

# Effects of global warming and ocean acidification on fertilization, larvae development and settlement of the sea urchins in the Canary Islands

Tesis doctoral  
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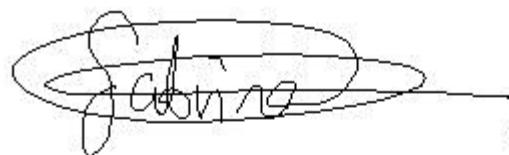
CERTIFICAN

Que la tesis doctoral titulada 'Effects of global warming and ocean acidification on fertilization, larvae development and settlement of the sea urchins in the Canary Islands', presentada por la licenciada Dña. Eliseba García Padrón, ha sido realizada bajo su dirección en la UDI de Ciencias Marinas del Departamento de Biología Animal de La Universidad de la Laguna y le otorgan un informe favorable para su lectura.

Y para que conste a los efectos oportunos, firman la presente en La Laguna a veinticuatro de octubre de dos mil catorce.



Fdo. Dr. José Carlos Hernández Pérez



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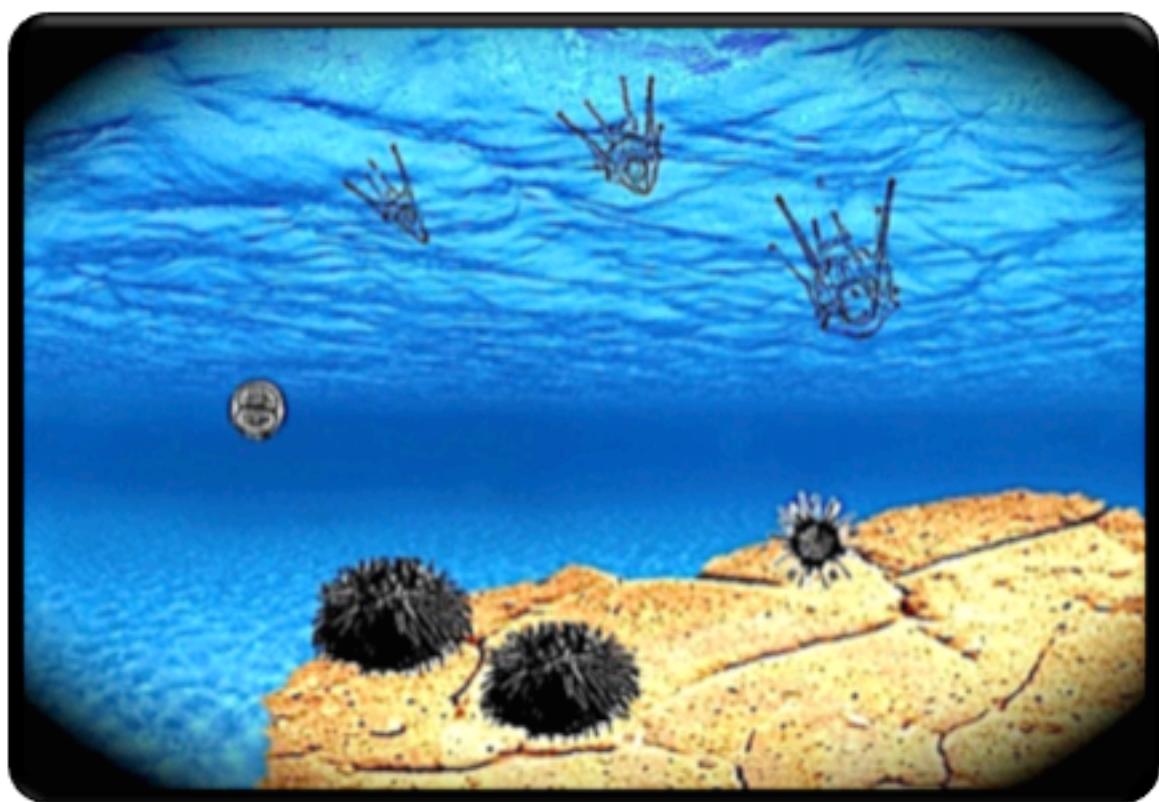


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# **CHAPTER 1 : INTRODUCTION**



## **CHAPTER 1: INTRODUCTION**

### **1.1. General context**

#### **1.1.1. Climate change: global warming and ocean acidification**

The Holocene has been an epoch in which human civilization has developed in an unusual period of stability. Living in a relative balance with nature, environmental and genetic factors have determined the ecology and evolution of species (Sørensen et al. 2003). However, human activities have become an important component in the climatic system in the last centuries (Vitousek 1994); the humankind now realizes that has the power to produce an impact at the global scale and we have entered into a new era, called the Anthropocene (Zalasiewicz et al. 2008).

In the mid-20<sup>th</sup> century, ecologists began to realize that human activities were damaging natural environments and they warned governments to take the appropriate steps to stop this phenomenon. It was in that moment when the society started to listen about climate change and global warming. However, it was not until 1972 when The United Nations' Scientific Conference, also known as 'The First Earth's Summit Meeting', was carried out in Stockholm. Here, participant states adopted a declaration that set out principles for the preservation and enhancement of the human environment, and an action plan containing recommendations for international environmental actions.

Since the beginning of the industrial period in the late 18<sup>th</sup> century, humankind has released huge quantities of pollutant gases into the atmosphere. These have been called greenhouse effect gases due to their capacity of taking up heat (Harley et al. 2006), being the most important the carbon dioxide (CO<sub>2</sub>) as it has been indiscriminately released into the atmosphere. This fact has occurred mainly as a result of fossil fuel burning, but also because of land use practices, such as agricultural activities and deforestation (Fig. 1.1).

Many measurements and reconstructions of the atmospheric CO<sub>2</sub> history have been done in the last decades. They reveal that less than half of the emissions remain in the atmosphere. Thus, the anthropogenic CO<sub>2</sub> that is not accumulated in the atmosphere must have been taken up by the oceans, by the land biosphere, or by a combination of both (Sabine et al. 2004).

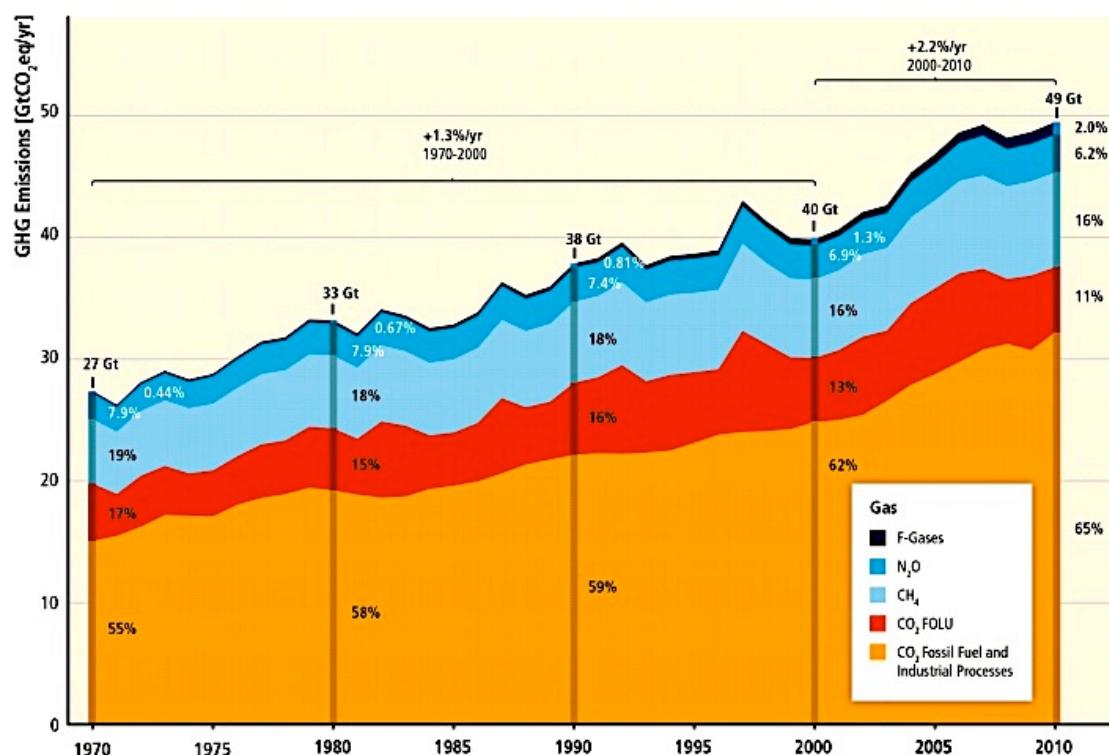


Figure 1.1. Total annual anthropogenic greenhouse gases emissions by groups of gases 1979-2010 (Source: IPCC 2013).

The variation of greenhouse effect gases concentration, as well as the increase in solar radiation, alters the energetic balance of climate system. CO<sub>2</sub> emissions have been significantly increased in the last decades. Before this unpredictable period, the atmosphere had a partial pressure of carbon dioxide (*p*CO<sub>2</sub>) of 267 parts per million (ppm). This moderated quantity has been roughly increased to 400 ppm from preindustrial period to nowadays (Fig. 1.2), and it is predicted that it could be increased to 1000 ppm at the end of 21<sup>st</sup> century (IPCC 2013). Global warming is the most obvious direct effect of greenhouse

effect gases emissions. It is not only noticeable in the temperature increase recorded during the 20<sup>th</sup> century, but also in the sea level rise as a consequence of melting glaciers and poles, eolian patterns alterations, increases in storms and desert areas, etc. (IPCC 2013).

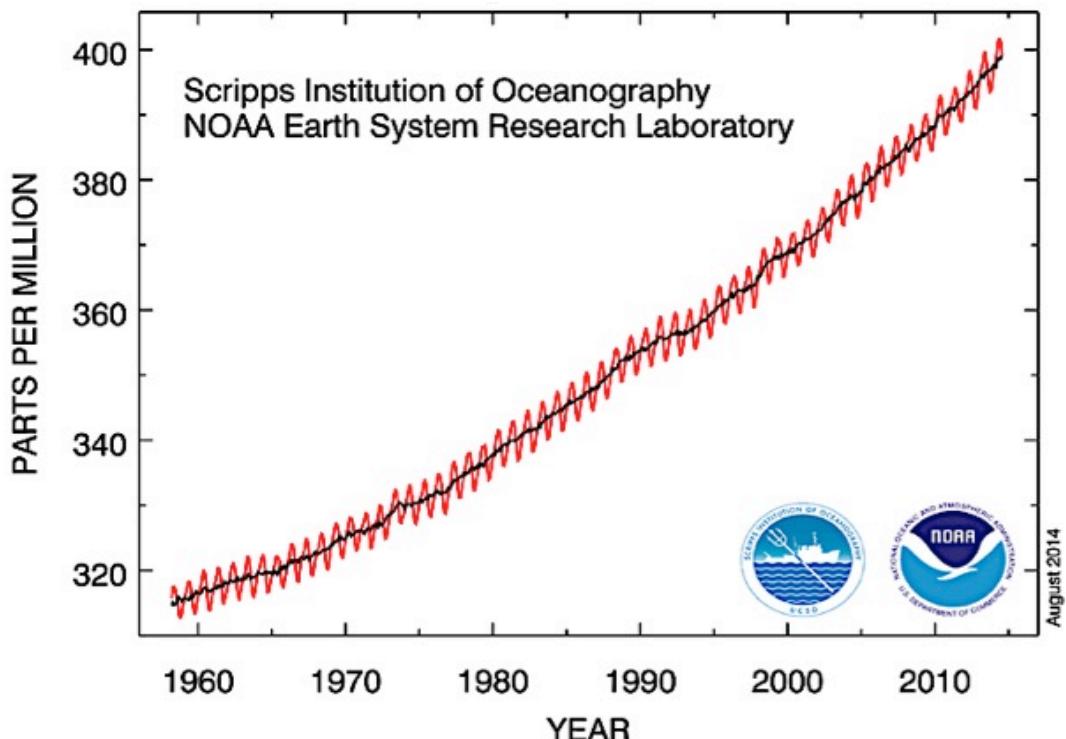


Figure 1.2. Concentrations of atmospheric CO<sub>2</sub> recorded at Mauna Loa Observatory (Hawaii) (Source: NOAA).

If greenhouse effect gases emissions continue in the same or higher levels than currently, then global warming would rise and the world climate system could undergo several changes during the 21<sup>st</sup> century, probably much more larger than those experimented in the 20<sup>th</sup> century (IPCC 2013) (Fig. 1.3).

Obviously, all these global changes are also triggering significant impacts on oceans. There are no doubts in the scientific community that the ocean is quickly changing, and the same warming trends of the Earth surface are also affecting the oceans. Global warming and increased atmospheric CO<sub>2</sub> are the most obvious effects of climate change

causing the oceans to become warmer and more acidic. In fact, oceans are taking up about 25% of the carbon generated by human activities since 1800 (Sabine et al. 2004).

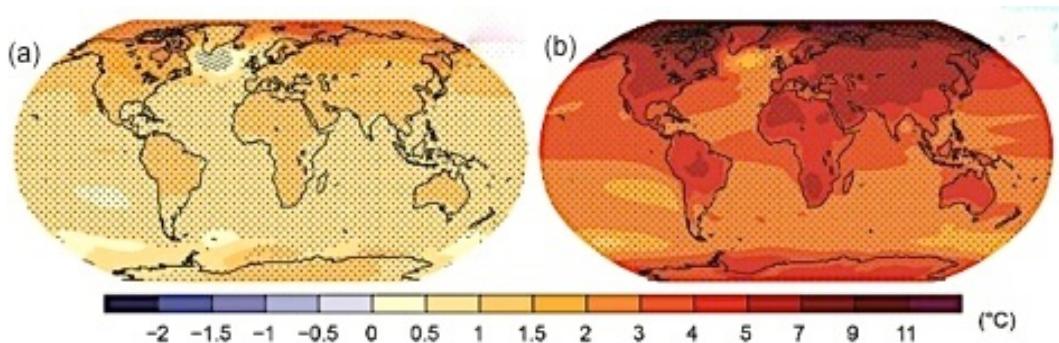


Figure 1.3. (a) Changes in average surface temperature from 1986 to 2005 and (b) changes predicted by the end of 21<sup>st</sup> century along the globe (Source: IPCC 2013).

Sea water warming is a well documented phenomenon affecting every ocean in the World. It is thought that sea water temperature will rise between 2-4.5°C at the end of the 21<sup>st</sup> century (IPCC 2013). The impact of global warming on oceans can have dramatic effects on marine ecosystems. The increase of the sea water temperature varies the distribution and adaptability of species and their survival can be compromised within a specific temperature range (Fields et al. 1993; Lubchenco et al. 1993; Harley et al. 2006). As a result, the redistribution of species can produce serious problems in specific regions, causing an imbalance on local ecosystems. Moreover, changes due to global warming, also affect trophic interactions and whole ecosystems' structure and functioning (Alheit 2009). Warmer ocean temperatures increase stratification of the surface mixed layer, which hinders the incorporation of nutrients from below to support ocean primary production (Sarmiento et al. 1998). Ocean phytoplankton is responsible for approximately half the global biospheric net primary production (Behrenfeld et al. 2001). Consequently, long-term changes in ocean primary production can potentially have important consequences for

the global carbon cycle. In the last decades, this production has fallen down at the same time that temperature has risen (Gregg et al. 2003).

The other most obvious effect of climate change on oceans is Ocean Acidification (OA). This phenomenon is referred to the ongoing decrease in ocean pH as a result of the uptake of anthropogenic CO<sub>2</sub> by the ocean. The ocean can be viewed as a dilute solution of sodium bicarbonate, together with other acid-base species at lower concentrations in a saltwater background (Riebesell et al. 2010). Seawater pH is the measurement of acid-base levels in seawater ( $\text{pH} = -\log[\text{H}^+]$ ). In normal conditions, the atmosphere keeps a balance with sea surface. When CO<sub>2</sub> dissolves in seawater, it forms carbonic acid (H<sub>2</sub>CO<sub>3</sub>) which dissociates to form an equilibrium with hydrogen ions (H<sup>+</sup>), bicarbonate ions (HCO<sub>3</sub><sup>-1</sup>) and carbonate (CO<sub>3</sub><sup>-2</sup>). When the inputs of anthropogenic CO<sub>2</sub> are increased, then it breaks the balance and this excess is taken up by the ocean. As a result, it increases the concentration of hydrogen ions, thereby reducing seawater pH (Fig. 1.4). As a consequence of these circumstances, recent global models predict that pH at the ocean surface will fall by an estimated 0.2 to 0.4 units by the year 2100, largely due to human driven emissions of CO<sub>2</sub> (Caldeira and Wickett, 2005; IPCC 2007).

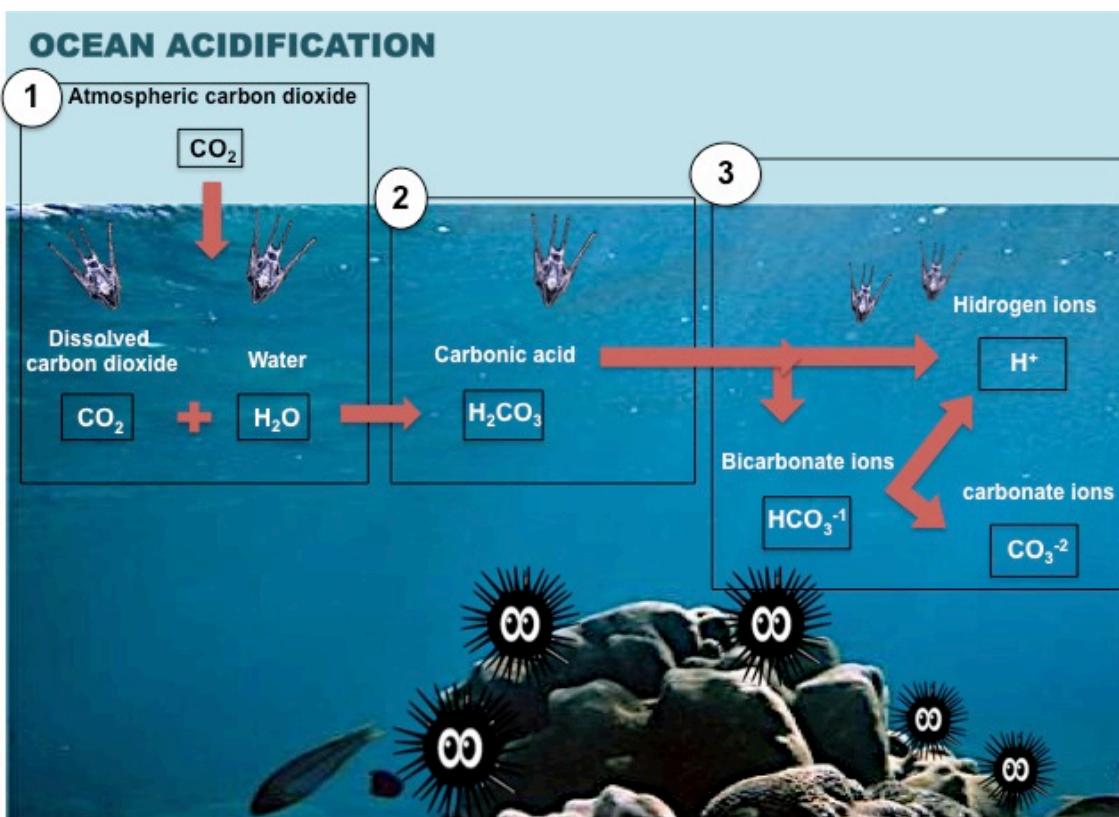


Figure 1.4. Diagram showing a summary of the carbonate system in the oceans.

These changes will have especial relevance on ectotherm organisms, as they should regulate their corporal fluids largely by exchanging with environment. Temperature and pH are the most important factors controlling the distribution, physiology, morphology and behavior of marine invertebrates (Doney et al. 2009). Any change in temperature affect all biological functions (Clarke 2003). Furthermore, ocean warming increases the possibility of taking diseases or parasite infections in adults of many marine invertebrates (Harvell et al. 1999, 2002) and it can trigger negative effects on reproduction (e.g. Byrne et al. 2010). It seems likely that early development stages of many species will suffer the same luck, although there are still many uncertainties (Alstatt et al. 1996; Friedman et al. 1997; Lester et al. 2007). On the other side, OA has also negative impacts on growth and development, mainly due to direct effects on metabolic processes (Pörtner 2008; Pörtner and Farrel 2008; Doney et al. 2009; Wittmann and Pörtner 2013).

Calcification is the production of calcium carbonate ( $\text{CaCO}_3$ ) structures, following this equation:  $\text{CO}_3^{2-} + \text{Ca}^{+2} \rightleftharpoons \text{CaCO}_3$ . The saturation state coefficient ( $\Omega = [\text{CO}_3^{2-}][\text{Ca}^{+2}] / K_{\text{sp}}^*$ ) is specific to the calcium carbonate polymorph formed, as calcite or aragonite, and express the chemical conditions driving calcification processes.  $K_{\text{sp}}^*$  is the stoichiometric solubility product that depends on temperature, pressure, salinity and calcium carbonate polymorph.  $[\text{CO}_3^{2-}]$  and  $[\text{Ca}^{+2}]$  are the *in situ* calcium and carbonate concentrations. When  $\Omega < 1$ , the seawater is corrosive to  $\text{CaCO}_3$  structures. Normally, surface tropical waters are supersaturated ( $\Omega > 1$ ), while deeper waters or waters at higher latitudes tend towards undersaturation ( $\Omega < 1$ ). It is predicted a lower saturation state in an OA context (Dorey 2013). Many marine organisms build their shells or skeletons with  $\text{CaCO}_3$ .  $\text{CO}_3^{2-}$  is one of the carbon forms used during calcification processes and its concentration decreases progressively with OA. This fact, make them potentially susceptible to dissolution in acidic waters (Orr et al. 2005; Keypas et al. 2006). When the equilibrium between bicarbonate and carbonate is broken, the pH decreases and there is less carbonate available for building up calcified structures. This process increases the rate of dissolution of deposited calcium carbonate. The rate of dissolution depends on the crystalline form of the  $\text{CaCO}_3$ : aragonite (found in corals and molluscs) is twice as soluble as calcite (found in crustaceans and echinoids) (Mucci 1983). Facing this new scenario, we can claim that OA decreases and dificulties the life of calcareous organisms, specially the skeletons building processes, by means of modifying the capacity of calcification in corals (Hoegh-Guldberg et al. 2007), mollusks (Comeau et al. 2009) and echinoderms (O'Donnell et al. 2010).

In conclusion, climate change represents itself a big challenge that should be overcome. This new scenario is the result of a human-driven contamination exceeding sustainable levels. Consequently, human emissions are likely to have huge impacts on

marine life since organisms will have to face conditions that their ancestors never had to face in the last 300 million years (Caldeira and Wickett 2003; Ruttimann 2006). These changes will have significant effects on marine organisms and, as a result, on the functioning of ecosystems.

### **1.1.2. Echinoderms as a target in marine research**

Echinoderms are exclusively marine animals that are widely distributed in every ocean and depths. About seven thousand species are known and they are subdivided in six classes, including crinoids (sea lilies and feather stars), asteroids (starfishes), ophiuroids (brittlestars), echinoids (sea urchins and sand dollars), holothuroids (sea cucumbers) and concentricycloids (sea daisies)

These animals have key positions in most ecosystems and are ecologically and economically important. As a typical benthic taxa, they are relevant key herbivores and bioturbators, as well as an important part of the food chain as prey for carnivorous fish and crustaceans (Bowmer and Keegan 1983; Sköld and Rosenberg 1996). Some of them represent an important grazing community such as sub-littoral sea urchins (Lawrence 1975). Furthermore in many areas echinoderms such as sea urchins and sea cucumbers are exploited for food, representing an important economic income (Micael et al. 2009).

Most of echinoderms have a planktonic larvae stage and an adult benthic stage. The planktonic period can last from hours to months and its duration differs between species. In sea urchins (target of this thesis) there is normally an external fertilization. When the correct environmental signal is present, spawning is synchronized and triggered (Mercier and Hamel 2009). Thereby, gametes are released into the water column where the eggs are fertilized and develop into embryos and larvae supported by 4 to 8, depending on the species, calcareous skeletal rods. Echinopluteus larvae are planktonic, and after roughly 3 weeks, the larvae build an embryonic juvenile rudiment inside the wall of an epidermic

invagination placed on the right-hand side of the body (Gosselin and Jangoux, 1998). This late development sets out the larvae to competent stage and look for an appropriate substrate to settle and metamorphose (Fig. 1.5). Usually, this pelagic period is in synchrony with the specific needs or lethal thresholds of each species, such as presence of food supply, optimal abiotic environment, etc. The newly settled post-larvae has the typical adult appearance but only develop the mouth and the rest of digestive tract during the following week, when it will reaches the true juvenile stage (Cameron and Hinegardner 1974; Gosselin and Jangoux 1996). Finally, adults can have a long-lived existence (Ebert 2008).

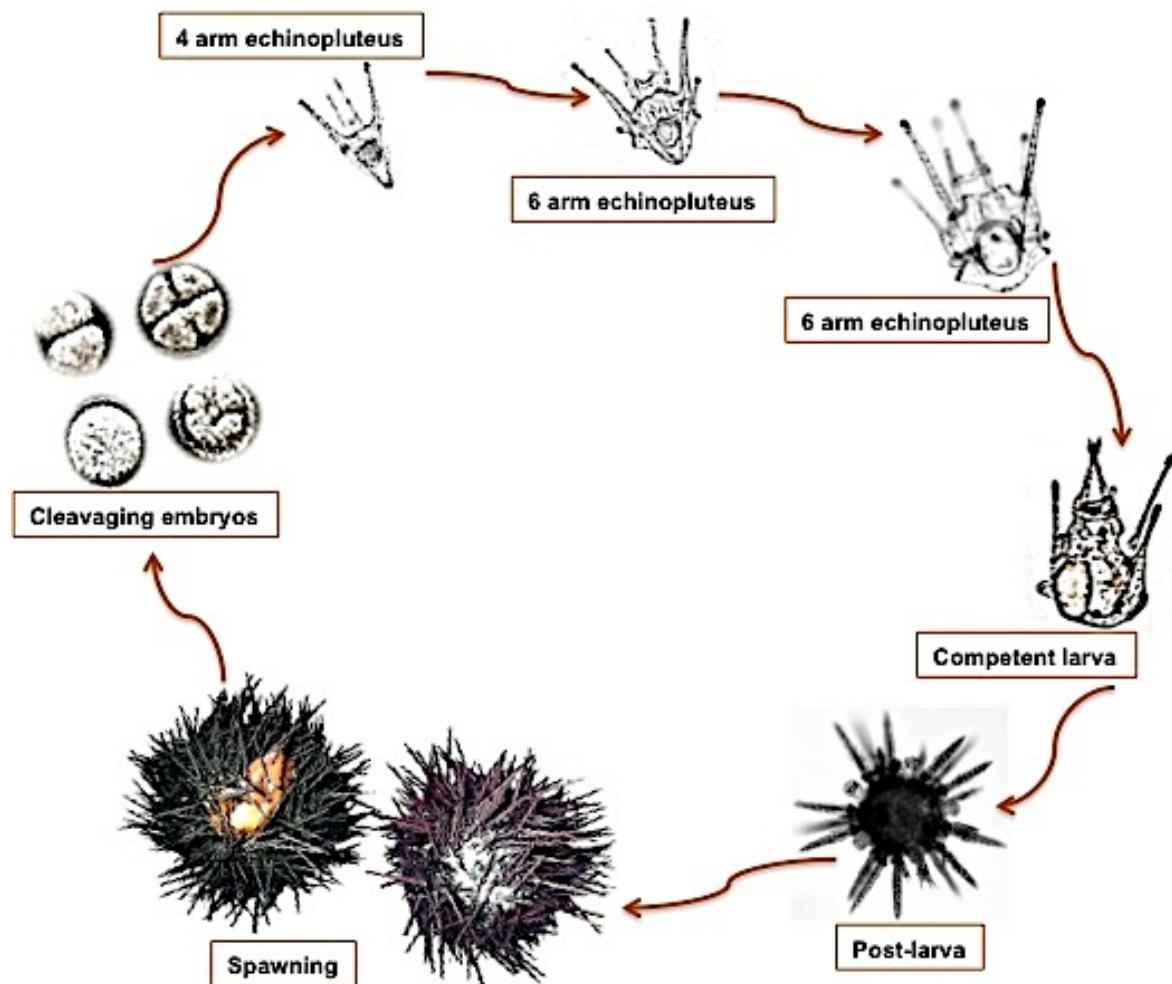


Figure 1.5. Representation of the life cycle of the sea urchins.

Most echinoderms species go then through three major transitions during their life cycle. A first ecological transition occurs when adults release gametes into the water column to ensure fertilization. This transition between the benthic and pelagic environment is also associated with complex developmental changes (fertilization, embryo and larval development). The second ecological transition is the settlement process in which individuals switch from the pelagic to the benthic environment, at about the same time as anatomical changes during the metamorphosis of the larva into a young juvenile. Finally, there is a post-metamorphic anatomical transition between the juvenile and the adult stages (Dupont and Thorndyke 2013).

Given their ecological relevance, echinoderms have been widely studied during a long time. However, in the last years they have become in target of several OA research due to their calcified nature. Echinoderms have two quite different and contrasting phases of skeletogenesis, one in the larval period and the other occurs during the adult period. The larval skeletal rods, adult test, jaws and spines are formed from an amorphous calcite crystal precursor, magnesium calcite ( $MgCO_3$ ), which is 30 times more soluble than normal calcite (Politi et al. 2004). Larval spicules are calcite structures of  $CaCO_3$  and  $MgCO_3$  embedded in a matrix of proteins. Primary mesenchyme cells build the spicules importing calcium from the seawater through calcium channels and precipitating it in the syncytium thanks to high saturation state conditions, probably maintained by ion pumps (Dorey 2013). Accordingly, skeletogenesis in echinoderms is predicted to be highly sensitive to climate change impacts. However, responses related to physiological and metabolic processes more than to calcification *per se* should not be neglected (Pörtner and Farrell 2008; Stumpp et al. 2011, 2012, 2013).

### 1.1.3. Global change impacts on echinoderms early development

Research assessing the impacts of predicted climate change processes on marine invertebrate larvae development has considerably increased during the last decade. However, there is still a lack in knowledge about the possible longer-term-impacts of these processes, the interaction between factors involved in environmental changes and the carry over effects on marine invertebrate life cycles.

The early life history stages of most marine invertebrates occur in the water column where climate change stressors are likely to have deleterious impacts on development. Early development stages such as fertilization, embryogenesis and larval development, are generally the most sensitive life phases due to environmental stresses (see reviews by Byrne et al. 2013; Byrne and Przeslawsky 2013; Dupont and Thorndyke 2013). However, recent studies report that settlement and juvenile stage is a critical phase that can be more affected by environmental changes than hitherto believed (Dupont et al. 2012; Dorey et al. 2013).

Temperature is considered to be the most important environmental factor controlling invertebrates' growth, reproduction, developmental rate, recruitment dynamics, and species' distributions (Pechenik 1987; O'Connor et al. 2007). In the last 15 years there has been a focus to quantify impacts associated with global change (see reviews by Byrne 2011; Callaway et al. 2012; Byrne and Przeslawsky 2013). The rise in sea water temperature has direct effects on gametes and embryos, producing for example acceleration of sperm swimming speeds, modification of fertilization kinetics, increased rate of development, reduced dispersal and induction or suppression of stress responses. Obviously, the increase of temperature has a lethal threshold (Clarke 2003; Staver and Strathmann 2002; Lee et al. 2004; O'Connor et al. 2007; Parker et al. 2009). The impacts of OA in these first stages are not so well known as temperature effects; nevertheless, low

pH ranges can trigger deleterious effects in metabolic processes (Pörtner 2008). Although, there are some contradictions between findings in different studies, recent research suggest a negative effect of OA on fertilization and early development (see reviews by Byrne et al. 2013; Dupont and Thorndyke 2013).

With regard to the larvae stage, the increase in seawater temperature speeds up growth, development and settlement, and also impacts on larval swimming behavior and duration of planktonic life, until a lethal threshold is achieved (see reviews by Byrne 2011; Byrne and Przeslawsky 2013). These facts, that at the beginning can seem positive because the time that the larvae is exposed to predation is lower, can, at the same time, reduce dispersal possibilities and cause alterations in the genetic connectivity and thereby in the dynamics of marine populations (López et al. 1998; O'Connor et al. 2007). It is thought that food availability will be reduced as a result of decreasing primary production resulting from stratification of the surface mixed layer, as a consequence of ocean warming (Gregg et al. 2003; Turley et al. 2013). Alough many studies have explored the impacts of food availability on the growth and survival of feeding larval forms (Olson and Olson 1989; Fenaux et al. 1994; Meidel et al. 1999; Vickery and McClintock 2000; Moran and Manahan 2004; Sewell et al. 2004; Meyer et al. 2007; McAlister 2007), few of them have linked them to environmental variability (McAlister 2008) and none has focused on the lack of food supply as an effect of climate change processes.

On the other side, although organisms' responses are highly species-specific (Wittmann and Pörtner 2013), OA has general negative effects on survival, development, growth and settlement (see reviews by Byrne et al. 2013; Dupont and Thorndyke 2013). The larvae of some species seem to be under a big threat (Dupont et al. 2008), however others such as sea urchin larvae are more robust to facing OA changes in the short-term than hitherto believed (see reviews by Byrne et al. 2013; Byrne and Przeslawsky 2013;

Dupont and Thorndyke 2013). However, organisms live in a multistressor environment, where stressors levels are exacerbated by global change (Feely et al. 2004; Caldiera and Wickett 2005; IPCC 2007). For example, it is hypothesized that the thermal windows of a species will be influenced by the interaction with other environmental stressors such as OA (Pörtner and Farrel 2008; García et al. in review).

Finally, recruitment success greatly depends on the survival of the embryos and larvae (López et al. 1998) and, consequently, any decrease in embryo and larval survival or any delay in their development can reduce population long-term viability (Morgan 1995).

#### **1.1.4. The Canary Islands in a context of climate change**

This Spanish archipelago is located in the Atlantic Ocean, off the Northwestern coast of Africa at 27°37'- 29°25' N and 13°20'- 18°10' W. Close to the south coast of Morocco and Sahara (95 km), it is at a distance of 1400 km from the European continent. The Islands have a volcanic environment and are part of the biogeographic region called 'Macaronesia', along with the archipelagos of Azores, Madeira, Salvages and Cape Verde.

Emerging from the oceanic basin as a result of successive overlays of volcanic material to form an independent set of islands, the Canarian Archipelago comprises of seven major islands and four islets. Despite its latitude, the Canary Islands have a subtropical weather that is moderated by the effect of the Trade Winds (Alisios winds). However, due to the altitude of the islands we can find different microclimates that generate a huge biological terrestrial diversity. Regarding the marine environment, the islands have a limited oceanic shelf as a result of their volcanic origin, showing the typical marine oceanic environment with oligotrophic waters. Its geographical location between the cool, nutrient-rich water from the north-west African coastal upwelling, and the warmer, nutrient-poor open ocean waters, means the Canary Islands are considered a

'Coastal Transition Zone', with strong implications for the productivity of the region (Barton et al. 1998). Upwelling filaments reach the Canary Islands waters stretching out from the NW Africa coastal upwelling system and supplying upwelled water to this oligotrophic region in the most oriental islands. At the same time the stronger winds in summer time produce local upwellings that are reduced during winter (Arístegui et al. 1997, 2004). In addition, the Canary Islands are immersed in the Canary Current, which dominates the water circulation in the North Atlantic Ocean. This current beside the Trade Winds generates the main currents in the region. The sea surface temperature fluctuates between 16-18°C during the winter months and 23-25°C during the summer (Fig. 1.6), although seasonal events can take place. The sea surface salinity has annual values that oscillate between 36.7 and 36.9‰ (Fig. 1.7), showing a higher mean salinity in the westernmost islands. All these circumstances make coastal ecosystems of the Canaries have a huge variability in a limited space; there are 5232 marine species of 18000 that have been catalogued for the Canary Islands (Hernández et al. 2012).

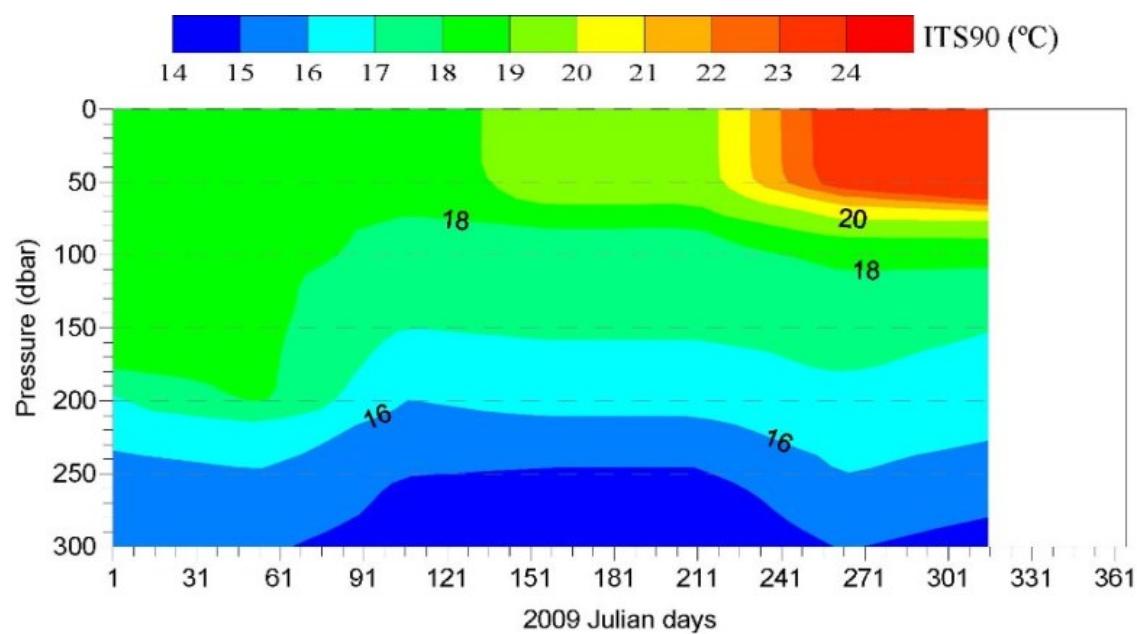


Figure 1.6. Temperature (°C) in the water column in the ESTOC station (29.167N-15.50W) during 2009 (Source: EuroSITES Project)

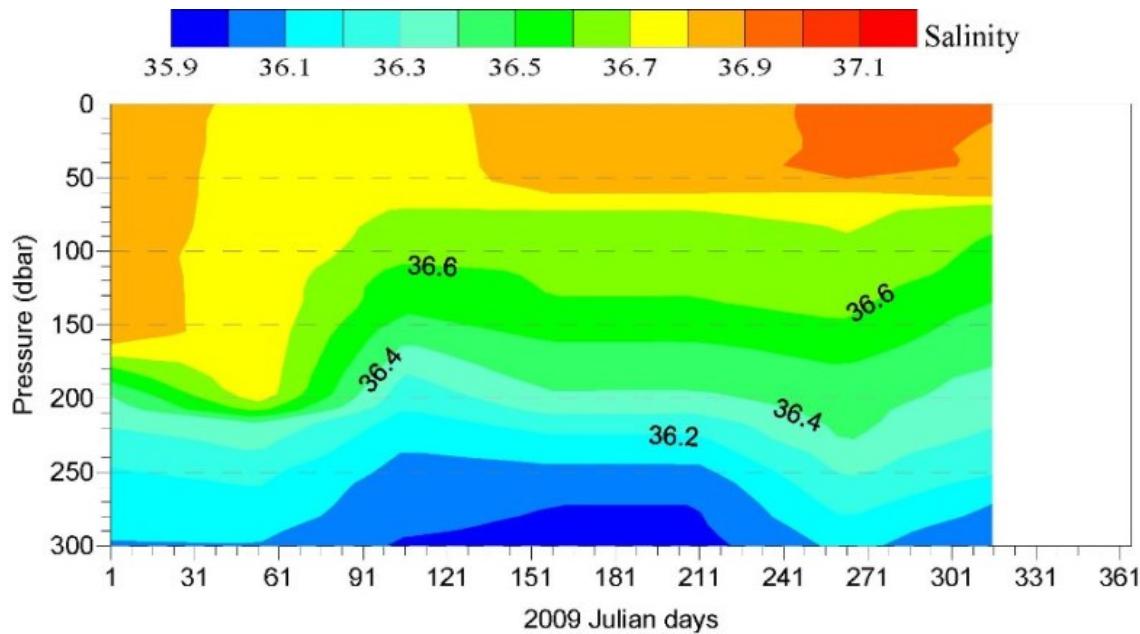


Figure 1.7. Salinity (‰) in the water column in the ESTOC station (29.167N-15.50W) during 2009 (Source: EuroSITES Project)

In a context of climate change, accordingly with the results for East Atlantic by Santana-Casiano et al. (2007), experts of the ‘QUIMA’ group (Marine Chemistry research group from ‘Universidad de Las Palmas de Gran Canaria’, O. Llinás pers. comm.) predicted an increase in ocean acidification as a result of a decrease of pH in 0.002 units by year for the Canary Islands. With respect to ocean warming, seawater temperature in the Canary Islands has already increased 1°C from 1985 to nowadays (AEMET 2008) and it is thought that is a tendency for the future (Fig. 1.8).

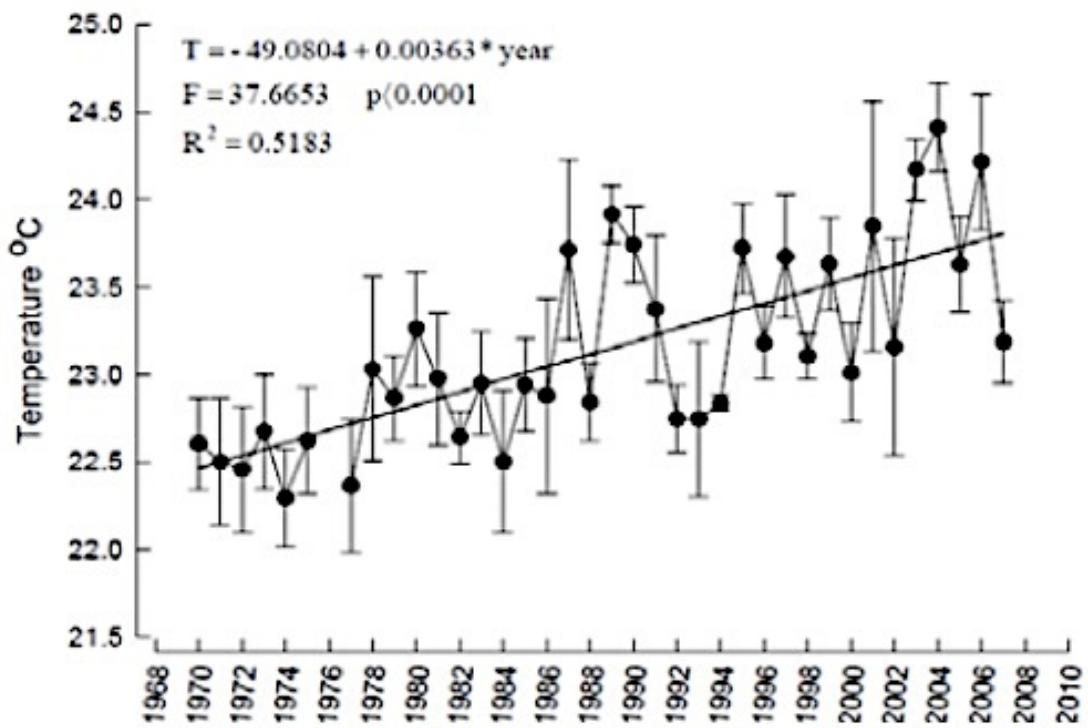


Figure 1.8. Trend of increasing seawater temperatures in The Canary Islands from the years 1968 to 2010 (Source: Hernández et al. 2010).

## 1.2. Objetives

This thesis is set within the framework of the Spanish scientific funding programme ‘Plan Nacional de I+D+I’, specifically within the project entitled ‘*Influence of phitocenosis at littoral rocky habitats on pH variability and its implications for calcifying invertebrate populations in a climate change scenario (ACIDROCK- CTM 2010-21724)*’. This project aims to evaluate the impact of several phitocenosis at coastal habitats on the natural variability of seawater pH, assessing the implications of these pH regimes and the ones foreseeable due climate change for the larval development, settlement, survival and growth of key calcifying invertebrates. Facing this scenario, this study tries to assess the impact of climate change processes on early development of key calcifying sea urchin species on the Canary Islands coastal ecosystems.

To reach this general aim, we established five specific objectives:

- 1) Evaluate how fertilization success, cleavage rate and early pluteus survival and development, in the main species of sea urchins from the Canary Islands, are affected by climate change processes (ocean warming and acidification).
- 2) Assess the combined effects of global warming and food availability on survival, growth and development of the larvae of the sea urchin *Paracentrotus lividus*.
- 3) Test the impact of ocean acidification on larval survival, growth, development and postlarval settlement of *Paracentrotus lividus*.
- 4) Evaluate whether the interaction of climate change related environmental factors (temperature and pH) has the potencial to change the responses of *Paracentrotus lividus* larvae and postlarvae performance.
- 5) Compare larval development and settlement of *Paracentrotus lividus* between constant pH conditions and daily natural fluctuations to a better understanding and forecast of its performance in more realistic future scenarios.

### 1.3. Structure of the chapters

This thesis has been structured in seven chapters. Five of them correspond to original research manuscripts, articles already published or submitted for publication to different scientific journals of the fields of Marine Biology and Ecology.

In Chapter 1, a review about marine global change and the impacts of climate change processes on marine invertebrates is showed, pointing out how early stages of marine calcifiers, especially echinoderms (target of this thesis), are affected. In this chapter the main objectives of the study and the structure of the thesis were also included.

In Chapter 2, the effects of combined stressors (ocean warming and acidification) on fertilization and early larvae development of the four main species of sea urchins in the Canary Islands (*Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*) were evaluated. Our results highlighted the higher susceptibility of subtidal species to environmental changes, and how intertidal species increased their resilience to OA at higher temperatures.

Chapter 3 includes a study assessing the effect of temperature and food availability on survival, growth and development of *Paracentrotus lividus* larvae. Our results suggested that larvae of this species are surprisingly robust facing these stressors in the short-term and the negative effects of decreasing food supply on larvae development will be significantly ameliorated by increasing seawater temperature.

In Chapter 4, we assessed the impact of ocean acidification on larval development and settlement of the sea urchin *Paracentrotus lividus*. Our outcomes revealed that *P. lividus* is robust to pH ranges covering present natural variability but sensitive to pH levels corresponding to near-future extremes.

Chapter 5 includes a study specifically testing the combined effect of ocean warming and acidification on survival, growth, development and settlement of

*Paracentrotus lividus* larvae. The most extreme near-future projections of decreasing pH narrowed the thermal window in the species. Larvae development and settlement performance of the sea urchin *P. lividus* was enhanced by a slight increase in temperature in the context of OA. However, the species showed sensitivities to ocean warming and acidification corresponding to near-future extremes.

In Chapter 6, we explored the sensitivities of *P. lividus* during its larvae development and settlement undergoing two different daily pH frequencies of oscillations that are currently taking place in the marine environment off the Canary Islands. *P. lividus* larvae development showed ecological strategies for inhabiting coastal areas covering present natural variability, being enhanced by a moderated pH fluctuation typical of intertidal environments that the sea urchin normally inhabits.

Finally, the general conclusions of this thesis are enclosed in Chapter 7.

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**CHAPTER 2: THE ROBUSTNESS OF INTERTIDAL  
SEA URCHIN SPECIES TO OCEAN WARMING AND  
ACIDIFICATION**



## **CHAPTER 2: THE ROBUSTNESS OF INTERTIDAL SEA URCHIN SPECIES TO OCEAN WARMING AND ACIDIFICATION**

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## Abstract

Ocean warming and acidification are the two most significant side effects of climate change in the world's oceans. By changing water, temperature and pH are the main environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates. This study evaluated the combined effects of predicted high temperature levels, and predicted low pH values, on fertilization and early development stages of the sea urchins *Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*. Twelve treatments, combining different temperatures (19, 21, 23 and 25°C) and pH values (8.1, 7.7 and 7.4 units), were tested in laboratory experiments. All of the tested temperatures and pH values were within the natural seawater range expected within the next century. We examined fertilization rate, cleavage rate, 3-day larvae survival, and development of the different sea urchin species at set time intervals after insemination. Our results highlight the susceptibility of subtidal species to environmental changes, and the robustness of intertidal species to ocean warming and acidification.

**Keywords:** Temperature, pH, climate change, fertilization, early development, *Paracentrotus lividus*, *Diadema africanum*, *Arbacia lixula*, *Sphaerechinus granularis*.

## Resumen

El calentamiento y la acidificación oceánica son los principales efectos del cambio climático en los océanos a nivel mundial. La distribución, fisiología, morfología y comportamiento de los invertebrados marinos va a estar determinada por multitud de factores ambientales pero, principalmente, por la temperatura y el pH. En este trabajo hemos evaluado los efectos que puede tener la interacción de estos dos factores, en los niveles esperados como resultado del cambio climático, sobre la fertilización y los primeros estados de desarrollo de las principales especies de erizos marinos de las islas Canarias: *Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*. Para ello, se ha llevado a cabo experimentos en el laboratorio, testeando 12 tratamientos combinados de temperatura (19, 21, 23 y 25°C) y diferentes valores de pH (8'1, 7'7 y 7'4 unidades), abarcando la variabilidad natural actual y los valores esperados para el próximo siglo. Hemos analizado el ratio de fertilización y el desarrollo embrionario a diferentes intervalos de tiempo después de la inseminación, así como la supervivencia y el desarrollo de las larvas a los tres días de vida. Nuestros resultados resaltan la susceptibilidad de las especies submareales a los cambios ambientales y la fortaleza de las especies intermareales al aumento de la temperatura y la disminución del pH.

*Palabras clave:* Temperatura, pH, Cambio climático, fertilización, desarrollo temprano, *Paracentrotus lividus*, *Diadema africanum*, *Arbacia lixula*, *Sphaerechinus granularis*.

## Introduction

Since the beginning of the industrial period in the late 18<sup>th</sup> century, anthropogenic modification of the environment has triggered global climate change processes (IPCC 2013). Mainly as a result of fossil fuel burning, huge quantities of CO<sub>2</sub> have been progressively released into the atmosphere. The partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) has increased from 267 to 390.5 ppm, since the beginning of the industrial revolution (IPCC 2013). Ocean warming is a direct effect of greenhouse gas emissions, due to the gases' direct absorption of heat (Harley et al. 2006). As a result, it is thought that sea surface temperature (SST) will rise by 2 to 4.5°C by the end of 21<sup>st</sup> century (IPCC 2007). However, not all anthropogenic CO<sub>2</sub> accumulates in the atmosphere; a proportion is also taken up by the oceans, by the land biosphere, or by a combination of both (Sabine et al., 2004). The oceans are thought to have taken up approximately 25% of the carbon generated by human activities since 1800 (Sabine et al. 2004). In seawater, an increase in absorption of atmospheric CO<sub>2</sub> leads to modification of the carbonate system, causing a decrease in ocean pH, known as ocean acidification (OA). When CO<sub>2</sub> dissolves in seawater, it forms carbonic acid (H<sub>2</sub>CO<sub>3</sub>), which dissociates to form hydrogen ions (H<sup>+</sup>), bicarbonate ions (HCO<sub>3</sub><sup>-1</sup>) and carbonate (CO<sub>3</sub><sup>-2</sup>). Since anthropogenic activities have caused a massive flux of extra CO<sub>2</sub> into the atmosphere, more CO<sub>2</sub> has also dissolved into the oceans. As a result, the concentration of H<sup>+</sup> ions has increases, thereby reducing the pH of the seawater. Mean surface ocean pH decreased by approximately 0.1 units between pre-industrial times and the end of the 20<sup>th</sup> century; a rate of acidification not otherwise seen within the last 60 million years. A further decrease of approximately 0.4 units is expected by the end of the 21<sup>st</sup> century (Gattuso and Hansson 2011; Turley et al. 2013). Ocean warming and acidification are causing substantial changes to marine physics, chemistry and biology, in ways that we are only beginning to understand (Turley et al.

2013).

