmental stressors associated with climate change. Altogether, our results suggest that future ocean conditions may affect the fitness of the sea slug *E. clarki* throughout different life stages, from adults to embryos, veligers and juveniles. The lower reproductive output of adults and the poorer condition of early life-stages will likely have a negative impact on recruitment and further affect the species persistence in a changing ocean.

Nevertheless, it is important to keep in mind that, despite the potentially negative effects of climate change, there will be some space for acclimation and adaptation. Recent studies have shown that some marine species have the capacity to acclimate to

warmer and more acidic environments across generations (Munday 2014, Donelson et al. 2012). This is particularly true for short-lived species such as *E. clarki*, which can evolve rapidly in an environment that is slowly changing. Till the end of the century, marine organisms will have the opportunity to acclimate and adapt over multiple generations to future changes in ocean's climate, potentially allowing the species to persist in a changing ocean.

**Acknowledgements.** This study was funded by the Portuguese Foundation for Science and Technology (FCT) through doctoral grants to G.D. and M.P. (SFRH/BD/73205/2010 and SFRH/BD/81928/2011, respectively), a post-doc grant to F.F. (SFRH/BPD/79038/2011), and Investigador FCT Consolidation Grants to R.C. and R.R. We acknowledge 3 anonymous reviewers for their comments to improve the manuscript, as well as Megan Walters for revising the manuscript.

#### LITERATURE CITED

- ✦ Allen RM, Buckley YM, Marshall DJ (2008) Offspring size plasticity in response to intraspecific competition: an adaptive maternal effect across life-history stages. *Am Nat* 171:225–237
- ✦ Anestis A, Lazou A, Pörtner HO, Michaelidis B (2007) Behavioural, metabolic and molecular stress responses of the marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. *Am J Physiol Regul Integr Comp Physiol* 293:R911–R921
- ✦ Beukema JJ, Honkoop PJC, Dekker R (1998) Recruitment in *Macoma balthica* after mild and cold winters and its possible control by egg production and shrimp predation. *Hydrobiologia* 375:23–34
- ✦ Bickell LR, Kempf SC (1983) Larval and metamorphic morphogenesis in the nudibranch *Melibe leonina* (Mollusca: Opisthobranchia). *Biol Bull* 165:119–138
- ✦ Byrne M, Przeslawski R (2013) Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integr Comp Biol* 53:582–596
- ✦ Byrne M, Ho M, Selvakumaraswamy P, Nguyen HD, Dworjanyn SA, Davis AR (2009) Temperature, but not pH, compromises sea urchin fertilization and early development under near-future climate change scenarios. *Proc Biol Sci* 276:1883–1888
- ✦ Cancino JM, Gallardo JA, Brante A (2011) The relationship between temperature, oxygen condition and embryo encapsulation in the marine gastropod *Chorus giganteus*. *J Mar Biol Assoc UK* 91:727–733
- ✦ Cruz S, Cartaxana P, Newcomer R, Dionísio G and others (2015) Photoprotection in sequestered plastids of sea slugs and respective algal sources. *Sci Rep* 5:7904
- ✦ Curtis NE, Pierce SK, Massey SE, Schwartz JA, Mangel TK (2007) Newly metamorphosed *Elysia clarki* juveniles feed on and sequester chloroplasts from algal species different from those utilized by adult slugs. *Mar Biol* 150: 797–806
- ✦ Davis AR, Coleman D, Broad A, Byrne M, Dworjanyn SA, Przeslawski R (2013) Complex responses of intertidal molluscan embryos to a warming and acidifying ocean in the presence of UV radiation. *PLOS ONE* 8:e55939

