UNIVERSITÉ LIBRE DE BRUXELLES

Faculté des Sciences
Département de Biologie des Organismes
Laboratoire de Biologie Marine

Temperate and cold water sea urchin species in an acidifying world: coping with change?

Ana Isabel DOS RAMOS CATARINO

Thèse présentée en vue de l'obtention du titre de Docteur en Sciences

Juin 2011

Promoteurs de thèse (ULB)

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QREN QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL PORTUGAL 2007.2013

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Dr Philippe Grosjean (UMons)
Dr Guy Josens (ULB)
Dr Denis Allemand (Centre Scientifique de Monaco)

JORGE CHAM©THE STANFORD DAILY

ACKNOWLEDGMENTS

It all started some years ago: the Belgian adventure. Moving to another country, enrolling in changeling projects, meeting new colleagues and friends was a challenging step. It has been a pleasure to meet, to work and to learn from you all.

I would like to start by thanking Prof. Michel Jangoux. Your knowledge and experience inspired me to start this adventure. It was still in Portugal that I came across with some of your work and that was how the idea "to leave" and come here was born. Your passion for these interesting little creatures, the echinoderms, has been of great inspiration for my research.

I wish to express my sincerest thanks to my supervisor, Philippe Dubois... or should I say, "thankful am I"! During these years you provided encouragement, sound advice and good company. Your guidance, accuracy, knowledge and patience kept me on the right path. You were not only a great advisor, but also a good and supportive friend. Thank you for trusting in me and for always keeping me looking *on the bright side of...* science (♪).

I would also like to thank my co-supervisor, Chantal De Ridder. I am grateful for the motivating discussions and collaborations. You always had a positive attitude whenever I came around with questions and it was a pleasure to work together.

I am also grateful to Dr. Lei Chou (ULB) and Dr. Philippe Grosjean (UMons), from my graduate committee. Your guidance and advice was precious and helped me structuring my ideas and work.

I am also thankful to all the BIANZO, MRAC, Erasme Hospital and Liège University collaborators. Working with you was gratifying and your expertise greatly improved my work. A special acknowledgement to Prof. Luc André and N. Dahkani (Royal Museum for Central Africa), Prof. Philippe Compère (Université de Liège) and Dr. De Bruyn (Hôpital Erasme).

I am grateful to the research groups that hosted me both in Chile and in Croatia. I was warmly welcomed and it was a pleasure to work in these two beautiful places.

A special thank you to the INACH (Instituto Antártico Chileno) research group, especially to Dr. Marcelo Gonzalez, Carla, Geraldine and Tania. Also to Emma, Carlos, César and to all the new good friends I made in Punta Arenas. I am grateful to the Laredo personnel, in particular to Dr. Pablo Gallardo.

Also, to all the Ruđer Bošković Institute (Croatia) research group a big thank you, especially to Prof. Davorin Medakovic, Dr. Daniel Lyons, Dr. B. Hamer and to sweet Adriana.

I am grateful for technical and theoretical advice to Prof Fred Mackenzie (University of Hawaii at Manoa), Dr. Alberto Borges (Université de Liège), Dr. Jerome Harlay (ULB), Dr. Caroline De Bodt (ULB), Dr. Pol Gosselin (UMons), Dr. Bruno Danis (Royal Belgian Institute of Natural Sciences), Prof. Maria Byrne (University of Sydney), Prof. N. Heisler (H-U Berlin), Prof. J. Rohlf (Stony Brooks University), Prof. P. Doncaster (University of Southampton) and Prof. J. H. McDonald (University of Delaware), Dr. N. (Ameziane, Muséum National d'Histoire Naturelle, Paris), Dr. D. Néraudeau (Université de Rennes).

Un très grand merci à toute l'équipe de BIOMAR. To Edith and Saloua, Mathieu and Philippe P., Thierry and now also to Jorge, Viviane, a big big THANK YOU! Your work and concern were priceless! Virginie, Laure, Thomas, Marie. It has been a pleasure to collaborate with you. Believe me when I say that I have learned a lot from you. And that it is a great pleasure to see Laure and Marie continuing in the group. I am also grateful to Lindsay and David for their help. Colin, Gauthier, Bea, Catherine, Bruno... It was a pleasure to meet great professionals, always seeking a good opportunity... to have a "scientific" chat! The lab was surely livelier with you. A special acknowledgement to the girls from the Biomineralization-Climate-Change-Ocean-Acidification-PhD-Gang! Claire, Julie, Steph, you were amazing! Thank you ever so much for answering all my questions, for the debates, for the lunch and tea time chats.

Of course this thesis would not have been possible without the.... (wait for it).... LEGENDARY Article-Portuguese-Mafia! Caros amigos mafiosos e traficantes de saber, β, Rui, Ju e Super Mário, não há nada melhor do que networking. Se algum dia precisarem seja do que for, seja (a)onde for, basta um email e eu estarei do outro lado para vos fornecer um qualquer "papel" sobre assuntos obscuros, mas de extremo interesse para O bem maior: a Ciência!

I am also grateful to the last minute "reviewers" and "advisers" of this thesis! Thank you Bea, Claire, Mathieu, Ju, Namita and Nikos.

Finally I am deeply grateful to my friends and family! What would I have done without you?!?!?

My friends, B-expats and some other expats all over the world, thank you for your time. I usually don't blabber much about work (thank God, right?), but the social and human part definitely helped me balancing the other half of my life. Um abraço apertado ao Gang Tuga de Bruxelas e um beijinho às Bioloucas Ju, Joaninha e β.

To my Parents and my Brother André! Vocês são pessoas extraordinárias. Além de terem sido em muitos momentos uma ajuda vital, foram os grandes incentivadores desta aventura! Foi o vosso exemplo de perseverança, trabalho árduo e coragem que em muitas ocasiões me serviu de bússola e me guiou. Pai e Mãe, obrigada por todas as oportunidades que me proporcionaram ao longo dos tempos e que me levaram a chegar aqui. Devo-vos tudo e por isso estou profundamente agradecida!

To Nikos. Who heroically dealt with my bad moods during the hardest times. Thank you for being by my side, always!

I would also like to thank Brussels, yeah... the city! Brussels and I had a tough start. The lack of sun is not something easy to deal with. Still, Brussels has an affectionate and caring side. You won't find this much diversity and easygoing life in many other places around the world. You need to have a couple of beers with Brussels, date Brussels, fall in love with Brussels, and then this small, intense and crazy city will slowly conquer your heart and eventually will become home.

There are not enough words to thank all the people that helped me building this project. To all that believed in me, motivated me, shared their knowledge with me, guided me, listened to me and took care of me: thank you, gracias, dank u wel, obrigada, hvala vam, merci, ευχαριστώ.

SUMMARY

Anthropogenic carbon dioxide (CO_2) emissions are increasing the atmospheric CO_2 concentration and the oceans are absorbing around 1/3 them. The CO_2 hydrolysis increases the H^+ concentration, decreasing the pH, while the proportions of the HCO_3^- and CO_3^{2-} ions are also affected. This process already led to a decrease of 0.1 pH units in surface seawater. According to "business-as-usual" models, provided by the Intergovernmental Panel on Climate Change (IPCC), the pH is expected to decrease 0.3-0.5 units by 2100 and 0.7-0.8 by 2300. As a result the surface ocean carbonates chemistry will also change: with increasing pCO_2, dissolved inorganic carbon will increase and the equilibrium of the carbonate system will shift to higher CO_2 and HCO_3^- levels, while CO_3^{2-} concentration will decrease. Surface seawaters will progressively become less saturated towards calcite and aragonite saturation state and some particular polar and cold water regions could even become completely undersaturated within the next 50 years.

Responses of marine organisms to environmental hypercapnia, i.e. to an excess of CO_2 in the aquatic environment, can be extremely variable and the degree of sensitivity varies between species and life stages. Sea urchins are key stone species in many marine ecosystems. They are considered to be particularly vulnerable to ocean acidification effects not only due to the nature of their skeleton (magnesium calcite) whose solubility is similar or higher than that of aragonite, but also because they lack an efficient ion regulatory machinery, being therefore considered poor acid-base regulators. Populations from polar regions are expected to be at an even higher risk since the carbonate chemical changes in surface ocean waters are happening there at a faster rate.

The goal of this work was to study the effects of low seawater pH exposure of different life stages of sea urchins, in order to better understand how species from different environments and/or geographic origins would respond and if there would be scope for possible adaptation and/or acclimatization.

In a first stage we investigated the effects of ocean acidification on the early stages of an intertidal species from temperate regions, the Atlantic *Paracentrotus lividus* sea urchin, and of a sub-Antarctic species, *Arbacia dufresnei*. The fertilization, larval development and larval growth were studied on specimens submitted through different pH experimental treatments. The fertilization rate of *P. lividus* gametes whose progenitors came from a tide pool with high pH decrease was significantly higher, indicating a possible acclimatization or adaptation of gametes to pH stress. Larval size in both species decreased significantly in low pH treatments. However, smaller *A. dufresnei* echinoplutei were isometric to those of control treatments, showing that size reduction was most likely due to a slower growth rate. In the pH 7.4 (predicted for 2300) treatment, *P. lividus* presented significantly more abnormal forms than control ones, but *A. dufresnei* did not. The latter does not seem to be more vulnerable than temperate species, most likely due to acclimatization/adaptation to lower pH seasonal fluctuations experienced by individuals of this population during spring time.

In a second stage, adult physiological responses of *P. lividus* and *A. dufresnei* to low pH seawaters were studied. Intertidal field *P. lividus* specimens can experience pH fluctuations

of 0.4 units during low tidal cycles, but their coelomic fluid pH will not change. During experimental exposure to low pH, the coelomic fluid (extracellular) pH of both species decreased after weeks of exposure to low seawater pH. However, it owned a certain buffer capacity (higher than that of seawater) which did not seem to be related to passive skeleton dissolution. In laboratory studies, the feeding rate of *P. lividus*, the RNA/DNA ratio (proxy for protein synthesis and thus metabolism) of both the gonads and the body wall of the studied species and the carbonic anhydrase activity in the body wall (an enzyme involved in calcification and respiratory processes) of *A. dufresnei* did not differ according to seawater pH. The same was true for spine regeneration (a proxy for calcification) of both species. This shows that both *P. lividus* and *A. dufresnei* are able to cope when exposed to mild hypercapnia (lowest investigated pH 7.4) for a mid-term period of time (weeks). In a different set of experiments, pH effects were tested on *P. lividus* individuals together with two temperatures (10ºC and 16ºC). The pH decrease of the coelomic fluid did not vary between temperatures, neither did its buffer response. The oxygen uptake rates of *P. lividus* (as a proxy for global metabolic state of the whole organism) increased in lower pH treatments (7.7 and 7.4) in organisms exposed to lower temperatures (10ºC), showing that this was upregulated and that organisms experienced a higher energetic demand to maintain normal physiological functions. For instance, gonad production (given by the RNA/DNA ratio) was not affected neither by temperature, nor pH.

Finally, possible morphological and chemical adaptations of cidaroid ("naked") spines, which are not covered by epidermis, to low magnesium calcite saturation states were investigated. Deep sea field specimens from the Weddell Sea (Antarctica), *Ctenocidaris speciosa* were studied. Cidaroid spines have an exterior skeleton layer with a polycrystalline constitution that apparently protects the interior part of the monocrystaline skeleton, the stereom (tridimensional magnesium calcite lattice). The cortex of *C. speciosa* was by its turn divided into two layers. From these, it presented a thicker inner cortex layer and a lower Mg content in specimens collected below the aragonite saturation horizon. The naked cortex seems able to resist to low calcium carbonate saturation state. We suggest that this could be linked to the important organic matrix that surrounds the crystallites of the cortex.

Some echinoid species present adaptive features that enable them to deal with low pH stresses. This seems to be related to the environmental conditions to which populations are submitted to. Therefore, organisms already submitted to pH daily or seasonal fluctuations or living in environments undersaturated in calcium carbonate seem to be able to cope with environmental conditions expected in an acidified ocean. Under the realistic scenario of a decrease of *ca*. 0.4 units of pH by 2100, sea urchins, and echinoderms in general, appear to be robust for most studied processes. Even thought, this general response can depend on different parameters such as exposure time, pH level tested, the process and the life stage considered, our results show that there is scope for echinoids to cope with ocean acidification.

RÉSUMÉ

Les émissions de dioxyde de carbone (CO_2) d'origine anthropique augmentent la concentration atmosphérique en CO_2 et sont absorbées, pour à peu près la moitié, par les océans. L'hydrolyse du CO_2 augmente la concentration en ions H^+ des océans, diminuant ainsi leur pH (acidification), et affectent également leurs proportions en ions HCO_3^- et CO_3^{2-}. Ce processus a déjà mené à une diminution de 0,1 unité de pH de l'eau de surface des océans. Selon l'IPCC, il est attendu que ce pH de surface diminue de 0,3-0,5 unités d'ici 2100 et de 0,7-0,8 unités d'ici 2300. Un des résultats de ce processus sera l'altération du système des carbonates; l'état de saturation de la calcite et de l'aragonite diminuera. Les eaux polaires et les eaux froides pourraient même se retrouver en sous-saturation d'ici les 50 prochaines années. Ces changements chimiques des océans pourraient avoir de sérieuses conséquences sur les organismes marins qui les habitent, en altérant des fonctions physiologiques majeures telles que la reproduction, la croissance et la calcification.