Temperature and pH are the two most important factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates (Doney et al. 2009). In echinoderms, temperature and pH have effects on several physiological responses, including respiration, feeding, fertilization and larval development (see reviews by Byrne et al. 2013b; Byrne and Przeslawski 2013; Dupont and Thorndyke 2013), as well as many genetic processes (Stumpp et al. 2011; Foo et al. 2012). However, few studies have tested the combined effects of temperature and pH, and those that have, produced contrasting findings (Byrne et al. 2009, Sheppard-Brennan et al. 2010; Byrne et al. 2010 a, b; Ericson et al. 2012; Foo et al. 2012; Byrne et al. 2013a; Padilla-Garmiño et al. 2013; Gianguzza et al. 2014; Hardy and Byrne 2014; Stavroff 2014). More research is therefore needed, to determine the effects of these two stressors on different species.

Responses to climate change stressors seem to be highly species-specific, even in closely related taxa, and effects on fitness-related parameters can be either negative or positive (Wittmann and Pörtner 2013). However, the habitat of the species seems to play an important role. It was recently hypothesize that scenarios, outside of present natural environmental variability, will induce more detrimental effects than conditions already experienced within the present natural range (Dorey et al. 2013). Species that are regularly exposed to a high level of environmental variability, could therefore withstand future climate change scenarios better than those inhabiting more stable environments (Moulin et al. 2011; Foo et al. 2012; see Byrne et al. 2013b for review). Those habituated to a wide range of temperature and pH scenarios already, are expected to possess greater resilience to seawater warming and/or acidification (Melzner et al. 2009; Talmage and Gobler 2009, 2011; Matson et al. 2012; Wolfe et al. 2013). The prime examples, are intertidal species. Intertidal organisms show ecological strategies that enable them to survive in coastal areas,

where environmental stress and disturbances are frequent. Their window of thermal tolerance is broad, which suggests significant phenotypic plasticity (Catarino et al. 2012; Calosi et al. 2013). Sea urchins living in extreme environmental conditions, have the ability to acclimatise to fluctuations in many seawater parameters, which means they are already partially equipped to cope with a range of environmental stresses (Hall-Spencer et al. 2008; Catarino et al. 2012). Hence, in our study, we have chosen to investigate the effects of temperature and pH on several different sea urchin species – two of which occupy habitat predominantly within the intertidal zone.

The Canary Islands are located within the subtropical region of the Eastern Atlantic Ocean. Tenerife is one of the central islands of the Archipelago, located at 28° N 16° W. In this area, there are four main species of sea urchins sharing the coastal ecosystems: *Arbacia lixula* (Linnaeus 1758), *Paracentrotus lividus* (Lamarck 1816), *Sphaerechinus granularis* (Lamarck 1816) and *Diadema africanum* (Rodríguez et al. 2013). Each of these species has different environmental preferences (Fig. 2.1), and some of them, such as *P. lividus*, are at the geographical limits of their distribution. Studying organisms in an environment close to their geographic limit provides an opportunity to assess their potential adaptation and/or selection capabilities; because these species are already acclimatising to temperature and pH conditions at the limit of what they can physiologically withstand.

Our study assessed the combined effects of ocean warming and OA on: (1) fertilization and cleavage rate; and (2) early larvae survival, growth and development, of the four main species of sea urchins in the Canary Islands. We hypothesized that intertidal species (*A. lixula* and *P. lividus*), would be more resilient to climate change processes than subtidal species that live in a more stable environment (*S. granularis* and *D. africanum*).

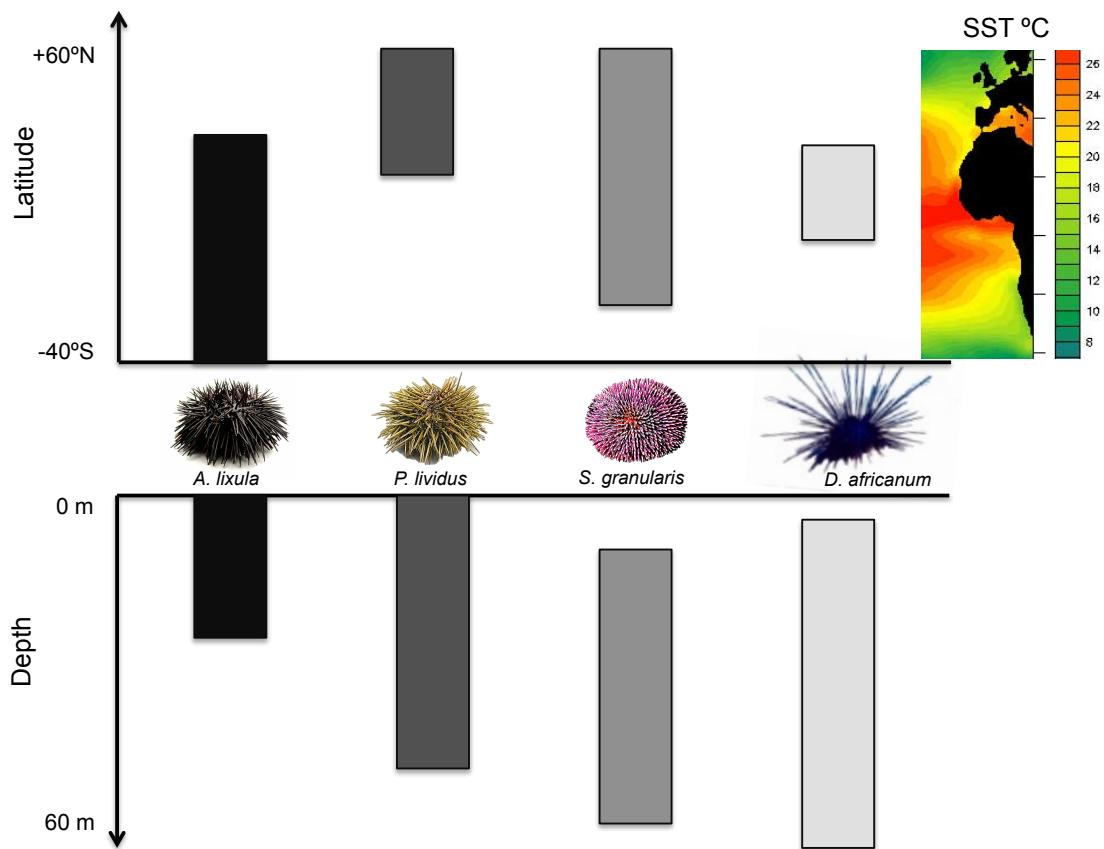


Figure 2.1. Range of latitudinal and bathymetrical distribution of the studied sea urchin species.

## Materials and methods

### *Seawater parameters*

Four different seawater temperature, and three different pH levels, were used in combination in this laboratory experiment. The temperature treatments were representative of the full range of water temperatures found in the Canary Islands within its current annual cycle. In addition, one higher temperature was incorporated, which the IPCC (2007) predict will be within the normal summer water temperature range by the year 2100. Overall the temperatures tested were: 19°C (SST in spring in the Canary Islands); 21°C (an exceptionally high value for March, but more typical SST in early summer); 23°C (normal SST in summer, also predicted during spring by the year 2100, IPCC 2007); and

25°C (predicted temperature for the summer period by 2100, IPCC 2007). Within each temperature treatment, three different treatments of pH were tested: pH 8.1 (control treatment; present average pH); pH 7.7 (present extreme of the natural variability, also average pH predicted for 2100 (IPCC 2007)); and pH 7.4 (extreme of natural variability predicted by 2100 (Caldeira and Wickett 2005)). In total, twelve different temperature-pH scenarios were tested.

To keep temperature conditions constant, thermostat coolers and heaters (EHEIM AQUATICS, 50 W) were used. In order to control pH, we used a computerised control system (AquaMedic) that regulated pH, by the bubbling pure CO<sub>2</sub> directly into the water (resolution ±0.01 pH units). In the three-day experiment, seawater was changed once on day 2. Temperature and pH were monitored using a Metrohm mobile meter, with a Primatrode NTC IP pH electrode, and temperature sensor. Salinity was measured using a handheld conductivity meter (COND 315i). Seawater total alkalinity (TA) was measured for each treatment, with a Tritration system. PCO<sub>2</sub>, calcite saturation state ( $\Omega_c$ ), and aragonite saturation state ( $\Omega_a$ ), were calculated from TA and pH using the software CO<sub>2</sub>sys 2011 (Lewis and Wallace, 1998). Calculations were based on a set of constants, K1 and K2, following Mehrbach et al. 1973 (refit by Dickson and Millero 1987).

Experiments were conducted with filtered seawater (FSW), which was purified within a recirculating system. The system used DRYDEN AQUA active filter media (AFM) bio-crystals; 50 µm, 10 µm and 1 µm UNICEL polyamide paper filters; and a UV-C AQUAEL 11W filter.

#### *Animal collection and bioassays*

Mature specimens of the sea urchin species *Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum* were collected by scuba divers, from subtidal rocky shores. Individuals were collected in 2013, during the spawning period for

each species: summer for *A. lixula* (Fénaux 1968; Wangensteen et al. 2013); late winter for *P. lividus* (Girard et al. 2008), spring for *S. granularis* (Guillou and Michel 1993) and summer for *D. africanum* (Hernández et al. 2011). All collections were made from a site on the east coast of Tenerife ( $28^{\circ}16'07''\text{N}$ ,  $16^{\circ}36'20''\text{W}$ ). Each species was tested in all twelve of the different temperature-pH scenarios.

Animals were induced to spawn by injection with 2 mL of KCl (0.5 M), through the peristomial membrane. Four males and five females of each species were randomly selected (Evan and Marshall 2005). Gametes were mixed before being put in contact with gametes of the opposite sex. Sperm was collected dry and kept on ice until usage, and eggs were collected in FSW. The same number of eggs were added to each of 144 separate experiment pots for each species. The appropriate quantity of sperm were then added to achieve the desired egg to sperm ratio of 1:1500. To carry out fertilization essays, eggs and sperm were added together in pots with 100 ml of FSW. The temperature and pH of the FSW was adjusted prior to its addition (twelve different combinations). To assess fertilization and early development, pots were incubated for different lengths of time after insemination (TAI): 15, 105 and 210 minutes, and 3 days. For all combinations of temperature, pH and TAI, 3 replicates were performed. Development was halted by adding 1 mL of buffered formaldehyde (4%). Total fertilization rate (FR) was checked by counting the number of fertilized eggs in aliquots of 0.2 ml (three aliquots per replicate). To evaluate cleavage rate (CR) we used only the 15, 105 and 210 minute after insemination (MAI) samples. We estimated the number of eggs in different stages of cell division: non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with  $> 4$  cells. To carry out this evaluation we observed three aliquots of 0.2 mL for each replicate, under a microscope.

Larvae survival, growth and development were assessed in the three-day samples. Counting was done by taking 3 aliquots of 1 mL from each replicate pot. In total 10 larvae were photographed and measured from each replicate, using ImageJ software (Rasband 1997-2012). Measurements of body length (BL), total length (TL) and post oral arm length (PL) were taken (Fig. 2.2). Overall, 30 larvae were measured and photographed for each combination of temperature and pH treatments.

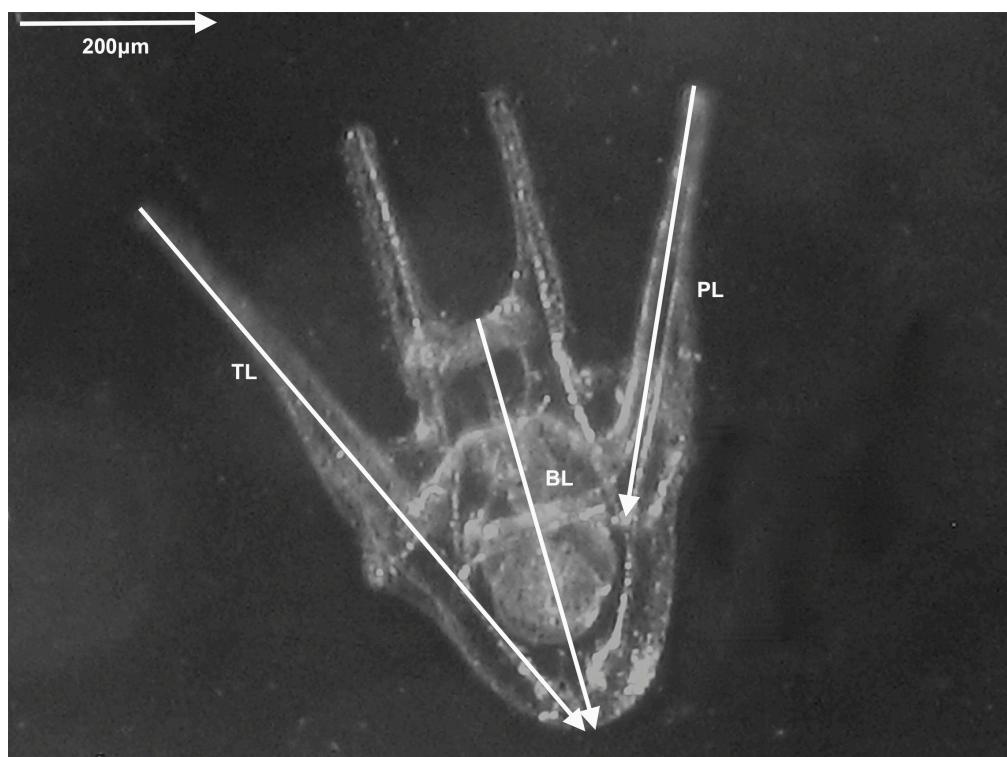


Figure 2.2. Morphometric measurements taken for each three-day larvae of the sea urchins *Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum* in laboratory experiments: Body length (BL), total length (TL) and post oral arm length (PL).

#### Data analysis

In order to assess the combined effects of seawater temperature and pH on FR, and to compare the different species, data were analysed by means of a four-way permutational

analysis of variance (PERANOVA) (Anderson 2001). A four-way permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was used to analyze the combined effects of temperature and pH on CR, including the five stages of division (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells). In both cases, four-way designs were conducted with species (4 levels), temperature (4 levels), pH (3 levels) and TAI (3 levels) used as fixed factors.

To evaluate the combined effects of temperature and pH on larvae survival in the studied species, data were analysed by means of a three-way permutational ANOVA. In order to analyze morphometric variables (BL, TL, PL) in three-day larvae development (3LD) data, a three-way PERMANOVA was performed. In each case, three-way designs were carried out using species (4 levels), temperature (4 levels), and pH (3 levels), as fixed factors.

In all analyses of variance, significant terms were examined using *a posteriori* pairwise comparisons by permutations (Anderson 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws, from the asymptotic permutation distribution. Principal Coordinates Analysis (PCO) ordinations of CR and 3LD data, were used to identify similarities between observations. All statistical analyses were carried out using PRIMER 6 & PERMANOVA+ v. 1.0.1 software.

## Results

The physico-chemical parameters of seawater, as measured during the experiments, are given in Table 2.1. The partial pressure of carbon dioxide ( $p\text{CO}_2$ ) increased at low pH in all temperature treatments, while saturation levels of calcite ( $\Omega_c$ ) and aragonite ( $\Omega_a$ ) decreased at low pH.

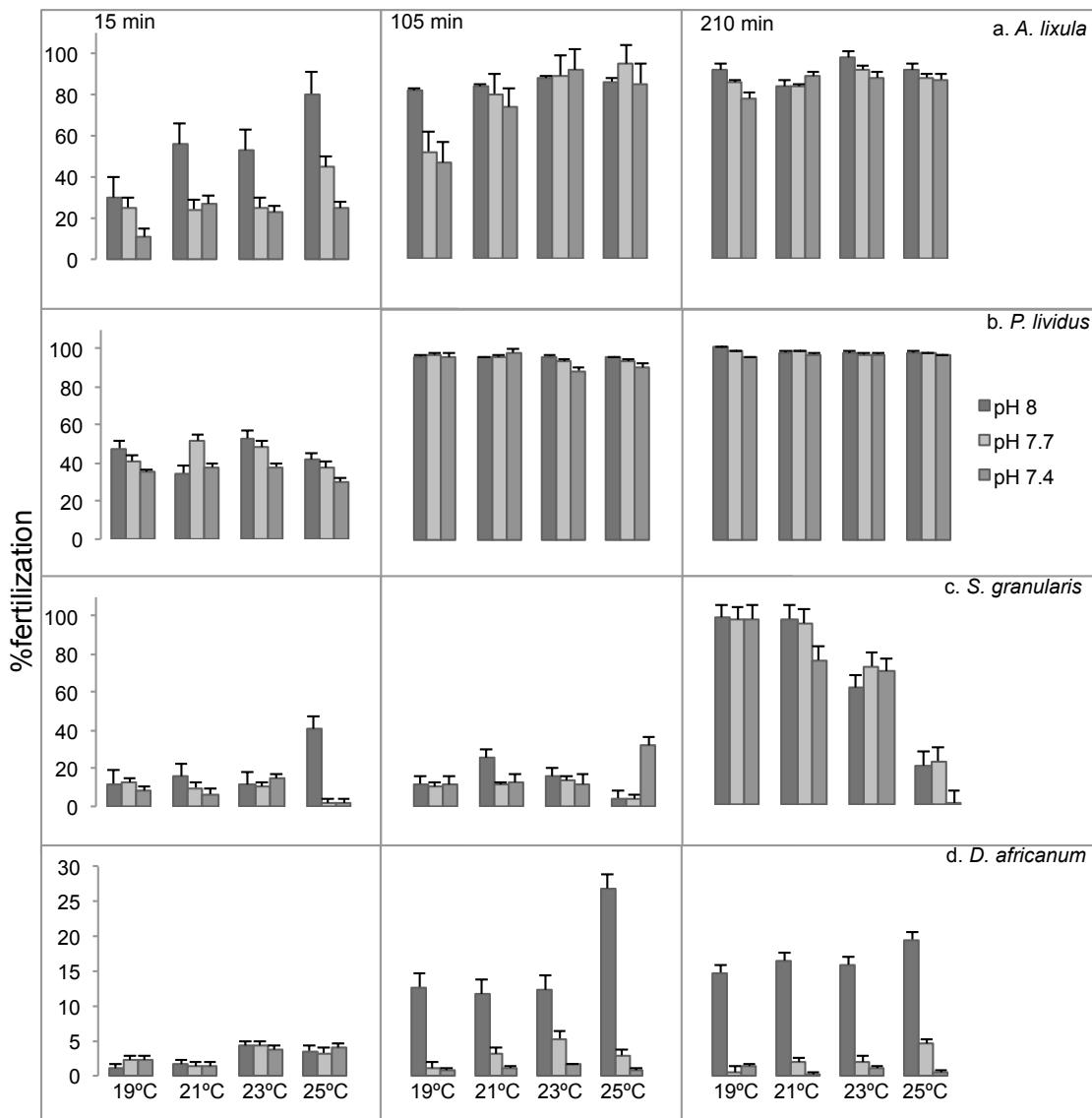


Figure 2.3. Percentage of fertilized eggs (mean  $\pm$  27.28, 6.63, 27.50, 33.47) of the sea urchin species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis*, (d) *Diadema africanum* in laboratory experiments testing the combined effects of seawater temperature and pH in different times after insemination (15, 105 and 210 minutes)

Table 2.1. Physico-chemical seawater parameters for each experimental treatment conducted with the different sea urchin species tested. T: seawater temperature (mean  $\pm$  SD), pH: seawater pH (mean  $\pm$  SD), S: salinity (mean  $\pm$  SD), pCO<sub>2</sub>: CO<sub>2</sub> partial pressure, TA: Total alkalinity,  $\Omega_c$ : Saturation level of calcite,  $\Omega_a$ : Saturation level of aragonite.

*A. lixula* experiment

	19°C			21°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	19.17 $\pm$ 0.15	19.27 $\pm$ 0.23	19.33 $\pm$ 0.06	21.20 $\pm$ 0.20	21.17 $\pm$ 0.23	21.23 $\pm$ 0.21
pH(n=3)	8.18 $\pm$ 0.01	7.72 $\pm$ 0.01	7.40 $\pm$ 0.02	8.14 $\pm$ 0.01	7.67 $\pm$ 0.03	7.40 $\pm$ 0.01
S (n = 3)	36.70 $\pm$ 0.10	36.63 $\pm$ 0.06	36.63 $\pm$ 0.15	36.70 $\pm$ 0.10	36.63 $\pm$ 0.06	36.63 $\pm$ 0.15
pCO <sub>2</sub>	394.40	1310.30	2858.40	448.20	1503.6	2903.30
TA (n = 1)	2375.52	2367.93	2365.54	2390.24	2358.43	2356.22
$\Omega_c$	4.64	1.85	0.93	4.58	1.76	0.98
$\Omega_a$	3.02	1.21	0.60	2.99	1.15	0.64
	23°C			25°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	22.97 $\pm$ 0.06	22.93 $\pm$ 0.06	22.97 $\pm$ 0.06	25.13 $\pm$ 0.11	25.17 $\pm$ 0.06	25.13 $\pm$ 0.23
pH(n=3)	8.08 $\pm$ 0.01	7.69 $\pm$ 0.02	7.41 $\pm$ 0.03	8.08 $\pm$ 0.01	7.67 $\pm$ 0.03	7.43 $\pm$ 0.00
S (n = 3)	36.77 $\pm$ 0.15	36.60 $\pm$ 0.26	36.70 $\pm$ 0.10	36.60 $\pm$ 0.46	36.73 $\pm$ 0.21	36.50 $\pm$ 0.40
pCO <sub>2</sub>	540.60	1484.30	2918.60	560.60	1599.60	2865.40
TA (n = 1)	2421.03	2407.12	2384.65	2474.20	2421.31	2402.01
$\Omega_c$	4.35	1.97	1.07	4.70	2.04	1.20
$\Omega_a$	2.86	1.30	0.70	3.11	1.35	0.79

*P. lividus* experiment

	19°C			21°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	19.17 $\pm$ 0.15	19.27 $\pm$ 0.23	19.33 $\pm$ 0.06	21.23 $\pm$ 0.06	21.3 $\pm$ 0.00	21.23 $\pm$ 0.21
pH(n=3)	8.09 $\pm$ 0.03	7.70 $\pm$ 0.05	7.40 $\pm$ 0.02	8.06 $\pm$ 0.01	7.67 $\pm$ 0.07	7.42 $\pm$ 0.02
S (n = 3)	36.63 $\pm$ 0.06	36.63 $\pm$ 0.11	36.63 $\pm$ 0.06	36.90 $\pm$ 0.00	36.80 $\pm$ 0.00	36.90 $\pm$ 0.00
pCO <sub>2</sub>	899.10	1591.0	2582.6	863.10	1293.20	2800.80
TA (n = 1)	2106.40	2069.53	2069.94	2081.02	2048.21	2070.60
$\Omega_c$	2.03	1.21	0.80	2.21	1.54	0.79
$\Omega_a$	1.32	0.79	0.52	1.45	1.01	0.52
	23°C			25°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	22.97 $\pm$ 0.06	22.92 $\pm$ 0.06	22.97 $\pm$ 0.06	25.20 $\pm$ 0.00	25.23 $\pm$ 0.11	25.07 $\pm$ 0.30
pH(n=3)	8.05 $\pm$ 0.03	7.73 $\pm$ 0.02	7.43 $\pm$ 0.03	8.06 $\pm$ 0.05	7.66 $\pm$ 0.05	7.39 $\pm$ 0.03
S (n = 3)	36.77 $\pm$ 0.21	36.60 $\pm$ 0.26	36.70 $\pm$ 0.10	36.60 $\pm$ 0.53	36.73 $\pm$ 0.11	36.50 $\pm$ 0.50
pCO <sub>2</sub>	1033.60	1265.70	2489.20	910.2	1367.1	2379.3
TA (n = 1)	2163.48	2121.63	2092.82	2220.17	2167.99	2148.82
$\Omega_c$	2.19	1.78	0.96	2.74	1.89	1.14
$\Omega_a$	1.44	1.17	0.63	1.81	1.25	0.76

*S. granularis* experiment

	19°C			21°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	19.27 $\pm$ 0.06	19.30 $\pm$ 0.10	19.33 $\pm$ 0.06	21.13 $\pm$ 0.23	21.23 $\pm$ 0.21	21.17 $\pm$ 0.15
pH(n=3)	8.06 $\pm$ 0.04	7.66 $\pm$ 0.06	7.36 $\pm$ 0.05	8.09 $\pm$ 0.01	7.65 $\pm$ 0.02	7.37 $\pm$ 0.02
S (n = 3)	36.50 $\pm$ 0.10	36.77 $\pm$ 0.23	36.33 $\pm$ 0.15	36.83 $\pm$ 0.15	36.60 $\pm$ 0.10	36.60 $\pm$ 0.02
pCO <sub>2</sub>	489.00	1342.70	2754.80	466.80	1432.90	2813.30
TA (n = 1)	2126.20	2096.35	2069.93	2181.52	2140.23	2127.23
$\Omega_c$	3.30	1.45	0.74	3.79	1.53	0.83
$\Omega_a$	2.15	0.94	0.48	2.48	1.00	0.54
	23°C			25°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	22.93 $\pm$ 0.06	23.00 $\pm$ 0.00	22.97 $\pm$ 0.06	25.20 $\pm$ 0.00	25.23 $\pm$ 0.11	25.06 $\pm$ 0.30
pH(n=3)	8.06 $\pm$ 0.05	7.70 $\pm$ 0.02	7.43 $\pm$ 0.04	8.02 $\pm$ 0.03	7.69 $\pm$ 0.04	7.40 $\pm$ 0.03
S (n = 3)	36.70 $\pm$ 0.10	36.70 $\pm$ 0.10	36.70 $\pm$ 0.10	36.83 $\pm$ 0.15	36.73 $\pm$ 0.15	36.77 $\pm$ 0.15
pCO <sub>2</sub>	518.10	1316.30	2539.30	592.80	1399.50	2829.1
TA (n = 1)	2203.48	2192.36	2179.13	2232.91	2229.36	2212.36
$\Omega_c$	3.79	1.84	1.02	3.79	1.96	1.04
$\Omega_a$	2.49	1.21	0.67	2.51	1.29	0.69

*D. africanum* experiment

	19°C			21°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	19.03±0.25	19.17±0.40	19.13±0.38	21.20±0.20	21.17±0.23	21.23±0.21
pH(n=3)	8.10±0.03	7.71±0.05	7.41±0.02	8.08±0.01	7.68±0.07	7.43±0.02
S (n = 3)	36.70±0.10	36.63±0.06	36.63±0.15	36.90±0.10	36.80±0.10	36.90±0.10
pCO <sub>2</sub>	456.40	1210.60	2465.60	511.80	1322.50	2311.60
TA (n = 1)	2215.50	2139.42	2096.50	2322.91	2132.44	2022.64
Ωc	3.70	1.63	0.83	3.98	1.63	0.90
Ωa	2.41	1.06	0.54	2.60	1.06	0.59
	23°C			25°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=3)	23.03±0.15	23.07±0.21	23.00±0.10	25.13±0.11	25.17±0.06	25.13±0.23
pH(n=3)	8.06±0.03	7.74±0.02	7.44±0.03	8.07±0.05	7.67±0.05	7.40±0.03
S (n = 3)	36.77±0.21	36.60±0.26	36.70±0.10	36.60±0.46	36.73±0.21	36.50±0.40
pCO <sub>2</sub>	574.00	1203.6	2416.0	580.60	1486.90	2722.70
TA (n = 1)	2432.84	2213.46	2124.01	2492.34	2253.50	2125.14
Ωc	4.22	2.02	1.02	4.65	1.89	0.99
Ωa	2.77	1.33	0.67	3.07	1.25	0.66

When analysing FR, a significant interaction of factors ‘Species x Temperature x pH x Time’ was found (Table 2.2A). In each combination of temperature and pH conditions, there were different responses for each of the studied species, at each of the different times after insemination (TAI). A *posteriori* pairwise test for *A. lixula*, revealed a significant decrease in FR at low pH, across all temperatures, at 15 MAI. The same trend was significant at all time points at the lowest temperatures, but not at the highest temperatures (Fig. 2.3a). Non-significant differences were found in FR of *P. lividus*, except in samples 15 MAI at 21°C when FR was significantly higher at pH 7.7 compared to pH 8 (Fig. 2.3b; Supplementary material 1, 2). *S. granularis* showed a significant increase in FR with time, especially at the lowest temperatures (19 and 21°C) (Fig. 2.3c; Supplementary material 1, 2). In *D. africanum* samples, FR significantly decreased with decreasing pH, and increased with increasing temperature, at 105 and 210 MAI (Fig. 3D; Supplementary material 1, 2). There were significant differences in FR between the species, particularly in the case of *D. africanum*, which exhibited the lowest fertilization percentages (Fig. 2.3; Supplementary material 3).

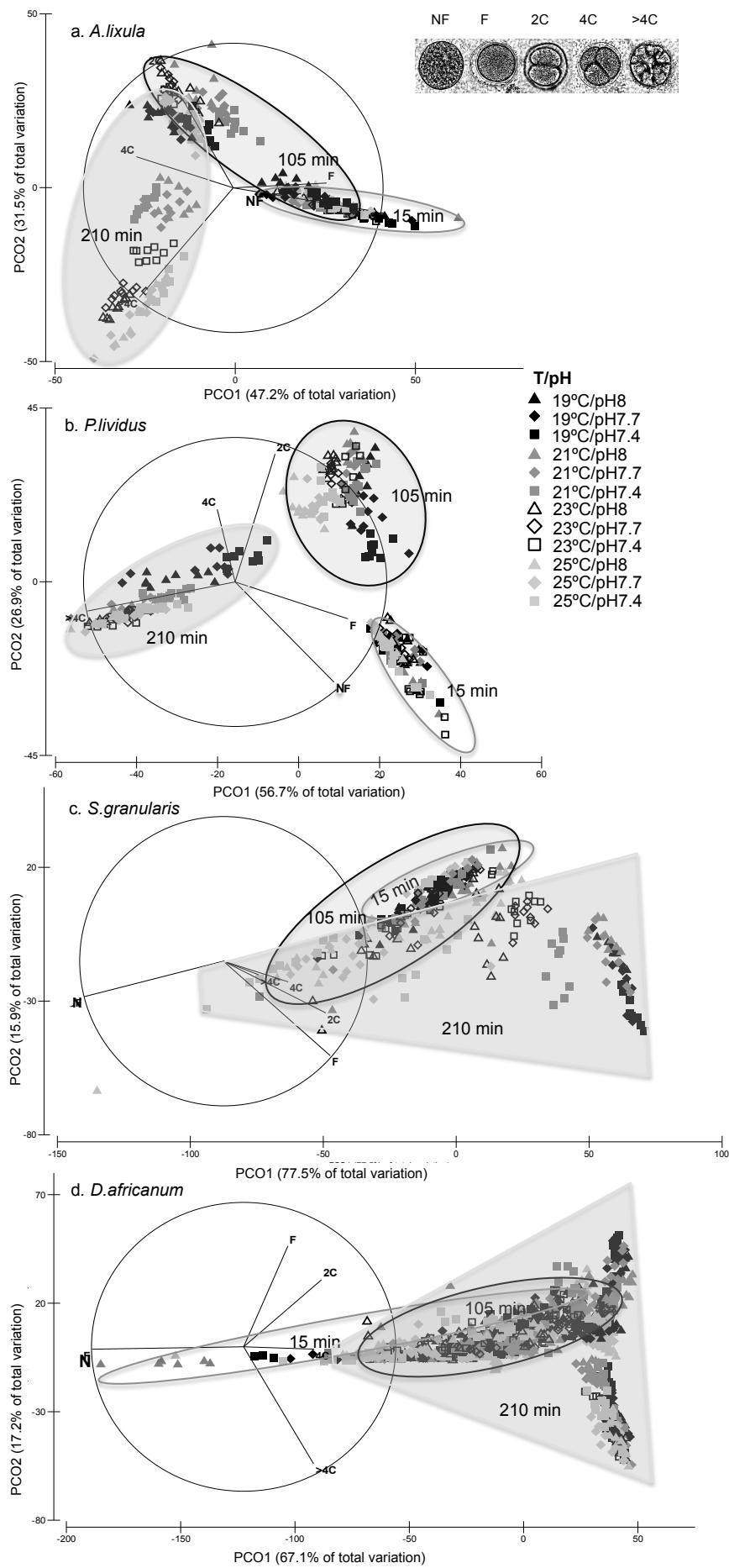
Results of the PERMANOVA analysing CR, showed a significant interaction of factors ‘Species x Temperature x pH x Time’ (Table 2.2B), indicating that the effects of

temperature and pH, on the rate at which eggs reached different stages of cleavage, varied significantly with TAI. Pairwise comparisons in *A. lixula*, showed that the proportion of non-cleaved eggs, or eggs in early stages of development (with only the fertilization membrane, or divided into 2 cells) was greater at low pH. This pattern was significant at all temperature and TAIs (Fig. 2.4a; Supplementary material 4). Also found with *A. lixula*, at higher seawater temperature, there were a greater the proportion of eggs in the advanced stages of cleavage (eggs divided into > 4 cells), but only in treatments at control pH, and only at 210 MAI (Fig. 2.4a; Supplementary material 5). With *P. lividus*, the lower the seawater pH, the greater the proportion of non-cleaved eggs or eggs in early stages (with only the fertilization membrane or divided into 2 cells). This pattern was seen at all time points, but only at temperatures of 19°C and 21°C (Fig. 2.4b; Supplementary material 4). At 210 MAI, higher seawater temperatures (23°C and 25°C) corresponded with a greater proportion of eggs in the advanced stages of cleavage (eggs into > 4 cells), and pH had no influence on this result (Fig. 2.4b; Supplementary material 5). In the species *S. granularis*, the most advanced egg stages were found at 21 and 23°C at pH 8.1. The greatest proportion of non-cleaved eggs or eggs in early stages of development (cells with fertilization membrane or divided into 2) coincided with the highest temperature (25°C) and the lowest pH conditions (Fig. 2.4c; Supplementary material 4, 5). Amongst *D. africanum*, the proportion of eggs in advanced stages of development (> 4 cells) was significantly lower at low pH (7.7 and 7.4), at all temperatures. The most advanced stages (4 cells and > 4 cells) were found in samples kept at the highest temperature and pH (Fig. 2.4d; Supplementary material 4, 5).

Three-day larvae survival was significantly affected by the interaction ‘Species x Temperature x pH ’(Table 2.2C). The density of *A. lixula* larvae was lower at low pH, at temperatures of 19°C, 21°C and 23°C. However, at 25°C larval density was significantly

higher in samples kept at pH7.7. At all levels of pH, the density of larvae was significantly higher in the highest temperature conditions (21, 23 and 25°C) (Fig. 2.5a; Supplementary material 6). Amongst *P. lividus*, larval survival decreased at low pH (7.7, 7.4) in all temperature treatments, except at 19°C where the difference between pH 8.1 and 7.7 was not significant. At all levels of pH, larval density was lower at temperatures above 21°C (Fig. 2.5b; Supplementary material 6).

Figure 2.4. PCO ordinations showing the combined effect of seawater temperature and pH on cleavage rate of the sea urchins species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis* and (d) *Diadema africanum*, at 15,105 and 210 minutes after insemination in laboratory experiments studying early development of the sea urchin. Vectors represent studied variables. NF: non-fertilized eggs, F: eggs only with the fertilization membrane, 2 C: eggs divided in 2 cells, 4 C: eggs divided in 4 cells, >4C: eggs divided in more than 4 cells. Percentages of variation explained by each of the axes are given in brackets.



*S. granularis* larvae were highest in density at pH 7.7, at temperatures of 19°C and 21°C. This pattern was not the same at higher temperature, where the number of larvae per ml was negligible at all pH levels (Fig. 2.5c; Supplementary material 6). The density of *D. africanum* larvae was lower at low pH (7.7, 7.4) in samples kept at all temperatures. However, the number of larvae per ml did increase with increasing temperature, across all levels of pH (Fig. 2.5d; Supplementary material 6).

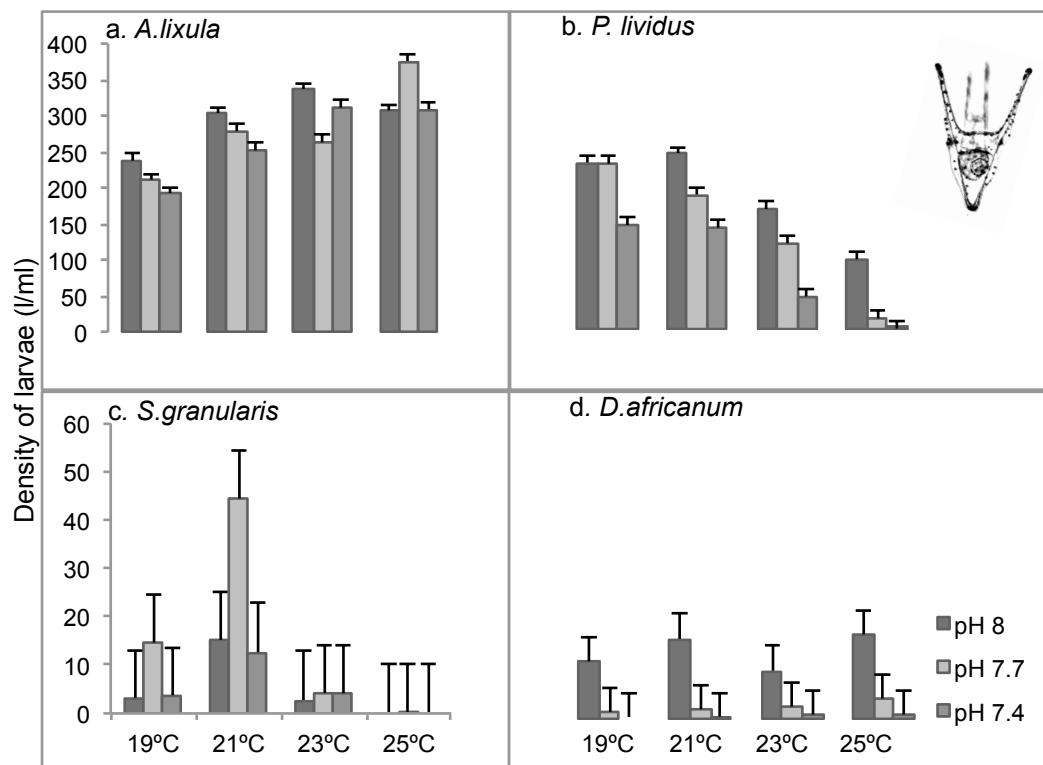


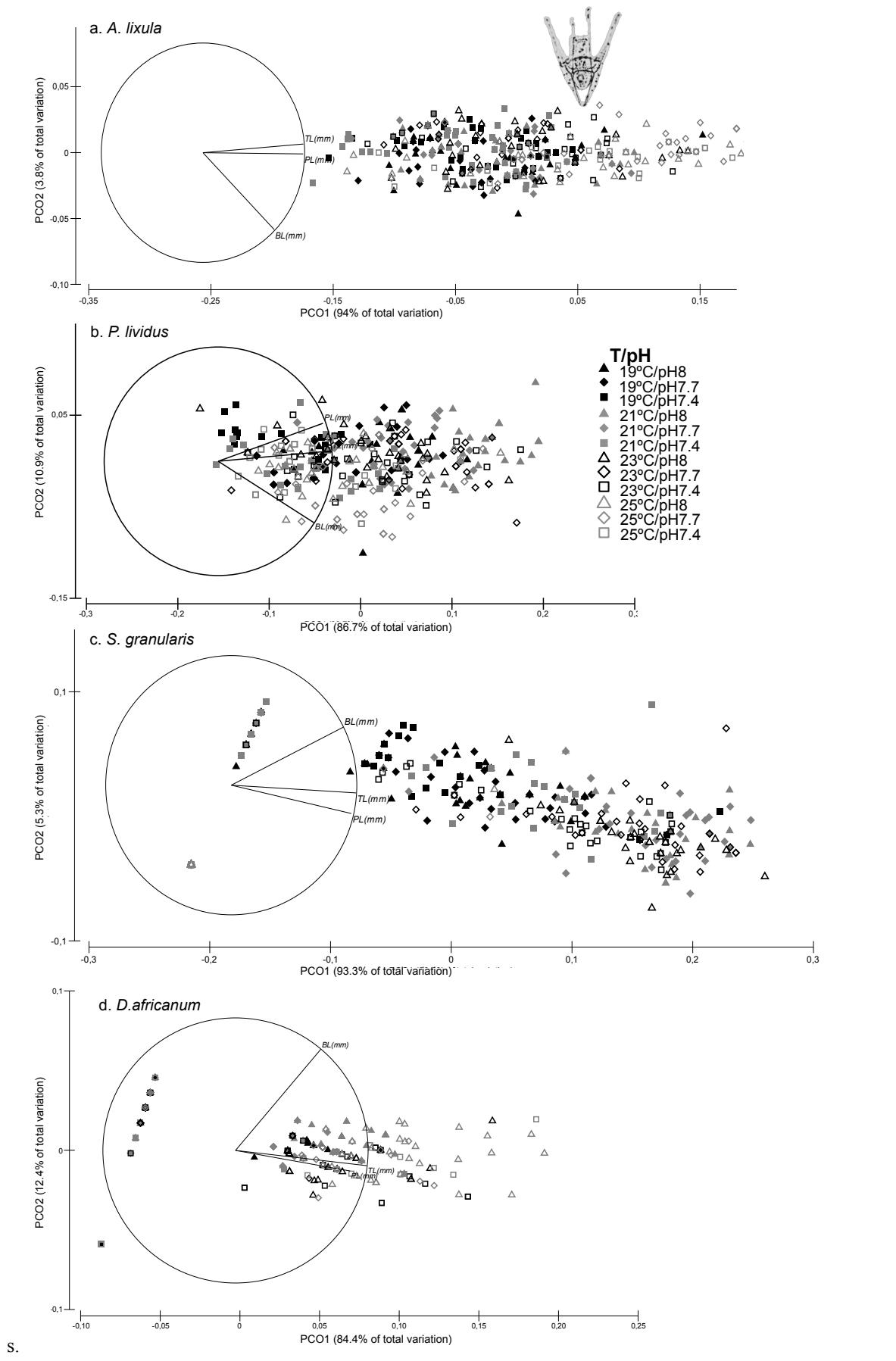
Figure 2.5. Density of larvae (mean  $\pm$  82.08, 6.39, 53.01, 12.52) of the sea urchin species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis*, (d) *Diadema africanum* at laboratory experiments testing the combined effects of seawater temperature and pH.

A significant interaction of ‘Species x Temperature x pH’ (Table 2.2D) was found when assessing three-day larvae morphology. This indicates that morphology is affected by both temperature and pH, but the effects are different for each of the four species. Pairwise comparisons in *A. lixula* showed that development of larvae was significantly higher at the highest temperatures (23, 25°C), across all levels of pH (Fig. 2.6a; Supplementary material 7). In *P. lividus* samples, the lower the seawater pH, the less developed the larvae (at 19

and 21°C). The most developed larvae were found at 21°C in the control pH (Fig. 2.6b; Supplementary material 7). In the species *S. granularis*, larvae were significantly more developed at high pH (pH 8.1 and 7.7), but only at temperatures of 21°C and 23°C. The most developed larvae were found at 21°C and 23°C across all pH levels (Fig. 2.6c; Supplementary material 7). No measurements of *S. granularis* larvae were able to be made at 25°C, because the number of larvae in the sample was too low. Amongst *D. africanum*, the most developed larvae were found at 25°C and pH 8.1. Larval development decreased at lower temperature and lower pH (Fig. 2.6d; Supplementary material 7).

Table 2.2. Results of (A) the four-way ANOVA analyzing fertilization rates; (B) the four-way PERMANOVA testing cleavage rate, including five studied variables (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells); (C) the three-way ANOVA analyzing larvae survival; and (D) the three-way PERMANOVA testing 3-day larvae development analysing morphometric variables (body length, total length and post oral arm length) of early stages of the studied sea urchin species. In the respective models the factors included are: Sp: species, T: temperature, pH, Ti: Time after insemination.

A. Fertilization Rate					B. Cleavage Rate					
Source of variation	df	SS	MS	Pseudo-F	P (perm)	df	SS	MS	Pseudo-F	P (perm)
Sp	3	3.64E5	1.21E5	4488.90	0.001	3	8.28E5	2.76E5	1580.40	0.001
T	3	1136	378.68	13.99	0.001	3	30164	10055	57.58	0.001
pH	2	6570.10	3285.10	121.36	0.001	2	10358	5178.80	29.66	0.001
Ti	2	1.31E5	65656	2425.40	0.001	2	3.31E5	1.66E5	948.90	0.001
Sp*T	9	17014	1890.50	69.84	0.001	9	1.06E5	11824	67.71	0.001
Sp*pH	6	2045.70	340.96	12.59	0.001	6	9582	1597	9.14	0.001
Sp*Ti	6	73517	12253	452.64	0.001	6	3.91E5	65237	373.59	0.001
T*pH	6	458.81	76.469	2.82	0.014	6	8156.70	1359.40	7.78	0.001
T*Ti	6	9750.70	1625.10	60.03	0.001	6	93193	15532	88.95	0.001
pH*Ti	4	807.44	201.86	7.46	0.001	4	8241.90	2060.50	11.80	0.001
Sp*T*pH	18	1739.60	96.64	3.57	0.001	18	25580	1421.10	8.14	0.001
Sp*T*Ti	18	20390	1132.80	41.85	0.001	18	1.84E5	10250	58.70	0.001
Sp*pH*Ti	12	5060.40	421.70	15.58	0.001	12	20115	1676.20	9.60	0.001
T*pH*Ti	12	1830	152.50	5.63	0.001	12	17159	1430	8.19	0.001
Sp*T*pH*Ti	36	5760	160	5.91	0.001	36	48070	1335.30	7.65	0.001
C. Larval survival					D. 3 day Larvae Development					
Source of variation	df	SS	MS	Pseudo-F	P (perm)	df	SS	MS	Pseudo-F	P (perm)
Sp	3	5.51E6	1.84E6	2071.70	0.001	3	15.75	5.25	1141	0.001
T	3	50832	16944	19.10	0.001	3	1.84	0.61	133.57	0.001
pH	2	94204	47102	53.09	0.001	2	0.79	0.40	86.40	0.001
Sp*T	9	6.25E5	69447	78.27	0.001	9	6.21	0.69	150	0.001
Sp*pH	6	1.12E5	18667	21.04	0.001	6	0.49	8.26E-2	17.95	0.001
T*pH	6	10178	1696.30	1.91	0.065	6	0.49	8.17E-2	17.76	0.065
Sp*T*pH	18	72575	4032	4.54	0.001	18	0.58	3.21E-2	6.98	0.001



S.

Figure 2.6. PCO ordinations showing the combined effect of sweater temperature and pH levels on larval development at 3 days after insemination at laboratory experiments of the sea urchin species (a) *Arbacia lixula*, (b) *Paracentrotus lividus*, (c) *Sphaerechinus granularis* and (d) *Diadema africanum*. Vectors represent studied morphological variables: TL: total length, BL: body length, PL: post-oral arm length. Percentages of variation explained by each of the axes are given in bracket

## Discussion

The present study confirmed that responses to climate change stressors in the marine environment are highly species-specific. The species tested seemed to be robust covering the present natural variability. However, subtidal species are more vulnerable to the environmental changes predicted to the end of the century.

Fertilisation rate in *Arbacia lixula* was lower at low pH, but the trend was only seen at low temperatures with TAI. Higher fertilization at higher temperatures ( $>21^{\circ}\text{C}$ ) has also been noted before in this species (Privitera et al. 2011). Though another study found no significant effects of temperature or pH (Gianguzza et al. 2014). *A. lixula* is a thermophilic species (Kempf 1962; Guidetti and Dulcić 2007), which may explain why its FR performance is better under warmer conditions (with no effect of pH). The species may be robust enough to withstand the future warming trend, as previously highlighted by Wangensteen et al. 2013. Low pH caused a moderate delay on CR, at all temperatures. Larval survival was enhanced at the highest temperature with moderate low pH (7.7). Wangensteen et al. (2013) also reported an increase in larval survival rates with rising temperature. In addition, larvae development was greater at higher temperatures, under all pH conditions, which is consistent with previous studies (Privitera et al. 2011; Wangensteen et al. 2013). Therefore, *A. lixula* performed best overall at higher temperatures, and in some cases at moderated lower pH. This suggests that *A. lixula* performance could be improved by near future climate change conditions, as suggested by

Gianguzza et al. (2014).

*Paracentrotus lividus* also showed great tolerance for warm and acidic waters. Previous studies have also found high fertilization success in this species (Moulin et al. 2011; Privitera et al. 2011; Martin et al. 2011; Cohen-Rengifo et al. 2013). However, CR was more sensitive than fertilization. Previous studies on this species have also noted a delay in CR at low pH and optimal temperatures (Moulin et al. 2011; Cohen-Rengifo et al. 2013). Though others found CR was only affected negatively under very low pH conditions (pH 7.0) (Martin et al. 2011). In our study, the negative effect of pH was neutralised by increasing the water temperature. However, this thermal tolerance was not apparent in the three-day larvae survival where the negative effect of low pH increased at warmer temperatures. The density of larvae dropped at temperatures above 21°C. Larvae development was also stunted by low pH, being slightly ameliorated at higher temperatures. Similar delays in development of larvae have been reported for *P. lividus* previously (Moulin et al. 2011; Martin et al. 2011; Cohen-Rengifo et al. 2013). Other studies have also reported a strong influence of high temperature on the species after blastulation (Ericson et al. 2012; Foo et al. 2012). Martin et al. (2011) suggested that the species has high molecular plasticity, as a result of gene up-regulation, which allows growth and skeletal calcification to be maintained at low pH. Our results showed that exposing *P. lividus* to a scenario of low pH alongside high temperature, can cancel out some of the negative effects of OA on FR, CR and larvae development. Similar trade-off between high temperature and low pH, has been found in recent studies (Sheppard-Brennand et al. 2010; Byrne et al. 2013). A genetic adaptability has also been reported, showing that certain species have genotypes tolerant of increased temperature that are also tolerant of decreased pH (Foo et al. 2012). However, when considering larvae survival, the combined effects of low pH and high temperature had deleterious impacts. This suggests

the species has a narrower thermal tolerance after blastulation.

For *Sphaerechinus granularis*, FR was dramatically impaired at higher temperatures, especially at 25°C. CR was drastically impaired by the most extreme combination of stressors (25°C and pH 7.4). *S. granularis* is a temperate species, with a single spawning period in spring (Guillou and Michel, 1993; Guillou and Lumingas, 1998). In the Canaries, the population occupies mainly subtidal habitats, and densities are rarely high. (pers. obs.). At higher temperatures the density of larvae dropped and at 25°C the number of larvae was too low to count accurately. These findings show the bad performance of early stages at high temperatures, suggesting a narrow threshold of the species to face global warming.

*Diadema africanum* was also significantly affected by low pH and high temperature. *D. africanum* is a thermophilic species that displays an annual reproductive cycle which peaks during summer in the Canary Islands, to coincide with the warmer SSTs (Hernández et al. 2011). This explains why its performance was best under warm conditions when at its optimal pH. However, at lower pH, its performance was unsuccessful demonstrating the species' likely vulnerability under expected OA conditions. *D. africanum* is mainly a subtidal species (Hernández et al. 2006), used to a stable environment, which may explain its lack of pH tolerance (Byrne et al. 2010b; Moulin et al. 2011).

Exposing sea urchins to two environmental stressors simultaneously, resulted in highly species-specific patterns. A broad thermo-tolerance of early stages of sea urchins has been shown by Byrne (2010), and it is well known that ocean warming can improve fertilization rates (Hagström and Hagström 1959; Mita et al. 1984). Some tolerate of acidification (~0,6 units) has also been shown previously (Khurihara and Shirayama, 2004; Byrne et al. 2009; Byrne et al. 2010a), perhaps a result of the natural acid environment to

which gametes are exposed during reproduction (Holland et al. 1984). Both temperature and pH are harmless, until a lethal threshold is reached. Their combined effect, in extreme conditions, may also have deleterious impacts. However, our study demonstrates that the effects of the interaction of climate change stressors differ among species identity and their life-history stages (see also reviews by Byrne 2011; Byrne et al. 2013; Dupont and Thorndyke 2013).