- Deschaseaux ES, Taylor A, Maher WA, Davis A (2010) Cellular responses of encapsulated gastropod embryos to multiple stressors associated with climate change. *J Exp Mar Biol Ecol* 383:130–136
- Dionísio G, Rosa R, Leal MC, Cruz S and others (2013) Beauties and beasts: a portrait of sea slugs aquaculture. *Aquaculture* 408–409:1–14
- Dionísio G, Cruz S, Seródio J, Calado R, Rosa R (2015) Ocean acidification promotes cellular burst on photosynthetic (kleptoplastic) sea slug. *Microsc Microanal* 21:32–33
- Dionísio G, Faleiro F, Rosa R (2017) Snails, slugs and cephalopods. In: Calado R, Olivotto I, Oliver MP, Holt GJ (eds) *Marine ornamental species aquaculture*. John Wiley & Sons, Chichester, p 536–563
- Donelson JM, Munday PL, McCormick MI, Pitcher CR (2012) Rapid transgenerational acclimation of a tropical reef fish to climate change. *Nat Clim Chang* 2:30–32
- Dupont S, Moya A, Bailly X (2012) Stable photosymbiotic relationship under CO<sub>2</sub>-induced acidification in the acoeel worm *Symsagittifera roscoffensis*. *PLOS ONE* 7:e29568
- Gianguzza P, Visconti G, Gianguzza F, Vizzini S, Sarà G, Dupont S (2014) Temperature modulates the response of the thermophilous sea urchin *Arbacia lixula* early life stages to CO<sub>2</sub>-driven acidification. *Mar Environ Res* 93: 70–77
- Glass N, Darby P (2009) The effect of calcium and pH on Florida apple snail, *Pomacea paludosa* (Gastropoda: Ampullariidae), shell growth and crush weight. *Aquat Ecol* 43:1085–1093
- Gosselin L, Qian PY (1997) Juvenile mortality in benthic marine invertebrates. *Mar Ecol Prog Ser* 146:265–282
- Hartmann DL, Klein Tank AMG, Rusticucci M, Alexander LV and others (2013) Observations: atmosphere and surface. In: Stocker TF, Qin D, Plattner G-K, Tignor M and others (eds) *Climate Change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge
- Havenhand JN, Buttler FR, Thorndyke MC, Williamson JE (2008) Near-future levels of ocean acidification reduce fertilization success in a sea urchin. *Curr Biol* 18: R651–R652
- Hettinger A, Sanford E, Hill TM, Russell A and others (2012) Persistent carry-over effects of planktonic exposure to ocean acidification in the Olympia oyster. *Ecology* 93: 2758–2768
- Hettinger A, Sanford E, Hill TM, Lenz EA, Russell AD, Gaylord B (2013) Larval carry over effects from ocean acidification persist in the natural environment. *Glob Change Biol* 19:3317–3326
- Honkoop PJC, van der Meer J, Beukema JJ, Kwast D (1998) Does temperature-influenced egg production predict the recruitment in the bivalve *Macoma balthica*? *Mar Ecol Prog Ser* 164:229–235
- Hurrell JW, Deser C (2009) North Atlantic climate variability: the role of the North Atlantic Oscillation. *J Mar Syst* 78:28–41
- Kriegstein AR, Castelucci V, Kandel ER (1974) Metamorphosis of *Aplysia californica* in laboratory culture. *Proc Natl Acad Sci USA* 71:3654–3658
- Kroeker KJ, Kordas RL, Crim R, Hendriks IE and others (2013) Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming. *Glob Change Biol* 19:1884–1896
- Kurihara H, Asai T, Kato S, Ishimatsu A (2008a) Effects of elevated pCO<sub>2</sub> on early development in the mussel *Mytilus galloprovincialis*. *Aquat Biol* 4:225–233
- Kurihara H, Matsui M, Furukawa H, Hayashi M, Ishimatsu A (2008b) Long-term effects of predicted future seawater CO<sub>2</sub> conditions on the survival and growth of the marine shrimp *Palaemon pacificus*. *J Exp Mar Biol Ecol* 367:41–46
- Lewis E, Wallace DWR (1998) CO<sub>2</sub>SYN-Program developed for the CO<sub>2</sub> system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, TN
- Li J, Jiang Z, Zhang J, Qiu JW, Du M, Bian D, Fang J (2013) Detrimental effects of reduced seawater pH on the early development of the Pacific abalone. *Mar Pollut Bull* 74: 320–324
- Lischka S, Büdenbender J, Boxhammer T, Riebesell U (2011) Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth. *Biogeosciences* 8:919–932
- Mackenzie C, Ormondroyd G, Curling S, Ball R, Whiteley N, Malham S (2014) Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation. *PLOS ONE* 9:e86764
- Mehrbach C, Culbertson C, Hawley J, Pytkowicz R (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol Oceanogr* 18:897–907
- Moran AL, McAlister JS (2009) Egg size as a life history character of marine invertebrates: Is it all it's cracked up to be? *Biol Bull* 216:226–242
- Munday PL (2014) Transgenerational acclimation of fishes to climate change and ocean acidification. *F1000Prime Rep* 6:99
- Muranaka MS, Lannan JE (1984) Broodstock management of *Crassostrea gigas*: environmental influences on broodstock conditioning. *Aquaculture* 39:217–228
- Naylor MA, Kaiser H, Jones CLW (2014) The effect of free ammonia nitrogen, pH and supplementation with oxygen on the growth of South African abalone, *Haliotis midae* L. in an abalone serial-use raceway with three passes. *Aquacult Res* 45:213–224
- Noisette F, Comtet T, Legrand E, Bordeyne F, Davoult D, Martin S (2014) Does encapsulation protect embryos from the effects of ocean acidification? The example of *Crepidula fornicata*. *PLOS ONE* 9:e93021
- Parker LM, Ross PM, O'Connor WA (2010) Comparing the effect of elevated pCO<sub>2</sub> and temperature on the fertilization and early development of two species of oysters. *Mar Biol* 157:2435–2452
- Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Portner HO (2012) Adult exposure influences offspring response to ocean acidification in oyster. *Glob Change Biol* 18:82–92
- Pechehnik JA (1979) Role of encapsulation in invertebrate life histories. *Am Nat* 114:859–870
- Pimentel MS, Faleiro F, Dionísio G, Repolho T, Pousão-Ferreira P, Machado J, Rosa R (2014) Defective skeletogenesis and oversized otoliths in fish early stages in a changing ocean. *J Exp Biol* 217:2062–2070
- Pörtner HO, Farrell AP (2008) Physiology and climate change. *Science* 322:690–692
- Pörtner HO, Karl DM, Boyd PW, Cheung WWL and others (2014) Ocean systems. In: Field CB, Barros VR, Dokken DJ, Mach KJ and others (eds) *Climate Change 2014: impacts,*