L'objectif du présent travail était d'étudier les effets d'une exposition à de l'eau à pH faible sur différentes étapes du cycle de vie des oursins, un groupe présentant des espèces-clés dans de nombreux écosystèmes marins. Les espèces d'oursins étudiées étaient issues de différents environnements et de différentes origines géographiques, de façon à pouvoir investiguer leur réponse différentielle, notamment leur possible adaptation et/ou acclimatation.

Dans un premier temps, nous avons étudié les effets de l'acidification des océans sur les premières étapes du cycle de vie d'une espèce intertidale de régions tempérées, l'oursin atlantique *Paracentrotus lividus*, et d'une espèce sub-antarctique, *Arbacia dufresnei*.

La fertilisation, le développement larvaire et la croissance larvaire ont été étudiés, sur des spécimens soumis à différents traitements de pH. Le taux de fertilisation des gamètes de *P.lividus* dont les géniteurs provenaient d'une mare caractérisée par une diminution de pH importante était significativement plus élevé, indiquant une possible acclimatation ou adaptation des gamètes à un stress de pH. La taille des larves soumises à des traitements de faible pH diminuait significativement pour les deux espèces. Par ailleurs, les plus petits echinoplutei d'*A. dufresnei* étaient isométriques à ceux de traitements-contrôles, ce qui montrait que la réduction de taille était probablement due à un taux de croissance plus faible. Les larves de *P.lividus* soumises à pH 7,4 (pH prédit pour 2300) présentaient significativement plus de formes anormales qu'au pH-contrôle, ce qui n'était pas le cas pour les larves d'*A. dufresnei*. La plus grande résistance de ces dernières proviendrait de l'acclimatation/l'adaptation aux importantes fluctuations de pH saisonnières vécues en conditions naturelles par *A. dufresnei*.

Dans un second temps, les réponses physiologiques d'adultes de *P.lividus* et *A. dufresnei* à une exposition à de l'eau à faible pH ont été étudiées. Les *P.lividus* intertidaux peuvent être soumis en conditions naturelles à des fluctuations de pH de 0,4 unités à marée basse, et le pH de leur liquide cœlomique ne s'en trouve pas en parallèle modifié. Après des semaines d'exposition expérimentale à pH faible, le liquide cœlomique (extracellulaire) des deux espèces étudiées diminuait. Ce liquide avait néanmoins une certaine capacité de tampon (plus

élevée que celle de l'eau de mer), qui ne semblait pas avoir de rapport avec une dissolution passive du squelette. En aquarium, le taux d'alimentation de *P.lividus*, l'activité de l'anhydrase carbonique (un enzyme impliqué dans la calcification et les processus respiratoires) du tégument d'*A.dufresnei*, et le rapport RNA/DNA (un indicateur de la synthèse des protéines et donc du métabolisme) des gonades et du tégument ainsi que la régénération des piquants (un indicateur de la calcification) de *P.lividus* et d'*A.dufresnei*, ne différaient pas en fonction du pH de l'eau de mer. Tout cela montre que les deux espèces étudiées sont capables de faire face à une légère hypercapnie (pH minimum étudié = 7,4) pendant une période moyenne (semaines).

Lors d'une autre série d'expériences en aquarium, l'effet du pH a été étudié sur l'oursin *P. lividus* à deux températures différentes, 10°C et 16°C. La diminution de pH du fluide cœlomique ne variait pas entre les températures, pas plus que les réponses de type tampon. Le taux de consommation d'oxygène (un indicateur de l'état métabolique global de l'organisme) de *P.lividus* augmentait pour les organismes soumis à des traitements de bas pH (7,7 et 7,4) à la température la plus basse (10°C), ce qui montrait qu'il était régulé à la hausse et que les organismes passaient par des périodes de haute demande énergétique pour maintenir à la normale leurs fonctions physiologiques. La production gonadique (évaluée par le rapport RNA/DNA) n'était affecté ni par la température, ni par le pH.

Finalement, une possible adaptation morphologique et chimique des piquants des cidaroïdes à des taux de saturation de calcite magnésienne faible a été investiguée. Ces piquants présentent la caractéristique de ne pas être recouverts d'épiderme comme ceux des autres oursins (piquants «nus»). Des spécimens d'eaux profondes de l'espèce *Ctenocidaris speciosa* provenant de la mer de Weddell (Antarctique) ont été étudiés. Les piquants de cidaroïdes présentent une couche extérieure de squelette polycristalline (le cortex) qui protège apparemment la partie intérieure du squelette, monocristalline (i.e. le stéréome classique des échinodermes, réseau tridimensionnel de calcite magnésienne). Le cortex de *C. speciosa* est divisé en deux parties. La partie intérieure était plus épaisse et présentait un taux de Mg plus élevé chez les spécimens collectés sous l'horizon de saturation de l'aragonite. Le cortex nu semblait capable de résister à des taux de saturation de carbonate de calcium bas. Nous suggérons que cela pourrait être lié à la quantité importante de matrice organique qui entoure les cristallites du cortex.

Notre travail indique que certaines espèces d'oursins présentent des caractéristiques adaptatives qui leur permettent de faire face à des stress de pH. Cela semble être lié aux conditions environnementales auxquelles ces populations sont soumises. Des organismes déjà soumis de façon journalière ou saisonnière à des fluctuations de pH, ou vivant dans des environnements sous-saturés en carbonate de calcium, semblent capables de faire face aux conditions environnementales attendues dans un océan acidifié.

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General Introduction

GENERAL INTRODUCTION

I - OCEAN ACIDIFICATION, THE OTHER CO_2 PROBLEM

1. ANTHROPOGENIC CO_2 EMISSIONS

The oceans contribute enormously to Earth's biodiversity. However, anthropogenic carbon dioxide (CO_2) emissions are changing them in many ways, posing a serious threat to marine organisms and ecosystems. Activities such as fossil fuel burning or cement production are largely associated with industrialization and are increasing the atmospheric CO_2 concentration (IPCC 2007). The analysis of ice core air bubbles showed that prior to industrial revolution the atmosphere contained approximately 280 ppm of CO_2 (Etheridge et al. 1998, Tyrrel 2011). This concentration had been fairly stable over hundreds of years until then (Fig. 1a, Etheridge et al. 1998).

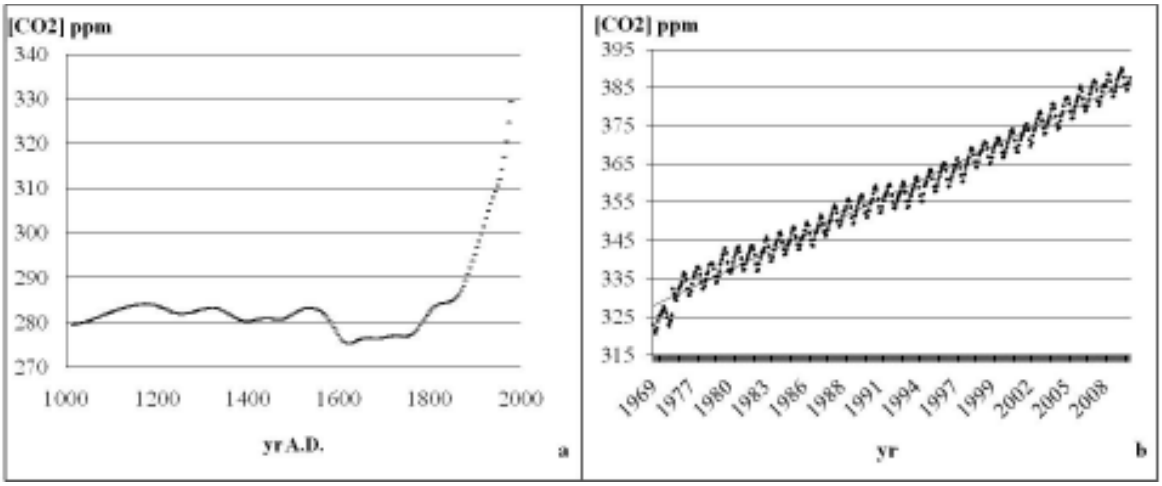

Fig. 1. a) Historical CO_2 record derived from the Law Dome DSS, DE08 and DE08-2 ice cores (Etheridge et al. 1998). b) The monthly mean CO_2 atmospheric concentration, measured at the Mauna Loa Observatory, NOAA, Hawaii (Tans 2011).

The Hawaiian NOAA station, the Mauna Loa Observatory, owns the longest data series of directly measured carbon dioxide atmospheric concentration (Fig. 1b, Tans 2011). By January 1979, the CO_2 concentration was already 336 ppm and the recent measures done in January 2011 revealed a value of 392 ppm (Trans 2011). This ca. 100 ppm increase since the industrial revolution represents more than the difference in atmospheric CO_2 between ice

ages and interglacial periods (Tyrrell 2011). Furthermore, the CO_2 concentration in the atmosphere is now higher than in the last 800,000 years (Lüthi et al. 2008).

One of the most well known consequences is the CO_2 greenhouse effect potentiation which is leading to an increase in the average temperature of the lower layer of the atmosphere and of the surface layers of the oceans, a phenomenon known as global warming. This has consequences such as melting of land-based ice, sea level rise, increased ocean stratification and climate change (Tyrrell 2011). Geographical distributions of aquatic and terrestrial animals have already been affected with enhanced risk of local or global extinction of species (IPCC 2007). Moreover, during the industrial era, the ocean has absorbed around a third of anthropogenic CO_2 emissions (Sabine et al. 2004, Royal Society 2005, Zeebe et al. 2008). The result is an increase in ocean acidity (pH decrease) and a shift in the chemistry of carbonates, a process known as ocean acidification. Only recently the scientific community has become aware of this parallel phenomenon to global warming. This process is not a climate change issue as it does not affect the weather patterns. However, known consequences for organisms and consequences for human well being can be as serious as global warming. In the Introduction section the chemical basis of ocean acidification and the current knowledge on its impacts on marine organisms will be presented.

2. SEAWATER CARBONATE SYSTEM IN AN OCEAN ACIDIFICATION CONTEXT

2.1 The carbonate system

In contrast with other atmospheric gases, like oxygen and nitrogen, when carbon dioxide dissolves in water it reacts with it forming bicarbonate and carbonate ions. In seawater, the essential components of the carbonate system are CO_2, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, hydrogen (H^+) and hydroxide (OH^-) ions. The carbonate species are related by the following equilibria (Zeebe and Wolf-Gladrow 2001):

$$CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

The concentration of the carbonic acid (H_2CO_3) is notably very low and the sum of the two electrically neutral forms, CO_2(aq) and H_2CO_3, is not chemically separable. Therefore, the previous equilibria are mostly represented in a simplified form (Zeebe and Wolf-Gladrow 2001):

$$CO_2(aq) + H_2O \overset{K_1}{\leftrightarrow} HCO_3^- + H^+ \overset{K_2}{\leftrightarrow} CO_3^{2-} + 2H^+ \quad (2)$$

The equilibrium constants K_1 and K_2 are the first and the second dissociation constants of carbonic acid, respectively, and they are dependent on temperature, salinity (Fig. 2) and pressure (Zeebe and Wolf-Gladrow 2001). In equilibrium, the net exchange of CO_2 between air and sea is zero: the partial pressure of CO_2 in the atmosphere equals the one of the surface seawater (pCO_2), related to the CO_2 concentration according to Henry's Law ($[CO_2]=K_0(T,S)\times pCO_2$, where $K_0(T,S)$ is the mainly temperature dependent solubility).

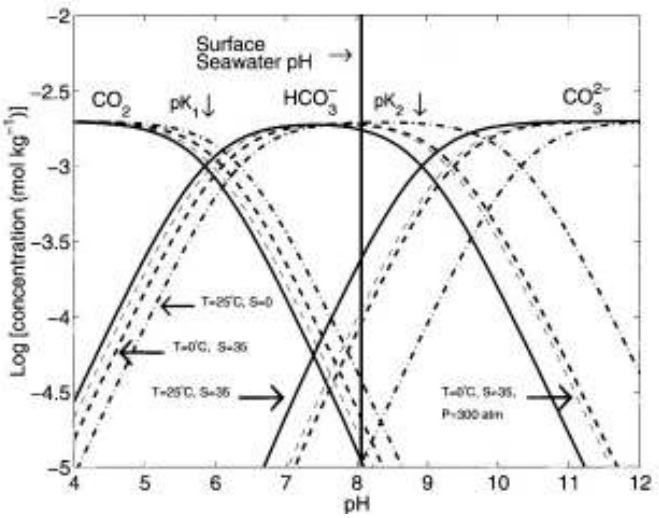

Fig. 2. Representation of the carbonate system equilibria and the effect of temperature, pressure and salinity on pK_1 and pK_2, i.e. the negative common logarithm of the dissociation constants of carbonic acid (after Zeebe and Wolf-Gladrow 2001). This figure shows the concentrations of the carbonate species as a function of the pH, but note that in seawater the proportions of CO_2, HCO_3^-, CO_3^{2-} control the pH and not vice-versa.

When $[CO_2(aq)] = [HCO_3^-]$, then pH=pK_1 and thus $K_1 = [H^+]$. Also, when $[CO_2(aq)] = [CO_3^{2-}]$, at that moment $K_2 = [H^+]$. When $[CO_2(aq)] = [CO_3^{2-}]$, the addition of HCO_3^- to the solution will not result in a pH change. As a result, the proportion of the carbon species can be calculated using the equilibrium constants (Morse and Mackenzie 1990, Zeebe and Wolf-Gladrow 2001).