Generally, temperature and pH had a greater impact on CR than FR. This can be explained by tolerance in fertilization, conveyed by maternal factors imprinted by ovary temperature (Fujisawa 1995; Yamada and Mihashi 1998), protective heat shock proteins (Hamdoun and Epel 2007), and the low natural pH associated with echinoderm reproduction (Holland et al. 1984). There is strong evidence that adult thermal history influences thermal tolerance in fertilization due to adaptive phenotypic plasticity with respect to prevailing temperatures (O’Conner and Mulley 1977; Fujisawa 1995; Johnson and Babcock 1994; Bingham et al. 1997). Also, the internal pH of activated sperm is ~7.6, and eggs also release acid at fertilization (Peaucellier and Doree 1981; Holland and Gould-Somero 1982); gametes are therefore made to withstand a certain level of pH variation. We observed a general trend towards delayed cleavage at low pH, as has been reported in the literature (Khurihara and Shirayama 2004; Moulin et al. 2011; Martin et al. 2011; Foo et al. 2012; Ericson et al. 2012; Cohen-Rengifo et al. 2013). A postponement of cleavage could compromise subsequent developmental stages. (O’Connor et al. 2007).

The occurrence of abnormal larvae has previously been found to increase at low pH (Moulin et al. 2011; Sheppard-Brennand et al. 2010; Martin et al. 2011; Cohen-Rengifo et al. 2013; Byrne et al. 2013; Padilla-Garmiño et al. 2013). We also observed reduced size at low pH, among the four species we studied. Reduced size, or arm length, can have lethal consequences, since post-oral arms enable food uptake and swimming (Strathmann 1971;

Chan et al. 2011). Mortality can therefore be increased by starvation and susceptibility to predators. The planktonic stages of many echinoids are also synchronized with the availability of food-blooms, so a delay in development can cause a mismatch and increase susceptibility to starvation even further (Reitzel et al. 2004). Such decreases in larval survival, among the key sea urchin species, can have cascading effects (Clark et al. 2009) that change the structure and functioning of entire ecosystems. The effects of climate change on early larvae development can also be explained at transcriptional responses level. It is thought that low pH can reduce body lengths as a result of impaired skeletogenesis (Padilla-Garmiño et al. 2013). Increases in temperature can increase expression of basic components of the cellular cytoskeleton, but low pH can decrease this expression (Padilla-Garmiño et al. 2013).

Our results suggest that intertidal species (*A. lixula* and *P. lividus*) may be less vulnerable to the effects of predicted climate change than the subtidal species (*S. granularis* and *D. africanum*), as hypothesized (Fig. 2.7). Intertidal species have a greater capacity to cope with environmental change due to the variability in temperature and pH that they are already exposed to. Based on a recent study, one of these intertidal species may have an advantage over the other. Calosi et al. (2013) found that *A. lixula* was more abundant than *P. lividus* at low pH, noting that the former has a greater ability to regulate its extracellular fluid under elevated pCO<sub>2</sub> conditions. Increases in densities of *A. lixula* populations could have serious consequences for the structure and functioning of the ecosystem (Wangensteen 2013; Gianguzza et al. 2014). Subtidal species are thought to be less resilient to changes in temperature and/or acidification (Melzner et al. 2009; McElroy et al. 2012; Nguyen et al. 2012; Melzner et al. 2013; Kelly et al. 2013). Of the four species investigated, *D. africanum* exhibited the lowest fertilization rates, and the percentage of fertilised eggs was dramatically impaired at low pH. Due to its highly specialized

reproductive strategy, which is also greatly affected by environmental factors (Hernández et al. 2011), *D. africanum* is particularly vulnerable to climate change processes. *S. granularis* was also significantly impacted by environmental stress. It is likely that survival of its populations will be constrained by the water temperature extremes predicted for the end of the century. We hypothesize that synergistic stressors like OA have the potential to narrow the thermal windows in the species, being highly dependent upon thermal conditions. Depending on the optimal temperatures of the species, warming may lead to increased resilience to OA at the lower end of the optimal temperature range, but it can enhance sensitivity when temperature is closer to the upper limit of tolerance.

This study highlights the importance of assessing the combined effects of stressors, and looking at their impacts across different life-history stages to understand the potential impacts of climate change processes on marine organisms. Intertidal species seem to be more resilient to near-future climate change scenarios than subtidal species.

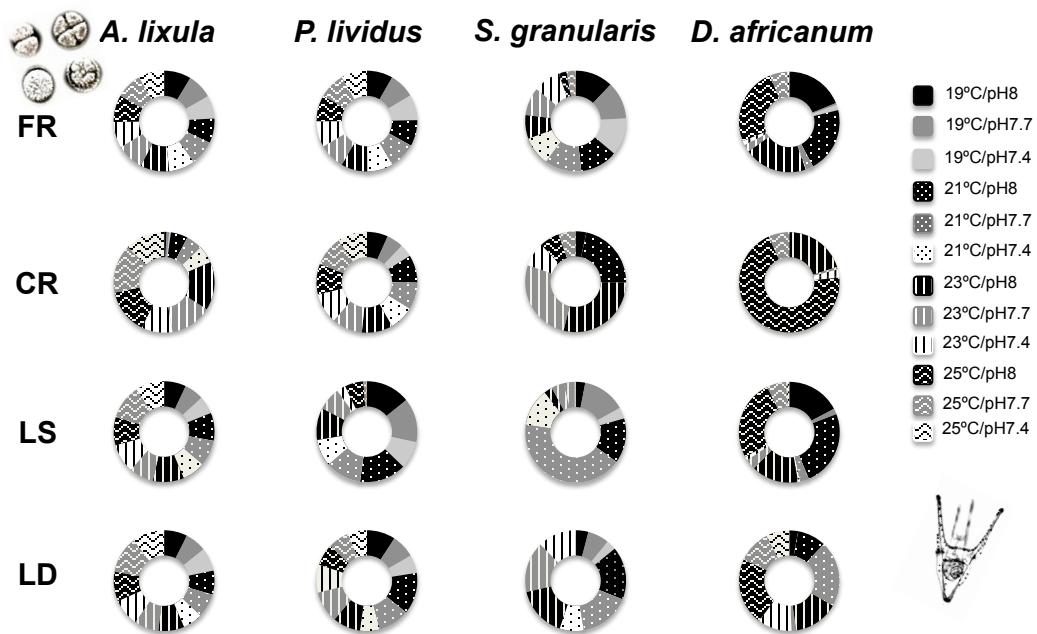


Figure 2.7. Proportional effects of each combined treatment on each species (*Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*) based on variables (FR: fertilization rate; CR: cleavage rate; LS: larvae survival; LD: larvae development) mean values that can be seen in supplementary

material 8.

Supplementary material 1. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH x time after insemination’ obtained in the ANOVA on fertilization rate of sea urchins in laboratory experiments. Combined effects of temperature and time after insemination (Ti) for pairs of levels of factor pH are shown for each studied species

*A. lixula*

Ti	19°C		21°C		23°C		25°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	0.76	0.487	4.70	0.015	4.84	0.009	13.32	0.002
	8 vs 7.4	3.04	0.039	3.87	0.024	4.34	0.013	4.80	0.007
	7.7 vs 7.4	4.65	0.014	0.40	0.682	0.61	0.567	1.72	0.172
105 min	8 vs 7.7	9.70	0.001	1.24	0.274	0.81	0.470	2.83	0.053
	8 vs 7.4	8.89	0.001	3.77	0.019	1.60	0.193	0.47	0.664
	7.7 vs 7.4	1.41	0.219	1.97	0.121	1.12	0.348	2.98	0.053
210 min	8 vs 7.7	2.19	0.083	0.14	0.897	3.71	0.018	1.87	0.140
	8 vs 7.4	4.44	0.012	2.08	0.108	5.33	0.008	2.71	0.055
	7.7 vs 7.4	2.29	0.085	6.12	0.002	2.67	0.071	0.41	0.718

*P. lividus*

Ti	19°C		21°C		23°C		25°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	0.43	0.708	3.73	0.016	0.51	0.630	1.29	0.265
	8 vs 7.4	1.12	0.333	0.66	0.554	0.83	0.441	2.50	0.071
	7.7 vs 7.4	0.51	0.612	2.20	0.082	0.64	0.535	1.99	0.123
105 min	8 vs 7.7	0.91	0.419	0.14	0.890	1.49	0.233	0.68	0.553
	8 vs 7.4	0.72	0.514	4.47	0.010	3.97	0.015	3.08	0.041
	7.7 vs 7.4	1.32	0.246	2.74	0.053	2.26	0.093	1.36	0.242
210 min	8 vs 7.7	0.43	0.698	1.90	0.134	0.63	0.557	0.47	0.695
	8 vs 7.4	1.55	0.187	1.47	0.211	0.34	0.720	1.34	0.273
	7.7 vs 7.4	0.80	0.482	2.49	0.057	0.11	0.915	0.84	0.446

*S. granularis*

Ti	19°C		21°C		23°C		25°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	0.18	0.849	3.31	0.029	0.21	0.838	26.31	0.001
	8 vs 7.4	1.78	0.141	5.82	0.010	0.55	0.572	25.53	0.001
	7.7 vs 7.4	1.85	0.136	3.10	0.035	0.76	0.492	0.11	0.925
105 min	8 vs 7.7	0.72	0.523	3.60	0.021	0.56	0.608	0.45	0.683
	8 vs 7.4	0.41	0.699	3.36	0.020	1.04	0.353	14.31	0.001
	7.7 vs 7.4	0.43	0.691	0.32	0.750	0.41	0.73	16.52	0.001
210 min	8 vs 7.7	2.41	0.076	1.34	0.247	7.67	0.002	0.34	0.734
	8 vs 7.4	1.35	0.250	32.36	0.001	5.61	0.009	3.16	0.031
	7.7 vs 7.4	1.11	0.355	11.16	0.001	1.92	0.127	5.90	0.009

*D. africanum*

Ti	19°C		21°C		23°C		25°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	1.42	0.232	0.40	0.705	5.63E-2	0.957	0.31	0.770
	8 vs 7.4	1.13	0.306	0.66	0.533	0.46	0.686	0.60	0.551
	7.7 vs 7.4	3.18E-2	0.974	0.20	0.870	0.41	0.708	1.13	0.336
105 min	8 vs 7.7	16.34	0.001	5.85	0.006	5.19	0.007	4.76	0.014
	8 vs 7.4	15.05	0.001	8.62	0.003	12.43	0.001	5.16	0.007
	7.7 vs 7.4	0.19	0.843	2.35	0.088	3.24	0.027	3.39	0.025
210 min	8 vs 7.7	14.15	0.001	8.72	0.001	7.57	0.002	6.46	0.004
	8 vs 7.4	13.52	0.001	9.75	0.003	9.19	0.002	30.59	0.001
	7.7 vs 7.4	3.26	0.021	6.61	0.003	0.86	0.434	1.72	0.172

Supplementary material 2. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH x time after insemination’ obtained in the ANOVA on fertilization rate of sea urchins in laboratory experiments. Combined effects of pH and time after insemination (Ti) for pairs of levels of factor temperature are shown for each studied species

<i>A. lixula</i>								
Ti	Comparisons	pH 8		pH 7.7		pH 7.4		
		t	P-perm	t	P-perm	t	P-perm	
15 min	19°C vs 21°C	3.23	0.034	0.19	0.868	2.88	0.059	
	19°C vs 23°C	2.80	0.050	4.79E-2	0.966	2.52	0.059	
	19°C vs 25°C	8.27	0.001	6.50	0.003	1.15	0.319	
	21°C vs 23°C	0.34	0.751	0.18	0.861	0.67	0.547	
	21°C vs 25°C	4.48	0.018	4.53	0.018	0.20	0.833	
	23°C vs 25°C	4.69	0.014	8.68	0.001	0.15	0.890	
	19°C vs 21°C	0.90	0.412	8.64	0.004	6.55	0.006	
	19°C vs 23°C	2.44	0.078	15.70	0.001	11.68	0.001	
	19°C vs 25°C	1.95	0.133	12.08	0.001	10.74	0.001	
	21°C vs 23°C	2.22	0.084	3.31	0.035	5.73	0.005	
105 min	21°C vs 25°C	1.55	0.196	3.88	0.017	4.14	0.011	
	23°C vs 25°C	0.84	0.413	1.90	0.151	2.52	0.083	
	19°C vs 21°C	2.70	0.047	0.95	0.379	4.24	0.011	
	19°C vs 23°C	2.43	0.073	2.84	0.047	3.71	0.025	
	19°C vs 25°C	7.18E-2	0.938	0.96	0.392	3.22	0.030	
	21°C vs 23°C	5.31	0.006	9.73	0.002	0.45	0.675	
	21°C vs 25°C	3.37	0.026	2.62	0.053	0.89	0.426	
	23°C vs 25°C	4.01	0.014	1.96	0.150	0.44	0.657	
	<i>P. lividus</i>							
	pH 8		pH 7.7		pH 7.4			
Ti	15 min	Comparisons	t	P-perm	t	P-perm	t	P-perm
		19°C vs 21°C	1.16	0.322	0.88	0.401	0.54	0.645
		19°C vs 23°C	0.37	0.708	0.62	0.564	0.19	0.868
		19°C vs 25°C	0.55	0.630	0.34	0.743	1.21	0.290
		21°C vs 23°C	1.99	0.118	0.69	0.509	1.38E-2	0.989
	105 min	21°C vs 25°C	1.98	0.107	3.24	0.034	1.36	0.259
		23°C vs 25°C	1.22	0.294	3.72	0.021	0.49	0.619
		19°C vs 21°C	1.14	0.314	1.32	0.251	2.59	0.051
		19°C vs 23°C	1.57E-2	0.992	2.24	0.086	3.87	0.017
		19°C vs 25°C	0.50	0.649	1.40	0.234	3.60	0.021
210 min	105 min	21°C vs 23°C	0.65	0.566	1.24	0.257	6.56	0.005
		21°C vs 25°C	0.10	0.933	0.74	0.505	7.63	0.002
		23°C vs 25°C	0.40	0.730	0.13	0.904	0.94	0.406
		19°C vs 21°C	0.80	0.486	9.74E-2	0.933	1.43	0.237
		19°C vs 23°C	0.91	0.410	0.41	0.700	0.95	0.409
	210 min	19°C vs 25°C	0.80	0.475	0.26	0.788	0.72	0.512
		21°C vs 23°C	0.62	0.558	1.80	0.133	1.12E-3	1.000
		21°C vs 25°C	7.71E-2	0.937	1.49	0.211	0.64	0.565
		23°C vs 25°C	0.36	0.708	0.42	0.693	0.40	0.714
		<i>S. granularis</i>						
Ti	pH 8		pH 7.7		pH 7.4			
	15 min	Comparisons	t	P-perm	t	P-perm	t	P-perm
		19°C vs 21°C	1.58	0.206	1.17	0.292	1.28	0.291
		19°C vs 23°C	0.21	0.835	0.74	0.486	1.27	0.276
		19°C vs 25°C	12.45	0.001	5.04	0.013	4.65	0.008
	105 min	21°C vs 23°C	1.34	0.246	0.34	0.776	1.65	0.187
		21°C vs 25°C	12.17	0.002	7.27	0.005	5.32	0.007
		23°C vs 25°C	8.82	0.002	4.88	0.007	2.62	0.046
		19°C vs 21°C	3.85	0.017	0.24	0.813	0.34	0.760
		19°C vs 23°C	1.36	0.219	0.87	0.469	0.14	0.888
210 min	105 min	19°C vs 25°C	5.32	0.009	3.88	0.022	10.48	0.002
		21°C vs 23°C	2.45	0.063	0.62	0.579	0.12	0.921
		21°C vs 25°C	6.07	0.005	3.34	0.038	7.26	0.001
		23°C vs 25°C	4.37	0.012	3.29	0.029	6.36	0.003
		19°C vs 21°C	1.18	0.274	0.96	0.380	33.24	0.001
	19°C vs 23°C	29.58	0.001	24.66	0.001	24.91	0.001	

19°C vs 25°C	12.50	0.001	19.71	0.001	204.23	0.001
21°C vs 23°C	29.59	0.001	11.89	0.001	5.15	0.008
21°C vs 25°C	12.38	0.001	17.68	0.001	134.61	0.001
23°C vs 25°C	6.48	0.006	12.91	0.002	65.78	0.001

*D. africanum*

		pH 8		pH 7.7		pH 7.4	
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm
15 min	19°C vs 21°C	0.73	0.503	1.31	0.267	1.14	0.308
	19°C vs 23°C	3.56	0.026	1.89	0.132	1.14	0.312
	19°C vs 25°C	2.54	0.066	1.46	0.213	2.43	0.066
	21°C vs 23°C	3.36	0.030	2.53	0.067	2.15	0.101
	21°C vs 25°C	2.17	0.101	2.40	0.074	9.17	0.002
	23°C vs 25°C	0.69	0.536	0.82	0.466	0.34	0.765
105 min	19°C vs 21°C	0.78	0.457	2.21	0.078	0.53	0.602
	19°C vs 23°C	0.47	0.666	3.71	0.027	1.15	0.306
	19°C vs 25°C	2.80	0.060	3.56	0.030	2.94E-2	0.981
	21°C vs 23°C	0.42	0.729	1.69	0.172	1.26	0.281
	21°C vs 25°C	2.94	0.039	0.15	0.882	0.59	0.568
	23°C vs 25°C	2.87	0.042	2.16	0.102	1.21	0.286
210 min	19°C vs 21°C	0.88	0.402	4.40	0.015	5.72	0.005
	19°C vs 23°C	0.60	0.599	1.49	0.215	2.21	0.088
	19°C vs 25°C	4.45	0.015	1.75	0.143	1.86	0.134
	21°C vs 23°C	0.24	0.813	4.80E-3	0.995	5.48	0.007
	21°C vs 25°C	1.76	0.147	1.14	0.325	0.62	0.568
	23°C vs 25°C	2.16	0.089	1.06	0.346	1.27	0.287

Supplementary material 3. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH x time after insemination’ obtained in the ANOVA on fertilization rate of sea urchins in laboratory experiments. Combined effects of temperature and time after insemination (Ti) for pairs of levels of factor species are shown for each level of pH studied

pH 8									
		19°C		21°C		23°C		25°C	
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	P.liv vs D.afri	4.20	0.015	22.65	0.001	5.37	0.004	12.02	0.001
	P.liv vs A.lix	1.42	0.235	3.79	0.026	2.73E-2	0.981	11.18	0.001
	P.liv vs S.gra	3.17	0.031	9.30	0.001	4.36	0.011	0.30	0.795
	D.afri vs A.lix	4.90	0.009	10.11	0.003	8.55	0.002	41.61	0.001
	D.afri vs S.gra	5.66	0.008	8.97	0.001	2.31	0.088	23.87	0.001
	A.lix vs S.gra	2.91	0.041	7.20	0.001	6.48	0.003	18.51	0.001
105 min	P.liv vs D.afri	109.83	0.001	62.29	0.001	56.48	0.001	13.25	0.001
	P.liv vs A.lix	6.82	0.001	9.17	0.001	4.68	0.015	5.41	0.007
	P.liv vs S.gra	70.72	0.001	20.71	0.001	30.29	0.001	55.52	0.001
	D.afri vs A.lix	31.00	0.001	42.30	0.001	47.52	0.001	11.52	0.001
	D.afri vs S.gra	0.67	0.552	3.87	0.019	1.27	0.266	4.40	0.013
	A.lix vs S.gra	28.96	0.001	16.42	0.001	26.45	0.001	55.73	0.001
210 min	P.liv vs D.afri	26.24	0.001	48.79	0.001	47.85	0.001	96.59	0.001
	P.liv vs A.lix	2.46	0.060	6.46	0.004	0.30	0.772	6.82	0.003
	P.liv vs S.gra	0.58	0.596	0.27	0.792	28.09	0.001	12.35	0.001
	D.afri vs A.lix	34.61	0.001	23.75	0.001	39.18	0.001	90.64	0.001
	D.afri vs S.gra	76.06	0.001	47.47	0.001	23.17	0.001	0.17	0.865
	A.lix vs S.gra	3.54	0.025	6.32	0.004	20.44	0.001	11.28	0.001
pH 7.7									
		19°C		21°C		23°C		25°C	
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	P.liv vs D.afri	3.42	0.023	11.53	0.001	15.02	0.001	24.41	0.001
	P.liv vs A.lix	1.36	0.262	4.66	0.010	8.11	0.002	3.11	0.035
	P.liv vs S.gra	2.46	0.071	9.40	0.003	11.74	0.001	26.23	0.001
	D.afri vs A.lix	10.62	0.003	5.77	0.004	15.23	0.001	18.93	0.001
	D.afri vs S.gra	4.99	0.011	8.14	0.003	3.12	0.037	1.64	0.184
	A.lix vs S.gra	4.28	0.008	3.55	0.030	7.65	0.004	19.82	0.001
105 min	P.liv vs D.afri	113.81	0.001	75.45	0.001	47.65	0.001	39.61	0.001
	P.liv vs A.lix	20.22	0.001	5.91	0.004	2.49	0.091	0.23	0.821
	P.liv vs S.gra	47.79	0.001	36.31	0.001	24.94	0.001	39.39	0.001
	D.afri vs A.lix	23.64	0.001	29.72	0.001	55.93	0.001	32.38	0.001

	D.afri vs S.gra	5.48	0.012	3.53	0.033	2.62	0.058	3.24	0.039
	A.lix vs S.gra	15.45	0.001	21.15	0.001	25.01	0.001	32.14	0.001
210 min	P.liv vs D.afri	26.88	0.001	243.18	0.001	70.35	0.001	38.31	0.001
	P.liv vs A.lix	3.18	0.033	30.24	0.001	4.24	0.014	4.97	0.005
	P.liv vs S.gra	0.33	0.742	1.89	0.105	17.64	0.001	19.38	0.001
	D.afri vs A.lix	38.97	0.001	183.90	0.001	74.25	0.001	28.78	0.001
	D.afri vs S.gra	232.06	0.001	55.79	0.001	54.55	0.001	4.21	0.020
	A.lix vs S.gra	5.60	0.009	7.38	0.004	15.28	0.001	15.59	0.001
<b>pH 7.4</b>									
		19°C		21°C		23°C		25°C	
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	P.liv vs D.afri	13.17	0.001	8.00	0.003	2.20	0.095	8.09	0.002
	P.liv vs A.lix	7.42	0.003	1.59	0.188	0.96	0.379	0.49	0.655
	P.liv vs S.gra	10.08	0.001	6.82	0.002	1.45	0.237	8.62	0.002
	D.afri vs A.lix	3.69	0.019	5.01	0.010	4.47	0.012	1.76	0.158
	D.afri vs S.gra	4.24	0.014	8.41	0.002	2.19	0.096	3.25	0.029
	A.lix vs S.gra	1.06	0.345	3.98	0.017	1.29	0.288	1.96	0.104
105 min	P.liv vs D.afri	81.64	0.001	382.89	0.001	59.53	0.001	79.03	0.001
	P.liv vs A.lix	14.33	0.001	10.17	0.001	1.08	0.332	2.95	0.037
	P.liv vs S.gra	59.10	0.001	40.12	0.001	25.46	0.001	30.08	0.001
	D.afri vs A.lix	13.83	0.001	29.59	0.001	43.42	0.001	52.82	0.001
	D.afri vs S.gra	9.08	0.001	5.16	0.015	3.73	0.024	17.54	0.001
	A.lix vs S.gra	10.34	0.001	18.89	0.002	23.72	0.001	23.70	0.001
210 min	P.liv vs D.afri	91.04	0.001	159.85	0.001	66.30	0.001	92.84	0.001
	P.liv vs A.lix	6.86	0.002	9.09	0.002	4.99	0.007	5.43	0.004
	P.liv vs S.gra	2.15	0.124	27.14	0.001	15.13	0.001	100.64	0.001
	D.afri vs A.lix	31.92	0.001	110.72	0.001	70.04	0.001	54.02	0.001
	D.afri vs S.gra	222.67	0.001	142.80	0.001	67.21	0.001	0.58	0.596
	A.lix vs S.gra	8.34	0.001	13.06	0.001	10.81	0.001	55.66	0.001

Supplementary material 4. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH x time after insemination’ obtained in the PERMANOVA on cleavage rate of sea urchins in laboratory experiments, including five studied variables (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells). Combined effects of temperature and time after insemination (Ti) for pairs of levels of factor pH are shown for each studied species.

<i>A. lixula</i>									
Ti		19°C		21°C		23°C		25°C	
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	1.00	0.347	3.61	0.001	6.02	0.001	6.68	0.001
	8 vs 7.4	3.56	0.006	3.48	0.001	3.73	0.001	9.22	0.001
	7.7 vs 7.4	3.13	0.004	0.34	0.881	0.73	0.511	2.03	0.034
105 min	8 vs 7.7	3.55	0.002	1.07	0.318	1.68	0.087	1.98	0.021
	8 vs 7.4	4.86	0.001	2.90	0.001	2.11	0.012	2.58	0.004
	7.7 vs 7.4	3.72	0.001	2.51	0.001	1.46	0.112	2.04	0.012
210 min	8 vs 7.7	1.51	0.094	2.93	0.002	2.59	0.010	1.57	0.121
	8 vs 7.4	3.97	0.001	4.09	0.001	7.67	0.001	2.36	0.017
	7.7 vs 7.4	2.69	0.002	3.36	0.001	5.72	0.001	3.34	0.001
<i>P. lividus</i>									
Ti		19°C		21°C		23°C		25°C	
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	0.63	0.631	3.4638	0.003	0.88	0.458	1.22	0.251
	8 vs 7.4	1.74	0.049	1.7571	0.069	1.80	0.084	3.39	0.001
	7.7 vs 7.4	0.99	0.393	2.8734	0.004	1.89	0.057	3.35	0.003
105 min	8 vs 7.7	2.71	0.008	3.5432	0.003	1.98	0.016	1.60	0.125
	8 vs 7.4	6.44	0.001	4.4161	0.001	2.29	0.006	3.62	0.001
	7.7 vs 7.4	2.44	0.006	1.8484	0.044	1.55	0.088	2.31	0.006
210 min	8 vs 7.7	1.81	0.035	1.106	0.290	0.47	0.728	0.40	0.730
	8 vs 7.4	4.83	0.001	3.1101	0.005	0.79	0.460	1.53	0.139
	7.7 vs 7.4	2.57	0.007	2.9096	0.011	1.19	0.264	1.36	0.172
<i>S. granularis</i>									
Ti		19°C		21°C		23°C		25°C	

	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	0.16	0.952	1.79	0.087	0.53	0.690	6.19	0.001
	8 vs 7.4	4.56	0.001	1.53	0.150	0.63	0.608	7.47	0.001
	7.7 vs 7.4	4.95	0.001	0.79	0.421	0.47	0.722	0.71	0.460
105 min	8 vs 7.7	0.12	0.964	0.82	0.454	3.38	0.007	1.03	0.333
	8 vs 7.4	2.24	0.043	1.18	0.257	1.19	0.259	6.17	0.001
	7.7 vs 7.4	1.44	0.183	0.93	0.365	2.61	0.018	5.64	0.001
210 min	8 vs 7.7	1.73	0.077	4.68	0.001	2.73	0.003	0.25	0.872
	8 vs 7.4	8.99	0.001	8.52	0.001	4.00	0.001	2.01	0.044
	7.7 vs 7.4	5.03	0.001	4.92	0.001	3.13	0.001	4.15	0.001
<i>D. africanum</i>									
Ti		19°C		21°C		23°C		25°C	
	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
15 min	8 vs 7.7	3.94	0.002	3.01	0.007	1.13	0.270	5.12	0.001
	8 vs 7.4	2.67	0.014	3.01	0.007	2.65	0.014	4.26	0.001
	7.7 vs 7.4	0.19	0.841	7.05E-2	0.969	1.38	0.158	0.47	0.666
105 min	8 vs 7.7	8.53	0.001	3.94	0.001	1.60	0.122	6.91	0.001
	8 vs 7.4	6.64	0.001	3.04	0.002	4.99	0.001	6.60	0.001
	7.7 vs 7.4	2.21	0.028	3.30	0.005	2.08	0.050	2.18	0.041
210 min	8 vs 7.7	7.70	0.001	2.59	0.007	3.33	0.003	4.37	0.001
	8 vs 7.4	5.59	0.001	3.13	0.002	2.64	0.012	5.94	0.001
	7.7 vs 7.4	0.25	0.813	0.74	0.473	1.39	0.188	1.72	0.125

Supplementary material 5. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH x time after insemination’ obtained in the PERMANOVA on cleavage rate of sea urchins in laboratory experiments, including five studied variables (non-fertilized eggs, cells with fertilization membrane, embryos with 2 cells, with 4 cells, and with > 4 cells). Combined effects of pH and time after insemination (Ti) for pairs of levels of factor temperature are shown for each studied species

<i>A. lixula</i>									
Ti	Comparisons	pH 8		pH 7.7		pH 7.4		t	P-perm
		t	P-perm	t	P-perm	t	P-perm		
15 min	19°C vs 21°C	3.17	0.002	0.74	0.554	4.92	0.001		
	19°C vs 23°C	4.19	0.001	0.89	0.431	2.58	0.021		
	19°C vs 25°C	9.44	0.001	2.69	0.003	5.39	0.001		
	21°C vs 23°C	1.14	0.245	2.26	0.016	0.96	0.371		
	21°C vs 25°C	3.41	0.003	2.97	0.002	1.88	0.038		
	23°C vs 25°C	5.56	0.001	4.51	0.001	1.64	0.116		
105 min	19°C vs 21°C	7.87	0.001	9.01	0.001	10.89	0.001		
	19°C vs 23°C	8.87	0.001	11.17	0.001	17.23	0.001		
	19°C vs 25°C	8.64	0.001	9.39	0.001	11.45	0.001		
	21°C vs 23°C	1.75	0.047	3.94	0.001	6.85	0.001		
	21°C vs 25°C	3.96	0.001	5.75	0.001	6.18	0.001		
	23°C vs 25°C	2.83	0.002	3.69	0.001	4.99	0.001		
210 min	19°C vs 21°C	6.36	0.001	7.41	0.001	9.37	0.001		
	19°C vs 23°C	17.42	0.001	15.96	0.001	11.55	0.001		
	19°C vs 25°C	13.69	0.001	13.92	0.001	13.28	0.001		
	21°C vs 23°C	11.17	0.001	11.63	0.001	6.83	0.001		
	21°C vs 25°C	8.96	0.001	10.80	0.001	11.23	0.001		
	23°C vs 25°C	2.52	0.009	3.11	0.001	5.45	0.001		
<i>P. lividus</i>									
Ti	Comparisons	pH 8		pH 7.7		pH 7.4		t	P-perm
		t	P-perm	t	P-perm	t	P-perm		
15 min	19°C vs 21°C	1.86	0.065	1.15	0.277	0.59	0.688		
	19°C vs 23°C	0.53	0.699	0.62	0.645	1.59	0.098		
	19°C vs 25°C	1.78	0.067	1.42	0.156	1.51	0.131		
	21°C vs 23°C	2.97	0.004	1.27	0.199	1.43	0.159		
	21°C vs 25°C	3.50	0.002	4.14	0.001	1.80	0.047		
	23°C vs 25°C	2.42	0.012	3.80	0.001	1.18	0.255		
105 min	19°C vs 21°C	3.72	0.001	4.05	0.001	7.14	0.001		
	19°C vs 23°C	5.70	0.001	6.15	0.001	8.53	0.001		
	19°C vs 25°C	10.35	0.001	8.34	0.001	13.27	0.001		
	21°C vs 23°C	7.58	0.001	4.66	0.001	3.43	0.001		

210 min	21°C vs 25°C	14.12	0.001	10.77	0.001	9.75	0.001
	23°C vs 25°C	8.41	0.001	8.47	0.001	6.07	0.001
	19°C vs 21°C	4.07	0.002	4.63	0.001	8.68	0.001
	19°C vs 23°C	5.31	0.001	6.56	0.001	13.75	0.001
	19°C vs 25°C	4.83	0.001	5.86	0.001	9.85	0.001
	21°C vs 23°C	2.26	0.032	2.12	0.033	6.02	0.001
	21°C vs 25°C	1.82	0.091	1.70	0.086	2.72	0.008
	23°C vs 25°C	0.14	0.963	0.70	0.527	2.55	0.022

*S. granularis*

		pH 8	pH 7.7		pH 7.4		
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm
15 min	19°C vs 21°C	2.52	0.018	0.96	0.330	1.55	0.150
	19°C vs 23°C	2.71	0.018	2.28	0.029	5.96	0.001
	19°C vs 25°C	5.92	0.001	1.73	0.074	6.05	0.001
	21°C vs 23°C	0.60	0.630	1.30	0.218	1.18	0.274
	21°C vs 25°C	3.49	0.001	0.99	0.347	0.65	0.538
	23°C vs 25°C	5.07	0.001	1.54	0.152	1.93	0.064
	105 min	0.96	0.364	0.37	0.738	2.96	0.006
	19°C vs 23°C	4.08	0.002	1.10	0.285	6.34	0.001
210 min	19°C vs 25°C	6.84	0.001	4.97	0.002	3.71	0.002
	21°C vs 23°C	3.06	0.005	1.47	0.158	2.87	0.010
	21°C vs 25°C	4.94	0.001	5.11	0.001	2.38	0.016
	23°C vs 25°C	2.50	0.010	5.82	0.001	3.24	0.002
	19°C vs 21°C	5.31	0.001	1.63	0.088	7.25	0.001
	19°C vs 23°C	9.23	0.001	7.72	0.001	16.27	0.001
	19°C vs 25°C	15.77	0.001	11.30	0.001	20.46	0.001
	21°C vs 23°C	7.86	0.001	7.78	0.001	7.82	0.001
	21°C vs 25°C	5.81	0.001	11.84	0.001	15.32	0.001
	23°C vs 25°C	3.05	0.008	8.85	0.001	14.61	0.001

*D. africanum*

		pH 8	pH 7.7		pH 7.4		
Ti	Comparisons	t	P-perm	t	P-perm	t	P-perm
15 min	19°C vs 21°C	4.36	0.001	1.31	0.207	0.69	0.511
	19°C vs 23°C	2.38	0.033	2.42	0.038	0.97	0.371
	19°C vs 25°C	2.92	0.014	0.93	0.365	0.50	0.592
	21°C vs 23°C	3.77	0.003	1.76	0.099	0.83	0.438
	21°C vs 25°C	3.60	0.004	0.98	0.345	0.84	0.426
	23°C vs 25°C	1.11	0.278	3.81	0.003	1.60	0.124
105 min	19°C vs 21°C	1.24	0.244	1.60	0.126	4.88	0.002
	19°C vs 23°C	6.09	0.001	4.31	0.001	1.38	0.191
	19°C vs 25°C	4.51	0.001	2.02	0.042	1.68	0.103
	21°C vs 23°C	3.18	0.003	3.02	0.009	1.46	0.146
	21°C vs 25°C	3.49	0.002	2.69	0.014	6.69	0.001
	23°C vs 25°C	2.18	0.031	4.87	0.002	2.42	0.025
210 min	19°C vs 21°C	4.08	0.001	2.41	0.028	1.51	0.156
	19°C vs 23°C	2.62	0.011	2.01	0.054	2.64	0.017
	19°C vs 25°C	3.91	0.001	5.26	0.001	2.54	0.022
	21°C vs 23°C	2.38	0.023	0.22	0.851	2.01	0.058
	21°C vs 25°C	6.70	0.001	3.16	0.007	1.79	0.090
	23°C vs 25°C	3.67	0.001	3.13	0.012	0.18	0.874

Supplementary material 6. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH’ obtained in the ANOVA on larvae survival of sea urchins in laboratory experiments. Combined effects of temperature and pH are shown for each studied species

		<i>A. lixula</i>		<i>P. lividus</i>		<i>S. granularis</i>		<i>D. africanum</i>	
Temp	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
19°C	8 vs 7.7	1.26	0.230	0.23	0.818	4.53	0.001	5.42	0.001
	8 vs 7.4	2.41	0.033	7.92	0.001	0.41	0.701	6.09	0.001
	7.7 vs 7.4	0.82	0.447	11.17	0.001	4.34	0.001	8.31	0.001
21°C	8 vs 7.7	0.88	0.402	4.77	0.001	10.22	0.001	12.65	0.001
	8 vs 7.4	1.86	0.075	9.16	0.001	0.59	0.561	14.13	0.001
	7.7 vs 7.4	1.25	0.218	7.37	0.001	7.66	0.001	4.60	0.001
23°C	8 vs 7.7	2.10	0.058	8.08	0.001	1.22	0.239	4.87	0.001
	8 vs 7.4	0.89	0.386	10.66	0.001	1.24	0.238	7.75	0.001
	7.7 vs 7.4	1.86	0.072	6.62	0.001	0.21	0.844	1.34	0.185
25°C	8 vs 7.7	2.52	0.029	6.17	0.001	1.00	0.334	7.72	0.001
	8 vs 7.4	0.14	0.892	7.51	0.001	0.00	-	9.99	0.001
	7.7 vs 7.4	2.29	0.032	3.91	0.001	1.00	0.324	4.57	0.002
		<i>A. lixula</i>		<i>P. lividus</i>		<i>S. granularis</i>		<i>D. africanum</i>	
pH	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
8	19°C vs 21°C	2.54	0.026	0.87	0.395	5.37	0.001	2.19	0.038
	19°C vs 23°C	3.40	0.004	6.34	0.001	0.36	0.756	0.75	0.482
	19°C vs 25°C	3.53	0.004	8.54	0.001	3.33	0.010	2.31	0.038
	21°C vs 23°C	0.97	0.360	6.64	0.001	5.77	0.001	4.06	0.002
	21°C vs 25°C	0.12	0.892	8.79	0.001	7.60	0.001	0.46	0.652
	23°C vs 25°C	0.99	0.305	5.15	0.001	3.57	0.004	3.77	0.004
	19°C vs 21°C	2.92	0.014	6.15	0.001	9.65	0.001	2.23	0.034
	19°C vs 23°C	1.78	0.075	18.68	0.001	4.18	0.002	0.94	0.373
	19°C vs 25°C	5.62	0.001	37.58	0.001	6.26	0.001	4.77	0.001
7.7	21°C vs 23°C	0.58	0.559	10.45	0.001	17.84	0.001	0.31	0.758
	21°C vs 25°C	3.49	0.006	27.27	0.001	21.40	0.001	3.45	0.001
	23°C vs 25°C	3.44	0.003	19.94	0.001	4.56	0.001	1.57	0.144
	19°C vs 21°C	2.81	0.020	0.48	0.639	2.39	0.032	2.53	0.028
	19°C vs 23°C	6.34	0.001	8.07	0.001	0.20	0.858	3.50	0.002
	19°C vs 25°C	5.14	0.001	22.94	0.001	4.08	0.004	1.41	0.166
7.4	21°C vs 23°C	3.07	0.017	8.56	0.001	2.38	0.026	1.18	0.247
	21°C vs 25°C	2.47	0.020	38.19	0.001	3.52	0.005	0.44	0.655
	23°C vs 25°C	6.97E-2	0.946	4.25	0.001	6.66	0.001	0.21	0.831

Supplementary material 7. Results of pair-wise tests examining the significant interaction of factors ‘Species x Temperature x pH’ obtained in the PERMANOVA on three-day larvae morphology of sea urchins in laboratory experiments, analysing morphometric variables (body length, total length and post oral arm length). Combined effects of temperature and pH are shown for each studied species.

		<i>A. lixula</i>		<i>P. lividus</i>		<i>S. granularis</i>		<i>D. africanum</i>	
Temperature	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
19°C	8 vs 7.7	1.15	0.247	2.89	0.002	2.65	0.011	3.66	0.002
	8 vs 7.4	0.37	0.829	6.78	0.001	0.43	0.754	9.99	0.001
	7.7 vs 7.4	1.28	0.180	3.67	0.001	2.65	0.007	73.17	0.001
21°C	8 vs 7.7	0.56	0.629	7.53	0.001	0.36	0.872	4.83	0.001
	8 vs 7.4	3.05	0.003	12.76	0.001	5.82	0.001	11.69	0.001
	7.7 vs 7.4	2.62	0.012	5.60	0.001	5.39	0.001	4.52	0.001
23°C	8 vs 7.7	1.10	0.266	1.83	0.063	0.47	0.739	8.27	0.001
	8 vs 7.4	0.52	0.657	0.76	0.514	3.20	0.003	1.26	0.207
	7.7 vs 7.4	1.62	0.100	1.61	0.101	2.99	0.003	4.98	0.001
25°C	8 vs 7.7	2.76	0.005	2.22	0.019	0.43	0.692	4.83	0.001
	8 vs 7.4	0.98	0.325	1.85	0.047	1.00	0.352	6.38	0.001
	7.7 vs 7.4	1.89	0.063	3.84	0.002	1.40	0.169	3.51	0.001
		<i>A. lixula</i>		<i>P. lividus</i>		<i>S. granularis</i>		<i>D. africanum</i>	
pH	Comparisons	t	P-perm	t	P-perm	t	P-perm	t	P-perm
8	19°C vs 21°C	0.65	0.558	7.34	0.001	10.34	0.001	5.69	0.001
	19°C vs 23°C	1.18	0.234	0.86	0.433	9.90	0.001	6.48	0.001

	19°C vs 25°C	2.41	0.027	3.01	0.001	8.95	0.001	10.24	0.001
	21°C vs 23°C	0.77	0.490	7.57	0.001	0.81	0.434	1.52	0.104
	21°C vs 25°C	1.99	0.050	10.50	0.001	26.52	0.001	4.83	0.001
	23°C vs 25°C	1.46	0.155	2.21	0.023	25.76	0.001	4.58	0.001
7.7	19°C vs 21°C	1.45	0.134	3.51	0.002	7.10	0.001	5.02	0.001
	19°C vs 23°C	1.67	0.098	3.65	0.001	6.87	0.001	2.83	0.006
	19°C vs 25°C	7.50	0.001	2.76	0.005	11.90	0.001	11.24	0.001
	21°C vs 23°C	0.70	0.552	1.12	0.276	0.33	0.874	1.99	0.046
	21°C vs 25°C	5.85	0.001	3.84	0.001	21.08	0.001	4.34	0.001
	23°C vs 25°C	6.14	0.001	3.19	0.003	20.72	0.001	6.64	0.001
7.4	19°C vs 21°C	2.38	0.010	2.13	0.027	2.06	0.035	25.64	0.001
	19°C vs 23°C	1.75	0.076	5.60	0.001	4.93	0.001	10.61	0.001
	19°C vs 25°C	3.87	0.001	2.08	0.037	8.93	0.001	4.30	0.001
	21°C vs 23°C	3.84	0.001	3.82	0.001	2.43	0.016	7.35	0.001
	21°C vs 25°C	5.63	0.001	0.21	0.970	9.69	0.001	3.92	0.001
	23°C vs 25°C	2.33	0.023	3.84	0.001	15.17	0.001	2.77	0.003

Supplementary material 8. Mean values (mean ± SD) of the variables tested: (FR, %) Fertilization rate; (CR, %) cleavage rate, measured here as the percentage of embryos divided in more than 4 cells after 210 min; (LS) larvae survival; (LD, mm) larvae development, measured here as mean values of post oral arm length.

<i>A. lixula</i>				
	FR	CR	LS	LD
19°C/pH 8	91.99±3.47	4.02±1.76	237.89±36.38	0.133±0.04
19°C/pH 7.7	85.53±3.76	4.11±1.00	210.56±53.73	0.121±0.03
19°C/pH 7.4	78.11±4.16	2.6±0.97	191.33±45.16	0.133±0.04
21°C/pH 8	83.78±3.96	27.59±3.46	302.67±67.36	0.137±0.04
21°C/pH 7.7	83.44±0.69	25.74±3.31	278.89±45.11	0.137±0.04
21°C/pH 7.4	88.82±1.36	28.38±3.82	251.89±46.34	0.113±0.03
23°C/pH 8	97.82±2.29	77.6±4.10	336.11±78.60	0.147±0.04
23°C/pH 7.7	92.09±1.38	66.66±3.59	262.78±69.53	0.139±0.03
23°C/pH 7.4	88.15±2.15	49.57±3.20	310.78±34.03	0.155±0.04
25°C/pH 8	91.84±1.19	72.21±10.03	306±44.90	0.169±0.06
25°C/pH 7.7	88.25±3.10	77.8±10.05	376.11±70.23	0.211±0.05
25°C/pH 7.4	87.28±2.66	63.73±6.69	309.33±52.04	0.182±0.05
<i>P. lividus</i>				
19°C/pH 8	97.83±0.33	66.26±8.54	229.78±27.13	0.131±0.03
19°C/pH 7.7	95.73±0.59	52.39±11.58	227.44±13.16	0.108±0.04
19°C/pH 7.4	96.03±1.78	40.23±4.22	143.22±18.39	0.083±0.02
21°C/pH 8	98.64±0.38	87.63±4.93	241.89±31.67	0.191±0.02
21°C/pH 7.7	99.42±0.60	84.35±8.41	185.89±15.42	0.143±0.03
21°C/pH 7.4	97.72±1.02	78.38±4.55	139.78±10.67	0.091±0.03
23°C/pH 8	98.25±1.01	96.91±6.81	166±13.17	0.126±0.03
23°C/pH 7.7	97.52±1.74	95.40±3.74	119.11±11.37	0.145±0.04
23°C/pH 7.4	97.72±2.52	95.09±5.64	46±31.09	0.128±0.04
25°C/pH 8	98.58±1.23	96.34±8.43	97±37.95	0.100±0.03
25°C/pH 7.7	98.07±1.45	95.25±8.00	15.89±10.58	0.097±0.04
25°C/pH 7.4	97.02±1.60	92.36±7.81	1.89±1.83	0.087±0.02
<i>S. granularis</i>				
19°C/pH 8	99.31±0.89	0.73±1.00	3.22±2.90	0.040±0.04
19°C/pH 7.7	97.83±0.58	0.00±0.00	14.56±6.91	0.067±0.05
19°C/pH 7.4	98.42±0.71	0.00±0.00	3.78±2.77	0.031±0.06
21°C/pH 8	98.50±0.77	4.63±2.03	15.11±5.97	0.166±0.03
21°C/pH 7.7	96.18±2.91	0.00±0.00	44.4±6.21	0.160±0.04
21°C/pH 7.4	76.61±0.88	0.00±0.00	12.67±10.78	0.076±0.06
23°C/pH 8	61.97±1.94	6.05±3.46	2.78±2.33	0.159±0.04
23°C/pH 7.7	73.34±1.62	5.60±2.53	4.22±2.68	0.160±0.04
23°C/pH 7.4	70.66±1.79	1.66±1.12	4.00±1.80	0.120±0.06
25°C/pH 8	20.64±10.86	1.35±1.12	0.00±0.00	-
25°C/pH 7.7	23.12±6.54	1.17±0.83	0.11±0.33	-
25°C/pH 7.4	0.78±0.42	0.00±0.00	0.00±0.00	-
<i>D. africanum</i>				
19°C/pH 8	14.82±1.70	0.00±0.00	11.44±5.64	0.008±0.02
19°C/pH 7.7	0.45±0.44	0.00±0.00	1.22±0.44	0.00±0.00
19°C/pH 7.4	1.39±0.24	0.00±0.00	0.00±0.00	0.00±0.00
21°C/pH 8	16.52±2.89	0.00±0.00	16.22±3.31	0.026±0.02
21°C/pH 7.7	1.86±0.34	0.00±0.00	1.89±0.78	0.006±0.01
21°C/pH 7.4	0.16±0.28	0.00±0.00	0.44±0.53	0.00±0.00
23°C/pH 8	15.95±2.80	3.95±1.01	9.98±3.42	0.041±0.02

23°C/pH 7.7	1.87±1.59	0.16±0.33	2.22±3.15	0.004±0.01
23°C/pH 7.4	1.08±0.05	0.53±0.50	0.78±0.67	0.031±0.03
25°C/pH 8	19.55±0.70	13.72±1.96	17.11±4.73	0.061±0.02
25°C/pH 7.7	4.50±3.97	1.33±1.09	4.11±1.76	0.03±0.02
25°C/pH 7.4	0.47±0.82	0.00±0.00	0.67±1.41	0.021±0.03

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**CHAPTER 3: OCEAN WARMING  
MITIGATES THE EFFECTS OF  
LIMITED FOOD AVAILABILITY ON  
PARACENTROTUS LIVIDUS LARVAL  
DEVELOPMENT.**



## **CHAPTER 3: OCEAN WARMING MITIGATES THE EFFECTS OF LIMITED FOOD AVAILABILITY ON PARACENTROTUS LIVIDUS LARVAL DEVELOPMENT.**

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## Abstract

Oceans are becoming warmer due to climate change processes. Marine invertebrates live within a limited range of body temperatures, and functional constraints result at temperature extremes. Furthermore, synergistic effects between global warming and other environmental stressors could have the potential to narrow the thermal windows of species. This study assessed the interactive effects of current and predicted conditions of ocean warming and food availability on the survival, growth and development of the sea urchin *Paracentrotus lividus*. Nine two-factor treatments of temperature (19, 20.5 and 22.5 °C) and food level (2000 cel mL<sup>-1</sup>, 1000 cel mL<sup>-1</sup> and 500 cel mL<sup>-1</sup>) were tested in laboratory experiments. The temperature and food level treatments were chosen based on current oceanographic data for seawater and from values predicted for the next century in the subtropical eastern Atlantic region. Our results indicated that the food levels examined in this study do not narrow the thermal window of the species. Our results suggest that the negative effects of decreasing food availability on the development of *P. lividus* larvae will be significantly ameliorated by increasing seawater temperature.

**Keywords:** Seawater temperature, food supply, climate change, larval development, *Paracentrotus lividus*.

## Resumen

Los procesos derivados del cambio climático están haciendo que el océano tenga una temperatura cada vez más elevada. Los invertebrados marinos desarrollan sus funciones dentro de unos rangos de temperatura que, si son sobrepasados, pueden ocasionar limitaciones a nivel funcional. Además, la sinergia del aumento de la temperatura con otros factores ambientales podría hacer que se reduzca el rango de temperatura en el que un organismo puede desarrollar sus funciones con normalidad. En este trabajo hemos evaluado cómo puede afectar la interacción de dos factores ambientales (la temperatura y la disponibilidad de alimento) en la supervivencia y el desarrollo larvario del erizo de mar *Paracentrotus lividus*. Para tal fin se han llevado a cabo experimentos combinando diferentes temperaturas (19, 20.5 y 22.5 °C) y cantidades de alimento (2000 cel  $\text{mL}^{-1}$ , 1000 cel  $\text{mL}^{-1}$  y 500 cel  $\text{mL}^{-1}$ ), en 9 tratamientos que abarcan la variabilidad ambiental actual y la prevista para final de siglo. Nuestros resultados mostraron que las limitaciones en la cantidad de comida empleadas en los tratamientos al combinarse con el aumento de temperatura no tienen el potencial de reducir el rango de temperatura en el que la larva de *P. lividus* puede desarrollarse con normalidad.

*Palabras clave:* Temperatura, ración de comida, cambio climático, desarrollo larvario, *Paracentrotus lividus*.

## Introduction

Ocean health is increasingly impacted by global climate change processes. These changes, triggered by interacting environmental and anthropogenic factors, will affect the ocean in ways that we are only now beginning to understand (Turley et al. 2013). Global warming, a consequence of the emission of gases with greenhouse effects, is one of the most prominent of these climate processes. The impact of global warming on the oceans can have dramatic effects on marine systems. It is thought that seawater temperatures will rise between 2.0-4.5 °C by the end of the 21<sup>st</sup> century (IPCC 2013). The increase of seawater temperature reduces ocean uptake of carbon dioxide, due to its decreased solubility. Warmer temperatures also tend to speed up chemical and biological processes (Clarke 2003), such as oxygen consumption and carbon dioxide production, and results in a decrease in oxygen transfer to the deep ocean (Turley et al. 2013). In addition, ocean warming determines the distribution and adaptability of species and their survival can be compromised within a specific temperature range (Fields et al. 1993; Lubchenco et al. 1993; Harley et al. 2006). As a result, the redistribution of species can produce serious ecological problems in specific regions causing imbalances within local ecosystems.

Changes related to global warming will likely affect the trophic structure of ecosystems. Warmer ocean temperatures increase stratification of the surface mixed layer, which hinders the incorporation of nutrients from below that support ocean primary production (Sarmiento et al. 1998), thereby causing a significant nutrient stress for phytoplankton. Ocean phytoplankton is responsible for approximately half the global biospheric net primary production (Behrenfeld et al. 2001), and thus long-term changes in ocean primary production can potentially have important consequences for the global carbon cycle. In recent decades, this production has declined, coincident with a pattern of rising seawater temperatures (Gregg et al. 2003).