- adaptation, and vulnerability. Part A: Global and sectoral aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge
- ✦ Przeslawski R, Webb AR (2009) Natural variation in larval size and developmental rate of the northern quahog *Mercenaria mercenaria* and associated effects on larval and juvenile fitness. *J Shellfish Res* 28:505–510
- ✦ Przeslawski R, Davis A, Benkendorff K (2005) Synergistic effects associated with climate change and the development of rocky shore molluscs. *Glob Change Biol* 11: 515–522
- ✦ Rawlings T (1994) Encapsulation of eggs by marine gastropods: effect of variation in capsule form on the vulnerability of embryos to predation. *Evolution* 48:1301–1313
- ✦ Rosa R, Seibel BA (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proc Natl Acad Sci USA* 105:20776–20780
- ✦ Rosa R, Baptista M, Lopes VM, Pegado MR and others (2014a) Early-life exposure to climate change impairs tropical shark survival. *Proc R Soc B* 281:1738
- ✦ Rosa R, Trübenbach K, Pimentel MS, Boavida-Portugal J and others (2014b) Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). *J Exp Biol* 217:518–525
- ✦ Rossiter M (1996) Incidence and consequences of inherited environmental effects. *Annu Rev Ecol Syst* 27:451–476
- ✦ Rumpho ME, Summer EJ, Manhart JR (2000) Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis. *Plant Physiol* 123:29–38
- ✦ Sarazin G, Michard G, Prevot F (1999) A rapid and accurate spectroscopic method for alkalinity measurements in sea water samples. *Water Res* 33:290–294
- ✦ Strathmann RR, Strathmann MF (1995) Oxygen supply and limits on aggregation of embryos. *J Mar Biol Assoc UK* 75:413–428
- ✦ Thompson TE (1967) Direct development in a nudibranch, *Cadlina laevis*, with a discussion of developmental processes in Opisthobranchia. *J Mar Biol Assoc UK* 47:1–22
- ✦ Todgham AE, Stillman JH (2013) Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. *Integr Comp Biol* 53:539–544
- ✦ Trowbridge CD (2000) The missing links: larval and post-larval development of the ascoglossan opisthobranch *Elysia viridis*. *J Mar Biol Assoc UK* 80:1087–1094
- ✦ Watson SA, Southgate PC, Tyler PA, Peck LS (2009) Early larval development of the Sydney rock oyster *Saccostrea glomerata* under near-future predictions of CO<sub>2</sub>-driven ocean acidification. *J Shellfish Res* 28:431–437
- ✦ Wieser W (1994) Cost of growth in cells and organisms: general rules and comparative aspects. *Biol Rev Camb Philos Soc* 69:1–33
- ✦ Wolfe K, Dworjanyn SA, Byrne M (2013) Effects of ocean warming and acidification on survival, growth and skeletal development in the early benthic juvenile sea urchin (*Heliocidaris erythrogramma*). *Glob Change Biol* 19: 2698–2707

Editorial responsibility: Steven Morgan,  
Bodega Bay, California, USA

Submitted: April 11, 2016; Accepted: June 15, 2017  
Proofs received from author(s): August 21, 2017