The main difference between fresh water and seawater is the concentration and relative proportion of dissolved ions in the solution. In this case, the ionic strength is used to characterize the seawater as an ionic solution. Its value is approximately 0.7, depending on the salinity. The effective concentration in solution of an ion differs according to the ionic strength of the solvent. In seawater it is described as its activity (Zeebe and Wolf-Gladrow 2001). The activity of the i^{th} dissolved species (a_i) is related to its concentration (m_i) by an activity coefficient (γ_i): $a_i = m_i\gamma_i$. Usually the activity of the gas phase is expressed as the

fugacity. However, as the fugacity coefficient for CO_2 is greater than 0.999 (except in extreme conditions), the partial pressure of CO_2 is usually used to replace this parameter (Morse and Mackenzie 1990). The pCO_2 is therefore a measure of the degree of saturation of the seawater with CO_2 gas (Dickson 2010).

The pH definition takes into account the activity, i.e. the effective concentration, of H^+: $pH_a = -\log_{10} a_{H^+}$. However, individual ion activities cannot be determined experimentally since the concentration of a single ion cannot be varied independently because electroneutrality is required (Zeebe and Wolf-Gladrow 2001). So, an operational definition was established by the International Union of Pure and Applied Chemistry (IUPAC), creating the scale of pH NBS (previous National Bureau of Standards), now known as NIST (National Institute of Standards and Technology): pH_{NIST}. This scale was established by a series of standard buffers with specific pH values with the best approximation of $-\log_{10} a_{H^+}$. Therefore, the pH_{NIST} is close, but not identical, to the pH_a. The γ_{H^+} approaches unity when $[H^+]$ approaches zero in pure water (Zeebe and Wolf-Gladrow 2001). Traditionally the pH_{NIST} is measured potentiometrically using a glass electrode (Dickson 1993), but there are other methods, such as spectrophotometry (Millero et al. 1993). Briefly, the glass electrode allows the measurement of the electrical potential difference that develops between the ion activity in two different solutions separated by an interface. The difference in electrical potential is measured by an electrode inside the glass bulb, usually placed inside a mantle, filled with a liquid of known, constant composition, which creates a potential difference across the glass membrane and a reference electrode outside (combined in the pH glass electrode) (Schuster et al. 2009).

When it comes to the measurement of the pH of seawater samples, the use of NIST standard buffer solutions is not recommended as they have a very low ionic strength (~0.1) (Zeebe and Wolf-Gladrow 2001). It is then possible to use an alternative convention to define pH standards in terms of their total hydrogen ion concentrations in seawater, where the activity coefficients of the various reacting acid-base species are denominated by the presence of the ionic medium (Dickson 1993). Hence, another scale should be applied: the pH total scale (pH_T), which is based on a set of standard buffers based on artificial seawater. With this new scale it is possible to measure the pH in an analogous fashion to the NIST scale, i.e. using a potentiometric procedure (Dickson 1993). In seawater, protonation of ions such as SO_4^{2-} occurs ($HSO_4^- = H^+ + SO_4^{2-}$), but this scale also accounts for the presence of sulphate ions (Millero et al. 1993, Zeebe and Wolf-Gladrow 2001). The pH_T is defined by:

$$pH_T = -\log m^*(H^+) \quad (3)$$

The total hydrogen ion is given by (Millero et al. 1993):

$$m^*(H^+) = m(H^+) + m(HSO_4^-) = m(H^+)[1 + m(SO_4^{2-})/K(HSO_4^-)] \quad (4)$$

Where $m(SO_4^{2-})$ is the stoichiometric concentration of sulfate and $K(HSO_4^-)$ is the dissociation constant of the bisulfate ion (Millero et al. 1993). In case the medium additionally contains fluoride ions the rpotonation of F^- will have to be taken into account and the seawater pH scale used.

There are other important concepts in order to describe the carbonate system such as the total dissolved inorganic carbon (DIC) and the total alkalinity (TA). The DIC is the sum of the following species (Zeebe and Wolf-Gladrow 2001):

$$DIC \equiv \Sigma CO_2 = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (5)$$

The total alkalinity (TA), also known as titration alkalinity, refers to the acid neutralization capacity of the solution and is therefore closely related to the charge balance in the seawater, being not only related to the carbonate system, but also with other solution components such as borates, sulfates, phosphates, among others (Morse and Mackenzie 1990, Zeebe and Wolf-Gladrow 2001). In open-ocean surface seawater the TA can be given approximately by the following expression (Dickson 2010):

$$TA \approx [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+] \quad (6)$$

The four carbonate system parameters that can be analytically determined are the DIC, TA, pH and pCO_2. The knowledge of any two of them, together with temperature, salinity, pressure conditions and the relevant equilibrium constants will allow the calculation of the carbonate chemistry in seawater (Pierrot et al. 2006).

When CO_2 dissolves in seawater, its concentration in solution changes only slightly because the system is buffered by CO_3^{2-} ions:

$$CO_2 + CO_3^{2-} + H_2O = 2HCO_3^- \quad (7)$$

The Revelle factor (RF_0), a dimensionless factor, is an expression that allows to quantify the CO_2-buffering, describing how the pCO_2 changes for a given change in the DIC. Its value is proportional to the ratio between DIC and TA (Zeebe and Wolf-Gladrow 2001, Sabine et al. 2004):

$$RF_0 := \left(\frac{d[CO_2]}{[CO_2]} / \frac{dDIC}{DIC}\right)_{TA=const} \quad (8)$$

Therefore, when the ocean takes up CO_2, the relative increase in DIC is of around one tenth of the relative increase in dissolved CO_2 (Zeebe and Wolf-Gladrow 2001).

Carbon dioxide dissolution can further influence other important processes in seawater such as the formation and dissolution of calcium carbonate ($CaCO_3$), playing an important role in the global carbon cycle (Zeebe and Wolf-Gladrow 2001). The $CaCO_3$ stoichiometric solubility product is defined as:

$$K_{sp}^{*} = [Ca^{2+}]_{sat} \times [CO_3^{2-}]_{sat} \quad (9)$$

where *sat* refers to the total equilibrium (free + complex) ion concentration in a seawater solution saturated with calcium carbonate. The K_{sp}^{*} depends on the temperature, salinity and pressure conditions. Calcium carbonate solubility increases at lower temperature and at higher pressure (or depth). The dissolution of marine carbonates buffers CO_2 via the reaction:

$$CaCO_3 + CO_2 + H_2O \leftrightarrow 2HCO_3^- + Ca^{2+} \quad (10)$$

Furthermore, the $CaCO_3$ saturation state (Ω) in seawater (SW) is given by:

$$\Omega = \frac{[Ca^{2+}]_{SW} \times [CO_3^{2-}]_{SW}}{K_{sp}^{*}} \quad (11)$$

Surface seawaters have a $\Omega > 1$, i.e. the solution is supersaturated with regard to $CaCO_3$ and the environment is favorable to the mineral precipitation. In the water column the saturation depth or the saturation horizon is reached when Ω = 1 and the solid and the solution are in equilibrium. Below this depth the environment becomes undersaturated and favorable to dissolution of calcium carbonate minerals (Morse and Mackenzie 1990, Zeebe and Wolf-Gladrow 2001). The majority of calcium carbonate can be found in the form of calcite or aragonite, both with the same chemical composition, but different mineralogy, with aragonite being more soluble than calcite. Consequently, both minerals have different K_{sp}^{*} and the saturation horizon of calcite is deeper than that of aragonite (Zeebe and Wolf-Gladrow 2001, Orr et al. 2005).

Calcium carbonate minerals can also incorporate other divalent ions such as magnesium, by replacing calcium in solid solution, forming in this case magnesium calcite (Morse et al. 2006, Andresson et al. 2008). Magnesium incorporation into the mineral crystal lattice will change the solubility of the magnesium calcite. Above 8-12 mol % of $MgCO_3$ its solubility

will be higher than both calcite and aragonite, although this also depends if the mineral origin is biogenic or abiotic (Morse et al. 2006, Andersson et al. 2008).

The general expression for magnesium calcite saturation state is given by:

$$\Omega_{Mg-calcite} = \frac{a_{Mg^{2+}}^{(x)} \times a_{Ca^{2+}}^{(1-x)} \times a_{CO_3^{2-}}}{K_x} \quad (12)$$

where x is the mol fraction of magnesium ions and K_x is the equilibrium constant with respect to a particular carbonate phase (K_x = IAP, ion activity product at equilibrium). The K_x represents a metastable equilibrium state obtained from the referred stoichiometric saturation but a true equilibrium cannot be achieved with respect to Mg-calcite minerals (Morse and Mackenzie 1990, Morse et al. 2006, Andersson et al. 2008). Thus the solubility of biogenic magnesium-calcite minerals is determined through the use of experimentally obtained curves where -log $IAP_{Mg\text{-}calcite}$ is expressed according to the magnesium content of the mineral (Morse and Mackenzie 1990). Now-a-days is not yet fully understood which solubility curve is most representative of the behavior of biogenic magnesium calcites, but there seems to be a consensus towards the use of the "biogenic curve of Plummer and Mackenzie", also known as the "minimally prepared curve" (Plummer and Mackenzie 1974, Morse and Mackenzie 1990, Morse et al. 2006, Andersson et al. 2008) (Fig. 3). This equilibrium constant, as any other, depends on parameters such as temperature and pressure. Therefore, it is still not yet possible to calculate $\Omega_{Mg-calcite}$ at higher depths, as measured values refer to ca. 1 atm.

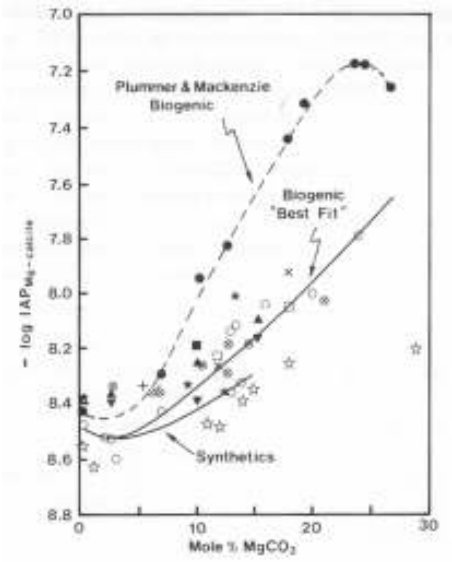

Fig. 3. Solubility of the magnesium calcites as a function of the magnesium content of the mineral ($MgCO_3$) expressed in terms of -log $IAP_{Mg\text{-}calcite}$ (after Morse and Mackenzie 1990). Different curves, determined experimentally, are represented, included the "biogenic curve of Plummer and Mackenzie" (see text for explanation).

2.2 Ocean acidification: present conditions and future changes

As atmospheric CO_2 concentration is increasing, carbon dioxide is partly absorbed by the oceans (following Henry's law), interfering with the carbonate chemistry (Fig. 4). During the past 200 years the oceans absorbed around a third of the anthropogenic carbon dioxide emitted to the atmosphere (Sabine et al. 2004, Royal Society 2005, Zeebe et al. 2008). The CO_2 hydrolysis increases the H^+ concentration, decreasing the pH, while the proportions of the HCO_3^- and CO_3^{2-} ions are also affected (Fig. 2). In fact, this process already led to a decrease of 0.1 pH units of surface seawater (Caldeira and Wickett 2003, Royal Society 2005, Zeebe et al. 2008). Time series data, from the Mauna Loa and ALOHA stations in Hawaii (North Pacific), show a clear link between the increase of atmospheric CO_2, the consequent increase of seawater pCO_2 and the decrease of surface seawater pH (Fig. 4) (Doney et al. 2009, Karl 2008, Tans 2011). The saturation states of calcite and aragonite are also declining (Fig. 4), due to the lower availability of the CO_3^{2-} ions (Fig. 5) (Royal Society 2005, Doney et al. 2009). This has already contributed to the shoaling of saturation horizons by 30-200m from the preindustrial period to the present (Feely et al. 2002, Sabine et al. 2002, Feely et al. 2004, Doney et al. 2009, Tyrrell 2011).

The majority of the anthropogenic CO_2 is confined by the thermocline to the upper layers of the ocean, due to density differences in water masses (Sabine et al. 2004). The capacity for ocean surface waters to uptake CO_2 is inversely proportional to the Revelle factor, as this gives an idea of the resistance of the oceans to absorb atmospheric CO_2. Regional differences in the Revelle factor lead to variations in surface CO_2 concentrations with warm tropical waters having lower RF_0 values, while polar waters have higher ones (Sabine et al. 2004).

The current surface ocean pH varies between 7.9 and 8.25 units according to season or region, due to temperature, biological production and freshwater input variations, but having an average value of 8.08 (Royal Society 2005). Local events can further disturb surface pH. For instance, low pH observed in the Southern Ocean is not only due to low surface temperature and carbonate system related thermodynamics, but also due to an important amount of upwelled water containing high [CO_2] (Orr et al. 2005). Other areas naturally enriched in CO_2, presenting intermittent or permanent lower pH conditions, are upwelling zones in general, intertidal pools, the coastal and estuarine areas and the deep sea.