Temperature is the most important environmental factor controlling the distribution, physiology, morphology and behaviour of marine invertebrates. This factor, more than any other variable, best explains developmental rates in marine invertebrates (Hoegh-Guldberg and Pearse 1995; Byrne et al. 2009). Early developmental stages such as fertilization, embryogenesis and morphogenesis, are generally the most sensitive life phases (Pörtner and Farrell 2008; see Byrne 2011 for review). Ocean warming improves fertilization (Hagström and Hagström 1959; Mita et al. 1984; Cohen-Rengifo et al. 2013), speeds up larval growth, development and settlement, and may also impact larval swimming behavior and duration of planktonic life, up to an organism's thermal threshold (Staver and Strathmann 2002; O'Connor et al. 2007; Sheppard-Brennan et al. 2010; see Byrne and Przeslawsky 2013 for review). Although a shorter planktonic period reduces exposure to predators, it also has the potential to limit dispersal distance, thereby altering the distribution and genetic connectivity of populations and thus the dynamics of marine populations, broadly considered (López et al. 1998; O'Connor et al. 2007).

Phytoplankton abundance in the ocean is both highly seasonal and spatially heterogeneous, and is strongly related to the timing of reproductive events of many species as well as the subsequent survival of feeding larval stages (Platt et al. 2003). During larval development, organisms typically encounter unpredictable feeding environments (Conover 1968). Furthermore, it is thought that food availability will be reduced as a result of decreasing primary production resulting from climate change processes (Gregg et al. 2003; Turley et al. 2013). For these reasons, the impacts of food availability on the growth and survival of feeding larval forms have been extensively studied (Olson and Olson 1989; Fenaux et al. 1994; Meidel et al. 1999; Vickery and McClintock 2000; Moran and Manahan 2004; Meyer et al. 2007). As feeding larvae grow, changes occur in a suite of correlated larval characters, including physiological rates, larval morphology, and the

magnitude of plasticity of feeding structures in response to different food levels (Fenaux et al. 1994; Sewell et al. 2004; McAlister 2007, 2008). Food-limited growth and development could have implications for the life-history, ecology, and evolution of species. Planktonic larvae have the potential to be food-limited, experience longer transport in the seawater, and may be subject to higher rates of mortality directly due to starvation or indirectly due to prolonged periods of exposure to predation (Lamare and Barker 1999). When an organism is stressed to the edges of its ecological niche, the energy required to maintain the necessary physiological mechanisms allowing survival, development and reproduction increases. Thus, a prolonged exposure to these challenging conditions can lead to unsustainable energetic costs (Dorey et al. 2013). If food limits growth rate, then larvae may often need to clear particles from suspension at higher rates, thereby increasing metabolic costs, and which may account for strikingly different features of larval form and behavior (Fenaux et al. 1994).

Marine organisms are clearly affected directly by changes in specific environmental factors (e.g. temperature, food availability, salinity, etc.) and in an interactive manner among multiple factors. Interactions between stressors can be additive, joining the effects of both stressors in isolation (Vinebrooke et al. 2004; Carilly et al. 2009; see Byrne and Przeslawsky 2013 for review); or antagonistic, where the combined effect is less than the additive expectation (Carilly et al. 2009; see Byrne and Przeslawsky 2013 for review). Organisms are constantly exposed to a range of abiotic and biotic stressors either unassociated, or indirectly associated, with global change. Stressors associated with warming are the most direct and pervasive of global change stressors for marine biota, but effects vary among regions, habitats, species, and life-history stages (Byrne and Przeslawsky 2013).

To examine the interactive effects of variation in multiple environmental variables on the development and growth of larvae, we examined the combined effects of food level and temperature using larvae of the sea urchin *Paracentrotus lividus*. *P. lividus* is widely distributed throughout the Mediterranean Sea and the NE Atlantic Ocean from Ireland to the Canary Islands. In the Canarian Archipelago, the species is found from the lowest intertidal, where it most commonly occupies crevices in tide-pools, to around 10 m depth in the subtidal and exceptionally down to about 20 m depth (Girard et al. 2012). In this region, the echinoid has the ability to extend its period of sexual maturity (late winter and late summer) and exhibits multiple spawning episodes during the year, likely an adaptation to the warmer seawater temperatures at its southernmost limit of geographical distribution. The planktonic larvae stage is estimated to last roughly one month and settlement occurs in late winter and early spring, when high phytoplankton abundance is found in the water column (Girard et al. 2008). Specifically, we aimed to explore, by means of manipulative laboratory experiments, the combined effects of ocean warming and different conditions of food availability on the larval development of *P. lividus*. We tested treatment conditions that were representative of both current and future levels of these environmental factors that are predicted to result from climate change processes.

## Material and methods

### *Animal collection and spawning*

Adult *Paracentrotus lividus* specimens (test diameter > 24mm) were collected by scuba divers from subtidal rocky shores between 5 and 10 m depth. Individuals were collected in March of 2011 off the north-east coast of Tenerife Island (Canary Islands; 28°24'N, 16°18'W), during the spawning season of the species (Girard et al. 2012).

Animals were induced to spawn by injection of 2 ml of KCl (0.5 M) through the peristomial membrane. Five males and seven females, randomly selected in order to reduce experimental bias (Evan and Marshall 2005), were used and their respective gametes were mixed prior to fertilization. Sperm was collected dry and kept on ice until usage. Eggs were collected in filtered seawater (FSW). Fertilization was done in a proportion of 1:2400 (eggs:sperm). Cleavaging embryos (two cell stage) were placed at a density of 15 individuals  $\text{mL}^{-1}$  in 20 L aquaria filled with FSW and constantly aerated.

#### *Experimental design and sea water chemistry*

When the embryos reached the gastrula stage, larvae were distributed into 2 L culture beakers at densities of 2 larvae  $\text{mL}^{-1}$ . Forty-five culture beakers were maintained in three seawater tables, which served to regulate temperature treatments. Seawater was replaced in each beaker twice per week. At day 4 post-fertilization, larvae were fed with the unicellular red alga, *Rhodomonas lens*. The microalgae strain was provided by ‘Spanish Oceanography Institute’ and cultured in the laboratory with enriched F/2 medium (Guillard and Ryther 1962) at 20°C and a 24h/0h light/dark cycle, with constantly aerated seawater. Algae were separated from the growth medium by centrifugation and then re-suspended in fresh FSW before use.

Larvae of *P. lividus* were raised in nine different replicated two-factor treatments of temperature and food ( $n=5$  for each treatment). Cultures were maintained at a salinity of 36.94‰ ( $\pm 0.38$ ), corresponding to the natural seawater conditions in March at the collection site. Temperature and food availability levels were chosen to cover the present and future natural variability at the sampling region. Therefore, the experiment included three treatments of temperature: 19 °C (control: SST in Spring in the Canary Islands), 20.5 °C (present extreme natural variability in Spring and predicted mean SST for the year 2050, IPCC 2007) and 22.5 °C (predicted SST for the year 2100, IPCC 2007). In each

treatment of temperature, three different treatments of food availability were carried out: T1: 2000 algal cells mL<sup>-1</sup>, corresponding to current average values in the seawater off the studied region (Baltar et al. 2009); T2: 1000 cells mL<sup>-1</sup> and T3: 500 cells mL<sup>-1</sup>, in order to simulate future conditions of a reduced primary production as predicted due to climate change effects (Gregg et al. 2003).

To keep constant temperature conditions in the experiments, thermostat coolers and heaters (EHEIM AQUATICS, 50 W) were used. Monitoring of temperature and salinity (handheld conductivity meter COND 315i) was performed daily. Experiments were conducted with FSW purified within a recirculating system provided with DRYDEN AQUA active filter media (AFM) bio-crystals; 50 µm and 10 µm UNICEL polyamide paper filters; and a UV-C AQUAEL 11W filter. The seawater was prepared with the proper temperature for each treatment before usage and the appropriate amount of food for each treatment was added every day.

#### *Biological measurements*

Larvae were sampled daily for a period of approximately one month in order to quantify survival, growth and larval development. Survival was estimated as the number of living larvae in each combined treatment at the end of the experiment. To account for larval growth and development, several larvae in each replicate beaker for each combination treatment of temperature and food were photographed using a digital camera mounted on a trinocular microscope. Morphological parameters were measured on each larva: body length (BL), post-oral arm length (PL) and two perpendicularly-oriented stomach diameters (S1 & S2) (Fig. 3.1). Stomach volume (SV) was then calculated as  $SV = \frac{4}{3} \pi ((S1+S2)/2)^3$  (Dorey et al. 2013).

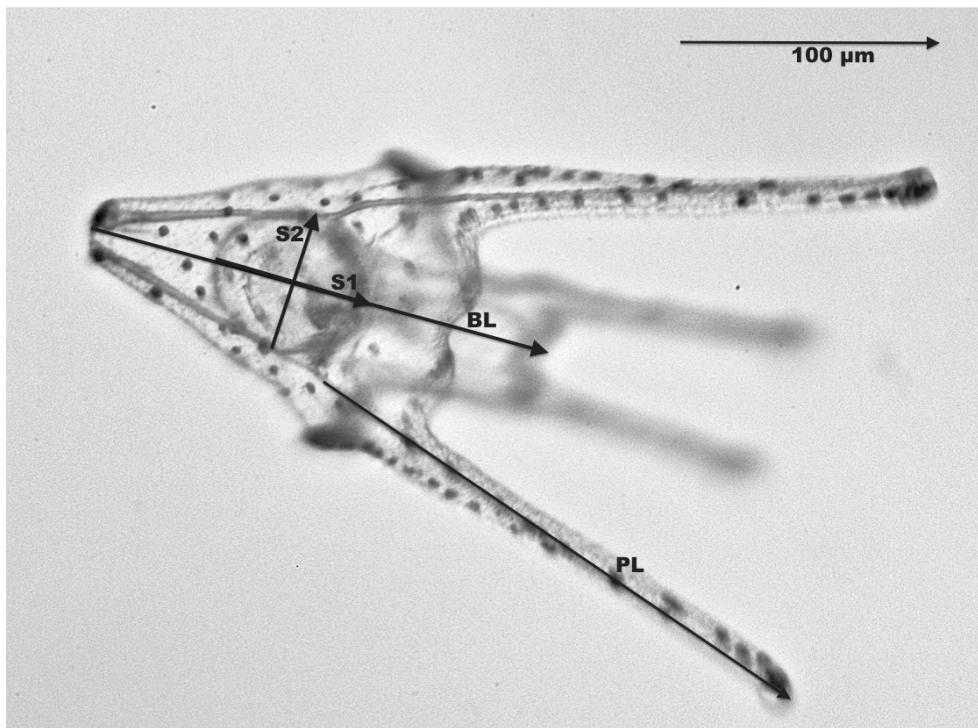


Figure 3.1 Morphometric measurements taken for each *Paracentrotus lividus* larvae: Body length (BL), Post oral arm length (PL) and stomach diameters (S1 & S2)

#### *Data analyses*

In order to assess the interactive effects of seawater temperature and food level on larval survival, data were analysed by means of a two-way permutational analysis of variance (PERANOVA) (Anderson 2001) with temperature (3 levels) and food (3 levels) as fixed factors.

To evaluate the interactive effects of temperature and food level on larval growth (as measured by BL and PL), three-way permutational multivariate analysis of variance (PERMANOVA) was performed. A three-way design was carried out with temperature (3 levels), food (3 levels) and time (7 levels) used as fixed factors. The same analysis and design, but with 6 levels of time, was used to analyse the interactive effects of temperature and food availability on stomach volume (SV).

In all analyses of variance, we used Euclidean distances of raw data and 4999 permutations of the appropriate exchangeable units (Anderson 2004). Significant terms in the full models were examined using *a posteriori* pairwise comparisons by permutations (Anderson 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws from the asymptotic permutation distribution. Principal Coordinates Analysis ordinations (PCO) of morphometric data were used to identify similarities between observations. All statistical analyses were carried out using PRIMER 6 & PERMANOVA+ v. 1.0.1 software.

## Results

Physico-chemical parameters of seawater during the larval experiments are given in table 3.1. Target temperature levels were achieved in each replicated treatment. A significant interaction of factors ‘Temperature x Food’ was found when analysing larval survival (Table 3.2), meaning that larvae exhibited different responses at each combination of temperature and food. An *a posteriori* pairwise test revealed that at 19 °C there was significantly higher survival at the control food treatment of 2000 cells mL<sup>-1</sup> (T1) than at the decreased levels of food availability. At 22.5 °C, only increased survival at food level T1 compared to T2 (1000 cells mL<sup>-1</sup>) was detected. No significant differences were detected at a seawater temperature of 20.5 °C (Table 3.3; Fig. 3.2). Comparison within temperatures revealed significantly higher larval survival between 19°C and the other temperature treatments, albeit only at control food conditions of 2000 cells mL<sup>-1</sup> (T1). No significant differences in survival were found in the other reduced food treatments T2 and T3 (500 cells mL<sup>-1</sup>) (Table 3.3; Fig. 3.2).

Table 3.1 Physico-chemical seawater parameters for each experimental treatment tested (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>). T: seawater temperature (mean ± SD), S: salinity (mean ± SD)

	19°C			20.5°C			22.5°C		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
T <sub>(n=28)</sub>	18.99±0.30	18.97±0.38	18.80±0.11	20.25±0.29	20.74±0.15	20.61±0.13	22.19±0.13	22.38±0.31	22.43±0.38
S <sub>(n=28)</sub>	36.67±0.40	37.24±0.41	37.02±0.45	36.82±0.38	36.93±0.24	36.86±0.35	37.00±0.33	36.90±0.38	37.01±0.29

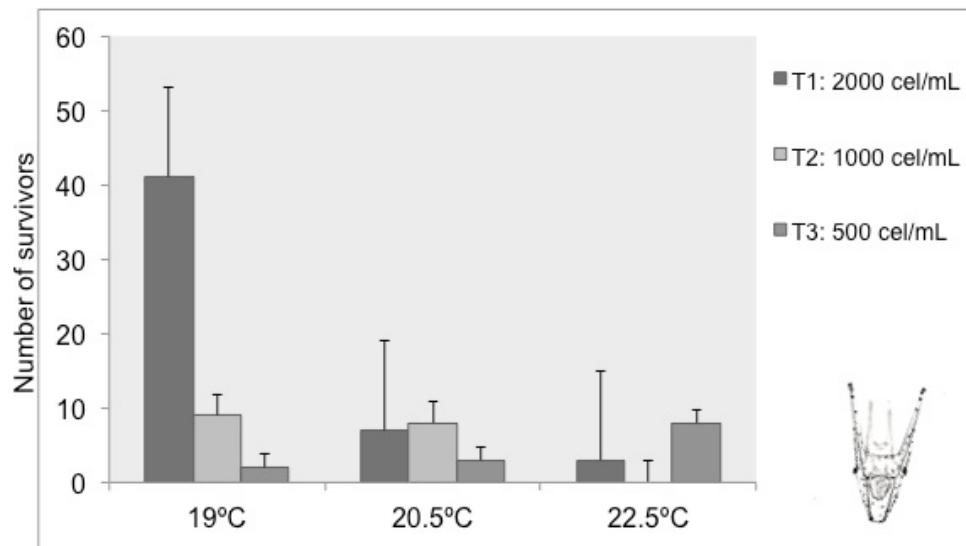


Figure 3.2 Number of survivors in each combined treatment of temperature (19, 20.5 and 22.5°C) and food availability conditions (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>) at the end of the combined experiment

Table 3.2 Results of the two-way ANOVA analyzing larval survival of *Paracentrotus lividus*. The factors included in the model are: T: temperature and Food availability

Source	df	SS	MS	Pseudo-F	p (perm)
T	2	64.13	32.07	3.49	0.023
Food	2	58.13	29.07	3.16	0.034
TxFood	4	124.13	31.03	3.38	0.011

Results of the PERMANOVA analysing the morphometric measurements showed a significant three-way interaction of factors ‘Temperature x Food x Time’ (Table 3.4A). These results indicate that the effects of temperature and food availability on body and post

oral arm length varied significantly across time during the larval period. Pairwise tests showed a significant trend toward shorter BL and PL with decreasing food availability at 19 °C (Table 3.5A, 7; Fig 3.3), particularly during the last days of larval development. On the contrary, a significant trend toward larger morphometric measurements was detected with increasing temperature at each food treatment (Table 3.5B, 7; Fig. 3.3).

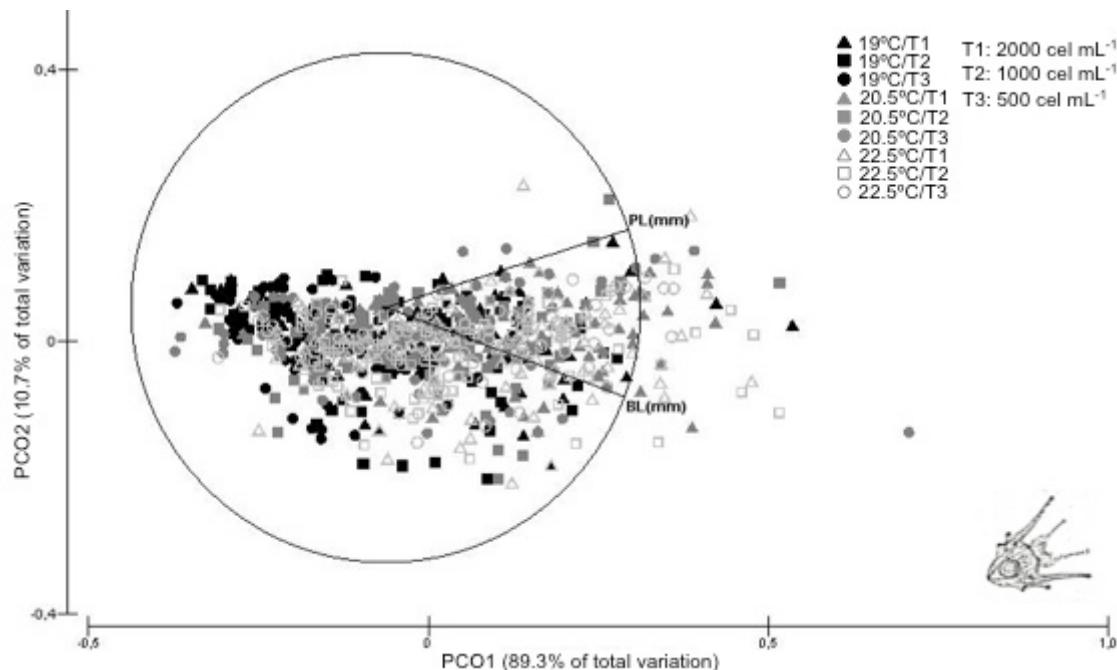


Figure 3.3 PCO ordinations showing the combined effect of seawater temperature and food availability conditions on *Paracentrotus lividus* larvae development. Vectors represent studied variables. BL: Body Length, PL: Post oral arm Length. Percentages of variation explained by each of the axes are given in brackets

Table 3.3 Results of pair-wise tests examining the significant interaction of factors ‘Temperature x Food’ obtained in the ANOVA on larval survival of the sea urchin *Paracentrotus lividus* in laboratory experiments. Combined effects of temperature (19, 20.5 and 22.5°C) for pairs of levels of factor Food (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>), and effects of Food treatments for pairs of levels of factor temperature are shown

	19°C		20.5°C		22.5°C	
Comparisons	t	p (perm)	t	p (perm)	T	p (perm)
T1 vs T2	1.87	0.062	0.14072	0.875	2.45	0.037
T1 vs T3	2.41	0.009	0.75593	0.460	0.62	0.534
T2 vs T3	1.23	0.257	0.90536	0.415	1.00	0.337
	T1		T2		T3	
Comparisons	t	p (perm)	t	p (perm)	T	p (perm)
19 vs 20.5	2.01	0.056	0.13	0.896	0.43	0.675
19 vs 22.5	2.34	0.011	1.62	0.149	0.74	0.477
20.5 vs 22.5	0.79	0.439	1.55	0.150	0.61	0.585

The analysis of SV showed a significant interaction of factors ‘Temperature x Food x Time’ (Table 3.4B). These results indicate that the influence of temperature and food availability on stomach volume varied significantly across time during the larval period. Pairwise analyses detected a trend toward larger SV with decreasing food at 20.5 and 22.5 °C, but not at 19 °C where no consistent differences between food treatments were found (Table 3.6A, 7; Fig. 3.4). Also a significant trend toward greater SV with increasing temperatures at each treatment of food was detected (Table 3.6B, 3.7; Fig. 3.4).

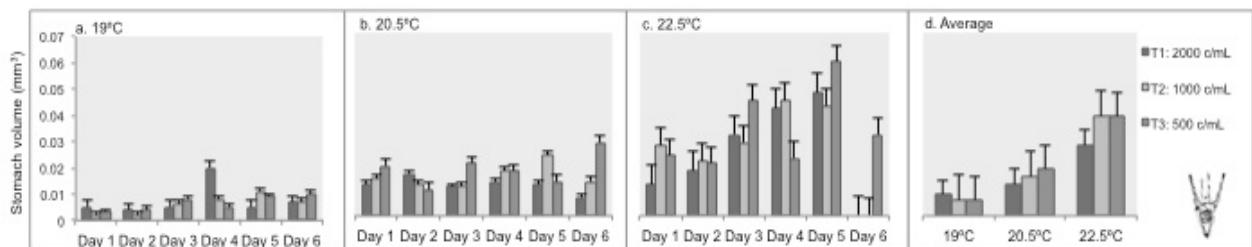


Figure 3.4 Stomach volume ( $\text{mm}^3$ ) of larvae (mean  $\pm$  SD) of the sea urchin *Paracentrotus lividus* at combined experiment testing the combined effects of seawater temperature (a. 19°C; b. 20.5°C; c. 22.5°C) and food availability conditions (T1: 2000 cel  $\text{mL}^{-1}$ ; T2: 1000 cel  $\text{mL}^{-1}$ ; T3: 500 cel  $\text{mL}^{-1}$ ). d. Overall mean values for the experiment are given

Table 3.4 Results of (A) the three-way PERMANOVA testing larvae development analysing morphometric variables (body length and post oral arm length), and of (B) the three-way ANOVA assessing stomach volume (SV) of the sea urchin *Paracentrotus lividus*. Factors included are: T: temperature, Food availability, and Ti: Time

A. Morphometric measurements					
Source	df	SS	MS	Peuso-F	p (perm)
T	2	1.76	0.88	57.78	0.001
Food	2	0.52	0.26	17.09	0.001
Ti	6	10.69	1.78	116.78	0.001
TxFood	4	0.32	8.02E-2	5.26	0.001
TxTi	12	0.50	4.16E-2	2.73	0.001
FoodxTi	12	0.37	3.10E-2	2.03	0.016
TxFoodxTi	24	0.95	3.97E-2	2.60	0.001
B. Stomach volume					
T	2	4.32	2.16	89.58	0.001
Food	2	0.17	8.45E-2	3.50	0.014
Ti	5	0.95	0.19	7.92	0.001
TxFood	4	0.18	4.60E-2	1.91	0.070
TxTi	10	1.73	0.17	7.16	0.001
FoodxTi	10	0.54	5.38E-2	2.23	0.003
TxFoodxTi	20	0.87	4.35E-2	1.80	0.004

Table 3.5 Results of pair-wise tests examining the significant interaction of factors ‘Temperature x Food x time’ obtained in the PERMANOVA on larvae morphology of the sea urchin *Paracentrotus lividus* in laboratory experiments: (A) Combined effects of temperature (19, 20.5 and 22.5°C) and time (Ti) for pairs of levels of factor Food (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>) on larvae development are shown. (B) Combined effects of Food (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>) and time (Ti) for pairs of levels of factor temperature (19, 20.5 and 22.5°C) on larvae development are shown

A. Effects of temperature							
		19°C		20.5°C		22.5°C	
Ti	Comparisons	t	p (perm)	t	p (perm)	t	p (perm)
Day 1	T1 vs T2	1.62	0.100	0.14	0.975	1.00	0.350
	T1 vs T3	0.89	0.392	0.77	0.493	0.89	0.418
	T2 vs T3	1.06	0.318	0.98	0.317	0.30	0.874
Day 2	T1 vs T2	0.58	0.701	1.52	0.110	0.54	0.716
	T1 vs T3	0.59	0.679	1.26	0.189	1.06	0.318
	T2 vs T3	0.54	0.740	0.22	0.922	1.72	0.056
Day 3	T1 vs T2	2.20	0.034	0.20	0.956	3.08	0.006
	T1 vs T3	3.13	0.003	0.94	0.392	1.67	0.083
	T2 vs T3	0.99	0.375	0.94	0.430	1.48	0.139
Day 4	T1 vs T2	0.77	0.537	1.72	0.096	2.88	0.006
	T1 vs T3	1.01	0.338	1.64	0.120	1.23	0.228
	T2 vs T3	0.43	0.823	0.42	0.776	2.49	0.018
Day 5	T1 vs T2	0.54	0.696	1.73	0.075	1.95	0.043
	T1 vs T3	3.79	0.001	2.42	0.009	0.94	0.374
	T2 vs T3	4.14	0.001	0.49	0.718	1.39	0.165
Day 6	T1 vs T2	1.66	0.117	0.75	0.455	0.30	0.886
	T1 vs T3	2.77	0.012	1.04	0.302	1.59	0.111
	T2 vs T3	1.47	0.169	0.34	0.841	1.40	0.162
Day 7	T1 vs T2	2.32	0.016	1.16	0.236	1.29	0.215
	T1 vs T3	4.67	0.001	9.19E-2	0.985	1.66	0.075
	T2 vs T3	3.47	0.001	0.97	0.384	1.62	0.104
B. Effects of food							
		T1		T2		T3	
Ti	Comparisons	T	p (perm)	T	p (perm)	t	p (perm)
Day 1	19 vs 20.5	1.52	0.129	4.50	0.001	1.89	0.055
	19 vs 22.5	2.33	0.023	3.52	0.002	2.99	0.006
	20.5 vs 22.5	0.57	0.639	0.52	0.716	0.78	0.489
Day 2	19 vs 20.5	4.40	0.001	3.16	0.002	1.56	0.115
	19 vs 22.5	3.77	0.001	4.12	0.001	5.79	0.001
	20.5 vs 22.5	0.78	0.508	1.05	0.331	2.11	0.035
Day 3	19 vs 20.5	2.02	0.029	4.38	0.001	5.66	0.001
	19 vs 22.5	5.08	0.001	4.27	0.001	7.43	0.001
	20.5 vs 22.5	2.97	0.003	0.42	0.805	2.17	0.030
Day 4	19 vs 20.5	3.48	0.004	2.07	0.030	2.03	0.037

	19 vs 22.5	4.32	0.001	0.82	0.448	3.39	0.001
Day 5	20.5 vs 22.5	1.15	0.261	1.45	0.137	1.84	0.069
	19 vs 20.5	1.18	0.240	0.19	0.946	4.43	0.001
	19 vs 22.5	2.23	0.023	1.25	0.222	6.78	0.001
	20.5 vs 22.5	1.46	0.133	1.15	0.275	3.43	0.001
Day 6	19 vs 20.5	0.83	0.411	1.29	0.222	2.25	0.029
	19 vs 22.5	1.08	0.324	0.77	0.491	3.42	0.004
	20.5 vs 22.5	1.61	0.142	0.70	0.546	1.20	0.205
Day 7	19 vs 20.5	0.80	0.480	0.28	0.909	3.55	0.004
	19 vs 22.5	2.31	0.019	1.18	0.243	2.84	0.004
	20.5 vs 22.5	1.66	0.100	0.82	0.422	1.78	0.074

Table 3.6 Results of pair-wise tests examining the significant interaction of factors ‘Temperature x Food x time’ obtained in the ANOVA on stomach volume of the sea urchin *Paracentrotus lividus* in laboratory experiments: (A) Combined effects of temperature (19, 20.5 and 22.5°C ) and time (Ti) for pairs of levels of factor Food (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>) on Stomach Volume are shown. (B) Combined effects of Food (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>) and time (Ti) for pairs of levels of factor temperature (19, 20.5 and 22.5°C) on Stomach Volume are shown

A. Effects of temperature							
		19°C		20.5°C		22.5°C	
Ti	Comparisons	t	p (perm)	t	p (perm)	T	p (perm)
Day 1	T1 vs T2	1.75	0.070	1.55	0.092	2.55	0.011
	T1 vs T3	1.21	0.235	2.97	0.003	0.78	0.478
	T2 vs T3	0.55	0.777	4.45	0.001	2.14	0.028
Day 2	T1 vs T2	1.23	0.222	1.92	0.050	0.25	0.930
	T1 vs T3	0.31	0.909	2.46	0.010	0.22	0.939
	T2 vs T3	0.78	0.506	0.77	0.465	0.31	0.873
Day 3	T1 vs T2	0.56	0.701	1.31	0.217	0.15	0.984
	T1 vs T3	0.77	0.538	2.30	0.028	0.77	0.533
	T2 vs T3	0.40	0.862	1.61	0.108	0.88	0.451
Day 4	T1 vs T2	2.15	0.023	1.75	0.059	2.01	0.037
	T1 vs T3	2.93	0.007	0.75	0.546	1.28	0.184
	T2 vs T3	1.27	0.186	1.13	0.253	1.27	0.199
Day 5	T1 vs T2	3.12	0.004	1.51	0.122	1.43	0.138
	T1 vs T3	2.07	0.039	1.99	0.044	0.39	0.854
	T2 vs T3	0.30	0.929	1.01	0.350	1.01	0.304
Day 6	T1 vs T2	0.64	0.642	0.42	0.837	2.41	0.007
	T1 vs T3	0.77	0.556	2.82	0.005	1.85	0.036
	T2 vs T3	0.80	0.516	1.93	0.049	0.87	0.508

B. Effects of food							
		T1		T2		T3	
Ti	Comparisons	t	p (perm)	T	p (perm)	T	p (perm)
Day 1	19 vs 20.5	4.71	0.001	5.99	0.001	2.80	0.008
	19 vs 22.5	4.12	0.001	6.69	0.001	2.79	0.005
	20.5 vs 22.5	0.79	0.476	2.53	0.004	2.08	0.039
Day 2	19 vs 20.5	3.51	0.001	4.73	0.001	3.95	0.001
	19 vs 22.5	3.78	0.001	4.13	0.001	3.48	0.003
	20.5 vs 22.5	2.23	0.024	0.95	0.351	0.96	0.352
Day 3	19 vs 20.5	3.54	0.002	4.80	0.001	3.75	0.001
	19 vs 22.5	4.30	0.002	4.01	0.001	3.71	0.001
	20.5 vs 22.5	2.08	0.039	1.51	0.136	1.00	0.342
Day 4	19 vs 20.5	0.88	0.426	4.85	0.001	4.04	0.001
	19 vs 22.5	1.37	0.171	4.71	0.001	4.11	0.001
	20.5 vs 22.5	1.20	0.223	1.69	0.079	1.07	0.304
Day 5	19 vs 20.5	1.00	0.367	0.80	0.518	0.21	0.983
	19 vs 22.5	3.02	0.005	2.84	0.001	1.58	0.124
	20.5 vs 22.5	3.22	0.003	1.47	0.123	1.94	0.041
Day 6	19 vs 20.5	1.01	0.364	0.92	0.386	2.60	0.014
	19 vs 22.5	1.79	0.059	4.82	0.001	3.57	0.001
	20.5 vs 22.5	2.46	0.006	3.44	0.001	3.24	0.004

Table 3.7 Mean values of body length (BL, mm), post oral arm length (PL, mm) and stomach volumen (SV, mm<sup>3</sup>) (mean±SD) of *Paracentrotus lividus* larvae for each combined treatment of temperature (19, 20.5 and 22.5°C) and food availability conditions (T1: 2000 cel mL<sup>-1</sup>; T2: 1000 cel mL<sup>-1</sup>; T3: 500 cel mL<sup>-1</sup>)

19°C			20.5°C			22.5°C			
	T1	T2	T1	T2	T3	T1	T2	T3	
BL	0.392±0.14	0.367±0.13	0.318±0.09	0.449±0.12	0.415±0.11	0.407±0.12	0.470±0.12	0.441±0.13	0.439±0.10
PL	0.335±0.13	0.302±0.10	0.257±0.08	0.400±0.12	0.357±0.13	0.366±0.13	0.391±0.14	0.356±0.13	0.388±0.13
SV	0.008±0.010	0.006±0.004	0.006±0.006	0.012±0.008	0.015±0.014	0.018±0.016	0.027±0.028	0.038±0.035	0.038±0.045

## Discussion

Our results suggest that *Paracentrotus lividus* larvae are widely tolerant to current levels of environmental variation in seawater temperature and food availability. However, *P. lividus* larvae appear to have greater sensitivity (detrimental effects on larval development) to near-future predicted levels for both of these parameters. The combined effects of the climate-change related environmental factors we examined were shown to narrow the thermal window of the species, thereby affecting larval survival and development. These detrimental effects may have striking consequences for the future performance of a key herbivore species (Pörtner and Farrell 2008) and thus for the stability of marine ecosystems.

Larval survival was highest at the species' optimal temperature (19° C) and within the highest food supply (2000 cells mL<sup>-1</sup>). Larval survival was reduced at the optimal raising temperature with lower levels of food. Fewer survivors were detected in seawater conditions representative of warming (20.5 and 22.5°C), with respect to the control temperatures, with non-significant effects of food availability treatments in these extreme conditions. Previous studies have reported the robustness of *P. lividus*, in terms of larval survival, against environmental factors such as ocean warming and acidification, in both cases up to a critical threshold (Martin et al. 2011; Privitera et al. 2011; García et al. in review). This sea urchin species has a wide latitudinal range, can be found in intertidal to subtidal environments, and thus has the capacity to cope with high environmental variability

(Moulin et al. 2011). Therefore, the species exhibits strategies for inhabiting coastal areas where stress and disturbance are frequent and its thermal tolerance window is broad, which suggests considerable plasticity for a number of different phenotypes (Catarino et al. 2012).

The fact that higher temperatures facilitate invertebrate larval growth and development up to a thermotolerance threshold is well known in the literature (Hoegh-Guldberg and Pearse 1995; see Byrne 2011 for review). We found a significant trend toward larger sizes for each of the morphometric variables (BL, PL and SV), with increasing seawater temperature at each treatment of food supply. Regarding food availability, it is known that changes in food supply would result in shifts in allocation and timing between ephemeral larval structures (paired arms) and structures that are retained in the post-metamorphic juvenile (echinus rudiment and stomach) (Strathmann et al. 1992). Within this study we observed a significant trend toward shorter larval BL and PL with decreasing food availability at the optimal temperature for the species (19°C).

Of note, our results suggest that warmer ocean temperatures, as predicted for future climate change scenarios, may compensate for lower food availability. Thus, the positive effects that result from more rapid development may erase the negative effects of low food, in part. Similar response trade-offs between increasing ocean warming and other environmental factors, i.e. ocean acidification, have been reported for other echinoid species (Sheppard-Brennand et al. 2010; Byrne et al. 2013). Likewise, in a previous study with *P. lividus* we found that a slight ocean warming (20.5°C) mitigated the negative effects of ocean acidification on larval growth and development, but enhanced the sensitivity at more extreme high temperature regimes (22.5°) (García et al. in review). However we did not observe the same pattern with food availability conditions in the present study.

Synergistic stressors may have the potential to narrow the thermal windows of species (Pörtner and Farrell 2008). Our results show however, that the food levels we tested do not have the potential to narrow the thermal window of the species close from its threshold, in terms of larvae development. If the baseline metabolism is far from its optimal, the organism is not energy limited and an increase in metabolism can lead to a positive response. In contrast, if the baseline metabolism is closer to its optimal, any increase in metabolism will lead to a negative response and under extreme chronic metabolic stress, the effect could even be lethal (Pörtner and Farrell 2008). In this sense, our results suggest that in the most extreme conditions of seawater temperature and food availability tested ( $22.5^{\circ}\text{C}/\text{500 cel mL}^{-1}$ ) the larvae is far from its critical threshold and the thermal window in this case is wider than in the case of other environmental factors such as ocean acidification (García et al. in review).

Some studies have shown that larvae with a consistent lack of food supply develop longer arms to increase the possibility of collecting food particles (Fenaux et al. 1994; McAlister 2008). The shift in allocation of resources from the stomach and echinus rudiment to the arms and ciliated band when food is scarce could therefore increase the larval capacity to successfully catch food and increase its growth rate (Strathmann et al. 1992; Miner 2005). In contrast to these findings, we did not detect evidence for an increase in size of post oral arm length of larvae exposed to food shortage. Plasticity of arm length may be an evolutionary strategy that results in greater food gathering capability for larvae inhabiting temperate habitats (McAlister 2008, Soars et al. 2009). In fact this pattern in pluteus larvae has been demonstrated primarily in temperate cold-water species (Boidron-Metairon 1988; Hart and Scheibling 1988; Sewell et al. 2004). Usually, this trend is not observed in ecosystems with a high variability of environmental factors, more typical in mean latitudes. Although there are certainly exceptions to the observation (e.g., Fenaux et

al. 1994), the general pattern suggests that there may be a latitudinal gradient in phenotypic plasticity of larval feeding structures (McAlister 2008). On the contrary, larvae living in environments with constantly low food availability conditions, which is the case of the oligotrophic waters off the Canary Islands, may express a constant long arm length phenotype likely increasing the food gathering capability of a given larva (McAlister 2008).

We hypothesize that in conditions of scarce food availability and with rising seawater temperatures, larvae could shift allocation of energetic reserves toward increasing stomach volume in order to maximize food digestion capacity and maintain its rate of growth (Strathmann et al. 1992; Miner 2005). In this sense, we detected a trend toward larger SV with the gradual shortage of food at 20.5 and 22.5 °C, but this pattern was not consistent at 19 °C. This result could be a consequence of a shift in the energy budget (uncompensated increased energy costs) as has been hypothesized by Stumpp et al. (2011).

In conclusion, with this study we have shown that increasing seawater temperatures, in ranges expected to occur over the next century, ameliorates the negative effects of decreasing food availability on *P. lividus* larval development. The sea urchin larvae seemed capable of shifting their energy budget to successfully grow and develop under the stressful conditions presented by the combined effects of environmental factors. While our study sheds light on the interactive effects of environmental stressors, experiments assessing novel and untested multiple stressors are needed to further evaluate organismal response. Understanding how multiple stressors interactively control thermal windows will provide useful information for making predictions about the adaptability of species to future climate-change conditions.

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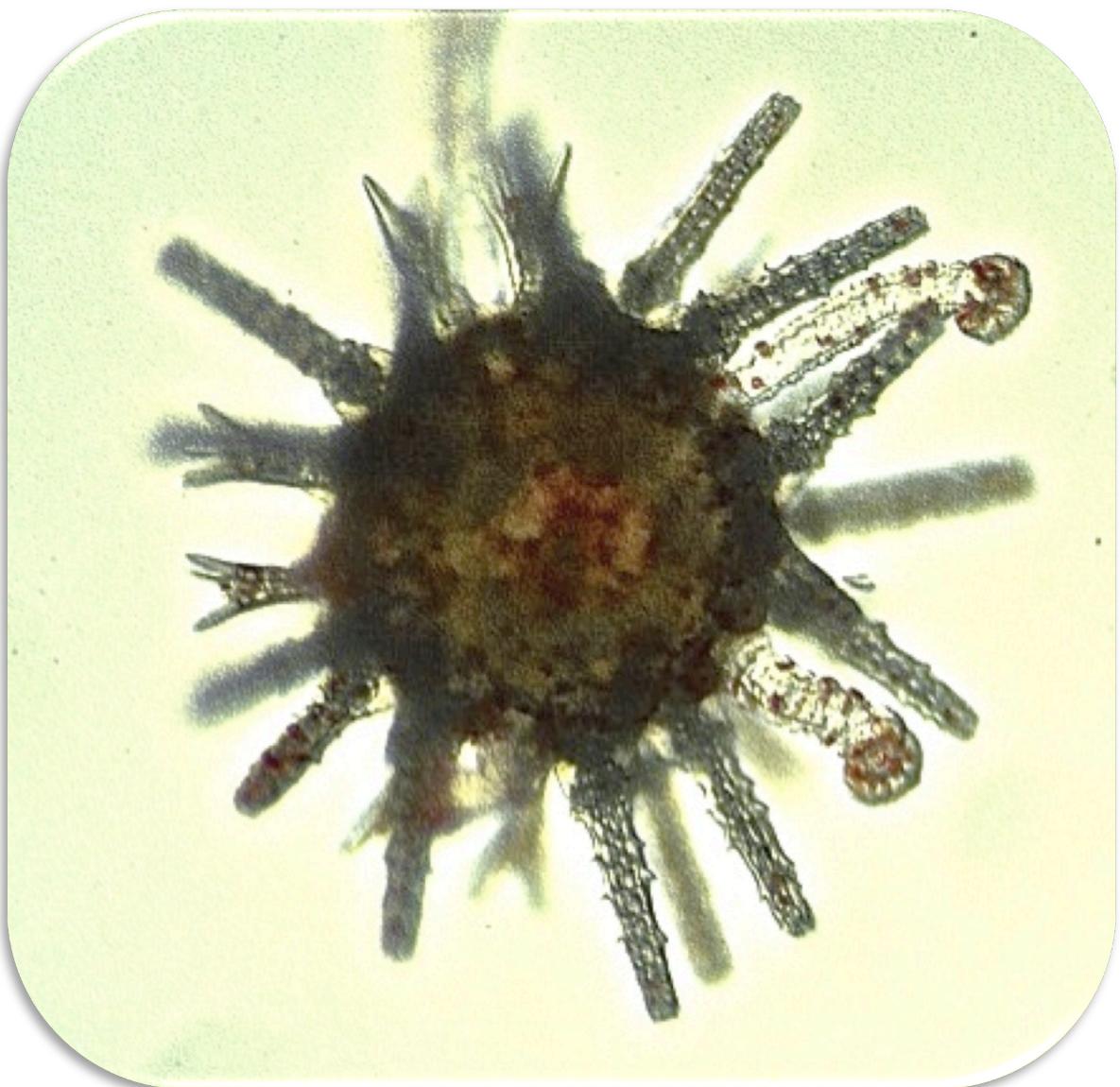
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**CHAPTER 4: RESPONSE OF  
PARACENTROTUS LIVIDUS LARVAL  
DEVELOPMENT AND POSTLARVAL  
SETTLEMENT TO PH LEVELS PREDICTED  
FOR THE TURN OF THE CENTURY**



## **CHAPTER 4: RESPONSE OF PARACENTROTUS LIVIDUS LARVAL DEVELOPMENT AND POSTLARVAL SETTLEMENT TO PH LEVELS PREDICTED FOR THE TURN OF THE CENTURY**

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## Abstract

Ocean acidification (OA) is causing changes to the physics, chemistry and biology of the marine environment, in ways that we are only just beginning to understand. Growing evidences demonstrates that ocean acidification can influence the survival, growth, development and physiology of marine invertebrates. Here, we assessed the impact of ocean acidification on the sea urchin *Paracentrotus lividus* larval development (35 days) and settlement. Samples were collected from the Canary Islands (28°24'N, 16°18'W) in March 2012. Three pH treatments were tested: (i) pH 8.1, the present average pH. (ii) pH 7.7, the average predicated for the year 2100, but already experienced in the natural environment during extremes of variability. (iii) pH 7.4, extremes of natural variability by 2100. The mortality rate was significantly increased by 40% at the lowest pH. Larval development speed increased by 10% at pH 7.7 (significantly faster growth rates but larval morphology maintained at a given size). Settlement was delayed by 8 days at pH 7.7 compared to pH 8.1, and no settlement was observed at pH 7.4. Overall, only sub-lethal effects were observed in larvae exposed to pH 7.7, while pH 7.4 induced both lethal and sub-lethal effects. Our results support the hypothesis that *P. lividus* is robust to survive in an environment with the present natural variation. However, the species is sensitive to more extreme levels of pH that are predicted within the next 90 years.

*Keywords:* pH, climate change, larvae development, settlement, *Paracentrotus lividus*.

## Resumen

La acidificación oceánica (AO) está ocasionando cambios en la física, química y biología de los ecosistemas marinos que apenas empezamos a comprender. Existen cada vez más estudios que demuestran que la acidificación puede influir en la supervivencia, crecimiento, desarrollo y fisiología de los invertebrados marinos. En este trabajo, hemos evaluado cuál es el impacto de la acidificación oceánica en el desarrollo larvario (35 días) y el asentamiento del erizo de mar *Paracentrotus lividus*. Las muestras fueron recolectadas en las islas Canarias ( $28^{\circ}24'N$ ,  $16^{\circ}18'W$ ) en marzo de 2012. Los cultivos fueron sometidos a tres tratamientos de pH, cubriendo la actual variabilidad natural y la esperada para final de siglo como consecuencia del cambio climático: (i) pH 8'1, (ii) pH 7'7 y (iii) pH 7'4. La mortalidad aumentó significativamente en el pH más bajo (40%). El desarrollo se incrementó en un 10% en los cultivos sometidos a 7'7 unidades de pH. El asentamiento se retrasó 8 días con respecto al tratamiento control (8'1), mientras que no hubo asentamiento a 7'4 unidades de pH. En general, podemos decir que se observaron efectos subletales en los cultivos sometidos a 7'7 unidades de pH, mientras que encontramos tanto efectos subletales como letales en los cultivos sometidos a 7'4 unidades. Nuestros resultados apoyan la hipótesis de que *P.lividus* es resistente y se puede desarrollar con normalidad en ambientes sometidos a la variabilidad ambiental actual. Sin embargo, la especie es sensible a los valores más extremos de pH previstos para final de siglo.

*Palabras clave:* pH, cambio climático, desarrollo larvario, asentamiento, *Paracentrotus lividus*.

## Introduction

The Earth's oceans are becoming more acidic, as a consequence of the rise in atmospheric carbon dioxide ( $\text{CO}_2$ ). The atmospheric partial pressure of  $\text{CO}_2$  ( $\text{pCO}_2$ ) has increased from 267 ppm, since the beginning of the industrial revolution, to 390 ppm in 2011 (IPCC 2013), leading to changes in climate processes and ecosystem functioning. The increase in atmospheric  $\text{CO}_2$ , mainly due to anthropogenic emissions, has led to modification of the seawater carbonate system, causing a decrease in ocean pH levels, a phenomenon known as ocean acidification (OA). When  $\text{CO}_2$  dissolves in seawater, it forms carbonic acid ( $\text{H}_2\text{CO}_3$ ), which dissociates into hydrogen ions ( $\text{H}^+$ ), bicarbonate ions ( $\text{HCO}_3^{-1}$ ) and carbonate ( $\text{CO}_3^{-2}$ ). The rise in  $\text{H}^+$  reduces the pH, making the ocean more acidic. Mean surface ocean pH has decreased by approximately 0.1 units since pre-industrial times, and a further decrease of approximately 0.4 units is predicted before the end of the 21<sup>st</sup> century (Gattuso and Hansson 2011). OA is expected to have profound impacts on marine ecosystems and many organisms suffer from the direct effects (Dupont and Pörtner 2013; Wittmann and Pörtner 2013).

Invertebrates are generally most sensitive to environmental stresses during their early development stages (Pörtner and Farrell 2008; Melzner et al. 2009; Dupont et al. 2010). A growing amount of research demonstrates that OA can impact survival, growth, development and settlement of marine invertebrates. Low seawater pH affects somatic and reproductive growth of many species (Dupont et al. 2008; Clark et al. 2009; Sheppard Brennand et al. 2010; Martin et al. 2011; Stumpp et al. 2011; Byrne et al. 2013; Dorey et al. 2013). However, responses to OA seem to be highly species-specific, even in closely related taxa, with the effects on fitness-related parameters ranging from negative to positive (Wittmann and Pörtner 2013). With respect to survival, most larvae of echinoderms are robust under moderate levels of acidification (Dupont et al. 2010; Byrne

2011; see reviews by Byrne et al. 2013; Dupont and Thorndyke 2013). There are some exceptions though; some studies have shown that OA can compromise survival, potentially leading to local species extinction (e.g. the brittlestar *Ophiothrix fragilis*; Dupont et al. 2008).

Sea urchins are generally the most effective benthic herbivores in shallow subtidal areas. Its carbonate structures such as skeleton and grazing apparatus are made up of the soluble high-magnesium calcite in adult, juvenile and larval stages (Dupont et al. 2010). During the settlement phase, sea urchins are very sensitive to environmental changes (Dupont and Thorndyke 2013). Yet few studies have focused on the effects of OA on settlement, despite the importance of this event in the life cycle (Byrne et al. 2008; Byrne et al. 2011; Dupont et al. 2012; Dorey 2013). Recruitment success is directly dependent on survival of embryos and larvae (López et al. 1998). A decrease in survival of embryos and larvae, or a delay in their development, can therefore reduce the long-term viability of a sea urchin population (Morgan 1995).

This study focused on the sea urchin *Paracentrotus lividus*, which is widely distributed throughout the Mediterranean Sea and the NE Atlantic Ocean from Ireland to the Canary Islands. *P. lividus* inhabits rocky intertidal pools, seagrass meadows and shallow subtidal rocky shores, from the low-water limit to about 20 m depth. The species acts as a key herbivore; an important benthic grazer that limits macroalgal growth and determines the species composition of algal assemblages (Hereu et al. 2005; Tomas et al. 2006). It is also economically important as an edible resource. In Spain, where *P. lividus* gonads are consumed, the species provides the largest economic return from sea urchin fisheries.

*Paracentrotus lividus* exhibits external fertilization; in the presence of the appropriate environmental signal, synchronized spawning is triggered. The gametes are released into the water column, where the eggs are fertilized and develop into embryos and larvae. Larval bodies are supported by 4 to 8 calcareous skeletal rods. The echinopluteus larvae of sea urchins are planktonic. After roughly 3 weeks, the larvae build a juvenile rudiment inside an epidermic invagination (Gosseling and Jangoux 1998). Once they reach this stage, the larvae become ‘competent’ and show behavior, which brings them close to the substratum. The newly settled post larvae have the appearance of miniature adults within hours. It then takes a further week for them to reach the true juvenile stage, as they must develop mouths and the rest of the digestive tract first (Cameron and Hinegardner 1974; Gosselin and Jangoux 1996).

In the Canary Islands, *P. lividus* inhabits an area from the lowest intertidal, where it most commonly occupies crevices in tidal pools, to around 10 m depth in the subtidal (Girard et al. 2012) and exceptionally down to about 20 m depth (Girard et al. 2012). As an adaptation to the warmer seawater temperatures in the Canary Islands, the echinoid has the ability to extend its maturity period (late winter and late summer), and have multiple spawning episodes during the year. Larval settlement occurs in late winter and early spring, when high phytoplankton abundance is found in the water column (Girard et al. 2008).

As discussed above, *P. lividus* larvae are usually thought to be robust to OA, but some studies have shown that low pH has negative effects on fertilization and early larval development (Moulin et al. 2010; Cohen-Rengifo et al. 2013). Documented effects have included a reduction in larval size, a delay in development, and plasticity at the gene expression level (Martin et al. 2011). However, no previous studies have tested the impact of OA on settlement of *P. Lividus* larvae. In this study, we investigated the effects of three

different seawater pH treatments, from 8.1 to 7.4, on *P. lividus* larval performance and settlement.

## Materials and Methods

### *Animal collection, spawning and larval culture*

Mature *Paracentrotus lividus* specimens (diameter >24mm) were collected by scuba divers from subtidal rocky shores, between 5 and 10 m depth, at a site off the north-east coast of Tenerife (Boca Cangrejo–28°24'N, 16°18'W). Individuals were collected in March 2012, during the known spawning period for the species.

Two males and seven females were induced to spawn by injection with 2 mL of KCl (0.5 M) across the peristomial membrane. Eggs were collected in filtered sea water (FSW), gently mixed and then fertilized with diluted sperm (egg:sperm ratio of 1:2400). Cleaving embryos (two cell stage), were placed at a density of 15 individuals  $\text{mL}^{-1}$ , in 20 L aquaria filled with FSW, and constantly aerated. When the embryos reached the gastrula stage, larvae were distributed into 2 L culture beakers at densities of 5 larvae  $\text{mL}^{-1}$ . Forty-five culture beakers were maintained in seawater tables to maintain a constant temperature. Seawater was replaced in each beaker twice per week. All seawater was FSW purified in a recirculating system with DRYDEN AQUA active filter media (AFM) bio-crystals, 50 and 10- $\mu\text{m}$  UNICEL polyamide paper filters, and a UV AQUAEL AS-11W filter. At day 4 post-fertilization, larvae were fed with red microalgae, *Rhodomonas lens*, at a concentration of 2000 cells  $\text{mL}^{-1}$ . The *R. lens* strain was provided by the ‘Spanish Oceanography Institute’, and cultured in the laboratory with enriched F/2 medium (Guillard and Ryther 1962) at 20°C, and in a cycle of 24h/0h light/dark. Algae were separated from the growth medium by centrifugation and then suspended in fresh FSW before use as a feed for cultured larvae. To maintain both food and larvae in suspension,

the seawater inside culture beakers was constantly aerated and homogenized using a paddle system (Strathmann 1987) moved by a micromotor.