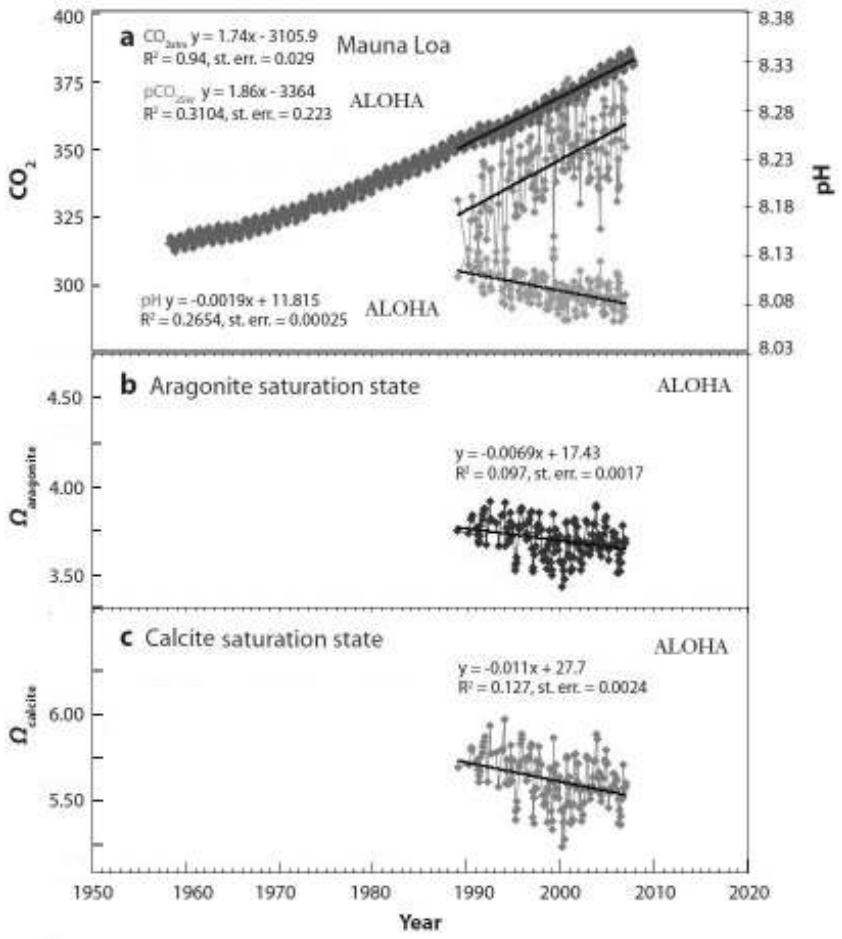

Fig. 4. Time series of the North Pacific Hawaiian Mauna Loa (Tans 2011) and ALOHA (Karl 2008) stations (after Doney et al. 2009). a) Atmospheric CO_2 (ppm) in red at Mauna Loa station, surface ocean pH in cyan and pCO_2 (μatm) in tan at ALOHA Station; b) aragonite saturation state ($\Omega_{aragonite}$), dark blue, and c) calcite saturation state ($\Omega_{calcite}$), gray, at ALOHA station.

Predictions of ocean chemistry changes have been derived from scenarios of CO_2 emissions and models of the carbon cycle. Six alternative Intergovernmental Panel on Climate Change (IPCC) scenarios (IS92a to f) were published in the 1992 Supplementary Report to the IPCC Assessment (IPCC 1992). This concerns the prediction of CO_2 emissions ranging from very high emissions of 35.8 GtC to very low emissions of 4.6 GtC by 2100 (corresponding to a six fold increase and a decline by a third compared to 1990 levels, respectively), based on assumptions such as climate policies and economic, social and environmental conditions. From these, the IS92a has been extensively used as a standard scenario for impact assessments and is usual referred as the "business-as-usual" scenario (IPCC 2000, IPCC 2007). According to this scenario the pH is expected to decrease 0.3-0.5 units by 2100 and 0.7-0.8 by 2300 (Figs. 5, 6) (Caldeira and Wickett 2003, IPCC 2007).

Despite past oscillations, in the previous 25 millions of years the oceans never suffered such a fast pH variation (Fig. 6, Pearson and Palmer 2000).

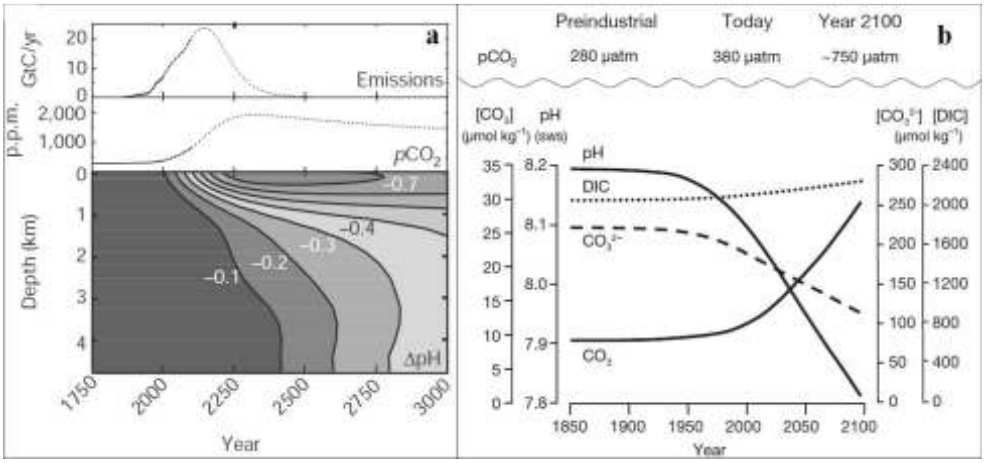

Fig. 5. Atmospheric content of carbon dioxide and relation with ocean acidification related changes in the carbonate system (after Caldeira and Wickett 2003, Rost et al. 2008, assuming the IS92a Scenario from IPCC 2007). a) Anthropogenic CO_2 emissions and atmospheric pCO_2 (historical - full line; predictions - dotted line) and consequent pH changes in seawater column. b) Historical and predicted changes in the surface ocean carbonate system chemistry in response to changes in atmospheric pCO_2.

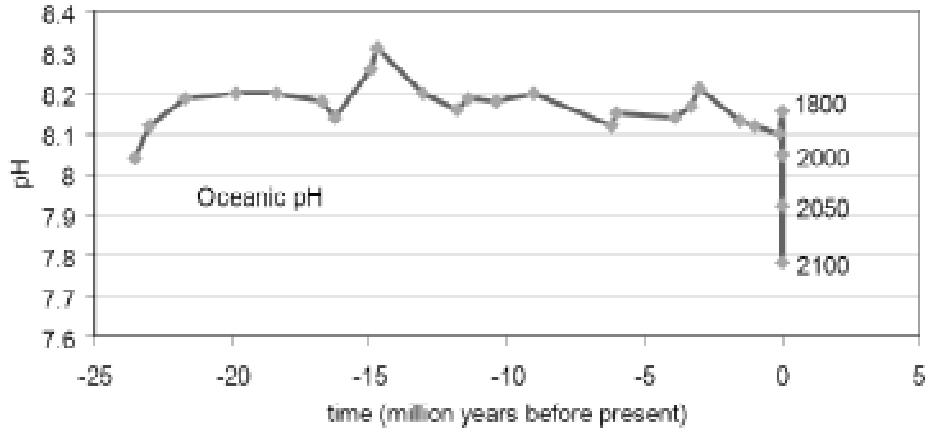

Fig. 6. Past and present variability of marine pH. Future predictions for years shown on the right-hand side of the figure are based on IPCC (2007) mean scenarios. Data from Pearson and Palmer (2000) and Turley et al.(2006) (after Eur-Oceans Fact Sheet No. 7 2007).

As a result, surface ocean carbonate system chemistry will also change: with increasing pCO_2, DIC will increase and the equilibrium of the carbonate system will shift to higher CO_2 and HCO_3^- levels, while CO_3^{2-} concentration will decrease (Fig. 5b). As previously seen, surface seawaters are becoming and will progressively become even less saturated in calcite and aragonite. Some particular polar and cold water regions can actually become completely undersaturated within the next 50 years (Orr et al. 2005).

According to Cao and Caldeira (2008), stabilization of atmospheric CO_2 levels should be lower than 450 ppm, otherwise the risk of deeply perturbation of the chemistry of the oceans will be significantly high. For instance, at this concentration, parts of the high latitude ocean will become undersaturated with respect to aragonite and they will experience a decrease in pH by more than 0.2 units. But at 550 ppm consequences will be even more serious and for instance there will be no water left in the open ocean with the kind of chemistry experienced by more than 98% of shallow-water coral reefs before industrial revolution. Such changes to ocean chemistry will adversely affect marine ecosystems.

II - BIOLOGICAL RESPONSES TO OCEAN ACIDIFICATION

1. OCEAN ACIDIFICATION EFFECTS THROUGHOUT DIFFERENT LIFE STAGES OF MARINE ORGANISMS

1.1 Early life stages

Many marine organisms have external fertilization, early hatching and/or free larval stages within the water column. Tolerance windows to stresses such as environmental hypercapnia (an exposure to extreme CO_2 levels) are species specific and vary throughout the life cycle, the early stages being potentially more susceptible to environmental change (Kurihara 2008, Pörtner 2008, Pörtner and Farrell 2008). These can therefore be particularly vulnerable to environmental perturbations and stressors, which can have serious downstream effects on populations (Byrne 2010). For instance, 55-85% of benthic invertebrates have a long pelagic larval stage that can spend weeks to months in the plankton (Thorson 1950, 1966) and so a prolonged exposure to adverse conditions can compromise their development. Recruitment success depends on the survival of the embryos and larvae (López et al. 1998) and a decrease in embryo and larval survival or delay in development can reduce population long-term viability (Morgan 1995).

As the ocean pH continues to decrease, environmental hypercapnia may be of concern for gamete production, function and fertilization. The gonads of many invertebrates have usually a low pH/high CO_2 content due to the inefficient internal oxygen transport to internal tissues (Elligton 1982). This also allows the gametes to be kept in a quiescent state (Byrne 2010). Hypercapnia has a narcotic effect on sperm, but this can be overcome by stimulatory

influence of compounds in the egg jelly coat (Byrne 2010). For instance, fertilization in oysters and mussels might not be affected by ocean acidification, while their larval development is (Table 1, Kurihara et al. 2007, Bechmann et al. 2011). Experimental evidences show that larval morphology and growth are in general affected by ocean acidification (Table 1). However, impacted responses are highly species specific and also related to the intensity of the exposure (Table 1). For instance, while at first glimpse the copepod *Acartia erythraea* seems to be more vulnerable to environmental hypercapnia than a close related species, *Acartia tsuensis*, the level of CO_2 exposure was higher in the first (Table 1, Kurihara et al. 2004a, Kurihara and Ishimatsu 2008).

Table 1. Examples of neutral (⇔) and negative (⇩) responses related to reproduction and early life stages of organisms submitted to low pH. pH in NIST scale except pH_{SWS} (seawater scale).

Group/Taxa	Studied Range	Effect	Reference
Cod fish *Gadus morhua*	pH 8.08-7.56 pCO_2 391-1365 µatm	Sperm motility: ⇔	Frommel et al. 2010
Clownfish *Amphiprion percula*	pH 8.15-7.6	Olfactory ability: ⇩	Munday et al. 2009
Coral larvae *Acropora digitifera*	pH_{SWS} 8.05-7.33 pCO_2 331-3100 µatm	Larval survival and metabolism: ⇔, metamorphosis: ⇩	Nakamura et al. 2011
Copepod *Acartia steueri*	pH 8.17-7.02 pCO_2 0.04-1.04 kPa	Egg production: ⇩	Kurihara et al. 2004a
Copepod *Acartia erythraea*	pH 8.11-6.96/6.82 pCO_2 0.04-1.04 kPa	Egg production, hatching and larval survival: ⇩	Kurihara et al. 2004a
Copepod *Acartia tsuensis*	pCO_2 380-2000 ppm	Egg production, hatching, larval survival and growth: ⇔	Kurihara and Ishimatsu 2008
Shrimp *Pandalus borealis*	pH 8.16/8.10-7.67/7.58 pCO_2 381/368-1332/1291 µatm	Larval survival: ⇔, larval growth: ⇩	Bechmann et al. 2011
Oyster *Crassostrea gigas*	pH 8.2-7.4 pCO_2 348-2268 µatm	Fertilization: ⇔, larval growth and morphology: ⇩	Kurihara et al. 2007
Mussel *Mytilus galloprovincialis*	pH 8.13-7.43 pCO_2 380-2000 ppm	Larval growth and morphology: ⇩	Kurihara et al. 2008
Mussel *Mytilus edulis*	pH 8.15-7.58 pCO_2 468-1929 µatm	Larval survival: ⇔, larval growth: ⇩, hatching: ⇔⇩	Gazeau et al. 2010
	pH 8.10/8.07-7.64/7.59 pCO_2 419/469-1940/2313 µatm	Fertilization: ⇔, larval growth: ⇩	Bechmann et al. 2011

It is during larval development or at settlement (attachment to the substratum) stages that the first calcifying structures appear in some taxa (Kurihara 2008). Ocean acidification has a negative effect on biomineralized structures (see section 2) influencing their size and production (Kurihara 2008). However, it is not always easy to identify the specific mechanism or group of processes being affected and not always smaller size or slower growth rate can be synonymous of lower calcification rates. So far, studies have not yet

addressed ways to distinguish or better understand these ambiguities. Nevertheless, the knowledge of the vulnerabilities and thresholds of early life stages is essential to better understand how ocean acidification will affect species and populations.