To induce settlement, once any larvae in the culture were observed to have developed tube feet extending from the rudiment (signs of competence) (Cameron and Hinegardner 1974), glass plates coated with natural biofilm were deployed into each experimental beaker.

#### *Experimental design and sea water chemistry*

Larvae of *P. lividus* were raised in 3 different pH treatment conditions (n=15 for each treatment). The three pH levels tested had no effect on growth of the algae used as a food source (Dupont et al. 2012). The cultures were maintained at a temperature of 19°C and a salinity of 37‰, equivalent to the natural conditions in seawater during March at the collection site. The three pH treatments were selected on the basis that they represent the present, and predicted future, natural variability of seawater pH within the sampling region. Group (i) larvae were treated with seawater at pH 8.1, the present average pH. Group (ii) larvae were treated at pH 7.7, the predicted average pH for the year 2100 (IPCC 2007). Records show that pH 7.7 is also encountered during occasional present extremes of natural variability at the site (Hernández et al. pers comm). Group (iii) were treated at pH 7.4, which is a very low pH level, not currently experienced at the site, but expected during extreme episodes by the year 2100 (Caldeira and Wickett 2005). In each culture beaker, pH was regulated using a computerized control system (AquaMedic) that adds pure gaseous CO<sub>2</sub> directly into the seawater, and is accurate to a resolution of ± 0.01 pH units. We performed daily monitoring of temperature, pH<sub>NBS</sub> (Metrohm mobile meter with a Primatrode NTC IP pH electrode and temperature sensor) and salinity (handheld conductivity meter COND 315i). Seawater total alkalinity (TA) was measured for each treatment by titration. Other parameters of the seawater carbonate chemistry (*p*CO<sub>2</sub>, calcite

saturation state ( $\Omega_c$ ) and aragonite saturation state ( $\Omega_a$ ) were calculated from TA and pH using CO2sys (Lewis and Wallace 1998). Calculations were based on a set of constants, K1 and K2, taken from Mehrbach et al. (1973) (refit by Dickson and Millero 1987).

#### *Biological measurements*

Larvae were sampled daily for a period of one month, to quantify survival, growth, development and settlement. In each replicate beaker, three 1-mL aliquots were collected every second day, and larvae were counted to estimate density. Several larvae in each treatment were photographed every two days (20 larvae), using a digital camera mounted on a binocular microscope. A selection of morphometric parameters was measured on each larva: body length (BL), post-oral arm length (PL) and stomach diameters (S1 and S2) (Fig. 4.1). Stomach volume (SV) was calculated as  $SV = \frac{4}{3} \pi ((S1+S2)/2)^3$ .

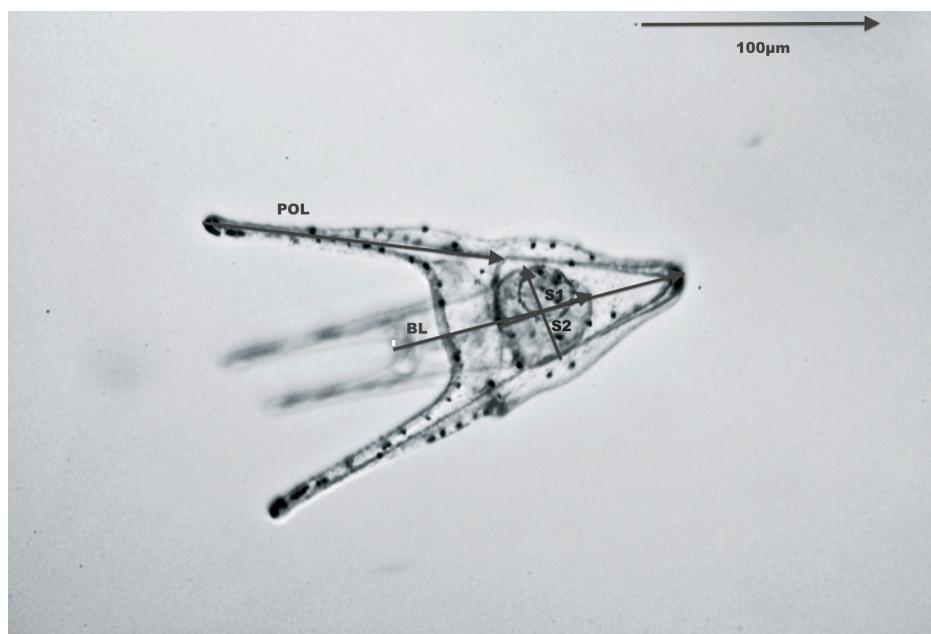


Figure 4.1 Morphometric measurements on *Paracentrotus lividus* larvae. Body length (BL); post-oral arm length (POL) and stomach diameter (S1, S2)

Recently settled postlarvae were counted on the experimental glass plates in each treatment. Settled larvae were also photographed and their test diameters and spine lengths were measured using ImageJ software.

#### *Statistical analyses*

All statistical analyses were performed using the SAS/STAT software (SAS Institute 1990). The normality of data distributions was checked with a Shapiro-Wilk test and the homoscedasticity was tested using the Bartlett test. Logarithmic, exponential and linear regressions were used to test for relationships between studied parameters. Differences between pH treatments were tested using ANCOVA, 1- and 2- way ANOVAs. Each mean value is expressed with the standard error of the mean (mean  $\pm$  SEM). A 5% significance level was applied.

## Results

Target pH levels were achieved in each replicate beaker (see in Supplementary Material 1) and mean pH<sub>NBS</sub> in each treatment were 8.10 $\pm$ 0.01 pH units, 7.73 $\pm$ 0.01 and 7.46 $\pm$ 0.01 respectively (Table 4.1). Between treatments, pH, pCO<sub>2</sub>, Ωc and Ωa were significantly different (ANOVA, p<0.0001, Table 4.1). Seawater was only under-saturated in respect to aragonite ( $\Omega_a < 1$ ) in the lowest pH treatment. Temperature did not vary significantly between pH treatments (ANOVA, p>0.05).

Relative mortality rates (RMR, day<sup>-1</sup>) for larvae were calculated, for each replicate, as the negative coefficient of the significant linear relationship between larval density and time. The results of each regression are presented for each culture replicate, in Supplementary Material 2. Larval RMR was significantly higher at pH 7.4 (0.042 $\pm$ 0.001

day<sup>-1</sup>) compared to the two other pH treatments ( $0.030 \pm 0.003$  day<sup>-1</sup>; Figure 4.2; ANOVA,  $F_{2,24}=3.39$ ,  $p=0.05$ ).

Table 4.1 Seawater carbonate chemistry parameters presented as mean  $\pm$  SD for 15 replicates (seawater carbonate chemistry for each replicate is available in Table S1, see Supplementary Material). Seawater pH on the NBS scale (pH<sub>NBS</sub>), temperature (T; °C), salinity and total alkalinity of 2440 mmol kg<sup>-1</sup> were used to calculate CO<sub>2</sub> partial pressure ( $p\text{CO}_2$ ; μatm) as well as aragonite and calcite saturation states (respectively Ω<sub>ar</sub> and Ω<sub>c</sub>). Results of ANOVA (F-value and p-value) testing the difference between the tested pH treatments are given for each parameter.

Measured			Calculated		
pH <sub>NBS</sub>	T (°C)	Salinity	$p\text{CO}_2$ (μatm)	Ω <sub>ca</sub>	Ω <sub>ar</sub>
8.10±0.01	18.93±0.02	37.36±0.02	499.00±1.27	4.13±0.01	2.69±0.01
7.73±0.01	18.93±0.02	37.34±0.02	1303.72±3.88	1.96±0.01	1.28±0.01
7.46±0.01	18.97±0.02	37.52±0.02	2568±9.51	1.09±0.01	0.71±0.01
$F=1.08$	$F=30.67$	$F=71741.40$	$F=30493.40$	$F=89846.70$	$F=89846.70$
$P<0.0001$	$P=0.34$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$

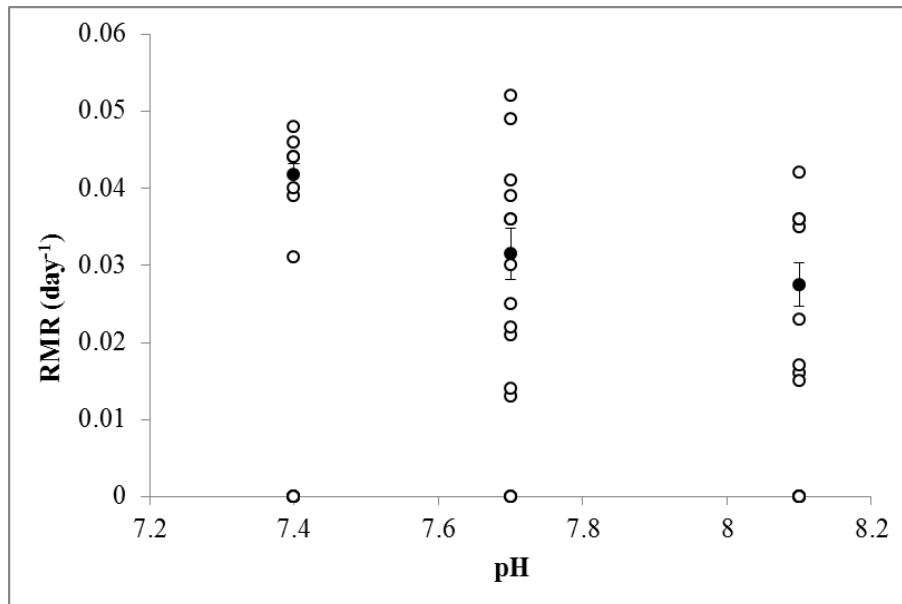


Figure 4.2 Relative mortality rate (RMR, day<sup>-1</sup>) in each tested pH and replicate (open circles; n=15 per pH treatment). Black circles represent average mean  $\pm$  SD

Body length growth rates (BL GR, mm log day<sup>-1</sup>) were calculated for each replicate as the coefficient of the significant logarithmic relationship between BL (mm) and time (day). The results of each regression are presented for each culture replicate in Supplementary Material 3. The BL GR was significantly higher at pH 7.7 (0.031±0.002 mm log day<sup>-1</sup>) compared to the two other pH treatments (0.026±0.001 mm log day<sup>-1</sup>; Figure 4.3; ANOVA,  $F_{2,42}=3.82$ ,  $p=0.03$ ).

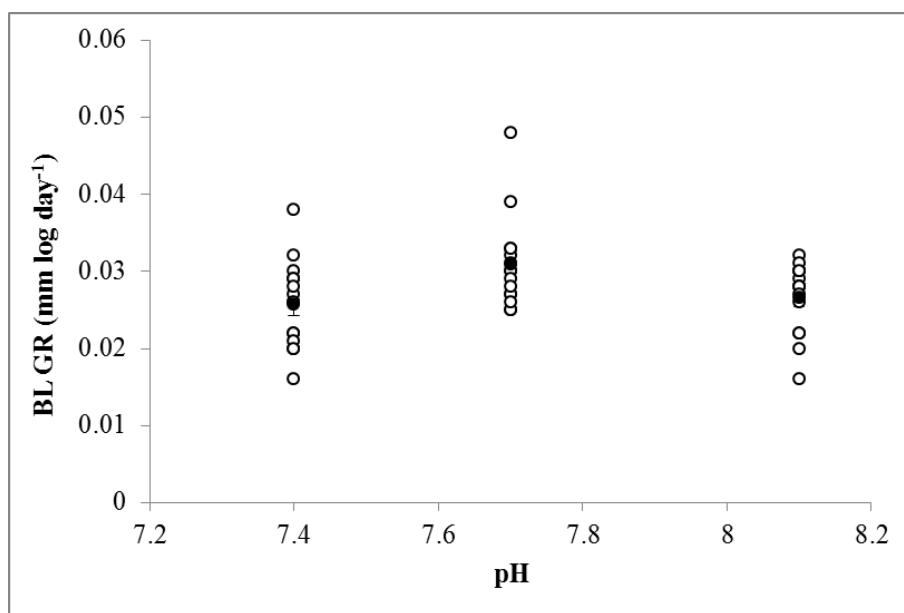


Figure 4.3 Body length growth rate (BL GR, mm log day<sup>-1</sup>) in each tested pH and replicates (open circles;  $n=15$  per pH treatment). Black circles represent average mean  $\pm$  SD

To account for any indirect impact through differences in BL GR, growth rates of the post-oral arm length (POL GR, mm mm<sup>-1</sup>) were calculated for each replicate as the coefficient of the significant linear relationship between POL (mm) and BL (mm). The results of each regression are presented for each culture replicate in Supplementary Material 4. The average POL GR was 1.30±0.14 and no effect of pH was observed on this parameter (Figure 4.4; ANOVA:  $F_{2,36}$ ,  $p=0.18$ ). It was not possible to measure the stomach diameter of all larvae, therefore too few data were available to perform analyses for each

replicate. Replicates were instead pooled, within treatments, to test the impact of pH on SV ( $\text{mm}^3$ ) (Figure 4.5). For each treatment, SV increased exponentially relative to BL (mm;  $p<0.0001$ ). However, no significant differences in the SV growth rate could be observed between pH treatments (ANCOVA on log transformed SV, model:  $F_{3,176}=217.70$ ,  $p<0.0001$ ; pH:  $p=0.33$ ).

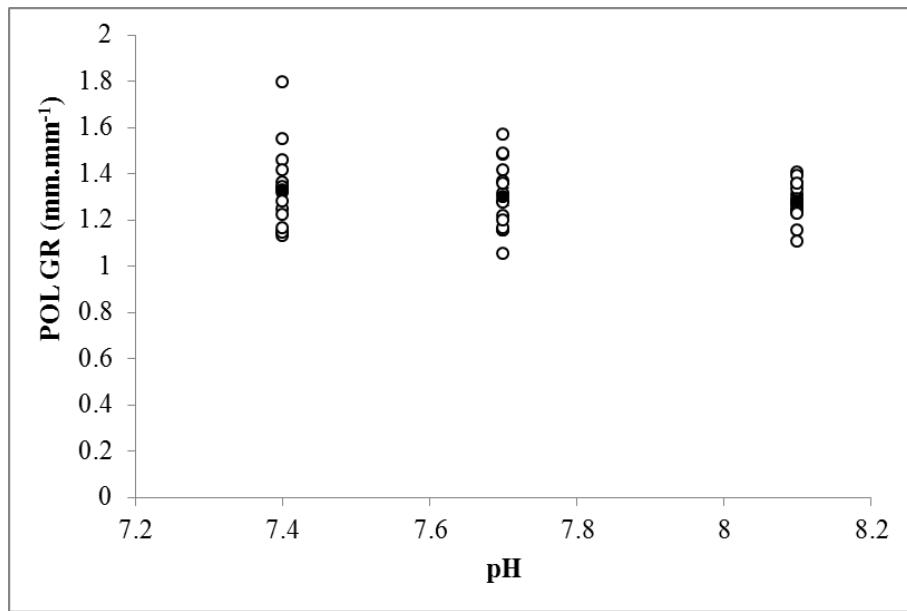


Figure 4.4 Post-oral rod length growth rate (POL GR,  $\text{mm mm}^{-1}$ ) in each tested pH and replicates (open circle;  $n=15$  per pH treatment). Black circles represent average mean  $\pm$  SD

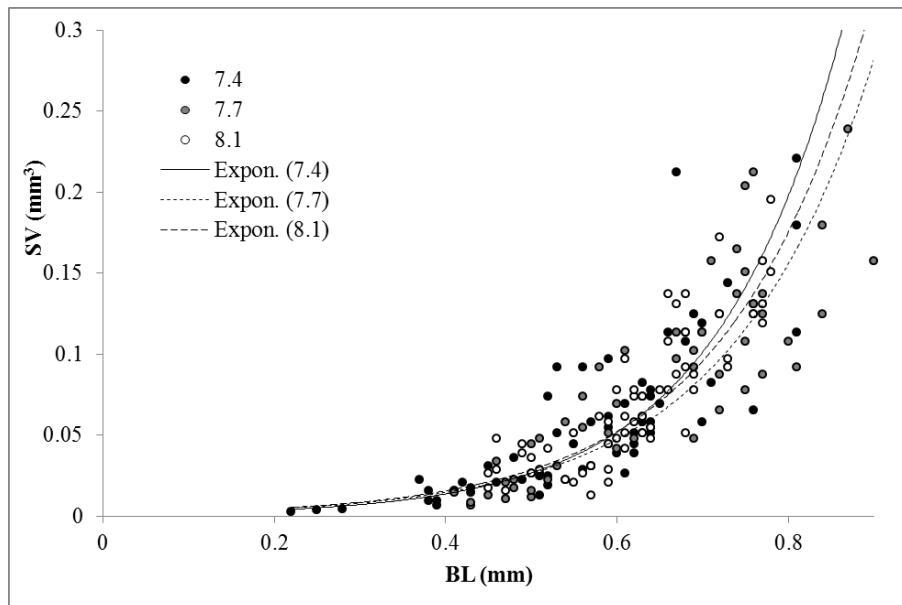


Figure 4.5 Significant exponential relationships between stomach volume (SV, mm<sup>3</sup>) and body length (BL, mm). pH 8.1:  $SV=0.0014 e^{6.06 BL}$ ;  $F_{1,58}=130.59$ ,  $R^2=0.69$ ,  $p<0.0001$ ; pH 7.7:  $SV=0.0013 e^{5.94 BL}$ ;  $F_{1,58}=208.76$ ,  $R^2=0.78$ ,  $p<0.0001$ ; pH 7.4:  $SV=0.0009 e^{6.69 BL}$ ;  $F_{1,58}=248.20$ ,  $R^2=0.81$ ,  $p<0.0001$

The first post-larva was observed after 27 days at pH 8.1, and after 35 days at pH 7.7. No successful settlement was observed in the pH 7.4 treatment. Data collected on the settled juveniles did not have enough power to allow analysis within each replicate. Data were therefore pooled, within pH treatments, for further analyses. Postlarvae treated at pH 7.7 had significantly larger test diameters ( $0.37\pm0.01$ ) compared to pH 8.1 ( $0.34\pm0.01$ ; ANOVA,  $F_{1,529}=25.85$ ;  $p<0.0001$ ). A significant linear relationship was observed between spine length (mm) and juvenile diameter (mm; Figure 4.6), but no difference was observed between the two pH treatments (ANCOVA, model:  $F_{2,528}=285.50$ ,  $p<0.0001$ ; pH:  $p=0.064$ ).

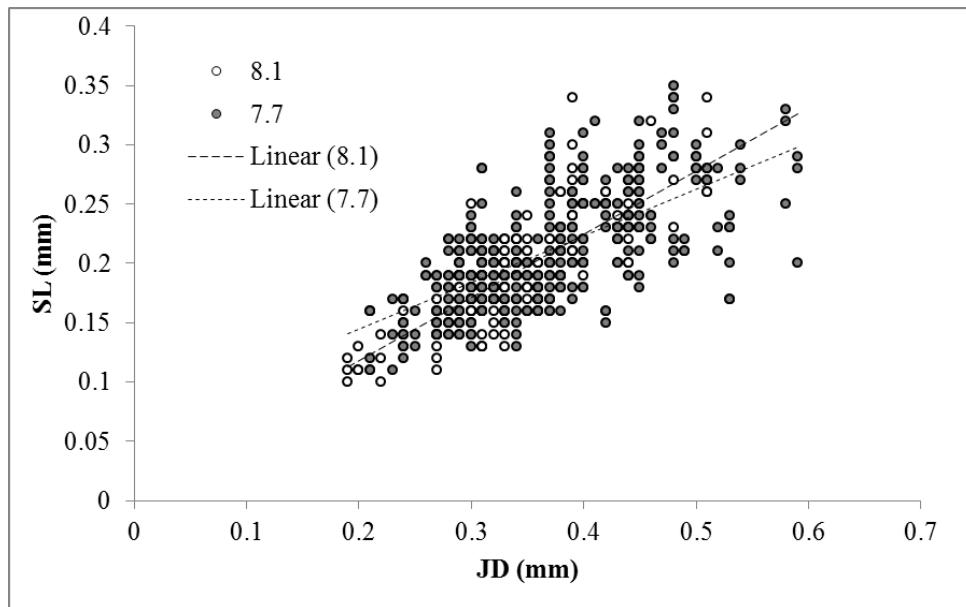


Figure 4.6 Significant linear relationships between spine length (SL, mm) and juvenile diameter (JD, mm).

pH 8.1:  $SL=0.053 \text{ JD} + 0.012$ ;  $F_{1,241}=388.80$ ,  $R^2=0.62$ ,  $p<0.0001$ ; pH 7.7:  $SL=0.039 \text{ JD} + 0.0067$ ;  $F_{1,286}=227.47$ ,  $R^2=0.44$ ,  $p<0.0001$

## Discussion

Our results support the hypothesis that *Paracentrotus lividus* is robust to pH levels within the range of present natural variability, but that the species is sensitive to some of the pH levels predicted in the near future (Dorey et al. 2013). When raised at pH 7.7, larvae only experienced a sub-lethal developmental delay (no increased mortality, 10% faster growth rate, 8 day delay in settlement) compared to larvae raised at pH 8.1. At pH 7.4, the lowest pH tested, lethal effects were induced in the larvae (40% increased mortality rate). In addition, no settlement was observed, within the course of the experiment, among larvae treated at pH 7.4.

Larval RMR was significantly higher at pH 7.4 compared to the two other pH treatments (8.1 and 7.7). In contrast, Martin and collaborators (2011) documented no effect of pH on *P. lividus* mortality, though this experiment was only performed over 3 days and may have underestimated the impact. Negative effects of low pH on survival of larvae have also been reported in different sea urchin species, including *Arachnoides placenta* and *Strongylocentrotus droebachiensis* (González-Bernat et al. 2012; Dorey et al. 2013). However, other studies have found no effect of low pH (Clark et al. 2009; Stumpp et al. 2011; Chan et al. 2011), or in the case of *Arbacia lixula*, even an increase in larval survival rates (Wangensteen et al. 2013). The different responses can be partly attributed to inter-specific variability in sensitivity (Dupont and Thorndyke 2013) but also to the tested scenarios. It was recently hypothesized that pH scenarios deviating from present natural variability are likely to induce lethal effects, while low pH scenarios within the natural variability range will induce a plastic sublethal response (Dorey et al. 2013). Our results support this hypothesis. Metabolism in sea urchin larvae increases at lower pH (Stumpp et al. 2011; Dorey et al. 2013), reflecting the additional energy costs associated with pH regulation (Stumpp et al. 2012) and digestion physiology (Stumpp et al. 2013). The

observed increase in mortality at pH 7.4 may be caused by energy limitation, leaving the larvae unable to sustain their basic physiological processes (Dorey et al. 2013).

The BL GR of *P. lividus* larvae was significantly higher at pH 7.7 compared to the two other pH levels. No effect of pH was observed on POL or SV growth rates, when corrected for the changes in growth rate. In general, low pH decreased larval size and/or growth rates in feeding larvae (see Dupont and Thorndyke 2013 for review). Stumpp et al. (2011) hypothesized that the scope for growth is limited as a consequence of the shift in energy budget (uncompensated increased energy costs). However, positive effects of low pH have also been observed among echinoderms larvae by other researchers (Dupont et al. 2010; Gianguzza et al. 2014). In such cases, increases in metabolism and growth rates during the lecithotrophic phase of development (non-energy limiting) may have been caused by compensated increases in energy costs. Our results show that *P. lividus* larvae can tolerate a wide range in pH (8.1-7.7). The species is known to have the capacity to acclimatize and withstand natural environmental variability (Moulin et al. 2010). For example, daily pH fluctuations of about ~ 0.2 units have been measured in shallow coastal areas of the Canary Islands (Hernández et al. pers comm).

In studies of the species *A. lixula* treated in pH 8.1-7.7, no delay in settlement was observed at lower pH (Wangensteen et al. 2013). Yet, we observed the first *P. lividus* postlarvae settle after 27 days at pH 8.1 and 35 days at pH 7.7 (an 8 day delay at the lower pH). A delay in larval settlement means a longer planktonic stage. During the planktonic stage larvae are exposed to potentially stressful conditions, and increased mortality due to predation rates. Conversely, a shorter period could potentially restrict dispersal, and connectivity of populations (O'Connor et al. 2007). No successful settlement was observed at pH 7.4 over the 35 days of this experiment. Reduced settlement rates have also been

reported for other organisms at low pH (Albright et al. 2010; Albright and Landon 2011; Doropoulos et al. 2012; Dorey 2013). Some studies suggest that low pH does not directly affect the ability of the larvae to settle, but affects them indirectly by altering the composition of settlement inducers such as crustose coralline algae or bacterial biofilms (Webster et al. 2013). Other researchers have suggested that if the baseline metabolism of larvae is already close to optimal, the effects of chronic metabolic stress (such as that caused by low pH) can be lethal, as larvae have to invest energy to regulate their internal conditions (Pörtner and Farrell 2008; Stumpp et al. 2011, 2012). Postlarvae were found to have significantly larger diameters when treated at pH 7.7 compared to control conditions at pH 8.1. As juveniles become exotrophic around eight days after metamorphosis (Gosseling and Jangoux 1998), the increased scope for growth could be caused by increased metabolism under non-limiting energy conditions (Pörtner and Farrell 2008). The larger juveniles could be less susceptible to predation as they possess larger defensive structures. Alternatively, their larger size could be a disadvantage, as the organisms require more food once the digestive tract is fully developed, and the mouth is open.

In conclusion, *P. lividus* larvae are tolerant to the present range of natural pH variability. However, populations encounter detrimental effects when their larvae are exposed to extreme conditions. As episodes of pH 7.4 have been projected in the natural environment within the next 90 years, our results raises questions about the future survival this, supposedly robust, species.

Supplementary material 1 Seawater carbonate chemistry ( $\text{pH}_{\text{NBS}}$ ,  $p\text{CO}_2$  and saturation states (calcite,  $\Omega_{\text{c}}$ , and aragonite,  $\Omega_{\text{a}}$ ) in each replicate and pH treatment.

Target pH	Replicate	Measured		Calculated	
		$\text{pH}_{\text{NBS}}$	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$\Omega_{\text{ca}}$	$\Omega_{\text{ar}}$
7.4	1	7.45±0.01	2582.67±35.54	1.08±0.01	0.70±0.01
	2	7.47±0.01	2471.49±37.16	1.12±0.01	0.73±0.01
	3	7.46±0.01	2516.27±23.18	1.10±0.01	0.72±0.01
	4	7.43±0.00	2719.60±33.68	1.03±0.01	0.67±0.01
	5	7.44±0.01	2671.23±24.59	1.04±0.01	0.68±0.01
	6	7.44±0.01	2658.74±41.24	1.05±0.01	0.68±0.01
	7	7.48±0.01	2432.18±41.30	1.14±0.02	0.74±0.01
	8	7.46±0.01	2539.46±35.70	1.10±0.01	0.71±0.01
	9	7.47±0.00	2471.84±25.93	1.12±0.01	0.73±0.01
	10	7.46±0.01	2569.30±33.48	1.08±0.01	0.70±0.01
	11	7.45±0.00	2624.30±25.81	1.06±0.01	0.69±0.01
	12	7.43±0.01	2709.47±38.41	1.03±0.01	0.67±0.01
	13	7.44±0.01	2630.88±34.63	1.06±0.01	0.69±0.01
	14	7.48±0.00	2439.45±26.64	1.14±0.01	0.74±0.01
	15	7.47±0.01	2481.40±30.45	1.12±0.01	0.73±0.01
7.7	1	7.72±0.00	1330.63±14.70	1.93±0.02	1.25±0.01
	2	7.71±0.01	1378.84±18.68	1.87±0.02	1.22±0.01
	3	7.72±0.01	1336.84±17.24	1.92±0.02	1.25±0.01
	4	7.72±0.00	1337.32±15.16	1.92±0.02	1.25±0.01
	5	7.75±0.00	1257.41±9.26	2.02±0.01	1.31±0.01
	6	7.72±0.01	1327.00±17.10	1.93±0.02	1.26±0.01
	7	7.74±0.00	1277.42±9.16	1.99±0.01	1.30±0.01
	8	7.74±0.00	1260.92±11.21	2.02±0.01	1.31±0.01
	9	7.73±0.00	1310.55±13.14	1.95±0.02	1.27±0.01
	10	7.73±0.00	1315.37±15.00	1.95±0.02	1.27±0.01
	11	7.75±0.01	1252.73±15.98	2.03±0.02	1.32±0.01
	12	7.74±0.02	1286.87±8.40	1.98±0.01	1.29±0.01
	13	7.74±0.00	1264.39±12.52	2.01±0.02	1.31±0.01
	14	7.73±0.00	1300.48±11.87	1.96±0.02	1.28±0.01
	15	7.73±0.00	1319.08±12.05	1.94±0.01	1.26±0.01
8.1	1	8.09±0.00	522.64±6.03	4.00±0.03	2.60±0.02
	2	8.10±0.00	505.14±4.90	4.10±0.03	2.66±0.02
	3	8.10±0.00	502.65±4.94	4.11±0.03	2.67±0.02
	4	8.11±0.00	492.61±4.51	4.17±0.02	2.71±0.02
	5	8.10±0.00	497.75±5.21	4.14±0.03	2.69±0.02
	6	8.10±0.00	506.59±5.14	4.09±0.03	2.66±0.02
	7	8.10±0.00	504.35±4.76	4.10±0.03	2.67±0.02
	8	8.10±0.00	496.89±4.57	4.14±0.03	2.69±0.02
	9	8.11±0.00	490.57±4.44	4.18±0.02	2.72±0.02
	10	8.11±0.00	488.26±3.99	4.19±0.02	2.73±0.01
	11	8.10±0.00	499.30±4.84	4.13±0.03	2.69±0.02
	12	8.10±0.00	500.00±4.59	4.13±0.03	2.68±0.02
	13	8.11±0.00	495.84±4.62	4.15±0.03	2.70±0.02
	14	8.11±0.00	491.92±4.29	4.17±0.02	2.71±0.02
	15	8.11±0.00	490.47±4.20	4.18±0.02	2.72±0.01

Supplementary material 2 Larval relative mortality rates (RMR, day<sup>-1</sup>) were calculated as the negative coefficient of the significant linear relationship between relative density (between 0=no mortality and 1=100% mortality) and time (day). Results of each regression are presented in this table: *p-value*, *R*<sup>2</sup>, *F-value* and *df*(degree of freedom). Data in bold (*p-value*>0.05) were removed from subsequent analyses.

Target pH	replicate	RMR	Intercept	<i>p-value</i>	<i>R</i> <sup>2</sup>	<i>F-value</i>	<i>df</i>
8.1	1	0.023	0.395	0.0063	0.68	14.77	7
	2	<b>0.013</b>	<b>0.683</b>	<b>0.0870</b>	<b>0.36</b>	<b>3.96</b>	7
	3	<b>0.044</b>	<b>-0.120</b>	<b>0.0773</b>	<b>0.43</b>	<b>4.53</b>	6
	4	<b>0.007</b>	<b>0.773</b>	<b>0.0604</b>	<b>0.42</b>	<b>5.00</b>	7
	5	<b>0.008</b>	<b>0.789</b>	<b>0.0966</b>	<b>0.34</b>	<b>3.68</b>	7
	6	0.042	0.071	0.0006	0.83	34.03	7
	7	0.016	0.660	0.0146	0.60	10.40	7
	8	<b>0.012</b>	<b>0.613</b>	<b>0.1586</b>	<b>0.26</b>	<b>2.49</b>	7
	9	0.015	0.650	0.0009	0.81	30.06	7
	10	<b>0.019</b>	<b>0.477</b>	<b>0.2218</b>	<b>0.20</b>	<b>1.80</b>	7
	11	0.035	0.286	0.0058	0.69	15.28	7
	12	0.036	0.245	0.0184	0.57	9.34	7
	13	<b>0.012</b>	<b>0.650</b>	<b>0.2311</b>	<b>0.20</b>	<b>1.72</b>	7
	14	0.017	0.611	0.0095	0.64	12.49	7
	15	0.036	0.179	0.0132	0.61	10.86	7
7.7	1	0.036	0.295	0.0012	0.79	27.11	7
	2	0.052	-0.091	0.0095	0.64	12.53	7
	3	<b>0.031</b>	<b>0.367</b>	<b>0.0736</b>	<b>0.39</b>	<b>4.42</b>	7
	4	0.025	0.535	0.0309	0.51	7.26	7
	5	0.013	0.696	0.0743	0.386	4.39	7
	6	0.030	0.444	0.0360	0.49	6.70	7
	7	0.036	0.308	0.0381	0.48	6.50	7
	8	<b>0.006</b>	<b>0.821</b>	<b>0.2467</b>	<b>0.19</b>	<b>1.60</b>	7
	9	0.014	0.698	0.0443	0.46	5.99	7
	10	0.021	0.579	0.0446	0.46	5.97	7
	11	<b>0.010</b>	<b>0.703</b>	<b>0.3363</b>	<b>0.13</b>	<b>1.07</b>	7
	12	0.041	0.198	0.0332	0.50	6.99	7
	13	0.022	0.519	0.0027	0.75	20.64	7
	14	0.039	0.234	0.0271	0.53	7.76	7
	15	0.049	0.059	0.0110	0.63	11.74	7
7.4	1	0.039	0.219	0.0383	0.48	6.49	7
	2	0.031	0.393	0.0069	0.67	14.32	7
	3	<b>0.030</b>	<b>0.208</b>	<b>0.1348</b>	<b>0.29</b>	<b>2.86</b>	7
	4	0.044	-0.005	0.0241	0.54	8.22	7
	5	<b>0.033</b>	<b>0.075</b>	<b>0.0586</b>	<b>0.42</b>	<b>5.09</b>	7
	6	<b>0.036</b>	<b>0.129</b>	<b>0.1248</b>	<b>0.30</b>	<b>3.04</b>	7
	7	<b>0.015</b>	<b>0.306</b>	<b>0.3892</b>	<b>0.11</b>	<b>0.84</b>	7
	8	0.048	-0.053	0.0159	0.59	9.99	7
	9	0.046	-0.049	0.0072	0.67	14.04	7
	10	0.044	0.057	0.0321	0.50	7.11	7
	11	<b>0.027</b>	<b>0.103</b>	<b>0.1367</b>	<b>0.27</b>	<b>2.82</b>	7
	12	0.040	0.040	0.0021	0.76	22.61	7
	13	<b>-0.011</b>	<b>0.683</b>	<b>0.9494</b>	<b>0.00</b>	<b>0.00</b>	7
	14	<b>0.025</b>	<b>0.242</b>	<b>0.1869</b>	<b>0.23</b>	<b>2.14</b>	7
	15	<b>0.009</b>	<b>0.609</b>	<b>0.6360</b>	<b>0.03</b>	<b>0.24</b>	7

Supplementary material 3 Body length growth rate (BL GR, day<sup>-1</sup>) were calculated as the coefficient of the significant logarithmic relationship between body length (BL, mm) and time (day). Results of each regression are presented in this table: *p-value*,  $R^2$ , *F-value* and *df* (degree of freedom).

Target pH	replicate	GR BL	Intercept	<i>p-value</i>	$R^2$	<i>F-value</i>	<i>df</i>
8.1	1	0.030	0.029	< 0.0001	0.85	87.62	16
	2	0.022	0.137	< 0.0001	0.72	31.61	12
	3	0.032	0.030	< 0.0001	0.91	137.73	14
	4	0.026	0.124	< 0.0001	0.74	34.51	12
	5	0.029	0.033	0.0049	0.76	18.77	6
	6	0.016	0.260	0.0023	0.50	13.81	14
	7	0.030	0.030	< 0.0001	0.69	39.53	18
	8	0.028	0.091	< 0.0001	0.77	54.01	16
	9	0.028	0.005	< 0.0001	0.83	56.83	12
	10	0.020	0.104	0.0051	0.76	18.52	6
	11	0.028	0.060	0.0079	0.72	15.26	6
	12	0.022	0.155	0.0028	0.61	15.53	10
	13	0.031	0.092	< 0.0001	0.87	69.39	10
	14	0.030	0.029	< 0.0001	0.82	56.52	12
	15	0.027	0.111	0.0071	0.73	16.02	6
7.7	1	0.033	0.006	< 0.0001	0.91	79.28	8
	2	0.025	0.144	0.0022	0.81	25.93	6
	3	0.030	0.054	0.0086	0.71	14.73	6
	4	0.039	-0.068	0.0001	0.79	37.94	10
	5	0.032	0.036	0.0005	0.99	2040.20	2
	6	0.027	0.079	< 0.0001	0.91	129.99	12
	7	0.048	-0.124	< 0.0001	0.89	65.23	8
	8	0.027	0.096	< 0.0001	0.74	45.27	16
	9	0.031	0.054	0.0002	0.76	31.62	10
	10	0.030	0.098	< 0.0001	0.81	79.16	18
	11	0.025	0.115	< 0.0001	0.74	33.39	12
	12	0.026	0.127	0.0002	0.63	24.37	14
	13	0.029	0.051	< 0.0001	0.82	82.84	18
	14	0.028	0.075	< 0.0001	0.74	45.22	16
	15	0.033	0.011	< 0.0001	0.88	106.02	14
7.4	1	0.026	0.038	0.0001	0.78	35.11	10
	2	0.027	0.013	0.0004	0.73	26.70	10
	3	0.022	0.001	0.0008	0.56	18.12	14
	4	0.030	-0.012	0.0110	0.57	10.82	8
	5	0.020	0.025	0.0288	0.47	7.08	8
	6	0.029	-0.012	< 0.0001	0.85	92.47	16
	7	0.032	-0.090	< 0.0001	0.81	60.08	14
	8	0.026	-0.024	0.0007	0.63	20.76	12
	9	0.022	0.018	< 0.0001	0.74	39.66	14
	10	0.038	-0.166	< 0.0001	0.75	35.47	12
	11	0.029	-0.004	0.0003	0.75	30.35	10
	12	0.021	0.009	0.0001	0.78	35.29	10
	13	0.028	-0.015	0.0001	0.72	30.89	12
	14	0.016	0.083	0.0003	0.74	28.82	10
	15	0.020	0.043	0.0050	0.56	12.85	10

Supplementary material 4 Post-oral rod growth rate (POL GR, day<sup>-1</sup>) were calculated as the coefficient of the significant linear relationship between post-oral rod length (POL, mm) and body length (BL, mm). Results of each regression are presented in this table: *p-value*,  $R^2$ , *F-value* and *df*(degree of freedom).

Target pH	replicate	BL RL	Intercept	<i>p-value</i>	$R^2$	<i>F-value</i>	<i>df</i>
8.1	1	1.356	-0.100	< 0.0001	0.91	172.35	16
	2	1.311	-0.093	< 0.0001	0.86	76.48	12
	3	1.408	-0.119	< 0.0001	0.96	363.60	14
	4	1.247	-0.010	< 0.0001	0.89	102.99	12
	5	1.391	-0.148	< 0.0001	0.96	145.65	6
	6	1.242	-0.151	0.0003	0.63	23.64	14
	7	1.232	-0.010	< 0.0001	0.90	167.00	18
	8	1.158	-0.036	< 0.0001	0.82	74.15	16
	9	1.265	-0.071	< 0.0001	0.89	96.97	12
	10	1.273	-0.077	< 0.0001	0.93	85.53	6
	11	1.333	-0.087	< 0.0001	0.98	369.70	6
	12	1.109	-0.083	0.0001	0.79	38.06	10
	13	1.230	-0.077	< 0.0001	0.87	67.80	10
	14	1.290	-0.084	< 0.0001	0.95	213.38	12
	15	1.359	-0.091	0.0003	0.90	57.37	6
7.7	1	1.572	-0.164	< 0.0001	0.98	502.38	8
	2	1.484	-0.198	0.0068	0.73	16.29	6
	3	1.219	-0.037	0.0001	0.92	73.86	6
	4	1.156	-0.006	< 0.0001	0.87	65.91	10
	5	1.156	-0.060	0.0021	0.99	471.61	2
	6	1.165	-0.063	< 0.0001	0.92	148.98	12
	7	1.054	-0.024	< 0.0001	0.92	97.99	8
	8	1.416	-0.110	< 0.0001	0.91	163.51	16
	9	1.283	-0.067	< 0.0001	0.97	364.16	10
	10	1.315	-0.087	< 0.0001	0.85	106.35	18
	11	1.366	-0.162	< 0.0001	0.78	42.81	12
	12	1.490	-0.184	< 0.0001	0.95	291.13	14
	13	1.276	-0.100	< 0.0001	0.92	209.44	18
	14	1.356	-0.179	< 0.0001	0.88	112.92	16
	15	1.200	-0.058	< 0.0001	0.82	62.56	14
7.4	1	1.246	-0.088	< 0.0001	0.90	86.17	10
	2	1.364	-0.150	< 0.0001	0.93	138.08	10
	3	1.337	-0.093	< 0.0001	0.90	130.41	14
	4	1.345	-0.127	< 0.0001	0.91	79.29	8
	5	1.147	-0.108	0.0002	0.84	42.85	8
	6	1.321	-0.095	< 0.0001	0.92	195.27	16
	7	1.131	-0.059	< 0.0001	0.88	104.84	14
	8	1.144	-0.117	0.0027	0.54	14.13	12
	9	1.461	-0.167	< 0.0001	0.77	46.15	14
	10	1.415	-0.184	< 0.0001	0.95	222.52	12
	11	1.279	-0.149	< 0.0001	0.85	55.06	10
	12	1.552	-0.112	< 0.0001	0.90	86.50	10
	13	1.166	-0.020	< 0.0001	0.76	37.56	12
	14	1.797	-0.207	< 0.0001	0.84	52.36	10
	15	1.224	-0.059	0.0008	0.69	22.45	10

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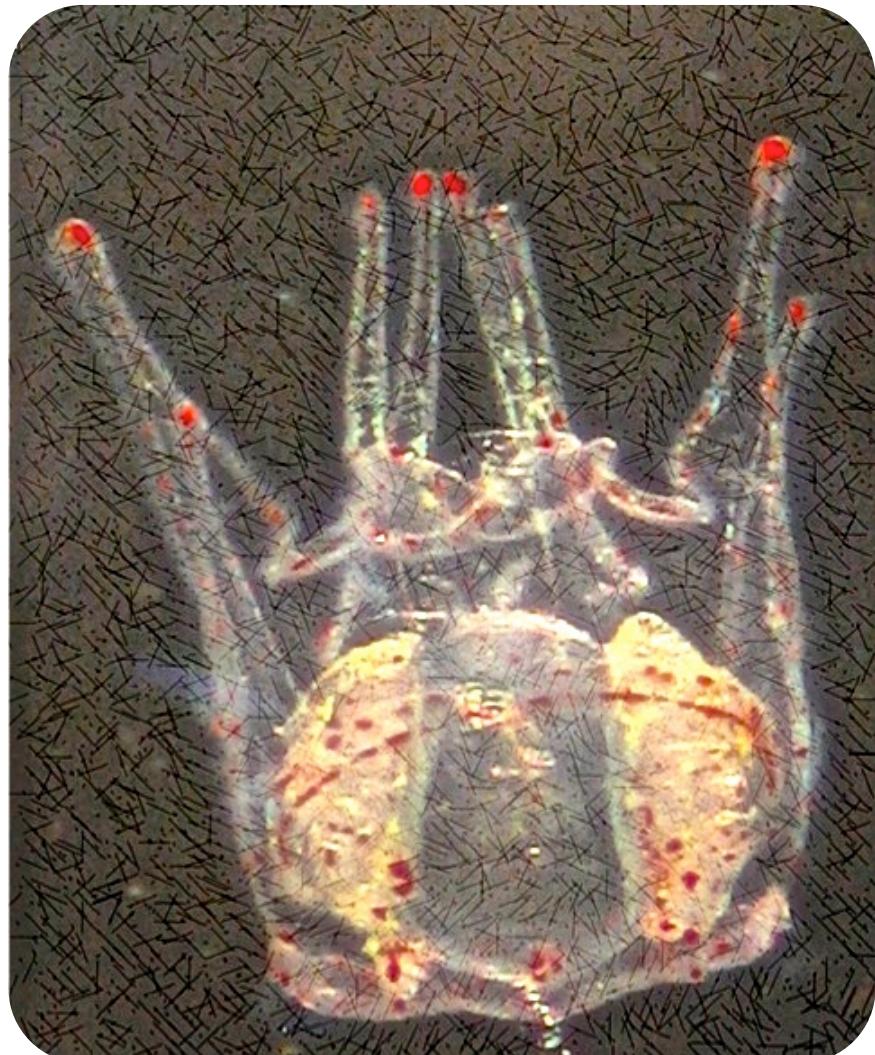
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**CHAPTER 5: GLOBAL WARMING AMELIORATES  
THE NEGATIVE EFFECTS OF OCEAN  
ACIDIFICATION ON PARACENTROTUS LIVIDUS  
LARVAL DEVELOPMENT AND SETTLEMENT**



**CHAPTER 5: GLOBAL WARMING AMELIORATES THE  
NEGATIVE EFFECTS OF OCEAN ACIDIFICATION ON  
PARACENTROTUS LIVIDUS LARVAL DEVELOPMENT AND  
SETTLEMENT**

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## Abstract

Ocean warming and acidification both impact marine ecosystems. All organisms have a limited body temperature range, outside of which they become functionally constrained. Beyond the absolute extremes of this range, they cannot survive. It is hypothesized that synergistic stressors, such as ocean acidification, have the potential to narrow the thermal windows of marine species. An organism's response to ocean acidification can therefore be highly dependent on thermal conditions. This study evaluated the combined effects of predicted ocean warming conditions and acidification, on survival, development, and settlement, of the sea urchin *Paracentrotus lividus*. Nine integrative treatments of temperature (19.0, 20.5 and 22.5°C) and pH (8.1, 7.7 and 7.4 units) were carried out. All of the conditions tested were either within the current natural ranges of seawater pH and temperature, or are within the ranges that have been predicted for the end of the century, in the sampling region (Canary Islands). Our results indicated that the negative effects of low pH on *P. lividus* larval development and settlement, will be mitigated by a rise in seawater temperature, up to a thermotolerance threshold. The most extreme pH conditions, narrowed the thermal window of this species. Larval development, and settlement performance, of the sea urchin *P. lividus* was enhanced by a slight increase in temperature, even under lowered pH conditions. However, the species did show sensitivities to the levels of ocean warming and acidification, which have been predicted by the turn of the century.

**Keywords:** Temperature, pH, climate change, larval development, settlement, *Paracentrotus lividus*.

## Resumen

Los ecosistemas marinos están siendo afectados por el aumento de la temperatura y la acidificación de los océanos. Todos los organismos desarrollan sus funciones con normalidad dentro de unos rangos de temperatura que, si son sobrepasados, pueden ocasionar limitaciones funcionales. Se cree que la acción sinérgica de ciertos factores medioambientales, como la temperatura y el pH, tiene la potencialidad de limitar el rango de temperatura en que un organismo desarrolla sus funciones con normalidad. Por tanto, las respuestas de un organismo a la acidificación oceánica van a depender, en gran medida, de las condiciones térmicas. En este trabajo hemos evaluado la acción combinada de la temperatura y el pH sobre la supervivencia, el desarrollo larvario y el asentamiento del erizo de mar *Paracentrotus lividus*, abarcando valores de ambos factores correspondientes a su variabilidad natural actual y la prevista para final de siglo, como consecuencia del cambio climático. Se llevaron a cabo 9 tratamientos combinando diferentes temperaturas (19.0, 20.5 y 22.5°C) y pHs (8.1, 7.7 y 7.4 unidades). Nuestros resultados mostraron que los efectos negativos de la disminución del pH en el desarrollo larvario y el asentamiento de *P. lividus* puede contrarrestarse, en parte, con un ligero aumento de temperatura. En cambio, cuando nos acercamos al umbral de temperatura de la especie, ésta muestra debilidades cuando es sometida a los niveles de pH previstos para final de siglo.

*Palabras clave:* Temperatura, pH, cambio climático, desarrollo larvario, asentamiento, *Paracentrotus lividus*.

## Introduction

Seawater warming and acidification are the two most significant side effects of climate change in the oceans. The partial pressure of CO<sub>2</sub> ( $p\text{CO}_2$ ) has already increased from 267.0 to 390.5 ppm, since the beginning of the industrial revolution (IPCC 2013). As a result of the direct heat absorption , caused by elevated levels of greenhouse gases in the atmosphere, ocean temperature has increased (Harley et al. 2006). It is thought that sea surface temperature (SST) will rise between 2.0 and 4.5°C by the end of 21<sup>st</sup> century (IPCC 2007).

In addition to heat, the ocean also takes up about 25% of anthropogenic CO<sub>2</sub>. The increase in CO<sub>2</sub> entering the marine environment leads to a modification of the seawater carbonate system, resulting in decreased ocean pH; this process is known as ocean acidification (OA). The ability of the ocean to take up CO<sub>2</sub> decreases as the CO<sub>2</sub> concentration of the atmosphere increases (Pörtner 2008). Surface ocean pH decreased by approximately 0.1 units between pre-industrial times and the end of the 20<sup>th</sup> century. A further decrease of approximately 0.4 units is predicted for the end of the 21<sup>st</sup> century (Caldeira and Wickett 2005; Gatusso and Hansson 2011; Turley et al. 2013).

Temperature and pH are the main environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates (Doney et al. 2009). It is clear that tolerances to climate-related factors may be very different between larvae and adult organisms (e.g. pelagic larvae versus benthic adults). Generally, the early development stages of invertebrates are the most sensitive to environmental stresses (Pörtner and Farrell 2008; Melzner et al. 2009; Dupont et al. 2010). Robustness to changing pH and temperature could also vary between species, and influence species interactions within marine ecosystems. The future distribution of organisms also depends on the availability of their required habitats, how these habitats are changed by climate-

related processes, and how fast each species can redistribute itself to follow that changing environment (Pörtner 2008). All organisms have a limited body temperature range, due to the optimized structural and kinetic coordination of their molecular, cellular, and systemic processes. At either end of that temperature range, organisms experience some functional constraints, and at extremes of temperature they cannot function at all. . It has been hypothesized that synergistic stressors like OA have the potential to narrow the thermal window of an organism. The response to OA can therefore be highly dependent on the temperature of the water in the surrounding environment (Pörtner and Farrell 2008). Consequently, different combinations of temperature and pH can then lead to different biological responses. For instance, if the baseline metabolism of an individual is below its optimum, the organism is not energy limited, and for this individual, an increase in metabolism, due to increased temperature or decreased pH, can create a positive response. However, if the baseline metabolism is already close to optimal, any increase in metabolism will lead to a negative response, and under extreme chronic metabolic stress, the result could be lethal (Pörtner and Farrell 2008). Climate scenarios that expose organisms to temperatures and pH levels outside of their natural range, could cause lethal or sub-lethal damage (Dorey et al. 2013). Species that live in habitats subject naturally to high levels of environmental variability (wide natural range), may have more resilience to warming and/or acidification of sea water, compared to those living in more stable habitats (narrow natural range) (Melzner et al. 2009; Talmage and Gobler 2009, 2011; Matson et al. 2012; Wolfe et al. 2013). Species exposed to highly variable environmental conditions, for example intertidal species, could therefore show greater robustness to future climate change scenarios, than those living in more constant environments (Sheppard-Brennan et al. 2010; Moulin et al. 2010; Martin et al. 2011; Foo et al. 2012; Byrne et al. 2013a; but see Byrne et al. 2013b for review). It seems likely that climate change will favour species with

wide thermal windows, short generation times, and a diverse range of genotypes in their populations (Pörtner and Farrell 2008).

This study focused on larval development and settlement, of the sea urchin *Paracentrotus lividus*. This species is widely distributed throughout the Mediterranean Sea, and the NE Atlantic Ocean, from Ireland to the Canary Islands. Populations are found in both intertidal and subtidal habitats. In the Canary Islands, *P. lividus* is found from the lowest intertidal, where it most commonly occupies crevices in tide-pools, to around 10 m depth in the subtidal. In exceptional cases, the species has been found as deep as 20 m (Girard et al. 2012). In the Canary Islands, the echinoid can extend its period of maturity (late winter and late summer), and have multiple spawning episodes during the year; an adaptation to the warm seawater temperatures in this region. The planktonic larval stage is estimated to last roughly 1 month, with settlement occurring in late winter and early spring, when high phytoplankton abundance is found in the water column (Girard et al. 2008).