1.2 Adults

Adult responses to environmental hypercapnia can be extremely variable and the degree of sensitivity varies between species (Table 2). Ocean acidification effects are generally negative, but it can be hard to establish patterns in responses and sensitivities (Kroeker et al. 2010). In general, calcifying organisms seem to be more sensitive to ocean acidification, but data is still missing in order to better understand how other physiological processes can be affected (see section 2, Fabry et al. 2008, Pörtner 2008, Doney et al. 2009, Hendriks et al. 2010, Melzner et al. 2009, Kroeker et al. 2010). Key survival processes are related to food acquisition (energy input), maintenance of body activity and energy through metabolism regulation, finally ensuring that energy will be allocated to growth and reproduction. Ocean acidification can potentially interfere with any of these processes and during all life stages of an individual (Table 1, 2). Organisms with longer generation times can actually experience ocean acidification effects within their life. It is thus important to know what are the thresholds of the organisms when it comes to hypercapnia and to better understand if there is scope for coping with these new environmental conditions.

At the present, reported impacts go from extracellular pH decrease (showing lack of compensation mechanisms in some species), metabolic rate increase (indicating a higher energetic demand), but also some cases of metabolic depletion, change in tissue metabolite production, change of growth rate and survival, among others (Table 2). These can have important consequences since organisms sensitivity for other stressors, for instance temperature increase due to global warming, can change and species vulnerability towards other impacts can increase (Pörtner 2008, Hoffman and Todgham 2010). Interestingly, in the examples shown on Table 2, feeding rate was maintained, possibly showing that if food is available these organisms will sustain this function in order to obtain energy and nutrients. Also, and as previously discussed, responses to environmental hypercapnia depend on the exposure time and intensity, as seen for the shrimp *Palaemon pacificus* (Kurihara et al. 2008, Table 2). Furthermore, a sudden exposure during a short term experimental period might not mimic real ongoing ocean acidification (Hendriks and Duarte 2010).

Table 2. Examples of positive (⇧), neutral (⇔) and negative (⇩) responses of physiological processes of adults submitted to low pH. V_{O2} stands for oxygen uptake, pH in NIST scale, manipulated by CO_2 bubbling. References (R.): 1) Marchant et al. 2010, 2) Beniash et al. 2010, 3) Lanning et al. 2010, 4) Thomsen et al. 2010, 5) Thomsen and Melzner 2010, 6) Gutowska et al. 2010b, 7) Small et al. 2010, 8) Kurihara et al. 2008.

Group/Taxa	Exposure time	Studied Range	R.	Physiological process	Response trend
Limpet (mollusk) *Patella vulgata*	5 days	pH 8.24-7.53, pCO_2 419-2804 µatm	1	Feeding rate	⇔
				Extracellular pH	⇔ (shell passive dissolution)
				Metabolic rate (V_{O2})	⇔
Oyster (mollusk) *Crassostrea virginica*	20 weeks	pH 8.3-7.5, pCO_2 385-3523 µatm	2	Metabolic rate (V_{O2}) (juveniles)	⇧
	2 weeks			Metabolic rate (V_{O2}) (adults)	⇔
				ADP:ATP (adults)	⇧
				Anaerobic end products (adults)	⇔
Oyster (mollusk) *Crassostrea gigas*	55 days	pH 8.07-7.68, pCO_2 0.059-0.15 kPa	3	Extracellular pH	⇩
				Metabolic rate (V_{O2})	⇧ (synergistic effect with temperature)
				Respiration rate of isolated gill cells	⇔
				Tissue metabolites	All ⇔ except: ⇩ alanine and ATP in muscle; ⇧ succinate in gills
Mussel (mollusk) *Mytilus edulis*	2 weeks	pH 8.05-7.08, pCO_2 464-4254 µatm	4	Extracellular pH	⇩ (no shell passive dissolution)
	8 weeks	pH 8.13-7.26, pCO_2 493-3898 µatm		Somatic growth	⇔ (but shell grew slower)
	2 months	pH 8.03-7.14, pCO_2 48-378 Pa	5	Somatic growth	⇔ (but shell grew slower)
				Metabolic rate (V_{O2})	⇧ until pH 7.38, but ⇩ at lowest tested pH 7.14)
				Ammonium excretion	⇧
Cuttlefish (mollusk) *Sepia officinalis*	48h	pH 8.12-7.10, pCO_2 0.05-0.6 kPa	6	Extracellular pH	⇩⇔ partially compensated
				Intracellular pH	⇔
				Arterial O_2	⇔
Crab (crustacean) *Necora puber*	30 days	pH 7.85-6.69, pCO_2 734-12341 µatm	7	Extracellular pH	⇔ (skeleton passive dissolution ?)
				Metabolic rate (V_{O2})	⇩
Shrimp (crustacean) *Palaemon pacificus*	30 weeks	pH 8.17-7.89, pCO_2 ?-1000ppm	8	Feeding rate	⇔
				Survival	⇩
				Total length	⇔
				Moulting frequency	⇧
				Metabolic rate (V_{O2})	⇔
	15 weeks	pH 8.15-7.64, pCO_2 ?-1900ppm		Feeding rate	⇔
				Survival	⇩
				Total length	⇩
				Moulting frequency	⇩

2. OCEAN ACIDIFICATION EFFECTS ON PHYSIOLOGICAL MECHANISMS

2.1 Ocean acidification effects on calcification

Calcification is the process whereby organisms use Ca^{2+} and CO_3^{2-} in order to precipitate calcium carbonate minerals, forming structures such as shells or skeletons. This is a tightly biologically controlled process, which usually occurs in either intra or extracellular confined compartments where ion transportation ensures supersaturation in order to promote mineral precipitation (Crenshaw 1990). Due to the reduction of the availability of the carbonate ion and of calcium carbonate saturation states, the scientific community was first concerned about ocean acidification effects on calcifiers. The first studies published on this thematic exposed how the calcification rates of corals and coccolithophores decreased when individuals were exposed to lower pH and/or low saturation states of calcium carbonate waters (Langdon 1993, Gattuso et al. 1998, Leclercq et al. 2000, Riebesell et al. 2000). In fact, evidence that calcification rates in some organisms are affected by ocean acidification conditions has been increasing over the years, with especial vulnerability reported for corals (e.g. Gattuso et al. 1998, Leclercq et al. 2000, Fabry et al. 2008, Marubini et al. 2008, Andersson et al. 2009, Doney et al. 2009) and polar pteropods (e.g. Orr et al. 2005, Comeau et al. 2009), but with other groups being affected such as coccolitophores, foraminifera, gastropods, mussels, oysters and echinoderms (see for review Royal Society 2005, Fabry et al. 2008, Doney et al. 2009, Dupont et al. 2010b, Hofmann et al. 2010). Field observations of the communities around a CO_2 volcanic vent showed that the abundance of calcifiers was significantly reduced in the lowest pH areas (Hall-Spencer et al. 2008). There is also evidence that extreme past events associated with ocean acidification, such as the Paleocene-Eocene Thermal Maximum (PETM), led to mass extinctions of calcifying organisms (Zachos et al. 2005, Kump et al. 2009, Ridgwell and Schmidt 2010). Calcified structures have an important role in protection against predators and stabilizing body form and function. Not only will this impact affects the organisms growth and well being, but also will interfere globally in the ocean carbon cycle. For instance, coccolithophores, foraminifera and pteropods are the major pelagic producers of calcite and aragonite and produce a large part of the particulate organic and inorganic carbon exported from the surface to deep ocean (e.g. Hohenegger 2000, Iglesias-Rodríguez et al. 2002, Jin et al. 2006). Furthermore, marine

calcifiers whose skeleton or shells contain magnesium-calcite, like echinoderms, foraminifera or coralline seaweeds, are considered to be at a higher risk since it can be more soluble than calcite and a have a similar or higher solubility than aragonite (Andersson et al. 2008).

Table 3. Examples of positive (⇧) or neutral (⇔) responses of calcifiers submitted to environmental hypercapnia.

Group	Species	Effect range	Calcification response	Reference
Coral (temperate sp)	*Cladocora caespitosa*	pH 8.10-7.84, [CO_2] 14.9-22.0 µmol.kg^{-1}, $\Omega_{aragonite}$ 3.04-2.78	⇔	Rodolfo-Metalpa et al. 2010
Cephalopod	*Sepia officinalis*	pH 8.01-7.10, [CO_2] 63.6-614.8 Pa, $\Omega_{aragonite}$ 1.78-0.27	⇧	Gutowska et al. 2010a
Limpet	*Crepidula fornicata*	pCO_2 605-903 ppm	⇧	Ries et al. 2009
Mussel	*Mytilus edulis*	pCO_2 605-2856 ppm	⇔	Ries et al. 2009

However, not all calcifying organisms present a negative response to high CO_2 levels in seawater (Table 3). As previously said, calcification is a biologically controlled process ocurring in confined compartments. Even though ocean acidification reduces availability of CO_3^{2-} in seawater, this ion hardly ever crosses biological membranes, entering cells via CO_2 diffusion or by HCO_3^- transport (Crenshaw 1990). The precipitation of calcium carbonate structures is therefore not directly dependent on seawater chemistry, but usually strongly modulated by the organisms (Pörtner 2008, Hofmann and Todgham 2010). Nevertheless, some structures can be exposed to passive dissolution and calcification can be impaired due to organism physiological status and throughout indirect processes. It is therefore important to understand how hypercapnia and seawater chemical changes can affect other physiological processes.

2.2 The buffering system and tolerance mechanisms towards hypercapnia

Maintenance of homoeostasis, regulated thanks to processes such as respiration, circulation, ionic regulation and acid-base balance, is a central feature in animals. Most of the mentioned processes are active and require energy for continued operation. Organism energy production, besides (in most of the cases) requiring oxygen supply, will also need the elimination of metabolic products such as CO_2, nonvolatile protons and organic acids responsible for internal fluids hypercapnia (Heisler 1989). This transport is provided by blood or other physiologic fluids such as hemolymph or coelomic fluid (Florey 1968, Heisler

1989). Large quantities of CO_2 can dissociate and produce further H^+, decreasing the pH, which may affect enzyme and other critical protein functioning and thus crucial processes in the organism (Cameron 1986, Boron 2004). Elimination of intracellular H^+ will depend on the extracellular one, its buffer capacity and available volume of extracellular fluid (Heisler 1989). The first line of defense of an organism against acidosis will be therefore the ability to buffer its external fluids pH (Fig. 7, Heisler 1989, Fabry et al. 2008, Pörtner 2008). The two extracellular buffering systems functional in all organisms are the CO_2-bicarbonate and the non CO_2-bicarbonate one which usually involves the presence of partially protonated amino acids, N-terminal α-amino groups of proteins or organic/inorganic phosphate groups (Melzner et al. 2009). The CO_2-bicarbonate buffer has a limited efficiency since its response to an excess of H^+ results is an increase of CO_2 in extracellular fluids that should be eliminated. As this depends on diffusion gradients between organism and surrounding water, this process can be extremely hard (Heisler 1989, Melzner et al. 2009). Other mechanisms might include passive buffering of extra and intracellular fluids or CO_2 transport on body fluids which own respiratory pigments (Heisler 1989, see Fabry et al. 2008 for review).

The mechanisms to deal with an environmental change of pH are limited and excretion and/or elimination of an excess of protons might require extra levels of energy through active transport (Fabry et al. 2008, Melzner et al. 2009). The main transporters are membrane Na^+-H^+ exchangers and Na^+-HCO_3^- co-transporters, which will further depend on ionic gradients, but other transporters can be associated with acid-base balance maintenance (Fig. 7, Heisler 1989, Pörtner 2008, Casey et al. 2010). Excessive extracellular acidosis can lead to metabolic suppression, reduction of ion exchange, reduction of protein synthesis, lowered oxygen supply, among others (Fig. 7, Pörtner et al. 2004, Fabry et al. 2008, Pörtner 2008, Melzner et al. 2009). Excess of protons in extracellular fluids can affect calcification processes either through metabolic depression or due to the increased cost of H^+ elimination (Pörtner 2008, Hofmann and Todgham 2010). The scale and magnitude of these responses depend on the intensity and duration of the exposure to a pH decrease. Whatever the mechanism, buffering will only mask the protons during an acidosis event, and it is then important to eliminate them via active transport usually in specialized epithelia (such as gills, renal and/or digestive tissue). These processes are therefore optimized whenever mechanisms such as osmoregulation and other ion-regulation are more developed (Fig. 7, Whiteley et al. 2001, Seibel and Walsh 2003, Pane and Barry 2007, Pörtner 2008, Melzner et al. 2009). The ability to tolerate acute CO_2 toxicity seems to depend on the capacity to compensate for acid-base

disturbances which is enhanced in hypermetabolic organisms owning highly specialized osmorregulatory and excretion structures in order to eliminate the large amounts of CO_2 produced in a high metabolism (Pörtner et al. 2004). Also, more tolerant taxa are usually characterized by high metabolic rates and high levels of activity (Melzner et al. 2009). For instance, this ability seems to be highly enhanced in crustaceans living in more variable habitats (coastal and intertidal) and that possess highly improved osmoregulation skills than those which live in more stable environments (Whiteley et al. 2001, Dissanayake et al. 2010). This can be due to adaptations (linked to genetic variability at a population level) and/or acclimatization (due to phenotypic plasticity at an individual level).

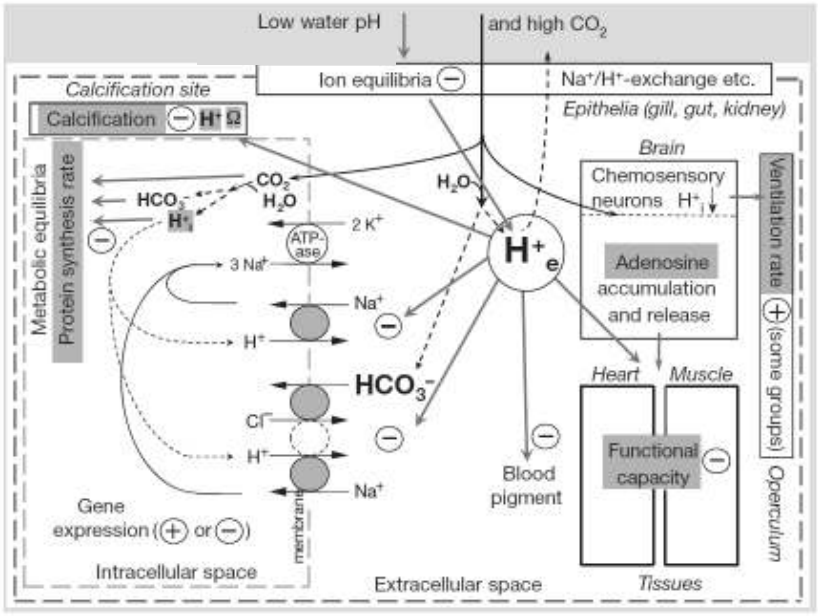

Fig. 7. Schematic representation of a water-breathing animal, showing the important role of extracellular pH and the positive (+) and negative (-) effects on processes and mechanisms in case of high environmental CO_2 and consequent internal fluids hypercapnia (after Pörtner 2008). Dark shaded areas indicate processes involved in changing energy budget. Gray arrows indicate signaling through water or body fluid physicochemistry, with a key role for intra (i) and extracellular (e) H^+ or other factors like adenosine, K^+, Na^+, or Cl^- (Pörtner 2008).

A wide variety of organisms can be found in environments where they experience seasonal or daily pH variations such as coastal, estuarine, intertidal and upwelling areas (Truchot and Duhamel-Jouve 1980, Morris and Taylor 1983, Denny and Gaines 2007, Feely et al. 2008, Wootton et al. 2008, Feely et al. 2010, Thomsen et al. 2010, Yu et al. 2011). Furthermore, deep sea and cold water regions are characterized by lower pH due to higher CO_2 concentrations (Park 1966, Valdenegro and Silva 2003, Yamamoto-Kawai et al. 2009).

These organisms can therefore be used as models to evaluate possible acclimatization and/or adaptation to ocean acidification.

III - ECHINODERMS IN AN ACIDIFIED OCEAN

1. ECHINODERMS

Echinoderms (phylum Echinodermata) are exclusively marine and they are widely distributed in all the oceans, from the intertidal to the abyssal regions. They are keystones species in many ecosystems having varied roles such as predators, grazers, deposit feeders and ecosystem engineers. This includes five extant classes: sea urchins (Echinoidea), starfish (Asteroidea), brittle stars (Ophiuroidea), sea cucumbers (Holothuroidea) and feather stars (Crinoidea) (Fell and Pawson 1966, Lawrence 1987).

Adults are benthic, but different life cycle patterns can be found with the presence of most commonly pelagic or benthic larvae. Development is planktotrophic or lecithotrophic; there are some brooding species or others with direct development. Larval development involves growth and elaboration of larval body and development is arrested when metamorphic competence is achieved, allowing settlement and consequent recruitment to occur (Ruppert and Barnes 2004, Balch and Scheibling 2001, McEdward and Miner 2001). Factors affecting the larval development and survival, which include temperature, predation, disease and environmental parameters fluctuations, can control larval supply and therefore influence settlement and recruitment success (Balch and Scheibling 2001). Post-metamorphic development will imply continuous growth and sexual maturation (McEdward and Miner 2001).

One of most important features of this group is the presence in postmetamorphic stages of an endoskeleton which, in most species, occupies a significant part of their dermis and which is separated from the environment by the epidermis. This endoskeleton is composed of magnesium calcite (Ca-Mg-CO_3), the solubility of which is similar or higher than that of aragonite (Morse and Mackenzie 1990, Morse et al. 2006, Andersson et al. 2008). This highly calcified group has thus an import role in the ocean carbon cycle, being their highest contribution (80%) done between 0-800m (Lebrato et al. 2010). Biomineralization in echinoderms is biologically mediated and involves ion transport pathways (Mitsunaga et al. 1987a, b, Dubois and Chen 1989). The skeleton is composed of a tridimensional meshwork

termed stereom and each individual strut is referred to as trabeculae (Dubois and Chen 1989, Smith 1990). Although it is a polycrystalline aggregate it behaves optically like a single crystal (Smith 1990). Furthermore, there is an organic phase composed of proteins, polysaccharides and lipids associated with the mineral one, named organic matrix, which strongly controls the skeleton magnesium content (see Hermans et al. 2011 and references therein). Larval spicules, present in ophiuroids and echinoids, are almost entirely reabsorbed and the skeleton of post-metamorphic organisms appears immediately before metamorphosis (Dubois and Chen 1989).

Echinoderms lack morphologically differentiated excretory organs and have a poor ability to osmo and ion regulate (Binyon 1966, Jangoux 1982, Stickle and Diehl 1987). Their metabolic wastes, essentially nitrogenous, including ammonia, urea and uric acid, are mainly eliminated in digestive organs (Jangoux 1982). Moreover, there is evidence that limited ionic regulation is possible in some fluid compartments (Binyon 1966, Bishop et al. 1994, Vidolin et al. 2007). The main circulatory medium, the coelomic fluid, together with the water vascular system ensure gas transportation (Farmanfarmaian 1966, Ruppert and Barnes 1994). Its ionic composition is similar to that of seawater, since echinoderms are osmoconformers, but its pH is usually 0.5-1.5 units lower because of a high CO_2 content, due to the slow elimination of this gas which is dependent on diffusion gradients for exchange with seawater (Farmanfarmaian 1966). With the exception of very few species of brittlestars and sea cucumbers, there are no respiratory pigments present in their body fluids (Shick 1983). Main respiratory surfaces, where gas exchange takes place, are tube feet on echinoids and asteroids, respiratory trees in holothurians, among others (Shick 1983).

These characteristics, low acid-base regulation linked to low excretory, respiratory and in regulation abilities together with a high soluble skeleton make echinoderms a group at risk in front of ocean acidification. However, echinoderms present significant morphological and physiological plasticity that allow them to function in different environments, presenting different growth rates, reproductive periodicity and environmental tolerances (Marcus 1983). For instance, transplantation studies showed that populations can acclimatize to new environmental conditions and change salinity tolerances or switch reproduction period, demonstrating high phenotypic plasticity (Marcus 1983). Genetic adaptation, e.g. to low salinity, was also demonstrated (Perrin et al. 2004). Interestingly, they can be found even in extreme environments such as estuaries, intertidal pools or the deep sea.

2. OCEAN ACIDIFICATION EFFECTS ON ECHINODERMS

2.1 Early life stages

Some of the first studies describing the effect of acids on echinoderm larvae development were those by Bouxin (1926) on the sea urchin *Paracentrotus lividus*. This author reported a "regression" of the skeleton influencing the larvae morphology. Furthermore, this author noticed that from pH 8.1 until 7.4-7.2 the larvae developed almost "normally", although their development was slowed down. A few other studies reported effects of acids on developing sea urchin embryos and larvae (e.g. Pagano et al. 1985a, b, Rand 1995, Byrne 2010 and references therein).

New studies on an ocean acidification context, now using CO_2 manipulation in order to reduce pH, flourished in the past five years (Table 4). They evidence that ocean acidification effects on echinoderm larvae are extremely variable (Table 4). Fertilization seems to be the less affected process. As previously discussed, this can be less susceptible most likely due to the stimulatory influence of compounds in the egg jelly coat that allow sperm to overcome the narcotic pH effect (Byrne 2010). Furthermore, fertilization success can be more dependent on sperm to egg proportion (sperm density) than on seawater pH (Byrne et al. 2009, 2010b, Ericson et al. 2010). Larval morphology and size (growth rate) seem to be generally affected (Table 4). However, the mechanisms behind these processes are yet poorly understood. Larval size reduction can be due to a combination of factors such as metabolism depression, development delay and calcification impairment (Dupont et al. 2010b). A decrease in the expression of genes involved in biomineralization and metabolism were reported (O'Donnell et al. 2008, 2010, Todgham and Hofmann 2009). Other species revealed high levels of gene expression plasticity, showing that development can be delayed but morphologically normal (Martin et al. 2011).

In fact, some species seem to be more resistant than others towards ocean acidification. For instance, Clark et al.(2009) observed that *Sterechinus neumayeri*, an Antarctic species, could be less vulnerable than temperate species possibly due to adaptive features, since Southern Ocean waters are already CO_2 enriched. Dupont et al. (2010b) also observed a series of positive responses on the starfish *Crassaster papposus* larval development, probably associated with its life cycle pattern (lecithotrophic larvae). However, even if early life stages

seem to develop well, other processes like metamorphosis and juvenile growth and development can be later affected (e.g. *Heliocidaris erythrogramma*, Table 4).

Table 4. Positive (⇧), neutral (⇔) and negative (⇩) responses to low pH waters of echinoderms reproduction and early life stages. pH in NIST. Only studies using CO_2 to manipulate pH were included.

Group	Taxa	Studied Range	Observed effects	Reference
Sea urchins	*Strongylocentrotus franciscanus*	pH 8.04, 7.81, 7.55, pCO_2 400, 800, 1800 ppm	Fertilized eggs: ⇩, fertilization success: ⇔, fertilization efficiency: ⇩, egg block to polyspermy: ⇩	Reuter et al. 2010
	Heliocidaris erythrogramma	pH 8.1, 7.7, pCO_2 370, 1000 ppm	Sperm speed and motility: ⇩, number of cleaved embryos and swimming larvae: ⇩	Havenhand et al. 2008
	Heliocidaris erythrogramma	pH 8.2, 7.9, 7.8, 7.6, pCO_2 230-1828 ppm	Fertilization success, % cleavage, % gastrulation: ⇔	Byrne et al. 2009, Byrne et al. 2010b
(lecithotrophic larvae)	*Heliocidaris erythrogramma*	pH 8.2, 7.8, 7.6, pCO_2 494-1861 ppm	Normal juveniles, spine numbers: ⇩	Byrne et al. 2010a
	Heliocidaris tuberculata	pH 8.25, 7.9, 7.8, 7.6, pCO_2 324-1828 ppm	Fertilization success: ⇔	Byrne et al. 2010c
	Tripneustes gratilla	pH 8.25, 7.9, 7.8, 7.6, pCO_2 324-1828 ppm	Fertilization success: ⇔	Byrne et al. 2010c
	Centrostephanus rodgersii	pH 8.25, 7.9, 7.8, 7.6, pCO_2 324-1828 ppm	Fertilization success: ⇔	Byrne et al. 2010c
	Tripneustes gratilla	pH 8.15, 7.8, 7.6, pCO_2 448-1990 ppm	Larvae morphology, larvae growth: ⇩, arm asymmetry: ⇔	Sheppard Brennand et al. 2010
	Hemicentrotus pulcherrimus	pH 8.01-6.83, pCO_2 0.04-1.04 kPa	% Fertilized eggs, % embryos: ⇔, ⇩(6.8), development speed: ⇩	Kurihara and Shirayama 2004
	Echinometra mathaei	pH 8.11-6.79, pCO_2 0.04-1.04 kPa	% Fertilized eggs: ⇔, ⇩(<7.2)	Kurihara and Shirayama 2004
	Sterechinus neumayeri	pH 8.0, 7.7, 7.3, 7.0, pCO_2 528-5806 µatm	% Fertilization (interaction with sperm density): ⇩, cleaved eggs: ⇔, normal embryos: ⇩, gastrula length ⇔⇩(7.0), delayed development	Ericson et al. 2010
	Strongylocentrotus purpuratus	pH 8.07-7.53, pCO_2 372-1469 ppm	Larvae size: ⇩, arm asymmetry: ⇔, development: ⇔	Yu et al. 2011
	Paracentrotus lividus	pH 8.1-7.0, pCO_2 397-6632 µatm	Fertilization, survival: ⇔; larvae size: ⇩, calcification was size dependent; development delay	Martin et al. 2011
Starfish	*Patiriella regularis*	pH 8.25, 7.9, 7.8, 7.6, pCO_2 324-1828 ppm	Fertilization success: ⇔	Byrne et al. 2010c
(lecithotrophic larvae)	*Crassaster papposus*	pH 8.1, 7.7, pCO_2 372, 930 ppm	Larvae survival: ⇔, larvae growth rates: ⇧, development: ⇔⇧, juveniles growth/size: ⇧	Dupont et al. 2010a
Brittle stars	*Ophiotrix fragilis*	pH 8.1, 7.9, 7.7	Larvae survival: ⇩, overall larvae size ⇔ with some parameters ⇩ after 8d, morphology ⇩	Dupont et al. 2008

2.2 Adults

Adult echinoderms seem to be more resistant to ocean acidification than early life stages (Dupont et al. 2010b), although a variety of physiological responses has been observed (Table 5). Sea urchins growth seems to be affected by lower pH waters (Table 5). Some aquaculture studies had already reported that lower pH (7.1-7.7) in rearing systems, due to poor aeration and respiratory CO_2 accumulation, reduced growth rates of the individuals (Grosjean et al. 1996, 1998). Also, extreme environmental hypercapnia (pH 6.98 compared to 8.0 in control levels) in such systems led to decreased feeding rates and consequent gonad growth reduction (Siikavuopio 2007). Indeed, extreme low pH caused metabolic depletion in echinoderms (Hiestand 1940). However, mild environmental hypercapnia in ophiuroids had as a consequence an up-regulation of the metabolism (Wood et al. 2008, 2010, 2011), which can indicate a higher energetic demand in order to maintain homeostasis.