We hypothesized that synergistic stressors, like OA, will narrow the thermal window of *P. lividus*. In individuals living at the lower end of their optimal temperature range, warming may lead to increased resilience to OA, because even with a narrowing of their thermal window, the increase in temperature means they will still be functioning within their optimum range. But in individuals currently exposed to the upper limit of thermal tolerance, OA could have harmful effects, because any increase in temperature will tip them outside of their optimal temperature range. We predicted that the negative effects of low pH, on *P. lividus* larval development and settlement, could be mitigated by raising the sea water temperature, up to thermotolerance threshold. Beyond this temperature threshold, we expected physiological processes to begin to break down.

## Materials and Methods

### *Animal collection and spawning*

Mature *P. lividus* specimens (diameter>24mm) were collected by scuba divers from subtidal rocky shores, between 5 and 10 m depth. Individuals were collected in March of 2013, during the spawning period, from the north-east coast of Tenerife (28°24'N, 16°18'W).

Animals were induced to spawn by injection with 2 ml of KCl (0.5 M) through the peristomial membrane. Five males and ten females, randomly selected in order to reduce experimental variability (Evan and Marshall 2005), were used to obtain sperm and eggs. Sperm was collected dry and kept on ice until usage. Eggs were collected in filtered seawater (FSW). Fertilization was carried out at a ratio of 1:1500 (eggs:sperm). Cleavaging embryos (two cell stage) were placed, at densities of 15 individuals mL<sup>-1</sup>, in 20 L aquaria filled with FSW, and constantly aerated.

### *Experimental design and sea water chemistry*

When the embryos reached the gastrula stage, larvae were distributed into 2 L culture beakers, at densities of 5 larvae mL<sup>-1</sup>. Forty-five culture beakers were maintained in three seawater tables to keep the temperature conditions constant. The seawater in each beaker was replaced twice per week. At day 4 post-fertilization, larvae were fed with the red alga *Rhodomonas lens* at a concentration of 2000 cells mL<sup>-1</sup>. The strain of microalgae was provided by the Spanish Oceanography Institute, and cultured in the laboratory with enriched F/2 medium (Guillard and Ryther 1962) at 20°C, and a cycle 24h/0h light/dark. The levels of pH tested in our study, do not have an effect on algal growth (Dupont et al. 2012). Algae were separated from the growth medium by centrifugation, and then suspended in fresh FSW before use. The water inside the culture beakers was constantly

aerated and homogenized, using a paddle system moved by a micromotor (Strathmann 1987), to keep food and larvae in suspension.

The larvae of *P. lividus* were incubated in 9 different combined treatments of temperature and pH ( $n=5$  for each treatment). The cultures were maintained at a salinity of 36.6 ‰. The range of temperatures and pH levels tested, represented current or future (predicted) conditions at the sampling site. The experiment included three treatments of temperature: 19°C (control: regular SST in spring in the Canary Islands); 20.5°C (exceptionally high but still within the natural range of water temperatures experienced in spring, also predicted as the regular SST for the year 2050, IPCC 2007); and 22.5°C (predicted regular spring SST for 2100, IPCC 2007). Within each treatment of temperature, 3 different treatments of pH were tested: pH 8.1 (present average); pH 7.7 (present extreme of pH, also the average predicted for the year 2100 (IPCC 2007) in this region); and pH 7.4 (extreme of the natural variability predicted for the year 2100 (Caldeira and Wickett 2005)).

When larvae within the cultures reached the competent stage, and were observed to have tube feet extending from the rudiment (Cameron and Hinegardner 1974), a settlement experiment was carried out. Forty-five competent larvae were placed in each beaker, and exposed to one of 9 different combined treatments of temperature and pH ( $n=3$  for each treatment). In each beaker, a glass plate with a natural biofilm was present to induce settlement. This part of the experiment was conducted without feeding or aeration.

To keep constant temperature conditions, thermostat coolers and heaters (EHEIM AQUATICS, 50 W) were used. To control pH, we used a computerised control system (AquaMedic) that bubbled pure CO<sub>2</sub> directly into the water, accurate at a resolution of  $\pm 0.01$  pH units. We carried out daily monitoring of temperature, pH<sub>NBS</sub> (Metrohm mobile meter with a Primatode NTC IP pH electrode and temperature sensor) and salinity

(handheld conductivity meter COND 315i). Seawater total alkalinity (TA) was measured for each treatment by titration. Other parameters of the seawater carbonate chemistry ( $p\text{CO}_2$ , calcite saturation state ( $\Omega_c$ ) and aragonite saturation state ( $\Omega_a$ )) were calculated from TA and pH using CO2sys (Lewis and Wallace 1998). Calculations were based on a set of constants K1 and K2 from Mehrbach et al. (1973) (refit by Dickson and Millero 1987).

Experiments were conducted with FSW, purified within a recirculating system using DRYDEN AQUA active filter media (AFM) bio-crystals; 50  $\mu\text{m}$ , 10  $\mu\text{m}$  and 1  $\mu\text{m}$  UNICEL polyamide paper filters; and a UV-C AQUAEL 11W filter. Seawater was prepared at the appropriate temperature and pH conditions for each treatment before using it.

#### *Biological measurements*

Larvae were sampled daily, for a period of a month, to quantify survival, growth, development and settlement. In each replicate, three 1mL aliquots were collected every second day, and larvae were counted to estimate density. Three larvae in each replicate beaker were photographed using a digital camera mounted on a binocular microscope. Several parameters were measured for each larva: body length (BL), post-oral arm length (PL), and stomach diameter (S1 & S2) (Fig. 5.1). Stomach volume (SV) was calculated as  $SV = \frac{4}{3} \pi ((S1+S2)/4)^3$  (Dorey et al. 2013)

In the settlement experiments, the number of swimming, dead and settled postlarvae were counted in each treatment, 4 days after competent larvae were added to the beakers. Settled postlarvae were photographed and their diameter lengths were measured using ImageJ software.

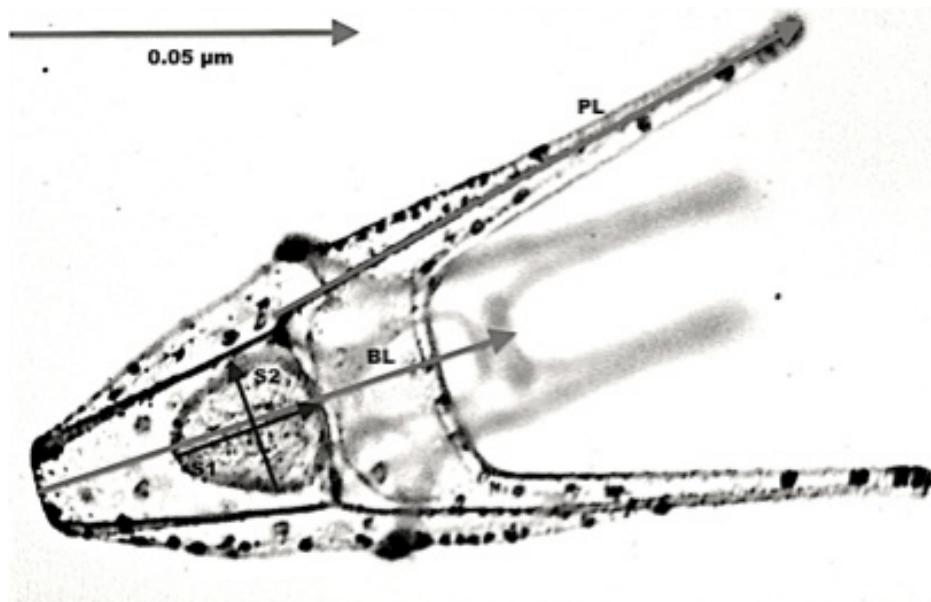


Figure 5.1. Morphometric measurements taken for each *Paracentrotus lividus* larvae: body length (BL), post oral arm length (PL) and stomach diameters (S1 & S2).

#### Data analyses

In order to assess the combined effects of seawater temperature and pH on larval survival, data were analysed by means of a three-way permutational analysis of variance (PERANOVA) (Anderson 2001). A three-way design was conducted with temperature (3 levels), pH (3 levels) and time (8 levels) as fixed factors.

To evaluate the effects on morphometric measurements (BL, PL, SV), three-way permutational ANOVAs were performed for each variable. In each case, three-way designs were carried out with temperature (3 levels), pH (3 levels) and time (10 levels) used as fixed factors.

The combined effects of temperature and pH, on both settlement and postlarval diameter, were analysed by means of two-way permutational ANOVAs. Two-way designs were conducted with temperature (3 levels) and pH (3 levels) used as fixed factors.

Euclidean distances were used for all analysis of variance, and respective significant terms were examined using *a posteriori* pairwise comparisons by permutations

(Anderson 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws from the asymptotic permutation distribution. All statistical analyses were carried out using PRIMER 6 & PERMANOVA+ v. 1.0.1 software.

## Results

The physico-chemical seawater parameters measured during larval experiments, are given in table 5.1. The partial pressure of carbon dioxide ( $p\text{CO}_2$ ) increased at low pH in all temperature treatments. Saturation levels of calcite ( $\Omega_c$ ) and aragonite ( $\Omega_a$ ) decreased at low pH. Seawater was only saturated in respect to aragonite ( $\Omega_a < 1$ ) in the lowest pH treatment, at all temperatures.

Table 5.1. Physico-chemical seawater parameters for each experimental treatment tested for larvae of *Paracentrotus lividus*. T: seawater temperature (mean  $\pm$  SD), pH: seawater pH (mean  $\pm$  SD), S: salinity (mean  $\pm$  SD),  $p\text{CO}_2$ :  $\text{CO}_2$  partial pressure, TA: Total alkalinity,  $\Omega_c$ : Saturation level of calcite,  $\Omega_a$ : Saturation level of aragonite.

	19°C			20.5°C			22.5°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=26)	18.8 $\pm$ 0.2	18.8 $\pm$ 0.2	18.8 $\pm$ 0.2	20.5 $\pm$ 0.2	20.5 $\pm$ 0.2	20.5 $\pm$ 0.2	22.5 $\pm$ 0.2	22.5 $\pm$ 0.2	22.5 $\pm$ 0.2
pH <sub>NB</sub> (n=26)	8.1 $\pm$ 0.0	7.7 $\pm$ 0.0	7.4 $\pm$ 0.1	8.1 $\pm$ 0.0	7.7 $\pm$ 0.0	7.4 $\pm$ 0.0	8.1 $\pm$ 0.0	7.7 $\pm$ 0.0	7.4 $\pm$ 0.0
S (n=26)	36.7 $\pm$ 0.3	36.7 $\pm$ 0.3	36.7 $\pm$ 0.3	36.6 $\pm$ 0.2	36.6 $\pm$ 0.2	36.7 $\pm$ 0.2	36.7 $\pm$ 0.3	36.8 $\pm$ 0.2	36.8 $\pm$ 0.3
$p\text{CO}_2$	310.6	885.4	1849.9	304.5	884.0	1838.4	313.6	915.6	1897.6
TA (n=3)	2165.8 $\pm$ 55.1	2125.6 $\pm$ 50.6	2110.6 $\pm$ 49.3	2131.3 $\pm$ 55.1	2113 $\pm$ 57.6	2083.1 $\pm$ 67.6	2205.7 $\pm$ 37.1	2180.7 $\pm$ 51.2	2133.8 $\pm$ 46.6
$\Omega_c$	4.6	2.1	1.1	4.7	2.2	1.1	5.2	2.4	1.2
$\Omega_a$	3.0	1.3	0.7	3.1	1.4	0.7	3.4	1.6	0.8

When analysing larval survival, there was a significant interaction of the factors 'Temperature x pH x Time' (table 5.2A). However, a *posteriori* pairwise tests revealed that, in general, there were no significant differences between treatments. The only significant differences were between pH 7.4 and the two higher pH levels, however this pattern was only apparent at 20.5 and 22.5 °C and was not consistent across all sampling times (Supplementary material 1, 2; Fig. 5.2).

Table 5.2. Results of the three-way permutational ANOVA analyzing (A) larval survival; (B) body length (BL); (C) post oral arm length (PL); and (D) stomach volume (SV) of *Paracentrotus lividus* larvae. In the respective models the factors included are: T: temperature, pH, Ti: Time.

A. Larval survival						B. Body Length					
Source	df	SS	MS	Pseudo-F	P(perm)	df	SS	MS	Pseudo-F	P(perm)	
T	2	11.45	5.72	4.38	0.012	2	1.36	0.68	26.95	0.001	
pH	2	0.92	0.46	0.35	0.689	2	2.18	1.09	43.28	0.001	
Ti	7	2257.60	322.51	246.98	0.001	9	68.17	7.57	300.57	0.001	
T*pH	4	15.27	3.82	2.92	0.028	4	0.24	5.96E-2	2.37	0.043	
T*Ti	14	50.33	3.59	2.75	0.001	18	1.15	6.42E-2	2.55	0.001	
pH*Ti	14	13.08	0.93	0.71	0.790	18	1.44	7.98E-2	3.17	0.001	
T*pH*Ti	28	58.11	2.07	1.59	0.029	36	1.89	5.26E-2	2.09	0.001	
C. Post oral arm Length						D. Stomach volume					
Source	df	SS	MS	Pseudo-F	P(perm)	df	SS	MS	Pseudo-F	P(perm)	
T	2	1.36	0.68	26.95	0.001	2	1.36	0.68	26.95	0.001	
pH	2	2.18	1.09	43.28	0.001	2	2.18	1.09	43.28	0.001	
Ti	9	68.17	7.57	300.57	0.001	9	68.17	7.57	300.57	0.001	
T*pH	4	0.24	5.96E-2	2.37	0.032	4	0.24	5.96E-2	2.37	0.038	
T*Ti	18	1.15	6.42E-2	2.55	0.001	18	1.15	6.42E-2	2.55	0.001	
pH*Ti	18	1.44	7.98E-2	3.17	0.001	18	1.44	7.98E-2	3.17	0.001	
T*pH*Ti	36	1.89	5.26E-2	2.09	0.001	36	1.89	5.26E-2	2.09	0.001	

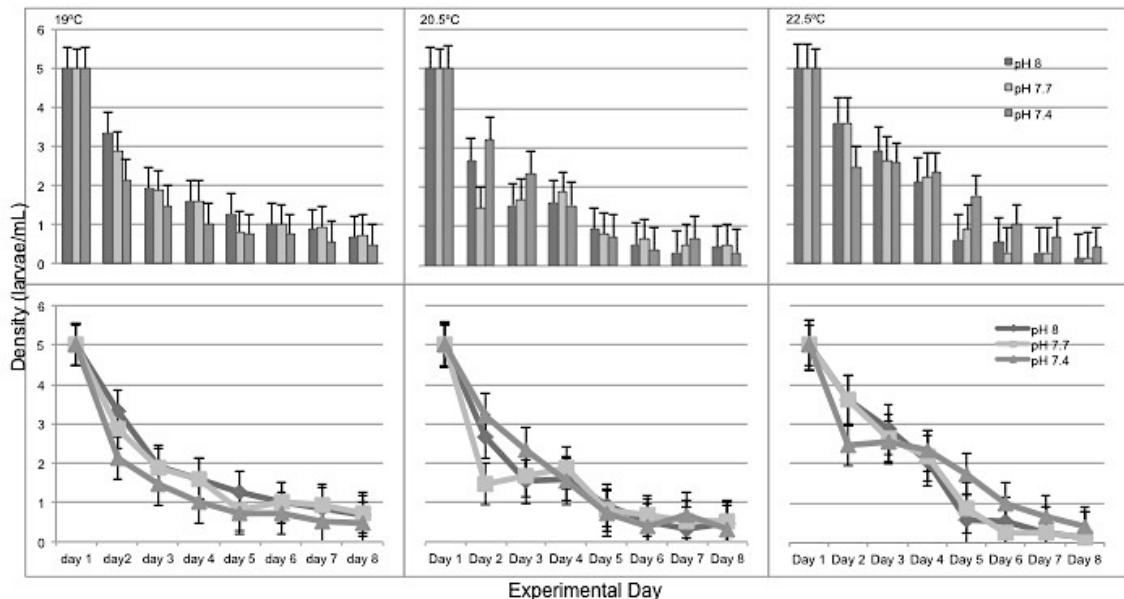


Figure 5.2 Density of larvae of the sea urchin *Paracentrotus lividus* (mean  $\pm$  SD) at laboratory experiments testing the combined effects of seawater temperature and pH.

Results of the PERMANOVA analysing morphometric measurements of planktonic larvae, showed a significant interaction of the factors ‘Temperature x pH x Time’ (Table

5.2B, C), indicating that the influence of temperature and pH on BL and PL varied significantly over time during the larval development cycle. Pairwise tests showed that BL and PL were significantly shorter at pH 7.4, but only at 19 and 22.5°C, and not at 20.5°C (Supplementary material 3, 4; Fig 5.3A; Fig. 5.3B). Both BL and PL were slightly longer at higher temperature, regardless of pH level (Supplementary material 5, 6; Fig 5.3A; Fig. 5.3B)

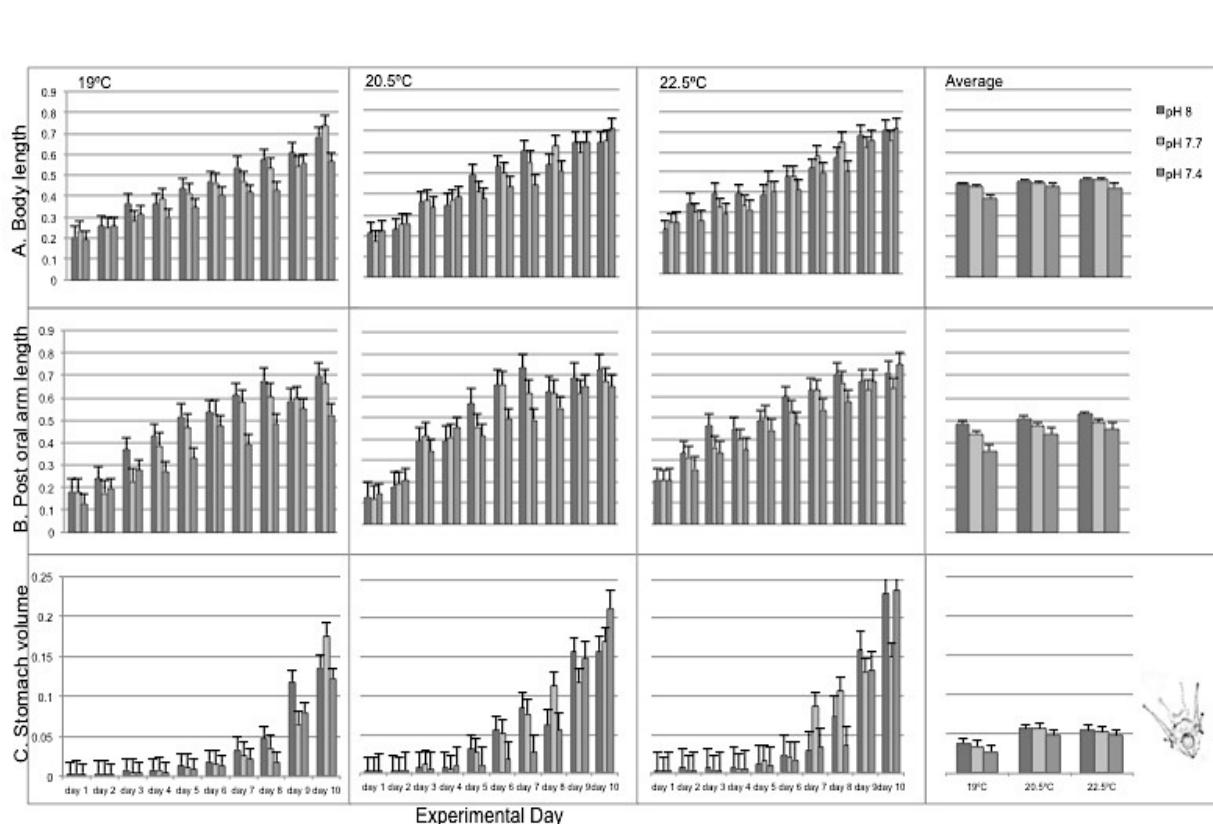


Figure 5.3. (A) Body length (mm), (B) Post oral arm length (mm) and (C) Stomach volume ( $\text{mm}^3$ ) of larvae of the sea urchin *Paracentrotus lividus* (mean  $\pm$  SD) at laboratory experiments testing the combined effects of seawater temperature at 19°C, 20.5°C, 22.5°C and pH levels of 8, 7.7 and 7.4 units. Overall mean values for the experiment are given.

Stomach volume results also revealed a significant interaction of the factors 'Temperature x pH x Time' (Table 5.2D), indicating that the influence of temperature and pH on the SV varied significantly over the lifecycle as well. SV was smallest at pH 7.4,

mainly at 19 and 22.5°C (Supplementary material 7; Fig 5.3C). Warmer temperatures tended to increase SV at all pH levels (Supplementary material 8; Fig 5.3C).

The physico-chemical seawater parameters measured during settlement experiments, are given in table 5.3. The partial pressure of carbon dioxide ( $p\text{CO}_2$ ) was higher at low pH in all temperature treatments. Saturation levels of calcite ( $\Omega_c$ ) and aragonite ( $\Omega_a$ ) were lower at low pH. Aragonite saturation ( $\Omega_a < 1$ ) only occurred at pH 7.4, across at temperature treatments.

Table 5.3. Physico-chemical seawater parameters for each experimental treatment tested for postlarvae of *Paracentrotus lividus*. T: seawater temperature (mean  $\pm$  SD), pH: seawater pH (mean  $\pm$  SD), S: salinity (mean  $\pm$  SD),  $p\text{CO}_2$ :  $\text{CO}_2$  partial pressure, TA: Total alkalinity,  $\Omega_c$ : Saturation level of calcite,  $\Omega_a$ : Saturation level of aragonite.

	19°C			20.5°C			22.5°C		
	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4	pH 8	pH 7.7	pH 7.4
T(n=7)	18.77 $\pm$ 0.11	18.83 $\pm$ 0.07	18.86 $\pm$ 0.09	20.44 $\pm$ 0.15	20.47 $\pm$ 0.19	20.46 $\pm$ 0.20	22.43 $\pm$ 0.23	22.40 $\pm$ 0.23	22.37 $\pm$ 0.27
pH <sub>NB</sub> (n=7)	8.06 $\pm$ 0.04	7.69 $\pm$ 0.04	7.40 $\pm$ 0.05	8.07 $\pm$ 0.02	7.69 $\pm$ 0.04	7.39 $\pm$ 0.05	8.07 $\pm$ 0.04	7.68 $\pm$ 0.04	7.40 $\pm$ 0.04
S (n=7)	36.78 $\pm$ 0.24	36.81 $\pm$ 0.42	36.77 $\pm$ 0.37	36.83 $\pm$ 0.23	36.84 $\pm$ 0.31	36.73 $\pm$ 0.35	36.78 $\pm$ 0.37	36.67 $\pm$ 0.33	36.74 $\pm$ 0.31
$p\text{CO}_2$	346.40	898.70	1825.60	326.10	895.70	1849.80	340.40	958.30	1874.20
TA (n=1)	2160.95	2104.47	2083.22	2101.96	2090.32	2046.62	2198.16	2167.54	2108.41
$\Omega_c$	4.24	2.01	1.07	4.41	2.11	1.09	4.90	2.28	1.23
$\Omega_a$	2.75	1.30	0.70	2.88	1.38	0.71	3.22	1.50	0.81

Postlarval settlement differed between treatments - demonstrated by a significant interaction of ‘Temperature x pH’ (table 5.4A). *A posteriori* pairwise tests revealed that settlement was lowest at pH 7.4 at 19 and 22.5°C. In fact, no successful settlement was observed in beakers incubated at pH 7.4 for either temperature. At 20.5°C, no significant effect of pH was detected on settlement (Supplementary material 9; Fig 5.4A). Settlement was significantly higher at 20.5°C across all pH treatments (Supplementary material 9; Fig 5.4A).

Table 5.4. Results of the two-way permutational ANOVA analyzing (A) percentage of settled *Paracentrotus lividus* postlarvae and (B) their test diameter. In the respective models the factors included are: T: temperature and pH.

Source	df	A. Settlement				B. Test diameter			
		SS	MS	Pseudo-F	P(perm)	df	SS	MS	Pseudo-F
T	2	2111.60	1055.80	40.96	0.001	2	0.31	0.15	63.31
pH	2	489.14	244.57	9.49	0.001	2	0.32	0.16	64.66
T*pH	4	457.69	114.42	4.45	0.003	4	0.33	8.31E-2	33.99

The results of the ANOVA analysing postlarval diameter showed significant interaction of ‘Temperature x pH’ (Table 5.4B). Temperature therefore influenced the size of the postlarvae differently depending on the level of pH. At 20.5°C postlarvae were larger at low pH, however at 22.5°C postlarvae were smaller at low pH (Supplementary material 10; Fig 5.4B).

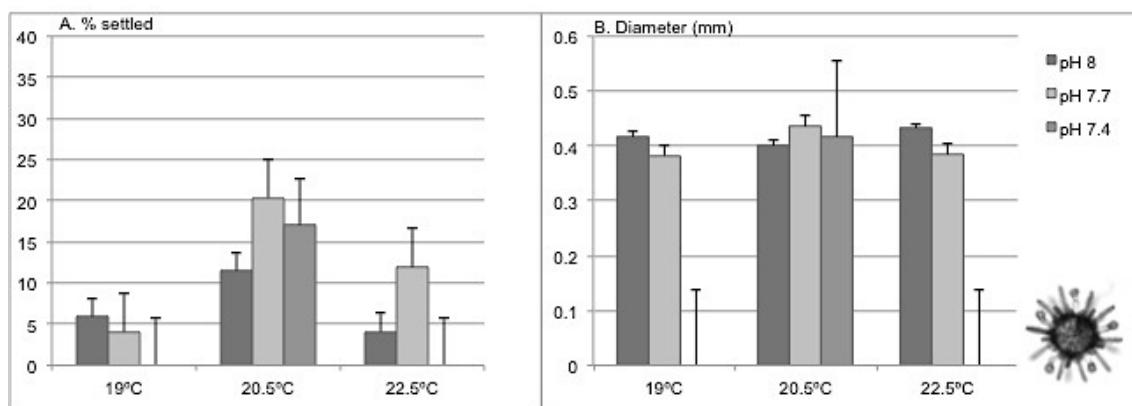


Figure 5.4. Settlement experiment of the sea urchin *Paracentrotus lividus* testing the combined effects of seawater temperature and pH. (A) Percentage of settled postlarvae in each treatment (mean  $\pm$  SD), and (B) their test diameter (mean  $\pm$  SD).

## Discussion

The present study supported the hypothesis that increasing the seawater temperature can counteract the negative effects of ocean acidification on *P. lividus* larval development and settlement, up to thermal threshold. At a temperature close to this species-specific thermal threshold (our results suggest this is around 22.5°C), a low pH (of 7.4) narrowed the thermal window of the species.

Larval survival was resilient to both current and predicted near-future values of temperature and pH. Previous combined stressor studies, on other intertidal sea urchin species, have also reported no effect on larval survival (Wolfe et al. 2013; Gianguzza et al.

2014). Although responses to environmental factors seem to be highly species-specific, even in closely related taxa (Wittmann and Pörtner 2013), most larvae of echinoderms survive under moderate changes of temperature and pH (see reviews by Byrne 2011; Byrne et al. 2013; Dupont and Thorndyke 2013). Scenarios deviating from present natural environmental conditions are more likely to induce lethal effects, compared to scenarios that expose organisms to temperatures and pH levels within their natural variability. It has been hypothesized that organisms exposed to treatments within the realms of their natural variability display a plastic sublethal response (Dorey et al. 2013). *P. lividus* is a species that exists in a variety of habitats, at a range of different latitudes, and is regularly exposed to environmental fluctuations (especially where it inhabits the intertidal zone) (Moulin et al. 2011). This fact could be playing an important role in the struggle for survival against climate change.

Both BL and PL were shorter at pH 7.4 at 19 and 22.5 °C, but not at 20.5 °C. Warmer temperatures increased the length of BL and PL at pH 7.7 and 7.4. The same pattern was observed with stomach volume results. Low pH is generally known to decrease size and/or growth rates in feeding larvae (see Dupont and Thorndyke 2013 for review). Stumpp et al. (2011) hypothesized that this is due to a shift in the energy budget, limiting the scope for growth (uncompensated increased energy costs). Reduced body size, stomach or arm length, as we have shown in this study, can affect survival of planktonic larvae, as echinoplutei morphology is tightly linked to its feeding ability. Post-oral arm length in particular, allows, food uptake and the capacity to swim (Strathmann 1971; Chan et al. 2011). Shorter PLs among these planktonic larvae may therefore increase rates of starvation and susceptibility to predators. However, increasing temperature could counteract the negative effects of low pH, up to a threshold, as has been reported for other sea urchin species (Sheppard-Brennand et al. 2010; Byrne et al. 2013). Our results

suggested that warming may lead to increased resilience to OA at the lower end of the optimal temperature range of *P. lividus* (20.5°C). We also noted an increased sensitivity to low pH when temperature was closer to the upper limit of the species' tolerance (22.5°C).

No settlement was observed at pH 7.4 at 19 °C. This result is consistent with previous data for the species, which has also showed unsuccessful settlement at extremes of pH (García et al. in review). Settlement has also been negatively affected at low pH in other organisms, including sea urchins, but also corals (Albright et al. 2010; Albright and Landon 2011; Doropoulos et al. 2012; Dorey 2013). Some studies suggest that low pH may not directly affect to the ability of the larvae to settle, but it affects them indirectly by altering the composition of settlement inducers (Webster et al. 2013). We found that sea urchin settlement was enhanced at 20.5°C at all levels of pH. However, when the temperature was higher (22.5°C), settlement was unsuccessful at pH 7.4. This finding lends further evidence to the idea that the baseline metabolism of *P. lividus* postlarvae may be at the top end of its range at around 22.5°C or pH 7.4. When water temperature is around 22.5°C and pH is as low as 7.4, this stressor is able to narrow the thermal window of the species. Below these conditions postlarvae have to invest a great amount of energy to regulate their internal conditions, and the narrowing of their thermal window induces chronic metabolic stress, which can be lethal (Pörtner and Farrel 2008; Stumpp et al. 2011, 2012).

Postlarvae were significantly larger in diameter at pH 7.7 and 7.4 compared to control conditions (8.1) at 20.5 °C. Hence more acidic conditions seem to have a positive impact on postlarvae at 20.5 °C. Juveniles become exotrophic around eight days after metamorphosis (Gosseling and Jangoux 1998), thus, this increased scope for growth could be a consequence of an increased metabolism under non-limiting energy conditions

(Pörtner and Farrel 2008). These larger juveniles have larger defensive structures so could be less susceptible to predation. However, being larger can also be a disadvantage as the requirement for food is greater once the digestive tract is fully developed and the mouth is open. At 22.5°C, low pH decreased the diameter of postlarvae, indicating this temperature is closer to the thermotolerance threshold of this life stage. The sea urchin *P. lividus* shows ecological strategies that allow it to inhabit coastal areas where environmental stress and disturbances are frequent. Its thermal tolerance window is therefore broad, which suggests a large phenotypic plasticity (Catarino et al. 2012). A recent study suggested that adults of *P. lividus* have a higher capacity than other urchin species to regulate their internal fluid pH, and can survive under conditions of moderate hypercapnia. This finding may be related to the acclimation ability of the species, that also allows it to withstand fluctuations in seawater parameters, which occur naturally in the intertidal (Catarino et al. 2012).

In terms of its larval development and settlement, the sea urchin *P. lividus* was widely tolerant of a range of temperature and pH scenarios, representative of current and future predicted conditions. The species' performance was enhanced by a slight increase in temperature, up from the control temperature of 19°C. A slight increase in temperature even mitigated the negative effects of low pH (7.4), which suggest that rising SST may counteract some of the negative effects of OA for this species. However, the species did show sensitivities to ocean warming and acidification levels at the more extreme end of the predicted values – when the highest temperature and lowest pH were tested in combination, the effects on larvae were harmful. This study highlights the importance of assessing the integrative effects of stressors across different life-history stages, when attempting to understand the potential impacts of climate change on marine organisms.

Supplementary material 1. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larval survival in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH are shown.

Ti		19°C		20.5°C		22.5°C	
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	8 vs 7.7	1E5	1.000	1E5	1	1E5	1.000
	8 vs 7.4	1E5	1.000	1E5	1	1E5	1.000
	7.7 vs 7.4	1E5	1.000	1E5	1	1E5	1.000
Day 2	8 vs 7.7	0.72	0.487	1.95	0.063	6.60E-9	1.000
	8 vs 7.4	2.09	0.040	0.81	0.398	1.89	0.058
	7.7 vs 7.4	1.38	0.193	2.77	0.008	1.63	0.118
Day 3	8 vs 7.7	9.53E-2	0.930	0.24	0.811	0.57	0.570
	8 vs 7.4	0.73	0.470	1.48	0.139	0.40	0.686
	7.7 vs 7.4	0.80	0.423	1.09	0.269	0.21	0.828
Day 4	8 vs 7.7	1E5	1.000	0.50	0.653	0.28	0.752
	8 vs 7.4	1.11	0.285	0.15	0.891	0.51	0.580
	7.7 vs 7.4	1.96	0.068	0.59	0.565	0.31	0.765
Day 5	8 vs 7.7	0.98	0.308	0.36	0.758	0.88	0.374
	8 vs 7.4	1.26	0.213	0.57	0.589	2.46	0.023
	7.7 vs 7.4	0.20	0.852	0.17	0.863	1.88	0.065
Day 6	8 vs 7.7	1.16	0.274	0.89	0.382	1.18	0.269
	8 vs 7.4	0.24	0.816	2.36	0.024	1.24	0.244
	7.7 vs 7.4	0.89	0.380	0.81	0.398	1.97	0.041
Day 7	8 vs 7.7	0.20	0.847	0.75	0.477	1E5	1.000
	8 vs 7.4	1.43	0.171	1.26	0.230	1.65	0.120
	7.7 vs 7.4	1.34	0.196	0.44	0.645	1.65	0.100
Day 8	8 vs 7.7	0.19	0.865	0.20	0.850	1.32E-9	1.000
	8 vs 7.4	0.70	0.496	0.58	0.586	1.43	0.145
	7.7 vs 7.4	0.95	0.366	0.66	0.509	1.43	0.177

Supplementary material 2. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larval survival in laboratory experiments. Combined effects of pH and time (Ti) for pairs of levels of factor temperature (T) are shown.

Ti		pH 8		pH 7.7		pH 7.4	
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	19 vs 20.5	1E5	1.000	1E5	1.000	1E5	1.000
	19 vs 22.5	1E5	1.000	1E5	1.000	1E5	1.000
	20.5 vs 22.5	1E5	1.000	1E5	1.000	1E5	1.000
Day 2	19 vs 20.5	1.00	0.330	2.33	0.032	1.90	0.061
	19 vs 22.5	0.38	0.702	0.98	0.356	0.73	0.445
	20.5 vs 22.5	1.39	0.164	2.90	0.009	1.27	0.204
Day 3	19 vs 20.5	0.57	0.565	0.39	0.696	1.7241	0.113
	19 vs 22.5	1.76	0.090	0.84	0.420	2.5347	0.023
	20.5 vs 22.5	2.93	0.004	1.30	0.228	0.58293	0.542
Day 4	19 vs 20.5	1E5	1.000	0.52	0.589	1.372	0.173
	19 vs 22.5	0.73	0.454	1.70	0.107	3.4518	0.003
	20.5 vs 22.5	0.97	0.310	0.63	0.540	1.6898	0.088
Day 5	19 vs 20.5	0.75	0.464	1E5	1	9.09E-9	1.000
	19 vs 22.5	1.52	0.147	0.19	0.869	2.24	0.032
	20.5 vs 22.5	1.07	0.281	0.18	0.845	2.05	0.052
Day 6	19 vs 20.5	1.16	0.240	0.89	0.400	1.36	0.194
	19 vs 22.5	0.53	0.631	2.75	0.010	0.67	0.490
	20.5 vs 22.5	1.70	0.087	1.18	0.259	1.65	0.099
Day 7	19 vs 20.5	2.14	0.045	1.17	0.229	0.53452	0.549
	19 vs 22.5	2.66	0.008	2.28	0.033	0.53452	0.600
	20.5 vs 22.5	0.34	0.745	1.08	0.274	8.46E-9	1.000
Day 8	19 vs 20.5	0.63	0.531	0.54	0.618	0.73	0.486
	19 vs 22.5	1.99	0.068	2.27	0.036	0.32	0.754

20.5 vs 22.5	1.57	0.139	1.39
			0.190
			0.32
			0.761

Supplementary material 3. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH on body length are shown.

Ti	19°C		20.5°C		22.5°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	8 vs 7.7	1.43	0.138	1.72	0.077	1.26	0.209
	8 vs 7.4	1.92	0.047	0.54	0.748	1.30	0.192
	7.7 vs 7.4	2.40	0.016	2.06	0.029	0.17	0.969
Day 2	8 vs 7.7	1.45	0.152	0.83	0.435	1.66	0.110
	8 vs 7.4	1.12	0.262	1.24	0.229	3.56	0.005
	7.7 vs 7.4	0.62	0.579	0.67	0.574	1.74	0.069
Day 3	8 vs 7.7	2.50	0.018	0.55	0.606	1.89	0.064
	8 vs 7.4	1.40	0.168	0.90	0.390	2.81	0.007
	7.7 vs 7.4	0.95	0.356	1.45	0.152	0.80	0.429
Day 4	8 vs 7.7	0.74	0.465	0.52	0.663	1.33	0.178
	8 vs 7.4	2.76	0.009	1.13	0.248	2.59	0.006
	7.7 vs 7.4	2.14	0.037	0.70	0.531	0.94	0.371
Day 5	8 vs 7.7	1.00	0.33	1.78	0.072	1.46	0.166
	8 vs 7.4	3.03	0.005	3.13	0.003	1.08	0.313
	7.7 vs 7.4	2.05	0.045	0.71	0.510	2.17	0.028
Day 6	8 vs 7.7	0.14	0.96	0.43	0.720	1.25	0.189
	8 vs 7.4	1.23	0.188	3.25	0.002	2.39	0.024
	7.7 vs 7.4	1.23	0.233	2.70	0.011	1.66	0.107
Day 7	8 vs 7.7	1.21	0.227	1.78	0.069	1.63	0.088
	8 vs 7.4	3.33	0.003	4.13	0.001	1.94	0.049
	7.7 vs 7.4	2.44	0.016	2.00	0.048	2.54	0.012
Day 8	8 vs 7.7	1.26	0.236	1.40	0.167	2.17	0.009
	8 vs 7.4	3.40	0.004	1.12	0.269	3.20	0.004
	7.7 vs 7.4	2.56	0.012	2.14	0.040	3.45	0.002
Day 9	8 vs 7.7	1.26	0.202	1.61	0.088	1.55	0.106
	8 vs 7.4	1.02	0.344	0.57	0.744	0.86	0.472
	7.7 vs 7.4	0.91	0.405	1.11	0.303	0.92	0.412
Day 10	8 vs 7.7	1.26	0.194	1.10	0.277	2.41	0.005
	8 vs 7.4	2.48	0.01	1.64	0.078	0.83	0.533
	7.7 vs 7.4	2.57	0.01	1.03	0.337	2.77	0.005

Supplementary material 4. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH on post oral arm length are shown.

Supplementary material 4

Ti	19°C		20.5°C		22.5°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	8 vs 7.7	1.43	0.133	1.72	0.067	1.26	0.215
	8 vs 7.4	1.92	0.043	0.54	0.707	1.30	0.182
	7.7 vs 7.4	2.40	0.018	2.06	0.040	0.17	0.972
Day 2	8 vs 7.7	1.45	0.155	0.83	0.435	1.66	0.117
	8 vs 7.4	1.12	0.262	1.24	0.203	3.56	0.001
	7.7 vs 7.4	0.62	0.570	0.67	0.573	1.74	0.085
Day 3	8 vs 7.7	2.50	0.014	0.55	0.641	1.89	0.065
	8 vs 7.4	1.40	0.163	0.90	0.400	2.81	0.010
	7.7 vs 7.4	0.96	0.352	1.45	0.146	0.80	0.417
Day 4	8 vs 7.7	0.74	0.484	0.52	0.624	1.33	0.192
	8 vs 7.4	2.76	0.013	1.13	0.257	2.59	0.014
	7.7 vs 7.4	2.14	0.045	0.70	0.511	0.94	0.350
Day 5	8 vs 7.7	1.00	0.334	1.78	0.079	1.46	0.135
	8 vs 7.4	3.03	0.005	0.71	0.004	1.08	0.285
	7.7 vs 7.4	2.05	0.048	3.13	0.525	2.17	0.029
Day 6	8 vs 7.7	0.14	0.962	0.43	0.741	1.25	0.217
	8 vs 7.4	1.23	0.211	3.25	0.006	2.39	0.012
	7.7 vs 7.4	1.23	0.203	2.70	0.014	1.66	0.110
Day 7	8 vs 7.7	1.21	0.238	1.78	0.067	1.63	0.074
	8 vs 7.4	3.33	0.003	4.13	0.001	1.94	0.057
	7.7 vs 7.4	2.44	0.021	2.00	0.057	2.54	0.004
Day 8	8 vs 7.7	1.26	0.195	1.40	0.164	2.17	0.013
	8 vs 7.4	3.40	0.001	1.12	0.249	3.20	0.001
	7.7 vs 7.4	2.56	0.013	2.14	0.026	3.45	0.001
Day 9	8 vs 7.7	1.26	0.191	1.61	0.083	1.55	0.092
	8 vs 7.4	1.02	0.360	0.57	0.714	0.86	0.508
	7.7 vs 7.4	0.91	0.372	1.11	0.260	0.92	0.407
Day 10	8 vs 7.7	1.26	0.195	1.10	0.287	2.41	0.007
	8 vs 7.4	2.48	0.019	1.64	0.079	0.83	0.521
	7.7 vs 7.4	2.57	0.007	1.03	0.301	2.77	0.001

Supplementary material 5. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of pH and time (Ti) for pairs of levels of factor temperature (T) on body length are shown.

Supplementary material 5

Ti	pH 8		pH 7.7		pH 7.4		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	19 vs 20.5	2.22	0.012	4.02	0.001	1.05	0.310
	19 vs 22.5	1.27	0.214	1.28	0.203	3.10	0.011
	20.5 vs 22.5	3.23	0.001	4.74	0.001	2.43	0.017
Day 2	19 vs 20.5	1.69	0.103	0.38	0.782	0.29	0.856
	19 vs 22.5	4.04	0.001	3.51	0.002	1.76	0.070
	20.5 vs 22.5	6.60	0.001	4.10	0.002	1.51	0.139
Day 3	19 vs 20.5	0.34	0.735	3.80	0.001	1.10	0.290
	19 vs 22.5	1.45	0.146	2.46	0.022	0.96	0.362
	20.5 vs 22.5	1.35	0.184	1.20	0.225	0.83	0.423
Day 4	19 vs 20.5	0.68	0.518	0.43	0.712	3.02	0.009
	19 vs 22.5	0.71	0.520	0.80	0.436	1.43	0.144
	20.5 vs 22.5	1.34	0.202	0.61	0.569	2.12	0.028
Day 5	19 vs 20.5	1.56	0.094	0.23	0.911	1.15	0.252
	19 vs 22.5	1.21	0.255	1.05	0.287	1.96	0.063
	20.5 vs 22.5	2.52	0.014	0.91	0.372	0.76	0.498
Day 6	19 vs 20.5	1.88	0.054	1.86	0.069	0.55	0.611
	19 vs 22.5	0.91	0.371	0.26	0.907	0.13	0.976
	20.5 vs 22.5	1.26	0.204	1.96	0.050	0.59	0.555
Day 7	19 vs 20.5	2.76	0.006	1.31	0.186	1.14	0.244
	19 vs 22.5	0.60	0.599	2.20	0.023	2.24	0.020
	20.5 vs 22.5	2.82	0.012	0.45	0.809	1.02	0.322
Day 8	19 vs 20.5	0.85	0.403	1.96	0.056	1.64	0.080

	19 vs 22.5	0.69	0.572	2.85	0.003	1.95	0.039
	20.5 vs 22.5	1.53	0.147	0.89	0.453	0.58	0.617
Day 9	19 vs 20.5	1.67	0.081	1.41	0.148	2.39	0.018
	19 vs 22.5	2.04	0.031	1.88	0.051	3.10	0.002
	20.5 vs 22.5	0.73	0.617	0.56	0.730	0.44	0.855
Day 10	19 vs 20.5	0.97	0.363	1.40	0.115	2.17	0.029
	19 vs 22.5	2.09	0.017	1.44	0.136	3.52	0.001
	20.5 vs 22.5	2.06	0.026	0.68	0.625	1.54	0.098

Supplementary material 6. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of pH and time (Ti) for pairs of levels of factor temperature (T) on post oral arm length are shown.

Ti	pH 8		pH 7.7		pH 7.4		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	19 vs 20.5	2.21	0.012	4.02	0.001	1.05	0.285
	19 vs 22.5	1.27	0.198	1.28	0.230	3.10	0.005
	20.5 vs 22.5	3.23	0.001	4.74	0.001	2.43	0.015
Day 2	19 vs 20.5	1.69	0.072	0.38	0.774	0.29	0.870
	19 vs 22.5	4.04	0.001	3.51	0.002	1.76	0.080
	20.5 vs 22.5	6.60	0.001	4.10	0.002	1.51	0.132
Day 3	19 vs 20.5	0.34	0.774	3.80	0.002	1.10	0.285
	19 vs 22.5	1.45	0.145	2.46	0.020	0.96	0.323
	20.5 vs 22.5	1.35	0.162	1.20	0.252	0.83	0.449
Day 4	19 vs 20.5	0.68	0.512	0.43	0.723	3.02	0.007
	19 vs 22.5	0.71	0.507	0.80	0.479	1.43	0.154
	20.5 vs 22.5	1.34	0.187	0.61	0.523	2.12	0.042
Day 5	19 vs 20.5	1.56	0.107	0.23	0.912	1.15	0.245
	19 vs 22.5	1.21	0.212	1.05	0.302	1.96	0.065
	20.5 vs 22.5	2.52	0.009	0.91	0.358	0.76	0.481
Day 6	19 vs 20.5	1.88	0.065	1.86	0.070	0.55	0.620
	19 vs 22.5	0.91	0.368	0.26	0.909	0.13	0.972
	20.5 vs 22.5	1.26	0.216	1.96	0.048	0.59	0.592
Day 7	19 vs 20.5	2.76	0.006	1.31	0.184	1.14	0.257
	19 vs 22.5	0.60	0.626	2.20	0.022	2.24	0.017
	20.5 vs 22.5	2.82	0.003	0.45	0.769	1.02	0.295
Day 8	19 vs 20.5	0.85	0.437	1.96	0.045	1.64	0.099
	19 vs 22.5	0.69	0.587	2.85	0.002	1.95	0.042
	20.5 vs 22.5	1.53	0.123	0.89	0.438	0.58	0.638
Day 9	19 vs 20.5	1.67	0.081	1.41	0.160	2.39	0.010
	19 vs 22.5	2.04	0.027	1.88	0.059	3.10	0.001
	20.5 vs 22.5	0.73	0.627	0.56	0.704	0.44	0.863
Day 10	19 vs 20.5	0.97	0.366	1.41	0.137	2.17	0.030
	19 vs 22.5	2.09	0.019	1.44	0.125	3.52	0.001
	20.5 vs 22.5	2.06	0.034	0.68	0.648	1.54	0.093

Supplementary material 7. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH on stomach volume are shown.

Supplementary material 7

Ti	19°C		20.5°C		22.5°C		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	8 vs 7.7	1.43	0.158	1.72	0.088	1.26	0.215
	8 vs 7.4	1.92	0.043	0.54	0.694	1.30	0.185
	7.7 vs 7.4	2.40	0.013	2.06	0.037	0.17	0.969
Day 2	8 vs 7.7	1.45	0.158	0.83	0.412	1.66	0.099
	8 vs 7.4	1.12	0.269	1.24	0.198	3.56	0.001
	7.7 vs 7.4	0.62	0.545	0.67	0.543	1.74	0.106
Day 3	8 vs 7.7	2.50	0.025	0.55	0.611	1.89	0.072
	8 vs 7.4	1.40	0.178	0.90	0.390	2.81	0.007
	7.7 vs 7.4	0.95	0.348	1.45	0.122	0.80	0.452
Day 4	8 vs 7.7	0.74	0.479	0.52	0.648	1.33	0.192
	8 vs 7.4	2.76	0.015	1.13	0.276	2.59	0.013
	7.7 vs 7.4	2.14	0.039	0.70	0.495	0.94	0.348
Day 5	8 vs 7.7	1.00	0.343	1.78	0.065	1.46	0.150
	8 vs 7.4	3.03	0.004	3.13	0.002	1.08	0.274
	7.7 vs 7.4	2.05	0.042	0.71	0.562	2.17	0.029
Day 6	8 vs 7.7	0.14	0.960	0.43	0.763	1.25	0.221
	8 vs 7.4	1.23	0.210	3.25	0.004	2.39	0.020
	7.7 vs 7.4	1.23	0.235	2.70	0.006	1.66	0.098
Day 7	8 vs 7.7	1.21	0.239	1.78	0.067	1.63	0.084
	8 vs 7.4	3.33	0.001	4.13	0.001	1.94	0.058
	7.7 vs 7.4	2.44	0.014	2.00	0.053	2.54	0.008
Day 8	8 vs 7.7	1.26	0.217	1.40	0.150	2.17	0.005
	8 vs 7.4	3.40	0.002	1.12	0.274	3.20	0.003
	7.7 vs 7.4	2.56	0.012	2.14	0.032	3.45	0.001
Day 9	8 vs 7.7	1.26	0.213	1.61	0.079	1.55	0.104
	8 vs 7.4	1.02	0.355	0.57	0.737	0.86	0.508
	7.7 vs 7.4	0.91	0.435	1.11	0.285	0.92	0.393
Day 10	8 vs 7.7	1.26	0.207	1.10	0.286	2.41	0.002
	8 vs 7.4	2.48	0.018	1.64	0.077	0.83	0.521
	7.7 vs 7.4	2.57	0.007	1.03	0.330	2.77	0.003

Supplementary material 8. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments. Combined effects of pH and time (Ti) for pairs of levels of factor temperature (T) on stomach volume are shown.

Ti	pH 8		pH 7.7		pH 7.4		
	Comparisons	t	P-perm	t	P-perm	t	P-perm
Day 1	19 vs 20.5	2.21	0.012	4.02	0.001	1.05	0.275
	19 vs 22.5	1.27	0.188	1.28	0.191	3.10	0.003
	20.5 vs 22.5	3.23	0.001	4.74	0.001	2.43	0.016
Day 2	19 vs 20.5	1.69	0.073	0.38	0.786	0.29	0.868
	19 vs 22.5	4.04	0.001	3.51	0.002	1.76	0.089
	20.5 vs 22.5	6.60	0.001	4.10	0.001	1.51	0.126
Day 3	19 vs 20.5	0.34	0.751	3.80	0.001	1.10	0.277
	19 vs 22.5	1.45	0.167	2.46	0.020	0.96	0.358
	20.5 vs 22.5	1.35	0.184	1.20	0.240	0.83	0.432
Day 4	19 vs 20.5	0.68	0.530	0.43	0.719	3.02	0.008
	19 vs 22.5	0.71	0.499	0.80	0.456	1.43	0.153
	20.5 vs 22.5	1.34	0.172	0.61	0.553	2.12	0.040
Day 5	19 vs 20.5	1.56	0.100	0.23	0.920	1.15	0.226
	19 vs 22.5	1.21	0.248	1.05	0.307	1.96	0.057
	20.5 vs 22.5	2.52	0.012	0.91	0.380	0.76	0.476
Day 6	19 vs 20.5	1.88	0.056	1.86	0.082	0.55	0.611
	19 vs 22.5	0.91	0.385	0.26	0.911	0.13	0.970
	20.5 vs 22.5	1.26	0.199	1.96	0.052	0.59	0.572
Day 7	19 vs 20.5	2.76	0.007	1.31	0.193	1.14	0.245
	19 vs 22.5	0.60	0.592	2.20	0.026	2.24	0.022
	20.5 vs 22.5	2.82	0.007	0.45	0.800	1.02	0.315
Day 8	19 vs 20.5	0.85	0.422	1.96	0.041	1.64	0.096

	19 vs 22.5	0.69	0.597	2.85	0.001	1.95	0.046
	20.5 vs 22.5	1.53	0.147	0.89	0.434	0.58	0.634
Day 9	19 vs 20.5	1.67	0.070	1.41	0.133	2.39	0.014
	19 vs 22.5	2.04	0.030	1.88	0.047	3.10	0.003
	20.5 vs 22.5	0.73	0.644	0.56	0.730	0.44	0.853
Day 10	19 vs 20.5	0.97	0.345	1.41	0.118	2.17	0.028
	19 vs 22.5	2.09	0.013	1.44	0.113	3.52	0.001
	20.5 vs 22.5	2.06	0.029	0.68	0.649	1.54	0.102

Supplementary material 9. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH' obtained in the permutational ANOVA on *Paracentrotus lividus* settlement in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH, and pH and time (Ti) for pairs of levels of factor temperature (T) are shown.