Table 5. Examples of positive (⇧), neutral (⇔) and negative (⇩) responses of physiological processes of adult echinoderms submitted to low pH. V_{O2} stands for oxygen uptake, pH in NIST scale, manipulated by CO_2 bubbling. References (R.): 1) Miles et al. 2007, 2) Shirayama and Thornton 2005, 3) Ries et al. 2009, 4) Gooding et al. 2009, 5) Hernroth et al. 2011, 6) Wood et al. 2008, 7) Wood et al. 2010, 8) Wood et al. 2011

Group	Taxa	Exposure time	Studied Range	R.	Response trend
Sea urchins	*Psammechinus miliaris*	8 days	pH 7.96, 7.44, 6.63, 6.16	1	Extracellular pH: ⇩, supposedly partly compensated by test dissolution
	Hemicentrotus pulcherrinus	6 months	pH 7.945-7.897, pCO_2 ?-560 ppm	2	Mortality at pH 6.63 and 6.16 Survival: ⇩ (experiment 1) and ⇔ (experiment 2) , growth rate: ⇩
	Echinometra mathaei	6 months	pH 7.945-7.897, pCO_2 ?-560 ppm	2	Survival: ⇩ (experiment 1) and ⇔ (experiment 2) , growth rate: ⇩
	Arbacia punctulata	60 days	pH 8.04-7.36, pCO_2 409-2856 ppm	3	Survival: ⇔, calcification: ⇧⇩ (parabolic response)
	Eucidaris tribuloides	60 days	pH 8.04-7.36, pCO_2 409-2856 ppm	3	Survival: ⇔, calcification: ⇔⇩ (threshold response)
Starfish	*Pisaster ochraceus*	70 days	pH 7.85/7.88-7.79/7.82, pCO_2 380-780 ppm	4	Feeding rate and somatic growth: ⇧, calcification ⇩ (interaction with temperature)
	Asterias rubens	6months/1 week	pH 8.1, 7.7, pCO_2 331-922 ppm	5	Extracellular pH: ⇩, impaired immune response
Brittle stars	*Amphiura filiformis*	40 days	pH 8.1, 7.7, 7.3, 6.8	6	Arm regeneration and V_{O2}: ⇧, but muscle wastage
	Ophiura ophiura	40 days	pH 7.99-7.38, pCO_2 553-2546 ppm	7	Arm regeneration: ⇩, V_{O2}: ⇧(interaction with temperature)
	Ophiocten sericeum	20 days	pH 8.31-7.32, pCO_2 260-1883 ppm	8	Arm regeneration: ⇩, V_{O2}: ⇧(interaction with temperature)

Within an ocean acidification context, pH values will not be as extreme as the ones tested in many of the studies (Table 5) and organisms might have scope to deal with such environmental changes. For instance, in natural carbon dioxide vents the abundance of sea urchins only seems affected bellow pH of 7.5-7.4 (Hall-Spencer et al. 2008), values that are below those predicted for 2100 and close to 2300 predictions (IPCC 2007). Nevertheless, it is difficult to establish a precise overall response to ocean acidification by echinoderms. For instance, calcification rates can either decline or increase with lower pH in closely related taxa (Table 5).

Objectives

OBJECTIVES

Sea urchins are key stone species in a variety of habitats and are considered to be particularly vulnerable to ocean acidification effects. This is not only due to the nature of their well developed skeleton (magnesium calcite) whose solubility is similar or higher than that of aragonite, but also because they lack an efficient ion regulatory machinery, being considered to be poor acid-base regulators. Populations from polar and sub-polar regions are expected to be at an even higher risk since the chemical changes that are occurring in these oceans are happening at a faster rate, with models predicting an earlier shallowing of the calcium carbonate saturation horizons at higher latitudes. Additionally, numerous species have an indirect development, with distinct larval and post-metamorphic forms which can respond differently to ocean acidification. Early live stages are considered to be more at risk in front of acidification, but it is not yet fully understood why even closely related taxa can respond differently to similar levels of pH. Nevertheless, several sea urchin species are able to cope with a wide range of ambient conditions, including enriched CO_2 habitats, most likely due to adaptive features and thanks to their phenotypic plasticity. However, physiological responses of adults to acidification are rather poorly investigated.

Therefore, the goal of this work was to study the effects of low seawater pH exposure of different life stages of sea urchins, in order to better understand how species from different environments and/or geographic origins would respond and if there would be scope for possible adaptation and/or acclimatization.

For that purpose the effects of ocean acidification experimental exposures to low pH on a series of developmental and/or physiological endpoints were investigated. In order to do so larvae and adults of a temperate intertidal species, *Paracentrotus lividus*, and of a subtidal sub-Antarctic one, *Arbacia dufresnei*, were studied. Furthermore, possible adaptations of sea urchins presently living in permanent low magnesium calcite saturation states, the Antarctic *Ctenocidaris speciosa*, and fluctuating envirnmental hypercapnia conditions, *P. lividus*, were assessed. The first two chapters of this thesis present the impact of ocean acidification on early life stages and the following three the adult responses.

Chapter I

Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816)

Chapter I

Effects of seawater acidification on early development of the intertidal sea urchin *Paracentrotus lividus* (Lamarck 1816)

Laure Moulin*, Ana Isabel Catarino*, Thomas Claessens, Philippe Dubois

Laboratoire de Biologie Marine, Université Libre de Bruxelles, Belgium

Published in Marine Pollution Bulletin *(2011) 62: 48-54*

Abstract: The effect of pH ranging from 8.0 to 6.8 (total scale - pH_T) on fertilization, cleavage and larval development until pluteus stage was assessed in an intertidal temperate sea urchin. Gametes were obtained from adults collected in two contrasting tide pools, one showing a significant nocturnal pH decrease (lowest pH_T = 7.4) and another where pH was more stable (lowest pH_T = 7.8). The highest pH_T at which significant effects on fertilization and cleavage were recorded was 7.6. On the contrary, larval development was only affected below pH_T 7.4, a value equal or lower than that reported for several subtidal species. This suggests that sea urchins inhabiting stressful intertidal environments produce offspring that may better resist future ocean acidification. Moreover, at pH_T 7.4, the fertilization rate of gametes whose progenitors came from the tide pool with higher pH decrease was significantly higher, indicating a possible acclimatization or adaptation of gametes to pH stress.

Keywords: Ocean acidification, Sea urchin, Intertidal, Early development, LOEC, Acclimatization/adaptation

* These authors contributed equally to this article.

1. Introduction

The continuous increment of anthropogenic carbon dioxide emissions is inducing changes in seawater carbon chemistry, lowering its pH. This phenomenon is known as ocean acidification. Since the industrial revolution, the average surface seawater pH has already been reduced by approximately 0.1 units. Expected surface pH reductions are of around 0.4 units by 2100 and 0.77 units by 2300 (Caldeira and Wickett 2003, 2005, IPCC 2007). Some particular environments already present lower pH such as upwelling zones (Feely et al. 2008), coastal areas (Wootton et al. 2008), the deep-sea (Park 1966, Millero 1996) and volcanic carbon dioxide vents (Hall-Spencer et al. 2008). Tide pools also undergo significant variations of their physicochemical conditions, including pH (due to pCO_2 fluctuations). Truchot and Duhamel-Jouve (1980) reported in rocky tidal pools (Roscoff, Brittany, France) a night increment of pCO_2, accompanied by a decrease of pH until 7.29. This is caused by the community respiration and absence of photosynthesis. During daytime, the tendency is inverted. These daily fluctuations depend on season, tide duration and algal cover (Truchot and Duhamel-Jouve 1980, Morris and Taylor 1983, Denny and Gaines 2007). The same authors also reported an increase of total alkalinity at night which was associated with calcium carbonate dissolution due to low pH. Thus, intertidal pools offer an interesting model where organisms are exposed to a succession of pH fluctuations and may possibly present adaptations (linked to genetic variability at a population level) and/or acclimatization (due to phenotypic plasticity at an individual level) processes to face them. Early development stages of marine invertebrates (fertilization, embryogenesis and larval development) are generally the most sensitive life phases to environmental stresses (Pörtner and Farrell 2008, Melzner et al. 2009, Dupont et al. 2010b). Recruitment success depends on the survival of the embryos and larvae (López et al. 1998) and, consequently, any decrease in embryo and larval survival or delay in development can reduce population long-term viability (Morgan 1995). Sea urchins are key species in many coastal ecosystems, being important grazers, and the sustainability of their populations is vital (Paine 1966, Harrold and Pearse 1987, Leblanc et al. 2005). Several studies showed that fertilization and early development stages of sea urchins can be negatively impacted by ocean acidification which causes a decrease of fertilization and cleavage rates and/or a reduction of the pluteus larva size (Kurihara and Shirayama 2004, Havenhand et al. 2008, Clark et al. 2009). A down

regulation of genes involved in calcification, cellular stress response, metabolism and apoptosis were reported in *Strongylocentrotus* spp larvae raised in low pH seawater (Todgham and Hofmann 2009, O'Donnell et al. 2010). However, larvae of *Strongylocentrotus droebachiensis* raised at lower pH (7.9 and 7.7) were more successful in reaching metamorphosis than those raised at control pH (8.0), although it took them longer to reach this stage (Dupont and Thorndyke 2008). It is noteworthy that a slower development can result in higher plantktonic mortality due to increased predation exposure and desynchronization with algal blooms, decreasing recruitment success (Morgan 1995, Elkin and Marshall 2007). Nonetheless, in other studies, no effect of ocean acidification was observed on fertilization and embryogenesis (Byrne et al. 2009, 2010b, c). These facts suggest that actually the response of early life-history stages appears to be highly speciesspecific and differs even in closely related taxa (Dupont and Thorndyke 2008, 2009, Clark et al. 2009). This fact emphasizes the need for a survey of specific effects of acidification as broad as possible and to understand the origin of the observed differences of sensitivity to ocean acidification between species. It is also essential not to disregard the fact that long-term exposure of adults to lower pH can affect gonad growth (Siikavuopio et al. 2007, Kurihara 2008), reproductive success and future larval performance (Dupont and Thorndyke 2008, Kurihara 2008). In this work, we studied the sea urchin *Paracentrotus lividus*, an important grazer species (Bulleri et al. 1999) with a broad distribution and that can be found in the whole Mediterranean and North Atlantic coasts of Europe (from Morocco to Scotland), inhabiting intertidal rock pools, seagrass meadows and shallow subtidal shores (Boudouresque and Verlaque, 2001). Furthermore, this species shows a high gene flow over extended distances (Duran et al. 2004, Calderón et al. 2008). The aim of this study was to understand if pH extreme oscillations to which *P. lividus* adults from rock tide pools are submitted could have an influence on fertilization, embryonic and larval development of their progeny. The strategy was to compare the effect of pH on the progeny of individuals collected from the same shore, i.e. same population, but from distinct tide pools: one where night pH was significantly reduced and the other where this decline was not so important.

2. Materials and methods

2.1. Study site and measure of physicochemical parameters

Observations were done in two tide pools, distant of around 2 m, in Aber, Crozon peninsula (48º14'N, 04º27'W, southern Brittany, France), in April 2009, i.e. during the spawning period of *Paracentrotus lividus* populations in this region (Mercier and Hamel, 2009). Intertidal adult individuals occurred in tide pools and showed a sedentary behavior in self burrowed holes. They are thus partially protected from wave action and never get emersed during low tide. Previous tagging experiments (data not shown) confirmed this population sedentary behavior. New recruits are found every year in this population (Catarino and Dubois, personal observation). The physicochemical parameters of two tide pools were measured every half an hour starting at pool individualization (ebb tide) until its cover (rising tide) during two night and two day low tides: temperature, salinity and pH_{NIST} (National Institute of Standards and Technology), also known as NBS (previous National Bureau of Standards, now NIST) scale. The temperature and pH_{NIST} were measured using a 827 pH Lab Metrohm meter (Switzerland) with a combined glass electrode (Metrohm 6.0228.010 with temperature sensor) calibrated with pH_{NIST} buffers 4 and 7 (Merck CertiPUR®, Darmstadt, Germany). Even though pH variation within each pool never exceeded 0.1 units, a pH cycle measurement was always done on the same spot. The salinity was measured using a conductivity meter pH/Cond 340i WTW (USA). Sea water samples were collected at the beginning and end of each low tide and immediately filtered (0.22 μm) in order to determine total alkalinity (TA). This was carried out by a potentiometric titration with HCl 0.1 M using a Titrino 718 STAT Metrohm (Switzerland), and calculated using the Gran function (Gran 1952). Our measurements had a deviation of 0.65 % of the standard certified material provided by Andrew G. Dickson's Oceanic Carbon Dioxide Quality Control laboratory. Aragonite and calcite saturation values (Ωar and Ωcal, respectively) and pCO_2 were determined from TA, pH_{NIST} and salinity data using the software CO2SYS (Pierrot et al. 2006) and by using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and K_{SO4} using Dickson (1990).