19°C		20.5°C		22.5°C		
Comparisons	t	P-perm	t	P-perm	t	P-perm
8 vs 7.7	1.13	0.274	2.02	0.090	2.65	0.025
8 vs 7.4	4.78	0.003	1.89	0.078	3.38	0.008
7.7 vs 7.4	3.84	0.008	0.89	0.399	4.44	0.003
pH 8		pH 7.7		pH 7.4		
Comparisons	t	P-perm	t	P-perm	t	P-perm
19 vs 20.5	1.89	0.094	4.44	0.004	13.74	0.001
19 vs 22.5	1.07	0.316	2.71	0.030	--	1.000
20.5 vs 22.5	2.54	0.022	1.93	0.071	13.74	0.001

Supplementary material 10. Results of pair-wise tests examining the significant interaction of factors 'Temperature x pH' obtained in the permutational ANOVA on postlarvae test diameter in laboratory experiments. Combined effects of temperature (T) and time (Ti) for pairs of levels of factor pH, and pH and time (Ti) for pairs of levels of factor temperature (T) are shown.

19°C		20.5°C		22.5°C		
Comparisons	t	P-perm	t	P-perm	t	P-perm
8 vs 7.7	1.57	0.116	3.17	0.006	2.97	0.004
8 vs 7.4	6.81	0.001	1.49	0.138	11.39	0.001
7.7 vs 7.4	7.48	0.001	1.95	0.071	7.97	0.001
pH 8		pH 7.7		pH 7.4		
Comparisons	t	P-perm	t	P-perm	t	P-perm
19 vs 20.5	1.08	0.310	3.05	0.001	8.59	0.001
19 vs 22.5	0.74	0.472	0.13	0.878	--	--
20.5 vs 22.5	2.16	0.035	4.47	0.001	8.59	0.001

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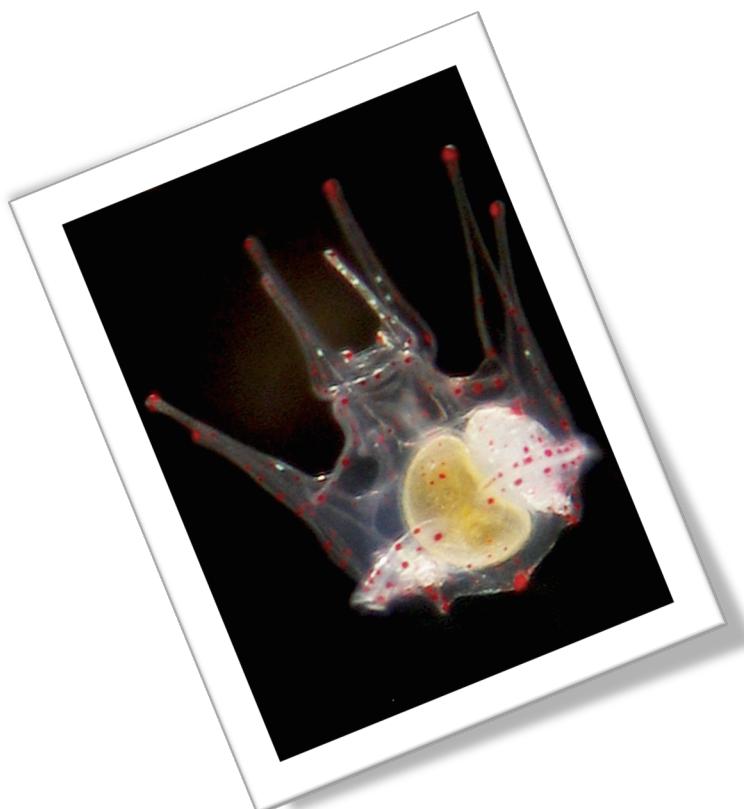
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**CHAPTER 6: EFFECTS OF NATURAL  
CURRENT PH VARIABILITY ON THE SEA  
URCHIN PARACENTROTUS LIVIDUS**



**CHAPTER 6: EFFECTS OF NATURAL CURRENT PH  
VARIABILITY ON THE SEA URCHIN PARACENTROTUS LIVIDUS  
LARVAE DEVELOPMENT AND SETTLEMENT.**

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## Abstract

One of the most important environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates is ocean pH. In the last decade, the effects of decreasing ocean pH as a result of climate change processes on marine organisms have been target of much research. However, the effects of natural pH variability in the species' niche have been largely neglected. Marine coastal habitats are characterized by a high environmental variability and, in some cases, organisms are already coping with pH values predicted by the end of the century. It is thought that because of adaptation or acclimation to natural environmental variability, intertidal species may have some resilience to future changes. In this study, we explored the sensitivities of the sea urchin *Paracentrotus lividus* during its larvae development and settlement undergoing two different daily pH frequencies (24h fluctuation from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH) that have been currently recorded in the sampling region (Canary Islands). Results showed that, despite larvae development was slightly enhanced by moderated fluctuating pH regimes, *P. lividus* larva was able to develop normally in both, fluctuating and constant, pH environments.

*Keywords:* pH, sea urchin, larvae development, settlement, *Paracentrotus lividus*.

## Resumen

El pH del océano es uno de los principales factores ambientales que controla la distribución, fisiología, morfología y comportamiento de los invertebrados marinos. En las últimas décadas, los efectos de la disminución del pH oceánico como consecuencia del cambio climático (Acidificación Oceánica) han sido objeto de muchas investigaciones. Sin embargo, la variabilidad que existe de manera natural en los ecosistemas, y a la que muchas especies están sometidas en sus nichos biológicos, ha sido ignorada. Los ambientes costeros marinos se caracterizan por una gran variabilidad ambiental y, en algunos casos, los organismos se enfrentan a fluctuaciones en los valores de pH que alcanzan valores que están previstos para final de siglo. Se piensa que, dado que tienen que convivir con una enorme variabilidad ambiental, las especies intermareales tendrán una mayor capacidad para adaptarse a los futuros cambios previstos como consecuencia del cambio climático. En este trabajo, hemos explorado las sensibilidades del erizo de mar *Paracentrotus lividus* durante su desarrollo larvario y asentamiento, sometiéndolo a dos frecuencias diarias de pH (fluctuación de 24h de 7'7 a 8'1 unidades de pH, y un tratamiento de pH constante a 8'1 unidades). Nuestros resultados mostraron que, a pesar de que el desarrollo larvario fue ligeramente favorecido por el ambiente fluctuante, la larva de *P.lividus* es capaz de desarrollarse con normalidad en ambos ambientes.

*Palabras clave:* pH, erizo de mar, desarrollo larvario, asentamiento, *Paracentrotus lividus*.

## Introduction

Marine coastal habitats are characterized by a high environmental variability. Especially in temperate regions, physico-chemical parameters such as temperature, pH, salinity, dissolved oxygen, turbidity, and other factors, suffer a wide variation that occurs on several spatial and temporal scales. These factors determine the features of coastal ecosystems and its variability will be the result of complex interactions between physical, chemical and biological processes (e.g. biological activity, currents, tidal excursions, background oceanography, freshwater inputs, upwellings, etc). Variations in physico-chemical parameters are therefore highly site-dependent with differences in their amplitude and frequency.

pH is, beside temperature, one of the most important environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates (Doney et al. 2009). In the last decade, the effects of decreasing ocean pH as a result of climate change processes (i.e. ocean acidification, OA) on marine organisms have been target of much research (see reviews by Byrne et al. 2013; Byrne and Przeslawski 2013; Dupont and Thorndyke 2013). Most laboratory experiments have been focused on expected future values of pH, pCO<sub>2</sub> and saturation states for calcite and aragonite for the near future. However, it has been reported that pH in the oceans is not constant temporal and spatial variations exist (Hofmann et al. 2011), and this natural pH variability effects on species' niche has been neglected in experimental studies. Although the levels of pH in a given environment may vary considerably, accurate records of this parameter are only very recent and/ or fragmented (Dorey et al. 2013). Variation in seawater pH is higher in shallow temperate coastal environments due to their inherent ambient heterogeneity and photosynthetic activity (Middelboe and Hansen 2007). A pronounced 24h pH cycle has

been recorded in coastal environments, spanning an average of 0.24 pH units during a day (Wooton et al. 2008), although this variation depends on numerous parameters and differs at different environments. For instance, Hofmann et al. (2011) recorded high pH variability in coastal temperate sites such as estuaries, near-shore habitats and kelp forest environments of up to 0.99, 0.50 and 0.54 pH units, respectively. In shallow coastal environments the surface seawater pCO<sub>2</sub> is already at levels significantly higher and pH lower than expected values from equilibrium with current atmospheric levels (Fagan and Mackenzie 2007; Bates et al. 2010; Thomsen et al. 2010; Shamberger et al. 2011; Yu et al. 2011; Hofmann et al. 2011).

Marine invertebrates, including calcifiers, can thrive in markedly heterogeneous environments. Thus, we have to highlight the importance of understanding species' niche to define their sensitivity to environmental changes. It is thought that species that naturally experience a high environmental variability, such as intertidal and shallow water organisms and species with a broad latitudinal distribution, may be more resilient to environmental changes than those species from relatively invariable habitats (Melzner et al 2009; Talmage and Gobler 2009 2011; Moulin et al. 2011; Matson et al. 2012; Wolfe et al. 2013; Byrne et al. 2013). Natural variability may occur at rates much higher than the rate at which carbon dioxide is decreasing ocean pH as a result of climate change processes (Dore et al. 2009; Byrne et al. 2010). Therefore organisms from natural low pH and fluctuating habitats may already be experiencing pH values forecasted for the end of this century (Kelly and Hofmann 2013). Variability in pH could potentially promote acclimation or adaptation to acidification through repeated exposure to low pH conditions; alternatively, transient exposures to high pH conditions could buffer the effects of acidification by momentary relieving physiological stress (Hofmann et al. 2011). In this sense, natural fluctuation in pH may improve the resilience of marine populations. On the contrary, OA

could shift such natural variations towards even lower pH levels, becoming these heterogeneous environments more vulnerable sites for near future climate change scenarios. In addition, some studies show that some intertidal invertebrates are already living near their physiological tolerance limits (Tomanek 2008; Somero 2010), and shifting of baseline environmental conditions may push extremes to suboptimal or lethal levels. Therefore, the combination of a wide natural variability with the steady effects of acidification could produce extreme events with large impacts (Joint et al. 2011).

This study focused on studying larvae development and settlement of the sea urchin *Paracentrotus lividus* under fluctuating regimes of seawater pH. This species is widely distributed, and is known to cross latitudes, throughout the Mediterranean Sea and the NE Atlantic Ocean from Ireland to the Canary Islands, and habitats, from the intertidal to the subtidal. In the Canary Islands, *P. lividus* is found from the lowest intertidal, where it most commonly occupies crevices in tide-pools, to around 10 m depth in the subtidal (Girard et al. 2012). In the Archipelago, this sea urchin mainly inhabits environments with a dense algal cover where *Cystoseira abies-marina* is the dominant species. This typical habitat of the species is characterized by a wide daily pH oscillation with minimum values occurring early in the morning and maximum records in the evening, mainly due to the biological activity of abundant benthic photosynthetic organisms (Hernández et al. unpublished data). This daily cycle of seawater pH variation is readily explained by daily variations in photosynthesis and respiration, as well as in sea water temperature. Seawater pH increases when CO<sub>2</sub> is captured by photosynthetic organisms (macroalgae and phytoplankton) during the day and decreases during the night when respiration and diffusion with the atmosphere regulate CO<sub>2</sub> (Bensoussan and Gatuso 2007). The special nature of the volcanic archipelago of the Canary Islands result in a variety of coastal habitats that experience huge environmental variability in a limited space. The planktonic larvae stage of *P. lividus*

is estimated to last roughly 1 month and then settlement occurs. Larvae of many benthic species display active habitat selection mechanisms by responding to abiotic environmental or chemicals cues of different sources, such as conspecifics, host plants, preys, or surface-associated bacterial communities (biofilms). Macroalgae and their associated biofilms have been appointed as one of the main inducers producing effective cues to trigger metamorphosis and settlement of sea urchins (Pearce and Scheibling 1999; Swanson et al. 2006).

In this study we explored the sensitivities of *P. lividus* during its larvae development undergoing two different daily pH frequencies that are currently taking place in the sampling region (Hernández et al. unpublished data): a 24h fluctuation of seawater pH from 7.7 to 8.1 vs. a constant pH regime of 8.1 units (which is normally used as a control for experimental trials). We also tested whether pH fluctuations *per se* or any other cue related with the algae *Cystoseira abies-marina* induce settlement.

## Material and methods

### *Animal collection and spawning*

Mature *P. lividus* specimens (test diameter >24mm) were collected by scuba divers from subtidal rocky shores between 5 and 10 m depth. Individuals were collected in November of 2013 at the south coast of Tenerife Island (28°5'57''N, 16°36'53''W), during the spawning period known for the species (Girard et al. 2012).

Animals were induced to spawn by injection of 2 ml of KCl (0.5 M) through the peristomial membrane. Five males and six females, randomly selected in order to reduce experimental variability (Evan and Marshall 2005), were used for fertilization and sexual products mixed before putting gametes of each sex in contact. Sperm was collected dry and

kept on ice until usage. Eggs were collected in filtered seawater (FSW). Fertilization was done in a proportion of 1:1500 (eggs:sperm). Cleavaging embryos (two cell stage) were placed at a density of 15 individuals  $\text{mL}^{-1}$  in 20 L aquaria filled with FSW and constantly aerated.

#### *Experimental design and sea water chemistry*

When the embryos reached the gastrula stage, larvae were distributed into 2 L culture beakers at densities of 5 larvae  $\text{mL}^{-1}$ . Six culture beakers were maintained in a seawater table to keep temperature conditions. Seawater was replaced in each beaker each 24h. At day 4 post-fertilization, larvae were fed with red algae *Rhodomonas lens* at a concentration of 2000 cells  $\text{mL}^{-1}$ . The microalgae strain was provided by Spanish Oceanography Institute and cultured in the laboratory with enriched F/2 medium (Guillard and Ryther 1962) at 20 °C and a cycle 24h/0h light/dark. The tested exposure pH levels within the experiments did not have effect on algal growth (Dupont et al. 2012). Algae were separated from the growth medium by centrifugation and then suspended in fresh FSW before usage. The seawater inside the culture beakers was constantly aerated and homogenized using a paddle system (Strathmann 1987) that was moved by a micromotor keeping food and larvae in suspension.

Larvae of *P. lividus* were raised in two different treatments of pH ( $n=3$  for each treatment). The cultures were maintained at a salinity 36.7 and a temperature of 19 °C, corresponding to the natural conditions of seawater during November at the collection site. Larvae were cultured at contrasting pH fluctuation frequencies over a period of one month: 24h fluctuation from 7.7 to 8.1 units of pH (Treatment 1, T1), and constant pH treatment of 8.1 units of pH (Treatment 2, T2), corresponding to the present variability of productive (high benthic macroalgae cover) and unproductive environments (low benthic macroalgae cover), respectively, at the sampling region (Hernández et al. unpublished data) (Fig. 6.1).

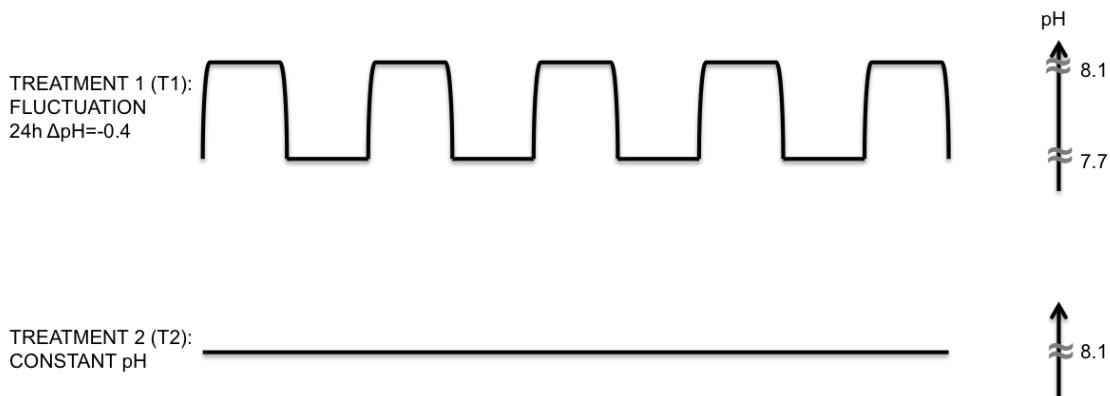


Figure 6.1 Diagram representing the seawater pH fluctuation frequencies used during the *Paracentrotus lividus* larvae experiment: (T1) 24h fluctuation from 7.7 to 8.1 units of pH and (T2) constant pH treatment of 8.1 units of pH.

When larvae within the cultures reached the competent stage and they were observed to have tube feet extending from the rudiment (Cameron and Hinegardner 1974), a settlement experiment was carried out. The settlement assays were conducted using 1L containers. Ten competent larvae were set in each beaker with glass plates with natural biofilm to induce settlement, corresponding to 6 different settlement treatments ( $n=3$  for each treatment): (t1) larvae that came from the 24h fluctuating treatment and continued in this pH regimen; (t2) larvae with the same conditions that in the previous treatment but with the addition of a covering of macroalgae (*Cystoceira abies-marina*) placed at the bottom of the beaker; (t3) larvae that came from the 24h fluctuating treatment and changed to constant pH treatment of 8.1 units; (t4) larvae that came from constant pH treatment (8.1 units) and changed to 24h fluctuating treatment; (t5) The same conditions that in treatment t4 but with the addition of a covering of algae (*Cystoceira abies-marina*) at the bottom of the beaker; (t6) larvae that came from constant pH treatment (8.1 units) and remained in these conditions.

The 50% treatment seawater was replaced every 24 h in order to create the fluctuation conditions, during which time any dead larvae were removed, minimising changes in water chemistry. This experiment was conducted without feeding and aeration.

To keep constant temperature conditions thermostat coolers (EHEIM AQUATICS, 50 W) were used. In order to control seawater pH, we used a computerised control system (AquaMedic) that regulated pH by the bubbling pure CO<sub>2</sub> directly into the water to a resolution of  $\pm 0.01$  pH units. Monitoring of temperature, pH<sub>NBS</sub> (Metrohm mobile meter with a Primatrode NTC IP pH electrode and temperature sensor) and salinity (handheld conductivity meter COND 315i) was performed daily. Seawater total alkalinity (TA) was measured for each treatment by titration. Other parameters of the seawater carbonate chemistry (*p*CO<sub>2</sub>, calcite saturation state ( $\Omega_c$ ) and aragonite saturation state ( $\Omega_a$ )) were calculated from TA and pH using CO2sys (Lewis and Wallace 1998). Calculations were based on a set of constants K1 and K2 from Mehrbach et al. (1973) (refit by Dickson and Millero 1987).

All experiments were conducted with FSW purified within a recirculating system provided with DRYDEN AQUA active filter media (AFM) bio-crystals; 50 µm, 10 µm and 1 µm UNICEL polyamide paper filters, as well as a UV-C AQUAEL 11W filter. FSW was prepared with the proper temperature and pH conditions for each treatment before using it.

#### *Biological measurements*

Larvae were sampled for a period of a month to quantify survival, growth, development and settlement. In each replicate beaker, three 2 mL aliquots were collected every second day and larvae were counted to estimate the density. Ten larvae in each replicate beaker were photographed every sampling day using a digital camera mounted on a binocular microscope. Several parameters were measured on each larva: body length

(BL), post-oral arm length (PL) and stomach diameter (S1 & S2) (Fig.6.2). Stomach volume (SV) was calculated as  $SV = \frac{4}{3} \pi ((S1+S2)/4)^3$  (Dorey et al. 2013).

After 7 days of competent larvae addition to the experimental containers for settlement experiments, the numbers of swimming, dead and settled postlarvae were counted in each treatment.

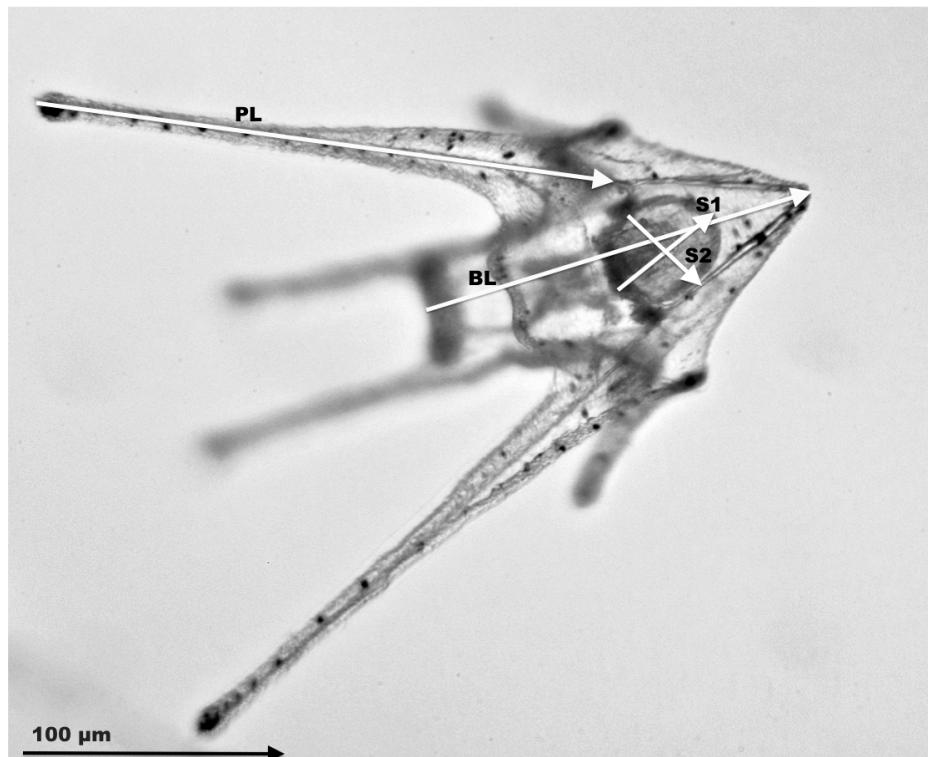


Figure 6.2 Morphometric measurements taken for each sea urchin *Paracentrotus lividus* larvae: body length (BL), post oral arm length (PL) and stomach diameters (S1 & S2).

#### Data analyses

In order to assess the effects of pH fluctuation on larval survival, data were analysed by means of a two-way permutational analysis of variance (PERANOVA) (Anderson 2001). A two-way design was conducted with pH (2 levels) and time (13 levels) as fixed factors.

To evaluate the effect on morphometric measurements (BL, PL), two-way permutational multivariate analysis of variance (PERMANOVA) was performed. A two-way design was carried out with factors pH (2 levels) and time (13 levels) as fixed factors.

To assess the impact on stomach volume (SV), a two-way permutational ANOVA was performed, using the same two-way design.

The effect of different settlement treatments (pH fluctuation frequencies and/or presence or lack of algae cover) on settlement and postlarvae test diameter was analysed by means of one-way permutational analyses of variance (PERANOVAs). One-way designs were conducted with factor settlement treatment (6 levels) used as fixed factor.

Euclidean distances were used for all analyses of variance and respective significant terms were examined using *a posteriori* pairwise comparisons by permutations (Anderson 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws from the asymptotic permutation distribution. Principal Coordinates Analysis (PCO) ordinations of morphometric measurements data were used to identify similarities between observations. All statistical analyses were carried out using PRIMER 6 & PERMANOVA+ v. 1.0.1 software.

## Results

Physico-chemical parameters of seawater during the larvae experiment are given in table 6.1. Carbon dioxide partial pressure ( $p\text{CO}_2$ ) was increased at the fluctuating pH treatment, while saturation levels of calcite ( $\Omega_c$ ) and aragonite ( $\Omega_a$ ) were decreased. However, seawater was not saturated in respect to calcite or aragonite ( $\Omega_c, \Omega_a < 1$ ) in either treatments. The daily pH fluctuation in each experimental treatment in the larvae experiment is shown in figure 6.3.

Table 6.1. Physico-chemical seawater parameters for (A) the 24h pH fluctuation treatment from 7.7 to 8.1 units, and (B) the constant treatment of 8.1 units of pH tested for larvae of *Paracentrotus lividus*. T: seawater temperature (mean  $\pm$  SD), S: salinity (mean  $\pm$  SD), pH: seawater pH (mean  $\pm$  SD), pCO<sub>2</sub>: CO<sub>2</sub> partial pressure, TA: Total alkalinity,  $\Omega_c$ : Saturation level of calcite,  $\Omega_a$ : Saturation level of aragonite.

	A. Fluctuating treatment		B. Constant treatment	
	0 h	12 h	0 h	12 h
T (n=23)	19.04 $\pm$ 2.18	19.08 $\pm$ 0.22	18.80 $\pm$ 0.22	19.02 $\pm$ 0.18
S (n=23)	36.57 $\pm$ 2.18	36.82 $\pm$ 0.09	36.87 $\pm$ 0.08	36.85 $\pm$ 0.08
pH (n=23)	7.67 $\pm$ 0.05	8.00 $\pm$ 0.04	8.02 $\pm$ 0.02	8.02 $\pm$ 0.02
pCO <sub>2</sub>	1038.10		415.5	
TA (n = 3)	2303.08 $\pm$ 53.01		2315.43 $\pm$ 38.57	
$\Omega_c$	2.12		4.24	
$\Omega_a$	1.38		2.75	

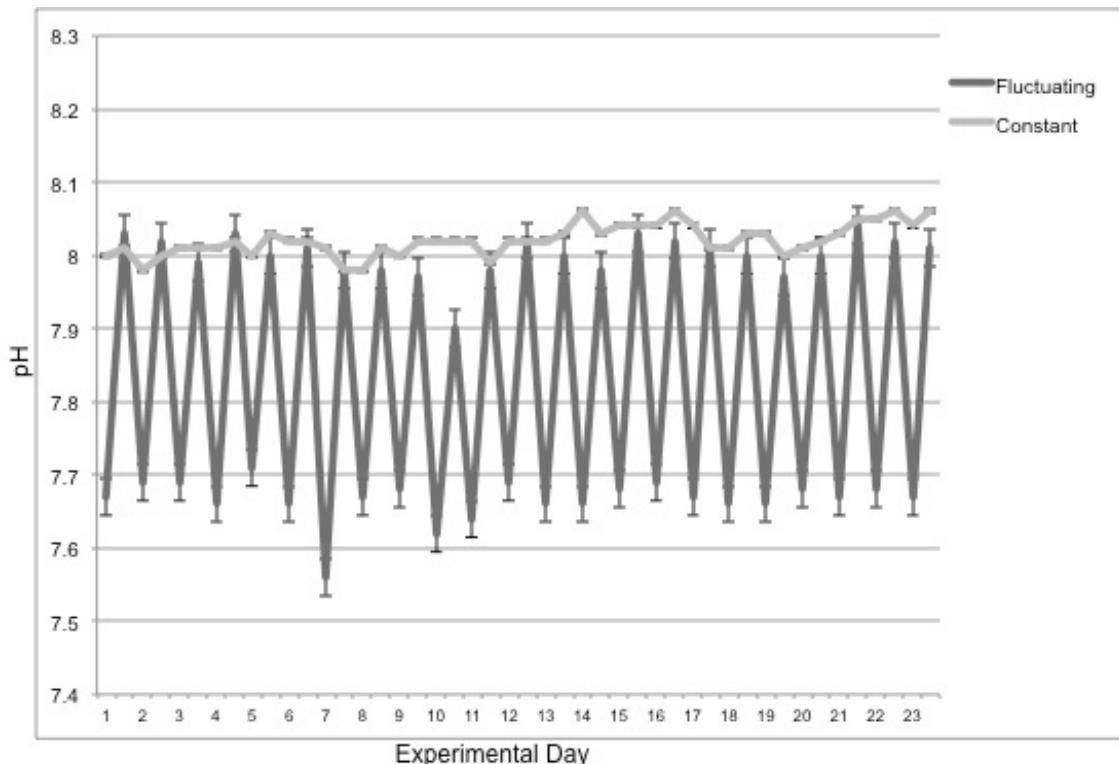


Figure 6.3. Daily measurements of seawater pH (mean  $\pm$  SD) recorded in both fluctuating and constant experimental treatments used for the larvae experiment of the sea urchin *Paracentrotus lividus*.

No effect on survival was detected between the different pH frequencies, neither in the interaction of factors ‘pH x Time’ (table 6.2), meaning that there were not different responses of larval survival at each treatment of pH tested with time (Fig. 6.4). The analysis only detected a clear effect of time on decreasing larval survival regardless of the pH regimen tested (table 6.2, Fig. 6.4).

Table 6.2. Results of the two-way permutational ANOVA analyzing larval survival of the sea urchin *Paracentrotus lividus* in laboratory experiments, testing the effects of seawater pH daily fluctuations by means of comparing a 24h pH fluctuation treatment from 7.7 to 8.1 units, and a constant treatment of 8.1 units of pH during the larval cycle of the species. The factors included in the model are: pH and Ti: Time.

Source of variation	df	SS	MS	Pseudo-F	P(perm)
pH	1	6.35E-2	6.35E-2	7.20E-2	0.800
Ti	13	646.92	49.76	56.44	0.001
pH *Ti	13	10.02	0.77	0.87	0.587

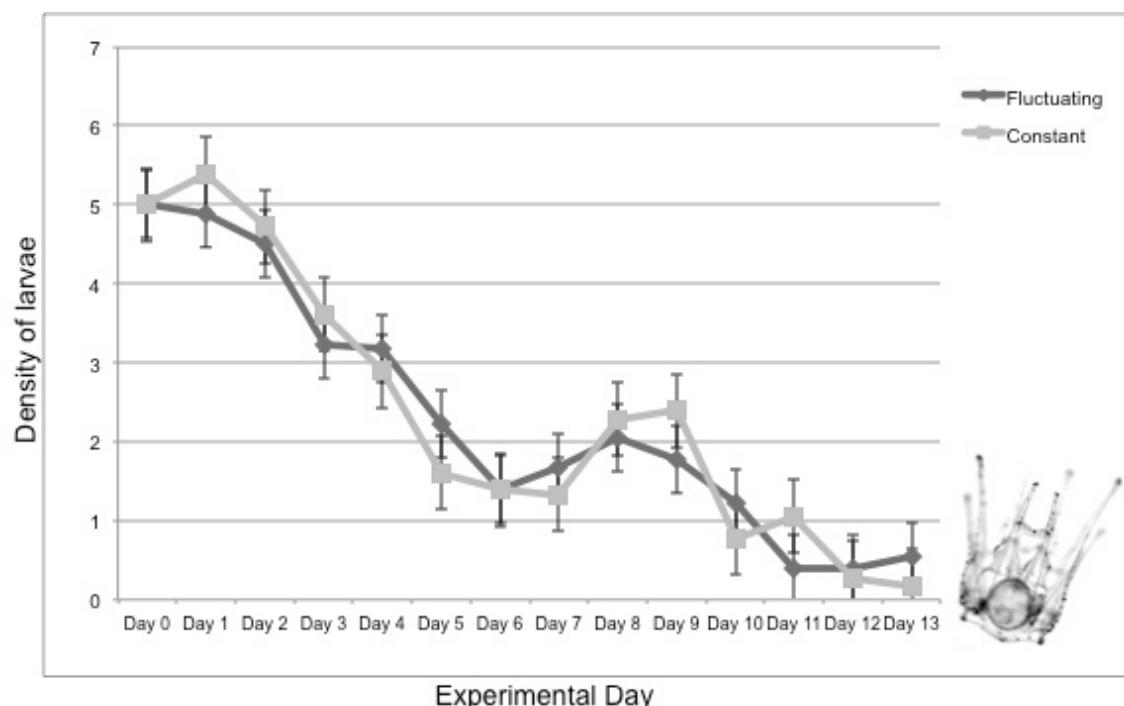


Figure 6.4. Density of larvae of the sea urchin *Paracentrotus lividus* (mean  $\pm$  SD) during the course of laboratory experiments testing the effects of contrasting treatments of pH frequencies: 24h fluctuation treatment from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH.

Results of the PERMANOVA analysing the morphometric measurements of the studied species showed a significant interaction of factors ‘pH x Time’ (Table 6.3A), indicating that the influence of pH on the BL and PL varied significantly across time during the larval development cycle in different manners in both pH treatments. However, *a posteriori* pairwise tests showed that in the majority of the sampling times there were not significant differences between treatments on larvae growth (Supplementary material 1; Fig 6.5).

Table 6.3. Results of the two-way (A) PERMANOVA analyzing body length and post oral arm length; and (B) permutational ANOVA testing stomach volume (SV) of larvae of the sea urchin *Paracentrotus lividus* in laboratory experiments testing the effects of seawater pH daily fluctuation, comparing a 24h pH fluctuation treatment (7.7 to 8.1 units), and a constant treatment (8.1 units) during the larval cycle of the species. In the respective models the factors included are: pH and Ti: Time.

Source of variation	A. Morphometric measurements				B. Stomach volume				Pseudo-F	P(perm)
	df	SS	MS	Pseudo-F	df	SS	MS	Pseudo-F		
pH	1	1.87E-2	1.87E-2	1.01	0.340	1	1.87E-2	1.87E-2	1.01	0.304
Ti	12	17.54	1.46	78.71	0.001	12	17.54	1.46	78.71	0.001
pH *Ti	12	0.61	5.05E-2	2.72	0.001	12	0.61	5.05E-2	2.72	0.002

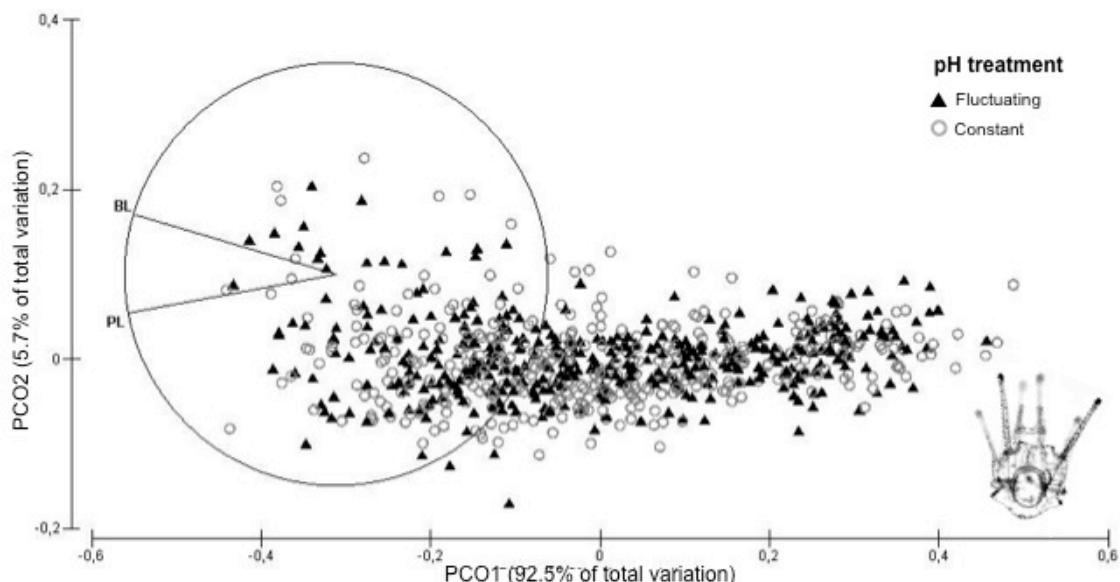


Fig. 5. PCO ordinations showing the effect of different treatments of pH frequencies (24h fluctuation treatment from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH).on *Paracentrotus lividus* larvae development. Vectors represent studied variables. BL: Body Length, PL: Post oral arm Length. Percentages of variation explained by each of the axes are given in brackets.

Stomach volume results revealed a significant interaction of factors ‘pH x Time’ (Table 6.3B), indicating that the influence of pH on the SV varied significantly across time during *P.lividus* larval development cycle. Nevertheless, pairwise analyses only showed significant differences between treatments in two of the thirteen sampling days and marginally significant differences in two additional sampling times (Supplementary material 2; Fig. 6.6).

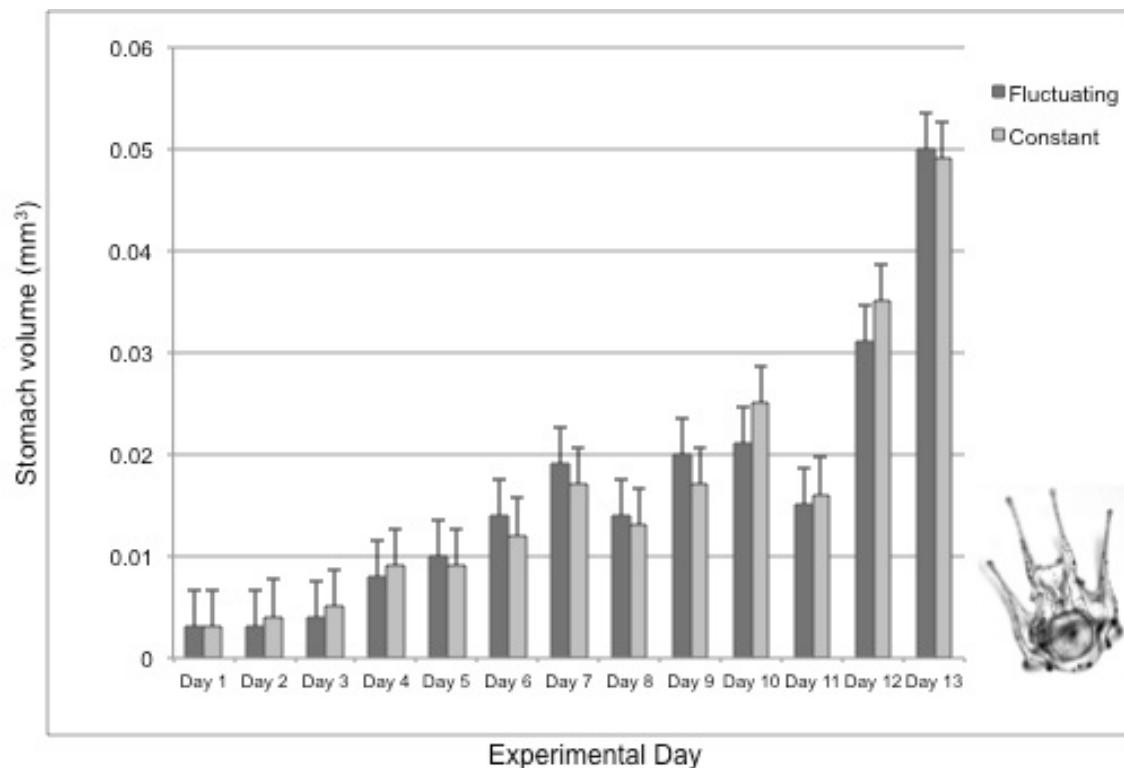


Figure 6.6. Stomach volume (mm<sup>3</sup>) of larvae of the sea urchin *Paracentrotus lividus* (mean ± SD) at laboratory experiments testing the effects of different treatments of pH frequencies: fluctuating (from 7.7 to 8.1 units of pH) and constant (≈8.1).

With regard to developmental dynamics, larvae with 6 arms appeared at day 11 of experiment in treatment 1 (fluctuating), and two days later on treatment 2 (constant). The same delay was observed with 8 arms larvae and the competent stage (Fig. 6.7).

Table 6.4. Physico-chemical seawater parameters for each experimental treatment testing the effects of seawater pH daily fluctuation on settlement *Paracentrotus lividus*. Experimental treatments included larvae that were raised at (t1) a 24h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoceira abies marina*), (t3) the 24h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 24h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions. T: seawater temperature (mean  $\pm$  SD), S: salinity (mean  $\pm$  SD), pH: seawater pH (mean  $\pm$  SD),  $p\text{CO}_2$ : CO<sub>2</sub> partial pressure, TA: Total alkalinity,  $\Omega_c$ : Saturation level of calcite,  $\Omega_a$ : Saturation level of aragonite.

	t1		t2		t3		t4		t5		t6	
	0h	12h										
T(n=7)	19.04 $\pm$ 0.30	19.13 $\pm$ 0.30	19.05 $\pm$ 0.30	19.03 $\pm$ 0.26	19.12 $\pm$ 0.32	19.12 $\pm$ 0.32	19.00 $\pm$ 0.26	19.02 $\pm$ 0.26	19.00 $\pm$ 0.27	19.10 $\pm$ 0.28	19.14 $\pm$ 0.27	19.10 $\pm$ 0.28
S(n=7)	37.01 $\pm$ 0.16	36.97 $\pm$ 0.12	36.78 $\pm$ 1.31	36.95 $\pm$ 0.12	37.02 $\pm$ 0.14	36.99 $\pm$ 0.13	37.03 $\pm$ 0.13	36.98 $\pm$ 0.11	37.06 $\pm$ 0.15	36.94 $\pm$ 0.11	37.02 $\pm$ 0.25	36.98 $\pm$ 0.13
pH(n=7)	7.67 $\pm$ 0.04	8.00 $\pm$ 0.04	7.69 $\pm$ 0.02	8.00 $\pm$ 0.03	8.11 $\pm$ 0.02	8.12 $\pm$ 0.01	7.69 $\pm$ 0.03	8.00 $\pm$ 0.04	7.69 $\pm$ 0.03	8.01 $\pm$ 0.03	8.11 $\pm$ 0.02	8.12 $\pm$ 0.02
$p\text{CO}_2$	1062.50		1018.40		319.00		1015.30		1012.70		332.00	
TA (n = 1)	2360.88		2377.46		2289.75		2373.47		2367.71		2378.70	
$\Omega_c$	2.19		2.29		4.99		2.29		2.28		5.20	
$\Omega_a$	1.42		1.49		3.24		1.49		1.48		3.38	

Physico-chemical parameters of seawater during the settlement experiment are given in table 6.4. Carbon dioxide partial pressure ( $p\text{CO}_2$ ) was increased at the fluctuating pH treatments (t1, t2, t4 and t5), while saturation levels of calcite ( $\Omega_c$ ) and aragonite ( $\Omega_a$ ) were decreased. However, seawater was not saturated in respect to calcite or aragonite ( $\Omega_c, \Omega_a < 1$ ) in either treatments. The daily pH fluctuation in each experimental treatment in settlement experiment is shown in figure 6.8.

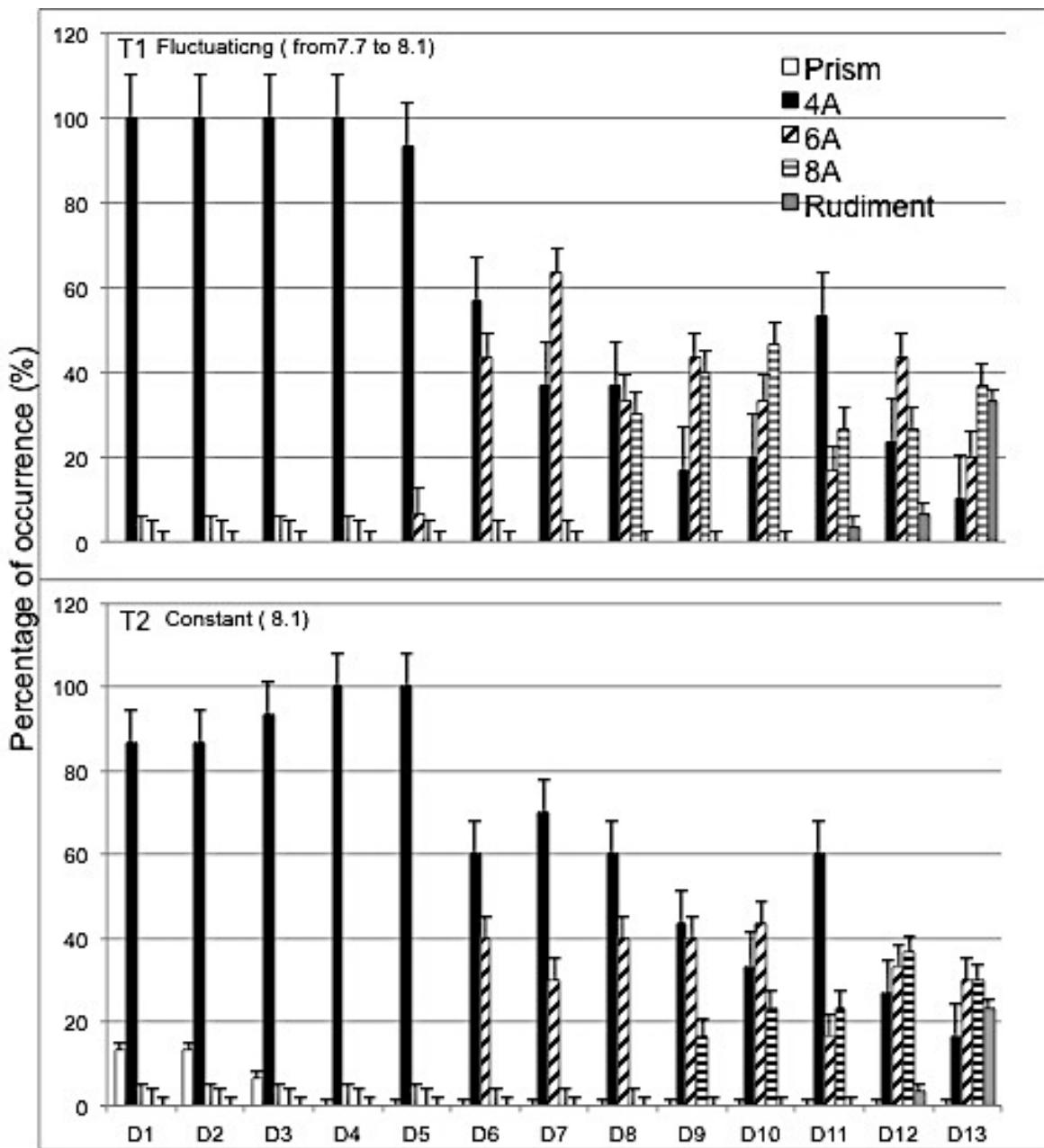


Figure 6.7. Developmental dynamics of *Paracentrotus lividus* larvae during the experiment showing the percentage of occurrence of the different larval stages (prism, 4 arms (4A), 6 arms (6A), 8 arms (8A) and rudiment) in the pH treatments tested: (T1) 24h fluctuation from 7.7 to 8.1 units of pH and (T2) constant pH treatment of 8.1 units of pH.

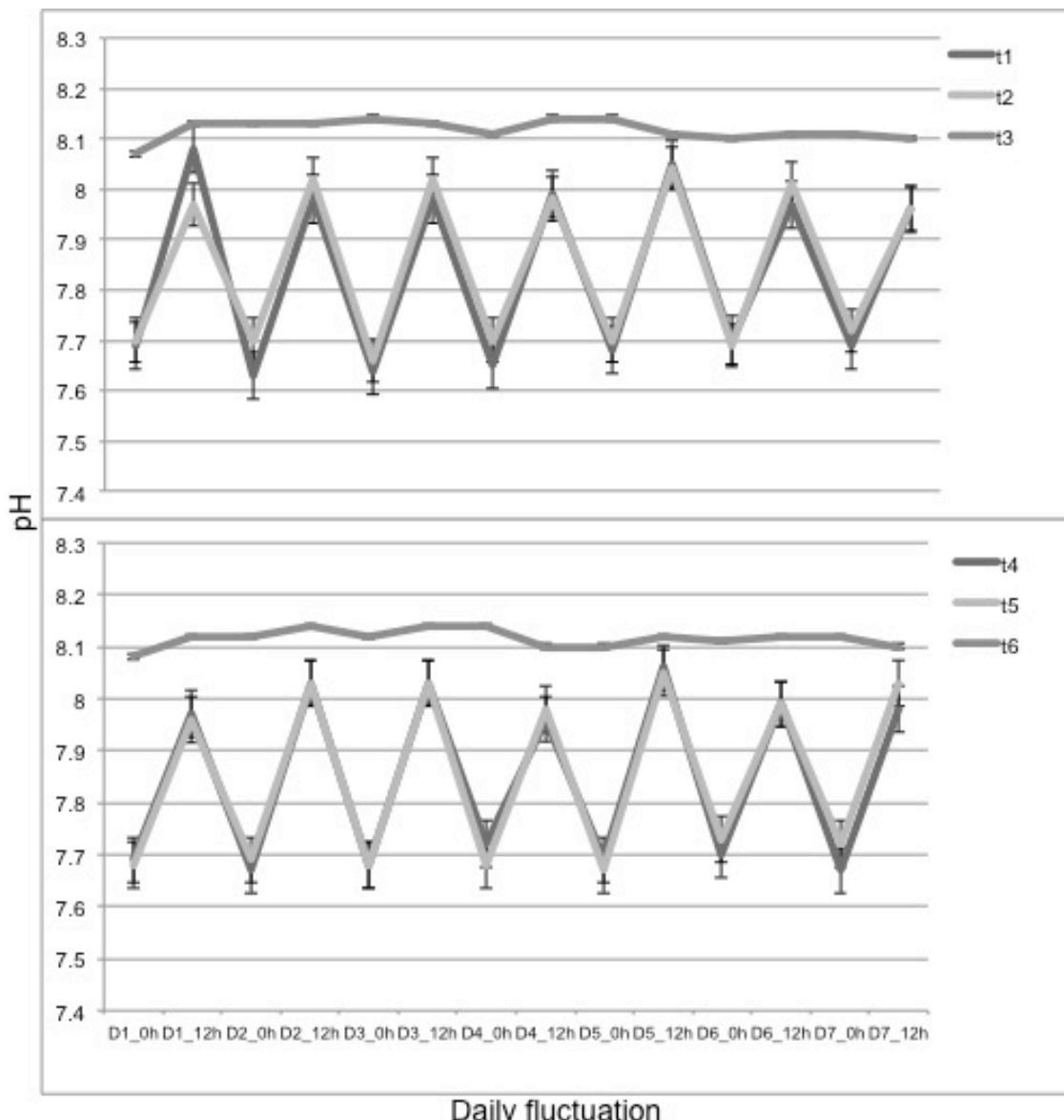


Figure 6.8. Daily measurements of seawater pH (mean  $\pm$  SD) recorded in each experimental treatment used for the settlement experiment of *Paracentrotus lividus*: larvae that were raised at (t1) a 24h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoceaera abies marina*), (t3) the 24h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 24h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions.

Postlarvae settlement showed a significant effect of factor settlement treatment (Table 6.5A), showing that settlement of the species varied with each treatment. *A posteriori* pairwise test revealed that treatments t2 and t5, which used an addition of a covering of algae (*Cystoceaera abies-marina*), had a similar response on settlement and was significantly different from the other treatments (Supplementary material 3; Fig. 6.9).

Table 6.5. Results of the one-way permutational ANOVA analyzing (A) settlement, and (B) test diameter of *Paracentrotus lividus* postlarvae, testing the effects of seawater pH daily fluctuations. The factor included in each model is pH.

**A. Settlement**

Source of variation	df	SS	MS	Pseudo-F	P(perm)
Settlement treatment	5	209.89	41.98	7.48	0.003
<b>B. Diameter</b>					
Source of variation	df	SS	MS	Pseudo-F	P(perm)
Settlement treatment	1	2.86E-5	2.86E-5	3.04E-2	0.875

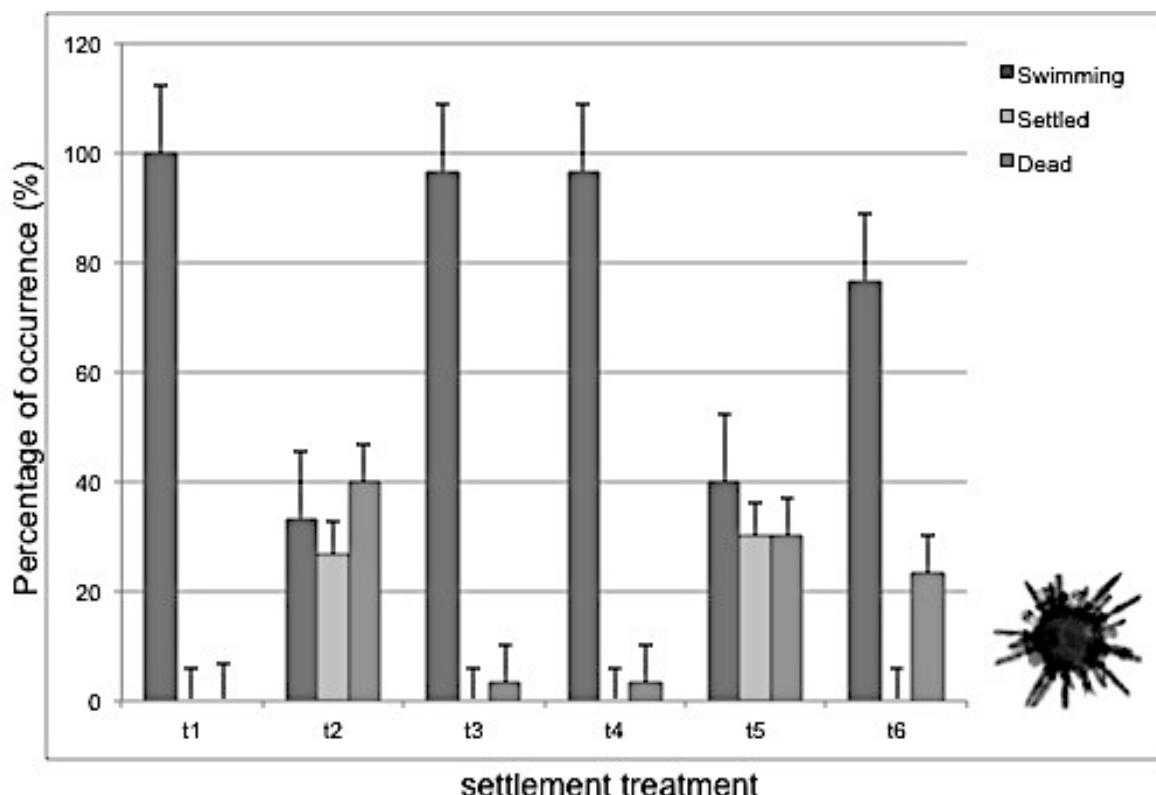


Figure 6.9. Percentage of occurrence of swimming, settled and dead postlarvae at the end of the settlement experiment in the different treatments tested: larvae that were raised at (t1) a 24h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoceaera abies marina*), (t3) the 24h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 24h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions.

When analysing postlarvae test diameter, no significant effect of pH treatments used in the experiment were detected (Table 6.5B). Post-larvae sea urchins in t2 showed a mean test diameter of 0.326 mm (SD± 0.020), and in t5, it showed a mean diameter of 0.328(SD± 0.030).

## Discussion

The present study explored the sensitivities of *P. lividus* larvae to different pH environments covering the current regional variability, showing that sea urchin larvae are able to develop normally in both daily fluctuating and constant pH environments. However, larvae performance was slightly enhanced by a moderated pH fluctuation.

We did not find clear effects of the pH fluctuation frequencies tested in the laboratory on the survival, growth or stomach volume of *P. lividus* larvae. Only one study has also explored natural pH variability on larvae development with similar results on the intertidal species *Strongylocentrotus droebachiensis* where no effect on the same larvae performance variables was found between the different current frequencies (from 7.7 to 8.1 vs constant treatment of 8.1) (Peeters 2013). Although responses to environmental factors seem to be highly species-specific, even in closely related taxa (Wittmann and Pörtner 2013), most larvae of echinoderms are robust under moderated changes in seawater pH with respect to survival and growth, despite growth is usually delayed by pH values deviating from the natural variability (see reviews by Byrne et al. 2013; Dupont and Thorndyke 2013). It is thought that the capacity for extracellular acid-base regulation is key in determining a species' ability to cope with high environmental variability (Pörtner 2008; Melzner et al. 2009; Calosi et al 2013). On the contrary to a previous view that echinoids have limited regulatory ability (Boolootian 1966; Binyon 1972), recent studies indicate the presence of a suite of responses to low pH conditions in some sea urchin species (Spicer et al. 2011; Stumpp et al. 2011; Catarino et al. 2012; Calosi et al. 2013). In this sense, species success may in part be determined by their homeostatic abilities and associated energy cost (Stumpp et al. 2011; Catarino et al. 2012; Calosi et al. 2013). Ion

regulation in echinoids is species-specific (Binyon 1966). This ability is even more important in sea urchins inhabiting coastal or shallow water environments where sharp environmental oscillations occur. The ability to buffer such fluctuations, even if partially, can be an adaptive feature that allows organisms to cope with environmental stresses (Catarino et al. 2013). A recent study suggests that adults of *P. lividus* have a higher capacity than other sea urchin species to compensate its internal fluid pH in cases of moderate hypercapnia of seawater. This fact is likely related to an acclimation ability of the species that allows it to cope with intertidal seawater parameters fluctuations (Catarino et al. 2012). In this sense, *P. lividus*'s adaptation potential, related to the great variety of habitats and environments that this species cope by crossing latitudes (Moulin et al. 2011), appears to play an important role in the species performance across a range of current environmental variability and it could be an important advantage in the struggle for survival in future climate change scenarios (García et al. in review).

Differences were detected with regard to sea urchin developmental dynamics. In this sense, larvae with 6 arms appeared at day 11 of experiment in fluctuating treatment (T1), while in constant treatment (T2) it appeared two days later. The same delay was observed with 8 arms larvae and the competent stage. A delay in developmental dynamics has been reported for other species when the larvae suffer stress of a low constant pH treatment with values expected for the end of the century (see review by Dupont and Thorndyke 2013). However, we found the delay in the constant treatment, corresponding to pH values that have typically been used as control treatments in OA experiments (see review by Byrne et al. 2013; Dupont and Thorndyke 2013). These results suggest that *P. lividus* larvae would perform better in environments with a moderate variability of seawater pH. A moderated pH fluctuation such as the one used in our experiments, corresponding to the current variability of the parameter in nature, does not lead larvae

near to its tolerance threshold. If there is a wide range between baseline metabolism and its optimal, then the organism is not energy limited, thus a moderate decrease in pH can increase metabolism and lead a positive response. This shift in the energy budget does not limit the scope for growth in *P. lividus*, suggesting that the species is far from its tolerance threshold (Pörtner and Farrell 2008; Stumpp et al. 2011). The repeated exposure to low pH conditions alternatively transient to high pH conditions could buffer the effects of acidification relieving physiological stress. This feature could potentially boost acclimation or adaptation to future more acidic environments (Hofmann et al. 2011).

Results of the settlement experiment showed very clear patterns since postlarvae settlement was only successful when a covering of algae was added to experimental units (t2 and t5), regardless of what was the frequency of pH that the larvae came from. Moreover, no differences in test diameter measurements were detected between juveniles settled at both fluctuating and constant pH treatments. Some studies suggest that low seawater pH does not affect directly to the ability of larvae to settle, but indirectly by the alteration in the composition of inducers at low pH ranges (Webster et al. 2013). However, a recent study points out the addition of an alga inducer as a strong cue for settlers of the sea urchin *Strongylocentrotus droebachiensis* regardless of pH (Dorey 2013). In our case, the daily oscillation in both fluctuating treatments (with absence or presence of alga cover, respectively) was similar. Thus, more than the fluctuation per se, our results suggest that the alga has a stronger component that induces postlarvae to settle.

In conclusion, *P. lividus* larvae development showed adaptive ecological strategies for inhabiting coastal areas covering present natural variability of seawater pH. The development of the species is surprisingly enhanced by a moderated pH fluctuation typical of intertidal environments that the sea urchin normally occupies. Considering the

multidimensional range of environmental conditions that species' ecological niche possesses (Pörtner 2002; Van Straalen 2003), our results highlight the importance of considering the natural current variability of pH in the species' niche to a better understanding and forecast of future scenarios.

Supplementary material 1. Results of pair-wise tests examining the significant interaction of factors 'pH x time' obtained in the PERMANOVA on larvae morphology in laboratory experiments testing the effects of seawater pH daily fluctuation, comparing a 24h pH fluctuation treatment (7.7 to 8.1 units; pH treatment 1), and a constant treatment (8.1 units; pH treatment 2) during the larval cycle of the species. Effects of time (Ti) for pairs of levels of factor pH on larvae morphology are shown.

	Comparisons	t	P-perm
Day 1	pH treatment 1 vs pH treatment 2	1.68	0.093
Day 2	pH treatment 1 vs pH treatment 2	0.58	0.687
Day 3	pH treatment 1 vs pH treatment 2	0.72	0.505
Day 4	pH treatment 1 vs pH treatment 2	3.33	0.001
Day 5	pH treatment 1 vs pH treatment 2	0.32	0.892
Day 6	pH treatment 1 vs pH treatment 2	0.72	0.504
Day 7	pH treatment 1 vs pH treatment 2	0.82	0.446
Day 8	pH treatment 1 vs pH treatment 2	1.13	0.226
Day 9	pH treatment 1 vs pH treatment 2	2.19	0.037
Day 10	pH treatment 1 vs pH treatment 2	1.25	0.195
Day 11	pH treatment 1 vs pH treatment 2	1.78	0.067
Day 12	pH treatment 1 vs pH treatment 2	1.44	0.157
Day 13	pH treatment 1 vs pH treatment 2	1.55	0.116

Supplementary material 2. Results of pair-wise tests examining the significant interaction of factors 'pH x time' obtained in the permutational ANOVA on larvae morphology in laboratory experiments testing the effects of seawater pH daily fluctuation, comparing a 24h pH fluctuation treatment (7.7 to 8.1 units; pH treatment 1), and a constant treatment (8.1 units; pH treatment 2) during the larval cycle of the species. Effects of time (Ti) for pairs of levels of factor pH on stomach volume are shown.

	Comparisons	t	P-perm
Day 1	pH treatment 1 vs pH treatment 2	1.68	0.067
Day 2	pH treatment 1 vs pH treatment 2	0.58	0.632
Day 3	pH treatment 1 vs pH treatment 2	0.72	0.476
Day 4	pH treatment 1 vs pH treatment 2	3.33	0.002
Day 5	pH treatment 1 vs pH treatment 2	0.32	0.893
Day 6	pH treatment 1 vs pH treatment 2	0.72	0.503
Day 7	pH treatment 1 vs pH treatment 2	0.82	0.444
Day 8	pH treatment 1 vs pH treatment 2	1.13	0.271
Day 9	pH treatment 1 vs pH treatment 2	2.19	0.023
Day 10	pH treatment 1 vs pH treatment 2	1.25	0.202
Day 11	pH treatment 1 vs pH treatment 2	1.78	0.053
Day 12	pH treatment 1 vs pH treatment 2	1.44	0.145
Day 13	pH treatment 1 vs pH treatment 2	1.55	0.094

Supplementary material 3. Results of pair-wise tests examining the significant effect of factor settlement treatment obtained in the permutational ANOVA on *Paracentrotus lividus* settlement in laboratory experiments. Experimental treatments tested included larvae that were raised at (treatment 1) a 24h fluctuating treatment and continued in this frequency, (treatment 2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoceira abies marina*), (treatment 3) the 24h fluctuating treatment and changed to constant pH treatment (8.1 units), (treatment 4) a constant pH treatment (8.1 units) and changed to 24h fluctuating treatment, (treatment 5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (ptreatment T6) the constant pH treatment (8.1 units) and remained in this conditions. Results for pairs of levels of factor settlement treatment are shown.

Comparisons	t	P-perm
treatment 1 vs treatment 2	5.51	0.001
treatment 1 vs treatment 3	1	0.379
treatment 1 vs treatment 4	1	0.366
treatment 1 vs treatment 5	2.78	0.043
treatment 1 vs treatment 6	1.83	0.146
treatment 2 vs treatment 3	4.98	0.002
treatment 2 vs treatment 4	4.98	0.003
treatment 2 vs treatment 5	0.41	0.837
treatment 2 vs treatment 6	2.50	0.056
treatment 3 vs treatment 4	0	1
treatment 3 vs treatment 5	2.58	0.038
treatment 3 vs treatment 6	1.60	0.188
treatment 4 vs treatment 5	2.58	0.046
treatment 4 vs treatment 6	1.60	0.152
treatment 5 vs treatment 6	2.97	0.043

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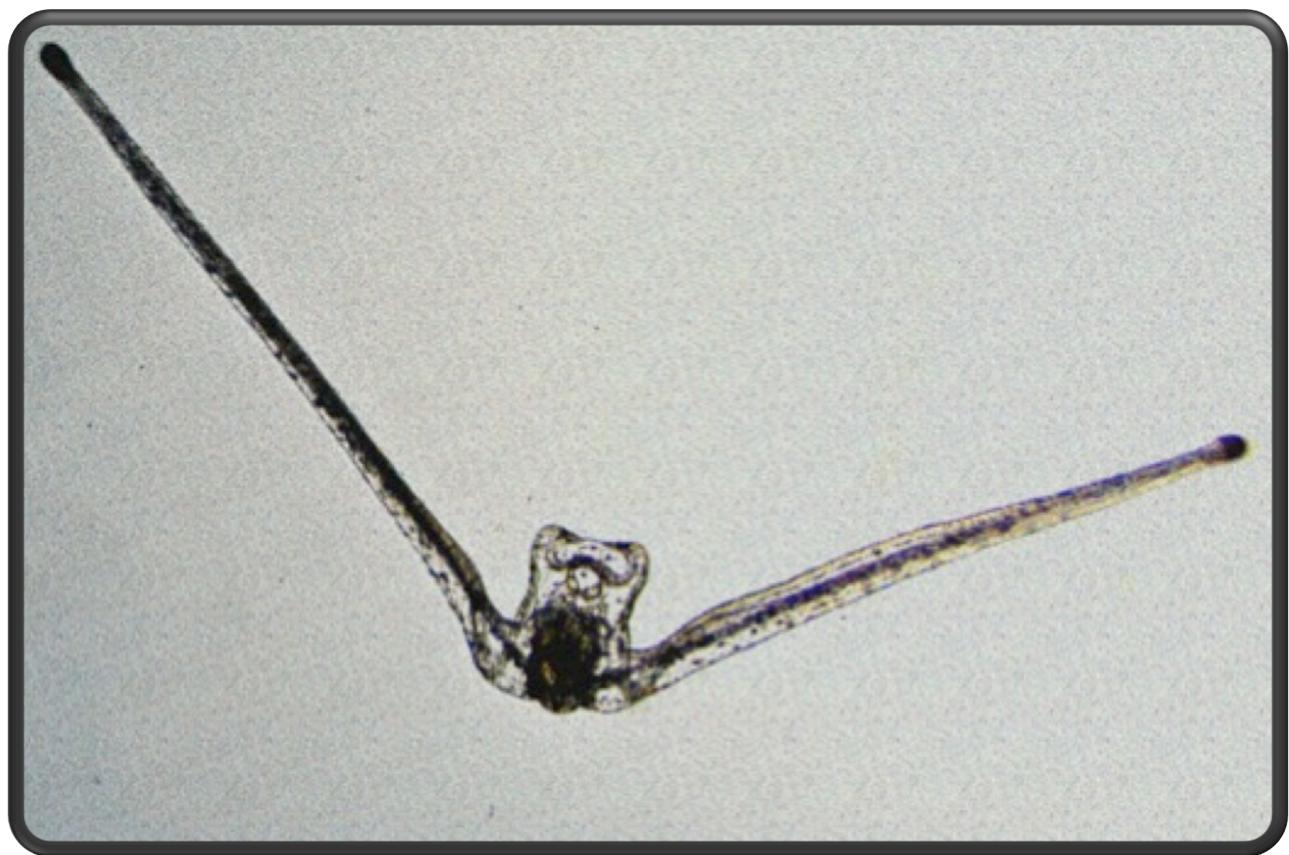
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## **CHAPTER 7: CONCLUSIONS**



## **CHAPTER 7: CONCLUSIONS**

1. The effects of the interaction of climate change stressors, ocean warming and acidification predicted for the Canary Islands region (temperature: 21, 23, 25 °C; pH: 7.7, 7.4 units), on sea urchins' fertilization, cleavage and early larval development differed among species identity (*Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*) and their life-history stages (embryos cleavage and early pluteus). However, we found a general higher impact on cleavage rate than in fertilization rate. Intertidal species were more resilient to environmental stressors than subtidal species, likely related to their capability to cope with environmental variability, suggesting a potential adaptation to climate change scenarios.

2. *Arbacia lixula* fertilization, cleavage and early larval development and survival were improved by near future climate change stressors. Ocean acidification negatively affected *Paracentrotus lividus* cleavage rate and larvae development, but these effects were countervailed by the increase of seawater temperature. Larvae survival showed deleterious impacts as a response of combined low pH and high temperature, proving a narrower thermal tolerance threshold after blastulation. In *Sphaerechinus granularis* fertilization, cleavage, early larval development and survival were drastically affected by increasing temperatures, showing a poor performance at high temperatures and a narrow threshold to face global warming. Finally, *Diadema africanum* performance during early stages was negatively affected by decreasing pH in seawater.

3. *Paracentrotus lividus* larvae were widely tolerant to present environmental variability of seawater temperature and food availability (19°C and 2000 cel mL<sup>-1</sup>, respectively). However, an interaction between the effects of temperature and food availability was detected. At control present seawater temperature food shortage decreased larvae development, while increasing temperatures, in ranges expected to occur over the

next century (20.5 and 22.5°C), ameliorated the negative effects of predicted food shortage (1000 and 500 cel mL<sup>-1</sup>). Larvae stomach volume was increased as a response to the gradual shortage of food at increasing temperatures. This result could be a consequence of a shift in the energy budget to increase stomach volume under unfavorable conditions in order to maximize food catch capacity and maintain the species rate of growth.

4. *Paracentrotus lividus* was robust to pH covering present natural variability but sensitive to pH corresponding to near-future extremes. Only sub-lethal effects were observed in larvae exposed to pH 7.7, leading to a 10% faster larval development but to a significant settlement delayed of 8 days. pH 7.4 induced both sub-lethal and lethal effects; the mortality rate was significantly increased by 40% while no settlement was observed.

5. In combined laboratory experiments testing climate change stressors (ocean warming and acidification), larvae development and settlement performance of the sea urchin *Paracentrotus lividus* was enhanced with a slight increase in temperature (+1.5°C) in the context of ocean acidification. However, the species showed sensitivities to ocean warming and acidification corresponding to near-future extremes (+3.5°C; -0.7 units of pH). The most extreme near-future projections of decreasing pH narrowed the thermal window of the species. The negative effects of decreasing pH on larvae development and settlement will be mitigated by increasing temperature up to a thermotolerance threshold of the species (22.5°C for the most extreme conditions of pH).

6. *Paracentrotus lividus* larvae development showed ecological strategies for inhabiting coastal areas covering present natural variability. Larvae development was slightly enhanced by a moderated pH fluctuation typical of intertidal environments and settlement was strongly induced by *Cystoseira abies-marina* cues regardless the pH oscillation frequencies conditions at which larvae were raised.

7. Within this thesis we have shown that the effects of climate change stressors differed among species identity and their life-history stages. We suggest that species facing higher environmental variability are more robust to climate change stressors. However, longer term research on different sea urchin species would be desirable to clarify these issues.



## **APPENDIX I: RESUMEN EN CASTELLANO**



# **Efectos del calentamiento global y la acidificación oceánica sobre la fertilización, desarrollo larvario y asentamiento de las especies de erizos marinos en las islas Canarias**

## **INTRODUCCIÓN**

### **1.2. Contexto general**

#### **1.1.1. Cambio climático: calentamiento global y acidificación oceánica.**

Durante el Holoceno la humanidad se desarrolló en un período de estabilidad climática. Los factores genéticos y medioambientales determinaron la ecología y evolución de las especies viviendo, en relativo balance, con la naturaleza (Sørensen et al. 2003). Sin embargo, en los últimos siglos, las actividades humanas se han convertido en una pieza fundamental en el sistema climático (Vitousek 1994). Como consecuencia, entramos a formar parte de una nueva era en la que la humanidad descubre que tiene poder para provocar cambios a escala global, el Antropoceno (Zalasiewicz et al. 2008).

A mediados del siglo XX, los ecologistas comenzaron a ser conscientes de que las actividades humanas estaban produciendo daños en el medioambiente y alertaron a los gobiernos para dar los pasos apropiados y detener este fenómeno. Es en este momento cuando la sociedad comienza a oír hablar del cambio climático y el calentamiento global, pero no es hasta 1972 cuando se celebra en Estocolmo La Conferencia Científica de las Naciones Unidas, también conocida como la Primera Cumbre para la Tierra, donde, los estados participantes aprueban una declaración de principios para la preservación del medioambiente y un plan de acción que contiene recomendaciones para llevar a cabo acciones medioambientales a nivel internacional.

Desde el comienzo del período industrial, a finales del siglo XVIII, la humanidad ha liberado a la atmósfera grandes cantidades de gases, conocidos como gases de efecto invernadero por su capacidad de absorber calor (Harley et al. 2006). El más importante de estos gases, en cuanto a su liberación indiscriminada al medio, es el dióxido de carbono ( $\text{CO}_2$ ). Esta liberación de  $\text{CO}_2$  se ha producido, principalmente, por la combustión del carbón pero, también, por las prácticas agrícolas y la deforestación. Aunque en las últimas décadas se han realizado numerosas medidas y reconstrucciones acerca de las cantidades de  $\text{CO}_2$  en la atmósfera, estos estudios revelan que menos de la mitad de las emisiones permanecen en la atmósfera, por lo tanto, el  $\text{CO}_2$  de origen antropogénico que no se acumula en la atmósfera es absorbido por la biosfera terrestre, por el océano o por ambos (Sabine et al. 2004).

El incremento en la concentración de los gases de efecto invernadero y de la radiación solar han alterado el equilibrio del sistema climático. Las emisiones de  $\text{CO}_2$  han aumentado sin precedentes desde el comienzo del período industrial. Antes de esta impredecible etapa, los valores de la presión parcial de  $\text{CO}_2$  ( $p\text{CO}_2$ ) en la atmósfera eran de 267 partes por millón (ppm). Desde ese momento y hasta nuestros días, esa moderada cantidad ha ido aumentando hasta alcanzar 400 ppm aproximadamente, aunque este valor se podría incrementar hasta 1000 ppm a finales del siglo XXI (IPCC 2013). El calentamiento global es el efecto más evidente de las emisiones de gases de efecto invernadero y no sólo es perceptible en el aumento de la temperatura terrestre producida durante el siglo XX sino, también, en el aumento del nivel del mar, la alteración de los patrones eólicos, la frecuencia en las tormentas, la amplificación de las áreas desérticas, etc. (IPCC 2007)

El hecho de que las emisiones de gases de efecto invernadero continúen en niveles iguales o superiores a los actuales, supondría someter al sistema climático mundial, durante

el siglo XXI, a cambios mucho más acusados que los experimentados durante el siglo XX (IPCC 2013).

Obviamente, todos estos cambios en el sistema climático están afectando a los océanos. El calentamiento global y el aumento en la concentración de CO<sub>2</sub> atmosférico están ocasionando que los océanos sean cada vez más calientes y ácidos. De hecho, los océanos están absorbiendo el 25% del CO<sub>2</sub> antropogénico generado desde el siglo XVII (Sabine et al. 2004).

Se cree que la temperatura aumentará entre 2- 4,5°C hacia finales del siglo XXI (IPCC 2013) y este calentamiento puede tener dramáticas consecuencias en los ecosistemas marinos.

El aumento de la temperatura del agua varía la distribución y la capacidad de las especies para adaptarse poniendo en compromiso la supervivencia de las mismas (Fields et al. 1993; Lubchenco et al. 1993; Harley et al. 2006) por lo que, esa redistribución de las especies puede provocar serios problemas, en ciertas regiones, ocasionando un desequilibrio en los ecosistemas locales. Es evidente que los cambios producidos por el calentamiento global va a afectar a las interacciones tróficas y a todo el funcionamiento y estructura de los ecosistemas (Alheit 2009). Este aumento de la temperatura incrementa la estratificación de la capa de mezcla, impidiendo la incorporación de nutrientes desde aguas más profundas para soportar la producción primaria (Sarmiento et al. 1998). El fitoplancton oceánico es el responsable de, aproximadamente, la mitad de la producción primaria neta global (Behrenfeld et al. 2001), por lo tanto, a largo plazo, cambios en la producción primaria oceánica pueden tener importantes consecuencias en el ciclo global del carbono. En las últimas décadas esa producción primaria ha disminuido a la vez que ha incrementado la temperatura (Gregg et al. 2003).

El segundo efecto más claro del cambio climático en los océanos es la acidificación

oceánica (AO), es decir, el progresivo descenso del pH en el agua de mar, como consecuencia de la absorción por el océano del CO<sub>2</sub> antropogénico. El océano puede ser visto como una solución diluida de carbonato sódico junto con otros compuestos ácidos-básicos en bajas concentraciones, todo ello en un contexto de agua salada (Riebesell et al. 2010). El pH marino se define como la medida de los niveles de acidez o basicidad del agua de mar ( $\text{pH} = -\log [\text{H}^+]$ ). En condiciones normales, la atmósfera guarda un equilibrio con la superficie del mar y cuando el CO<sub>2</sub> se disuelve en ella se forma ácido carbónico (H<sub>2</sub>CO<sub>3</sub>) que se disocia para crear un equilibrio en iones hidrógeno (H<sup>+</sup>), iones bicarbonato (HCO<sub>3</sub><sup>-1</sup>) y carbonato (CO<sub>3</sub><sup>-2</sup>). De modo que, cuando el océano tiene que absorber un exceso de CO<sub>2</sub>, este equilibrio se rompe y se incrementa la concentración de H<sup>+</sup>, lo que provoca una reducción en el pH. Modelos globales predicen una disminución en el pH marino entre 0,2 y 0,4 unidades hacia final del siglo XXI, especialmente como consecuencia de las emisiones de origen antropogénico (Caldeira y Wickett 2005; IPCC 2007).

Estos cambios tendrán una especial relevancia en los organismos ectotermos que tienen que regular sus fluidos corporales en función de los ambientales. La temperatura y el pH son los dos factores más importantes que controlan la distribución, fisiología, morfología y comportamiento de los invertebrados marinos (Doney et al. 2009). Los cambios en la temperatura afectan a todas las funciones biológicas (Clarke 2003) y, además, los incrementos en la temperatura aumentan la posibilidad de sufrir enfermedades e infecciones por parásitos en muchos invertebrados (Harvell et al 1999, 2002) pudiendo llegar a tener consecuencias negativas en la reproducción (e.g. Byrne et al. 2010). Probablemente los estados de desarrollo temprano sufrirán la misma suerte, aunque existen aún muchas lagunas de conocimiento (Alstatt et al. 1996; Friedman et al. 1997; Lester et al. 2007). Por otra parte, la AO tiene impactos negativos en el crecimiento, desarrollo y

reproducción de los organismos debidos, principalmente, a efectos directos sobre los procesos metabólicos (Pörtner 2008; Pörtner and Farrell 2008; Doney et al. 2009; Wittmann and Pörtner 2013).

La calcificación es la creación de estructuras de carbonato cálcico ( $\text{CaCO}_3$ ) siguiendo la siguiente ecuación:  $\text{CO}_3^{2-} + \text{Ca}^{+2} \rightleftharpoons \text{CaCO}_3$ . El coeficiente del estado de saturación ( $\Omega = [\text{CO}_3^{2-}][\text{Ca}^{+2}]/K_{sp}^*$ ) es específico de las formaciones polimórficas de  $\text{CaCO}_3$ , como la calcita y el aragonito y expresa las condiciones químicas en que se lleva a cabo el proceso de calcificación.  $K_{sp}^*$  es el producto estioquiométrico de solubilidad y depende de la temperatura, presión, salinidad y carbonato cálcico polimorfo.  $[\text{CO}_3^{2-}]$  y  $[\text{Ca}^{+2}]$  son las concentraciones *in situ* de calcio y carbonato. Cuando  $\Omega < 1$ , el agua de mar es corrosiva para las estructuras de  $\text{CaCO}_3$ . Normalmente, la superficie de las aguas tropicales suele estar muy saturada ( $\Omega > 1$ ), mientras que aguas más profundas o en latitudes más altas tienden a permanecer por debajo del nivel de saturación ( $\Omega < 1$ ). En un contexto de acidificación oceánica se espera un estado de saturación más bajo (Dorey 2013).

Muchos organismos marinos construyen sus conchas y/o esqueletos a partir del  $\text{CaCO}_3$ .  $\text{CO}_3^{2-}$  es una de las formas del carbonato utilizadas durante el proceso de calcificación y sus concentraciones disminuyen progresivamente con la acidificación oceánica. Este hecho los hace potencialmente más susceptibles a la acidificación, que podría provocar la disolución de sus estructuras calcáreas (Orr et al. 2005; Keypas et al. 2006). Cuando el equilibrio del carbono se rompe y desciende el pH, existe menos carbonato disponible y este proceso incrementa el ratio de disolución del carbonato depositado. Ese ratio de disolución depende de la forma cristalina del  $\text{CaCO}_3$ : el aragonito (encontrado en corales y moluscos) es dos veces más soluble que la calcita (encontrado en crustáceos y equinoideos) (Mucci 1983). Ante este nuevo escenario, podemos afirmar que la AO disminuye y dificulta la vida de los organismos calcáreos, especialmente en el

proceso de construcción de sus estructuras, modificando la capacidad de calcificación en corales (Hoegh-Guldberg et al. 2007), moluscos (Comeau et al. 2009) y equinodermos (O'Donnell et al. 2010).

En conclusión, el cambio climático representa en sí mismo un gran reto. El nuevo escenario con el que nos encontramos es el resultado de que la contaminación de origen humano ha excedido los límites sostenibles y, como consecuencia, es probable que los organismos marinos tengan que enfrentarse a grandes impactos en su medio, mucho mayores que los que tuvieron que superar sus antecesores en los últimos 300 millones de años (Caldeira and Wickett 2003; Ruttimann 2006). Estos cambios tendrán importantes efectos sobre los organismos marinos y, por tanto, sobre el funcionamiento de los ecosistemas.

### **1.1.2 Equinodermos como objetivo en la investigación marina.**

Los equinodermos son animales exclusivamente marinos que están ampliamente distribuidos en todos los océanos y profundidades. Son conocidas alrededor de 7000 especies que se subdividen en seis clases; los crinoideos, asteroideos, ofiuroideos, equinoideos, holoturioideos y concentricicloideos.

Estos animales ocupan posiciones claves en la mayoría de los ecosistemas y son ecológica y económicamente, relevantes. Como un típico taxón bentónico, son importantes creadores de ecosistemas y parte fundamental de la cadena trófica (Bowmer y Keegan 1983; Skołd and Rosenberg 1996). Muchos de ellos representan una importante comunidad ramoneadora como los erizos de mar sublitorales (Lawrence 1975). En muchas áreas, los equinodermos como los erizos de mar o las holoturias son explotados como alimento suponiendo ésto un importante ingreso económico (Micael et al. 2009).

La mayoría de los equinodermos tiene un periodo larvario de vida planctónica y un período adulto de vida bentónica. El período planctónico puede durar de horas a meses dependiendo de las especies. Los erizos marinos (objeto de esta tesis), suelen tener una fertilización externa. Normalmente el periodo pelágico está sincronizado con necesidades específicas o umbrales de supervivencia de las especies como, por ejemplo, la presencia de alimento, factores ambientales óptimos, etc.

Cuando las condiciones medioambientales son las adecuadas es cuando se produce el desove (Mercier y Hamel 2009). En ese momento los gametos son liberados en la columna de agua y se produce la fertilización, dando lugar a embriones y posteriormente a larvas con 4 a 8 brazos, dependiendo de la especie, con esqueleto calcáreo. Las larvas pluteus son planctónicas, y aproximadamente después de tres semanas, crean una invaginación epidérmica en la pared derecha del cuerpo (Gosseling and Jangoux 1998) que dará lugar a un rudimento que coloca a la larva en estado competente para asentarse en el sustrato y metamorfosearse al estado de postlarva. Normalmente, este período pelágico está sincronizado con necesidades y umbrales específicos de cada especie, como la presencia de alimento, la idoneidad de ciertos factores abióticos, etc. La postlarva tiene el aspecto de un adulto en miniatura pero solo desarrollará la boca y el resto del aparato digestivo durante la siguiente semana al asentamiento, cuando alcanza el estado de juvenil propiamente dicho (Cameron and Hinegardner, 1974; Gosselin and Jangoux, 1996). Finalmente, los adultos pueden tener una vida muy longeva (Ebert 2008).

La mayor parte de las especies de equinodermos pasan por tres transiciones ecológicas a lo largo de su ciclo de vida. La primera tiene lugar cuando los adultos liberan los gametos en la columna de agua para asegurar la fertilización. Esta transición entre el ambiente bentónico y el pelágico lleva asociada cambios complejos a nivel de desarrollo (fertilización, embriones y desarrollo larvario). La segunda transición va asociada al

proceso de asentamiento en el sustrato en el que el organismo pasa del ambiente pelágico al bentónico, al mismo tiempo que sufre transformaciones anatómicas durante la metamorfosis de larva a juvenil. Finalmente, hay una transición anatómica post-metamórfica desde juvenil al estado adulto (Dupont y Thorndyke 2013).

Por su relevancia ecológica, los equinodermos han sido estudiados durante mucho tiempo, sin embargo, en los últimos años se han convertido en el objetivo de múltiples trabajos acerca de la AO debido a su naturaleza de organismos calcáreos. Los equinodermos tienen dos fases bien diferenciadas en la esqueletogénesis; una se corresponde con el periodo larvario y la otra con el estado adulto. Los brazos, la cubierta, los dientes y las púas están formadas por un precursor amorfo del cristal de calcita, la calcita de magnesio, que es 30 veces más soluble que la calcita normal (Politi et al. 2004). De acuerdo con esto, es previsible que estos organismos sean muy sensibles a los impactos del cambio climático.

Las espículas de las larvas están compuestas de  $\text{CaCO}_3$  y  $\text{MgCO}_3$  embebidos en una matrix proteica. Las células mesenquimáticas primarias construyen las espículas trasladando el Ca desde el agua de mar, a través de canales de calcio y este precipita gracias al mantenimiento de un alto estado de saturación, probablemente por bombas iónicas (Dorey 2013). De acuerdo con esto, es muy probable que la esqueletogénesis se vea muy afectada por los efectos del cambio climático. Sin embargo, además de la calcificación, existen otros efectos fisiológicos y metabólicos que no deben ser ignorados (Pörtner and Farrel, 2008; Stumpp et al. 2011, 2012, 2013).

### **1.1.3 Efectos del cambio climático en las primeras etapas de desarrollo de los equinodermos.**

En los últimos años muchas investigaciones se han centrado en averiguar cómo afectan los procesos derivados del cambio climático en el desarrollo larvario de los invertebrados marinos, pero aún quedan muchas incógnitas por resolver en cuanto al alcance de estos impactos a largo plazo y cómo pueden afectar a las siguientes fases de su ciclo de vida.

Las primeras fases de desarrollo de la mayoría de invertebrados marinos tienen lugar en la columna de agua, como miembros planctónicos de la comunidad pelágica y susceptibles de sufrir deletéreos impactos en su desarrollo como consecuencia del cambio climático. Esas primeras etapas como son la fertilización, embriogénesis y el desarrollo larvario, son generalmente las más sensibles al estrés medioambiental (ver revisiones de Byrne et al. 2013; Byrne y Przeslawsky 2013; Dupont y Thorndyke 2013). Sin embargo, estudios recientes apuntan a que el asentamiento y el estado juvenil pueden ser fases muy críticas que se pueden ver más afectadas por los cambios medioambientales de lo que se ha pensado hasta ahora (Dupont et al. 2012; Dorey et al. 2013).

Se considera que la temperatura es el factor ambiental más importante ya que controla el crecimiento, reproducción, ratio de desarrollo y dinámicas de reclutamiento en los invertebrados marinos, así como la distribución de las especies (Pechenik 1987; O'Connor et al. 2007). En los últimos 15 años se han hecho muchos estudios para evaluar los impactos de la temperatura en relación con el cambio climático (ver revisiones de Byrne 2011; Callaway et al. 2012; Byrne y Przeslawsky 2013). El aumento de la temperatura del agua tiene un efecto directo en los gametos y los embriones, acelerando la velocidad de natación de los espermatozoides, modificando la cinética de fertilización, incrementando el ratio de desarrollo, reduciendo la dispersión e induciendo o suprimiendo

las respuestas de estrés. Obviamente, el incremento de temperatura tiene un umbral letal para los organismos (Clarke 2003; Staver y Strathmann 2002; Lee et al. 2004; O'Connor et al. 2007; Parker et al. 2009). Aunque los efectos de la AO en estos primeros estadíos de desarrollo no están tan estudiados como los de la temperatura se sabe que los rangos bajos de pH pueden provocar efectos deletéreos en los procesos (Pörtner 2008). Aunque existen algunas contradicciones entre los resultados de los diferentes estudios, alguna investigaciones recientes sugieren que, de una manera general, la AO tiene efectos negativos sobre la fertilización y las primeras fases de desarrollo (ver revisiones de Byrne et al. 2013; Dupont y Thorndyke 2013).

En cuanto al estado larvario, el aumento de la temperatura del agua de mar acelera el crecimiento, desarrollo, asentamiento y también tiene un efecto sobre la capacidad de natación y la duración del estado planctónico, hasta alcanzar el umbral de supervivencia (ver revisiones de Byrne 2011; Byrne y Przeslawsky 2013). Estos hechos, que en principio podrían parecer positivos ya que al estar menos tiempo en la columna de agua son menos susceptibles a la acción de los depredadores, al mismo tiempo pueden reducir las posibilidades de dispersión, producir alteraciones en la conectividad genética y, finalmente, alterar la dinámica de poblaciones (López et al. 1998; O'Connor et al. 2007). Además, se cree que la disponibilidad de comida se verá reducida como consecuencia de la disminución de la producción primaria debida a la estratificación de la capa de mezcla. Como ya hemos apuntado con anterioridad en el texto, ésto es una consecuencia del calentamiento del océano (Gregg et al. 2003; Turley et al. 2013). Aunque muchos trabajos han investigado el efecto de la disponibilidad de comida en el medio en el crecimiento y supervivencia de las formas larvarias (Olson y Olson 1989; Fenaux et al. 1994; Meidel et al. 1999; Vickery y McClintock 2000; Moran y Manahan 2004; Sewell et al. 2004; Meyer et al. 2007; McAlister 2007), solo algunos lo han relacionado con la variabilidad ambiental

(McAlister 2008) y, ninguno de ellos, enfoca la escasez de alimento en el medio como un efecto del cambio climático.

Por otra parte, con respecto al efecto de la AO sobre el estado larvario, aunque las respuestas de los organismos dependen en gran medida de la especie (Wittmann y Pörtner 2013), en general existe un efecto negativo sobre la supervivencia, desarrollo, crecimiento y asentamiento (ver revisiones de Byrne et al. 2013; Dupont y Thorndyke 2013). Las larvas de algunas especies parecen estar bajo una gran amenaza (Dupont et al. 2008), sin embargo otras como las larvas de erizo de mar parecen tener una fortaleza mayor de la que se pensaba para superar los efectos de la AO a corto plazo (ver revisiones de Byrne et al. 2013; Byrne y Przeslawsky 2013; Dupont y Thorndyke 2013).

Pero no hay que olvidar que los organismos viven en un ambiente multifactorial, donde los niveles de estrés ambiental son aumentados por el cambio climático (Feely et al. 2004; Caldiera y Wickett 2005; IPCC 2007). Por ejemplo, se cree que el rango de temperatura en el que una especie vive de manera óptima, puede estar influenciado por la interacción de otros factores como la AO (Pörtner y Farrel 2008).

Finalmente, el éxito del reclutamiento depende de la supervivencia y la calidad de los embriones y las larvas (López et al. 1998), por tanto, cualquier disminución en la supervivencia o retraso en el desarrollo puede reducir la viabilidad de las poblaciones a largo plazo (Morgan 1995).

#### **1.1.4 Las Islas Canarias en un contexto de cambio climático**

Este archipiélago español está situado en el océano Atlántico, al norte de África a 27°37'- 29°25' N y 13°20'- 18°10' W, próximo a la costa sur de Marruecos y el Sáhara. Las Islas Canarias, que tienen una naturaleza volcánica, forman parte de la región de la Macaronesia junto a los archipiélagos de Azores, Madeira, Salvajes y Cabo Verde.

Emergiendo desde el fondo del océano como resultado de sucesivas erupciones volcánicas que dieron lugar a un conjunto de islas independientes entre sí, el archipiélago canario está compuesto de siete islas y cuatro islotes. A pesar de su latitud, las Islas Canarias tienen un clima subtropical moderado gracias a la acción de los vientos Alisios. Sin embargo, debido a la altitud, podemos encontrar diferentes microclimas que confieren al archipiélago una gran biodiversidad terrestre. Con respecto al medio marino, las islas tienen una plataforma oceánica reducida como consecuencia de su origen volcánico, mostrando el típico ambiente oceánico con aguas oligotróficas.

Su localización geográfica entre las aguas frías y ricas en nutrientes del afloramiento africano y las aguas más cálidas y pobres en nutrientes de mar abierto, sitúan a Canarias en una zona de transición con fuertes repercusiones para la productividad de la región (Barton et al. 1998). Los filamentos del afloramiento alcanzan las islas enriqueciendo así las aguas de las islas más orientales. Así mismo, los fuertes vientos que soplan en verano producen afloramientos locales que se reducen durante el invierno (Arístegui et al. 1997, 2004).

Las Islas Canarias están inmersas en la corriente fría de Canarias, que domina la circulación del agua en el océano Atlántico Norte. Esta corriente, junto con los vientos Alisios, genera las principales corrientes en la región. La temperatura de la superficie del mar fluctúa entre 16-18°C durante el invierno y 23-25°C durante el verano, aunque pueden tener lugar algunos eventos estacionales. La salinidad en superficie oscila entre 36.7 and 36.9 ‰, con salinidades medias más elevadas en las islas más occidentales. Todas estas circunstancias, confieren a las islas una enorme variabilidad de ecosistemas costeros en un espacio muy limitado. De las 18000 especies catalogadas para Canarias, 5232 son marinas (Hernández et al. 2012).

En un contexto de cambio climático, y en concordancia con los datos presentados por Santana-Casiano et al. (2007) para el Atlántico Este, los expertos del QUIMA (O. Llinás comunicación personal, Universidad de Las Palmas de Gran Canaria) predicen un incremento en la AO como resultado del descenso del pH en 0,002 unidades por año. Con relación al calentamiento, la temperatura en aguas del archipiélago canario ha aumentado 1°C desde 1985 (AEMET 2008) y se cree que esta tendencia se mantendrá en el futuro.

## 1.2 Objetivos

Esta tesis está contextualizada en el marco del proyecto del Plan Nacional de I+D+I “*Influencia de las fitocenosis del litoral rocoso en la variación del pH y su relevancia para las poblaciones de invertebrados calcáreos en un contexto de cambio climático (ACIDROCK- CTM 2010-21724)*”. Este proyecto tiene por objeto evaluar el papel de las fitocenosis de zonas litorales rocosas sobre la variabilidad natural del pH de agua de mar y evaluar las implicaciones de esos regímenes de alcalinidad, así como los previstos como consecuencia del cambio climático en el desarrollo larvario, asentamiento, supervivencia y crecimiento de invertebrados calcáreos clave. En este contexto, el presente estudio trata de evaluar el impacto de los procesos derivados del cambio climático sobre las primeras etapas de desarrollo de las especies de erizo de mar de las islas Canarias.

Para lograr ese objetivo general, establecemos cinco objetivos específicos:

- 1) Evaluar cómo afectan los procesos derivados del cambio climático (calentamiento y acidificación oceánicos) sobre la fertilización, la embriogénesis y la primera fase larvaria de las principales especies de erizo marino en las islas Canarias.
- 2) Analizar el efecto combinado de la temperatura y la disponibilidad de alimento en la supervivencia, crecimiento y desarrollo de la larva de *Paracentrotus lividus*.

- 3) Testear el impacto de la acidificación oceánica en la supervivencia, crecimiento, desarrollo y asentamiento de la larva de *Paracentrotus lividus*.
- 4) Evaluar si la interacción de los factores medioambientales (temperatura y pH) tiene la potencialidad de cambiar las respuestas de la larva y postlarva de *Paracentrotus lividus* a la acidificación oceánica.
- 5) Comparar el desarrollo larvario y el asentamiento de *Paracentrotus lividus* en condiciones de pH constante y fluctuaciones naturales diarias, para tener una mejor comprensión y poder predecir, con más asertividad, cómo podrá la especie enfrentarse a futuros escenarios en un contexto de cambio climático.

### 1.3. Estructura de los capítulos

Esta tesis ha sido estructurada en siete capítulos, cinco de los cuales se corresponden con manuscritos originales, artículos publicados o enviados para su publicación en diferentes revistas científicas en el campo de la biología y ecología marina.

En el Capítulo 1 se muestra una revisión sobre el cambio global en los océanos, y el impacto de los procesos derivados del cambio climático sobre los invertebrados marinos, remarcando cómo son afectados los primeros estados de desarrollo, especialmente en los equinodermos (objeto de esta tesis). En este capítulo se incluyen, así mismo, los objetivos y la estructura de la tesis.

En el Capítulo 2 se evalúa el efecto combinado del calentamiento y la acidificación oceánica en la fertilización, embriogénesis y primera fase larvaria de las cuatro especies principales de erizos de las islas Canarias (*Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Diadema africanum*). Nuestros resultados resaltan la mayor susceptibilidad de las especies submareales a los cambios medioambientales y cómo las

especies intermareales aumentan su resiliencia a la acidificación oceánica a mayor temperatura.

El Capítulo 3 incluye un estudio evaluando el efecto combinado de la temperatura y la disponibilidad de comida sobre la supervivencia, crecimiento y desarrollo larvario de *Paracentrotus lividus*. Nuestros resultados sugieren que la larva de esta especie es fuerte para enfrentar estas agresiones a corto plazo y que el efecto negativo de la escasez de comida en el desarrollo larvario puede ser contrarrestado con el aumento de temperatura.

En el Capítulo 4 hemos analizado el impacto de la acidificación oceánica en la supervivencia, desarrollo larvario y asentamiento de *Paracentrotus lividus*. Nuestros resultados revelan que *P. lividus* es resistente a rangos de pH que abarcan la presente variabilidad natural, pero la especie muestra sensibilidades en los niveles de pH previstos en el futuro.

En el Capítulo 5 se muestra un estudio que analiza, específicamente, el efecto combinado de temperatura y pH sobre la supervivencia, crecimiento, desarrollo larvario y asentamiento del erizo de mar *Paracentrotus lividus*. Los valores más extremos de pH proyectados para final de siglo tienen el potencial de estrechar el rango funcional de temperatura de la especie. El desarrollo larvario y el asentamiento se mejora ligeramente con un pequeño aumento de la temperatura en un contexto de AO. Sin embargo, la especie muestra sensibilidades a los valores más extremos de temperatura y pH previstos para final de siglo.

El Capítulo 6 explora las sensibilidades de *P. lividus* durante su desarrollo larvario y su asentamiento, sometido a diferentes frecuencias diarias de fluctuación del pH que tienen lugar actualmente en aguas de Canarias. La larva de *P. lividus* mostró que tiene estrategias para desarrollarse con normalidad y habitar ambientes costeros que presentan distinta variabilidad ambiental. El desarrollo larvario se vio ligeramente favorecido por una

moderada fluctuación del pH típica de los ambientes intermareales donde suele habitar la especie.

Finalmente, en el Capítulo 7 se recogen las conclusiones generales de la tesis.

## CONCLUSIONES

1. Los efectos de la interacción entre el calentamiento y la acidificación oceánica, previstos para la región de las islas Canarias como consecuencia del cambio climático (temperatura: 21, 23, 25°C; pH: 7'7, 7'4), sobre la fertilización, desarrollo embrionario y los primeros días de desarrollo larvario, varía entre las distintas especies de erizo de mar (*Arbacia lixula*, *Paracentrotus lividus*, *Sphaerechinus granularis* y *Diadema africanum*) y el estado de desarrollo dentro de su ciclo de vida (división embrionaria y primera fase de larva pluteus). Sin embargo, encontramos un impacto mayor sobre el desarrollo embrionario que sobre la fertilización. Las especies intermareales fueron más resilientes que las submareales a las agresiones medioambientales, probablemente debido a la mayor capacidad de las primeras de lidiar con la variabilidad ambiental, sugiriendo una potencial adaptación a futuros escenarios a consecuencia del cambio climático.
2. La fertilización, el desarrollo embrionario y la supervivencia y desarrollo larvario fueron beneficiados por las futuras condiciones de cambio climático en *Arbacia lixula*. La acidificación oceánica afectó negativamente al desarrollo embrionario y larvario en *Paracentrotus lividus*, sin embargo, estos efectos fueron contrarrestados con el aumento de temperatura. La supervivencia fue claramente afectada por la combinación de bajo pH y alta temperatura, mostrando un umbral de termo tolerancia más reducido después del estado de blástula. En *Sphaerechinus granularis*, la fertilización, el desarrollo embrionario y la supervivencia y

desarrollo larvario fueron dramáticamente afectados por el aumento de la temperatura, mostrando las debilidades de la especie para enfrentar el futuro calentamiento oceánico. Finalmente, *Diadema africanum* fue negativamente afectada por la reducción de pH en el agua en todas las fases de desarrollo analizadas.

3. *Paracentrotus lividus* fue tolerante a la variabilidad medioambiental actual con respecto a la temperatura y la disponibilidad de comida en el agua de mar ( $19^{\circ}\text{C}$  y  $2000 \text{ cel mL}^{-1}$ , respectivamente). Sin embargo, se detectó una interacción entre los efectos de ambos factores. A la temperatura utilizada como control ( $19^{\circ}\text{C}$ ) la disminución de la disponibilidad de alimento afectó negativamente al desarrollo larvario, mientras que el incremento de la temperatura en los niveles esperados para final de siglo ( $20.5$  and  $22.5^{\circ}\text{C}$ ) contrarrestó el efecto de la disminución en la disponibilidad de comida prevista ( $1000$  and  $500 \text{ cel mL}^{-1}$ ). El volumen del estómago de las larvas se incrementó como respuesta a la gradual disminución de alimento a altas temperaturas. Este resultado podría explicarse como un incremento de la energía empleada para maximizar la captación de comida y mantener el ratio de crecimiento, bajo condiciones desfavorables.
4. *Paracentrotus lividus* fue tolerante a la variabilidad natural de pH que tienen lugar en la actualidad, pero mostró debilidades a los valores de pH extremos previstos en un futuro. Sólo se detectaron efectos subletales en las larvas expuestas a  $7.7$  unidades de pH, donde el desarrollo se aceleró en un  $10\%$  pero el asentamiento se retrasó  $8$  días. A pH  $7.4$  se dieron efectos tanto subletales como letales; el ratio de mortalidad se incrementó en un  $40\%$  y no se observó asentamiento.
5. En los experimentos combinados, analizando los efectos del calentamiento y la acidificación oceánica , el desarrollo larvario y el asentamiento de *Paracentrotus*

*lividus*, se vio favorecido por un ligero aumento de la temperatura (+1'5°C) en un contexto de acidificación. Sin embargo, la especie mostró debilidades a los cambios de temperatura y pH extremos previstos (+3'5°C; -0'7 unidades de pH). Los valores más extremos de pH previstos para final de siglo, estrecharon el rango de temperatura funcional de la especie. Los efectos negativos provocados por el descenso del pH en el desarrollo larvario y el asentamiento serán mitigados por el aumento de temperatura hasta llegar al umbral de termo tolerancia de la especie (22'5°C)

6. *Paracentrotus lividus* mostró estrategias ecológicas para habitar en áreas que abarcan la variabilidad natural presente. El desarrollo larvario se vio ligeramente favorecido por una moderada fluctuación del pH, típica de los ambientes intermareales. El asentamiento fue fuertemente inducido por *Cystocea abies-marina*, sin influencia de las frecuencias de oscilación de pH a la que habían sido cultivadas las larvas.
7. Esta tesis ha mostrado que los efectos de los procesos derivados del cambio climático difieren entre las especies y los estados de desarrollo. Sugerimos que las especies que tienen que enfrentar normalmente una amplia variabilidad natural son más fuertes para enfrentar las condiciones futuras. Sin embargo, son necesarios más estudios a largo plazo analizando los comportamientos de las distintas especies para poder clarificar estas ideas.