2.2. Gonad maturity

Ten *P. lividus* individuals with a minimum diameter of 30 mm were collected from each tide pool, transported to the laboratory in tide pool water and dissected on the same day. A piece of gonad was removed and fixed in Bouin's fluid. The gonads were then dehydrated, embedded in paraffin, cut in 7 µm sections (Leica RM 2155 microtome) and stained with Masson's trichrome. The gonad maturity was estimated on a scale of 1-8 based on morphological characteristics according to the method of Spirlet et al. (1998).

2.3. Fertilization and larval development experiments

Thirty individuals were collected from each tide pool and kept at pH_{NIST} 8.13, 12.7 ºC and 32.1 PSU until the beginning of the experiment 14 days later. All experiments were conducted in a temperature controlled room at 14 ºC and in filtered seawater (0.22 µm) from the study site. The pH of the seawater was adjusted by bubbling CO_2 (Air Liquide) until the required pH was obtained. The pH_{NIST}, the electromotive force (e.m.f) and the temperature of the seawater were measured at the start and at the end of each experiment with the same pH meter as previously described. These values and sequential measurements of the e.m.f. of the cell using standard buffers of known pH, 2-aminopyridine/HCl (AMP) and tris/HCl (TRIS) were applied on the calculation of the pH expressed in total scale (pH_T) (DOE 1994, Del Valls and Dickson 1998, Dickson et al. 2007). The salinity was measured using a conductivity meter pH/Cond 340i WTW (USA). The TA, pCO_2, Ωar and Ωcal were determined in each vial as described in Section 2.1. To induce spawning, ca. 1 ml of 0.5 M KCl was injected into the perivisceral cavity of individual sea urchins. Gametes of 5 males and 5 females from each tide pool, selected according to their gamete quantity and quality (shape), were collected in control pH seawater. Gametes of the same sex were gently mixed in order to have a homogeneous batch and to avoid individual variations. The Lowest Observed Effect Concentration (LOEC, results in nominal pH), i.e. the highest pH at which the considered end point significantly differed from that at control pH (see e.g. Rand 1995), was calculated for the fertilization rate, cleavage rate, larval morphology and rod size. The LOEC was also determined for other sea urchin species from contrasting environments, based on literature data (Byrne et al. 2009a, 2010b, c, Clark et al. 2009, Havenhand et al.

2008, Kurihara et al. 2004b, Kurihara and Shirayama 2004, O'Donnell et al. 2010). Only works in which pH was manipulated by CO_2 addition and for which data for determining a LOEC were available were included.

2.3.1. Fertilization and cleavage

Fertilization was conducted in Petri dishes by mixing diluted sperm and eggs at selected pH_T (control 8.0, 7.6, 7.4 and 6.8). Three replicates by pH for each tide pool were produced. Embryos were randomly sampled from each treatment at different times (10, 20, 30, 60, 90 and 120 min) and fixed in Bouin's fluid and 200 embryos were observed in each replicate using an optical microscope. Fertilization was defined as the presence of an elevated normal fertilization membrane (15 min after gamete mixing) and cleavage as the presence of minimum 2 blastomeres (1 h after gamete mixing). At pH_T 6.8, embryos presenting a very thin membrane not clearly visible or with a fertilization membrane not surrounding completely the embryo were observed. They were counted as abnormal and they were considered as neither ‘‘fertilized” nor ‘‘cleaved”.

2.3.2. Larval development

Eggs were suspended as a homogeneous layer in a Petri dish containing control seawater and a drop of diluted sperm was added. Twenty minutes later, the presence of the fertilization membrane was checked. Fertilization rate was always higher than 95 %. Two hours after insemination, three replicates of ca. 500 embryos were transferred into 125 ml vials at each selected pH_T (control 8.0, 7.8, 7.6, 7.4, 7.2, 7.0 and 6.8). Vials were completely filled to avoid air spaces and thus preventing CO_2 exchanges. After 72 h of incubation, the echinopluteus stage was reached and the 4 arms were visible in control larvae with an optical microscope. The larval development was stopped at this moment (before the feeding stage). Some larvae were fixed in Bouin's fluid without acetic acid (for skeleton preservation) and the remaining ones in ethanol 70 %. One hundred pluteus larvae fixed in Bouin's fluid were observed in each replicate using an optical microscope and the number of normal and abnormal larvae was counted. The criteria of abnormality were: absence of one or several arms, absence of stomach or totally abnormal bowl-like shape (adapted from Warnau and Pagano 1994). Larvae preserved in ethanol 70 % were observed in phase contrast inverted

microscopy and photographed using a digital camera (QImaging, Micropublisher, software Qcapture). Sixty larvae per vial were measured from the apical extremity to the extremity of an anal arm (skeletal rod) using the software ImageJ (Fig. 1).

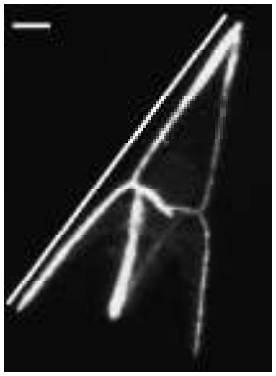

Fig. 1. Pluteus larvae observed in phase contrast inverted microscopy. The long bar indicates the morphometric measurement of skeletal rod. Small bar = 50 µm (upper left corner).

2.4. Data analysis

Differences of pH, salinity and temperature during night and day tidal cycle between tide pools at the end of the low tide as well as comparison of gonad maturity between sea urchins of the two tide pools were investigated using a one way ANOVA (fixed factor tide pool). The fertilization rate was analyzed using a repeated measurement ANOVA (fixed factors nominal pH and tide pool and repeated factor time). All mean multiple comparisons were performed using Tukey tests. The analysis of the cleavage rate was carried out using a two way ANOVA (fixed factors tide pool and nominal pH) followed by a post hoc Dunnett test. To test for possible differences in pH condition in the vials between the beginning and the end of the experiment after 72 h, a repeated measurement ANOVA was done. The size of the calcareous rods of pluteus larvae was analyzed for each tide pool separately using a model III nested ANOVA (fixed factor nominal pH and random factor replicate nested in factor nominal pH) followed by a Tukey test on the pH variable. The analysis of the percentage of normal larvae (arcsin transformed) was performed using a two way ANOVA (fixed factors tide pool and nominal pH) followed by a post hoc Dunnett test. All test were conducted according to Zar (2005) using the software Systat 9 (Systat Software Inc.). The level of significance α was set at 0.05.

3. Results

3.1. Tide pools parameters

The pH_{NIST} of coastal seawater was 8.14. At the end of the night low tides, tide pools 1 (TP1) and 2 (TP2) had a pH_{NIST} of, respectively, 7.8 and 7.4, i.e. a pH decrease of respectively, 0.34 and 0.74 (Table 1, Fig. 2a). There was a significant difference of pH drop between the two tide pools during the night tidal cycle (p_{Tukey} = 0.021), but not of temperature and salinity (p_{ANOVA} > 0.178). During the day tidal cycle, the tendency was inverted with the pH increasing up to 8.83 in TP1 and 8.63 in TP2 (Table 1, Fig. 2b). However, the pH increase was not significantly different between the two tide pools as well as the temperature and the salinity (p_{ANOVA} > 0.333).

Table 1: sea water conditions at the beginning (sea) and at the end of representative night and day tidal cycles in the two tide pools

Tidal cycle	Location	Salinity (psu)	Temperature (°C)	pH_{NIST}	TA (µmol/kg)	DIC (µmol/kg)	pCO_2 (µatm)	Ωcal	Ωar
Night	Sea	34.7	13.0	8.14	2321	2125	423	3.44	2.20
Night	Tide pool 1	34.7	12.3	7.80	2315	2252	999	1.67	1.07
Night	Tide pool 2	34.7	11.4	7.40	2397	2467	2696	0.70	0.45
Day	Sea	35.0	11.5	8.21	2322	2100	346	3.79	2.42
Day	Tide pool 1	35.4	15.0	8.83	2244	1579	51	10.55	6.78
Day	Tide pool 2	35.0	14.7	8.63	1935	1483	84	6.87	4.41

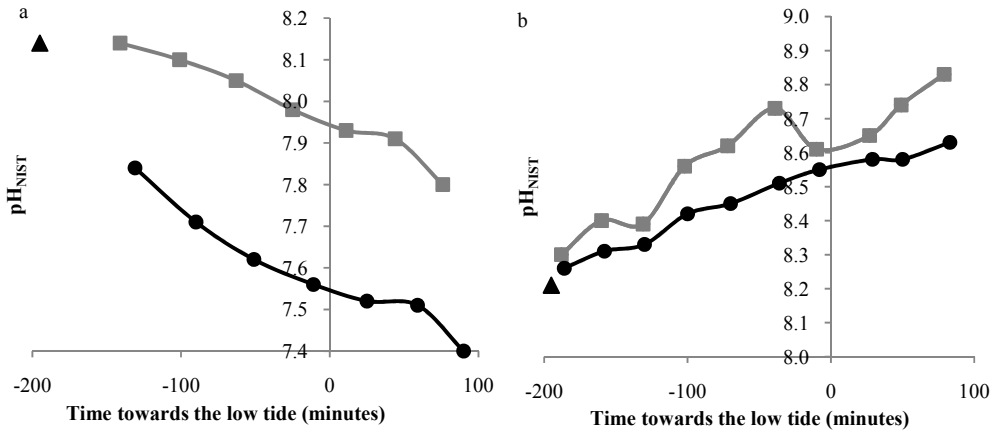

Fig. 2: Evolution of pH in tide pool 1 (grey squares) and 2 (black points) according to time towards the low tide during representative night (a) and day (b) tidal cycles in April. The pH of seawater at the beginning of the tidal cycle is represented by the black triangle

3.2. Gonad maturity

There was no significant difference in gonad maturity stage between individuals from both tide pools (p_{ANOVA} = 0.331). Most individuals were in premature stage (stage V according to Spirlet et al. 1998), i.e., immediately before the mature stage and spawning, ova and spermatozoa were accumulated in the center of the gonad acini.

3.3. Fertilization and larval development experiments

The initial experimental water conditions (pH_T, carbonate and pCO_2 parameters) are presented in Table 2. These conditions were the same for fertilization, cleavage and larval development experiments.

Table 2. Initial experimental seawater conditions (mean ± SD, n = 3) for fertilization, cleavage and larval development experiments (t_0) and after 72 h of incubation for larval development experiment (t_f) according to origin of genitors (tide pool 1 and 2). Initial and final conditions did not differ significantly (see text). For TA, DIC and pCO_2 units please see Table 1.

	pH nominal	pH_T (t_0)	pH_T(t_f)	TA (t_0)	TA (t_f)	DIC (t_0)	DIC (t_f)	pCO_2 (t_0)	pCO_2 (t_f)	Ωcal (t_0)	Ωcal (t_f)	Ωar (t_0)	Ωar (t_f)
1	6.8	6.80 ± 0.01	-	2208.37	-	2513.99	-	8108.88	-	0.22	-	0.14	-
	7.0	7.07 ± 0.01	7.10 ± 0.00	2209.87	2214.66	2353.81	2347.57	4292.58	4050.29	0.41	0.44	0.26	0.28
	7.2	7.27 ± 0.02	7.24 ± 0.04	2215.62	2234.33	2282.28	2315.17	2686.36	2972.84	0.65	0.60	0.41	0.38
	7.4	7.47 ± 0.00	7.46 ± 0.06	2243.80	2251.68	2247.34	2258.04	1690.81	1743.88	1.03	1.02	0.66	0.65
	7.6	7.67 ± 0.00	7.60 ± 0.02	2242.98	2268.74	2186.78	2234.29	1037.37	1254.82	1.59	1.38	1.02	0.88
	7.8	7.83 ± 0.01	7.76 ± 0.03	2211.17	2233.37	2109.95	2152.73	697.22	829.64	2.14	1.89	1.36	1.20
	8.0	8.14 ± 0.01	7.98 ± 0.02	2216.54	2390.59	1990.34	2224.33	310.66	507.99	3.98	3.18	2.53	2.02
2	6.8	6.78 ± 0.01	-	2208.37	-	2523.25	-	8335.25	-	0.22	-	0.14	-
	7.0	7.07 ± 0.01	7.14 ± 0.01	2209.87	2249.24	2353.90	2366.79	4294.44	3733.06	0.41	0.49	0.26	0.31
	7.2	7.29 ± 0.01	7.25 ± 0.02	2215.62	2235.18	2277.94	2311.67	2604.74	2885.64	0.67	0.62	0.42	0.39
	7.4	7.47 ± 0.00	7.38 ± 0.01	2243.80	2248.27	2247.17	2279.76	1688.46	2113.87	1.03	0.84	0.66	0.54
	7.6	7.67 ± 0.02	7.56 ± 0.02	2242.98	2274.66	2187.88	2250.91	1047.35	1373.17	1.58	1.28	1.01	0.82
	7.8	7.85 ± 0.02	7.73 ± 0.04	2211.17	2203.29	2102.50	2134.31	660.09	895.15	2.24	1.73	1.43	1.10
	8.0	8.13 ± 0.00	8.00 ± 0.04	2216.54	2226.14	1995.12	2057.88	319.16	444.55	3.91	3.10	2.48	1.97

3.3.1. Fertilization and cleavage

Fertilization rate significantly decreased with pH_T ($p_{pH} < 10^{-3}$) (Fig. 3). The observed LOEC was 7.6 for embryos from parents of both tide pools ($p_{Tukey} < 10^{-3}$). However, the fertilization rate at pH_T 7.4 of gametes from TP2 parents was significantly higher than that of gametes from TP1 parents ($p_{pH \times tide\ pool} = 0.026$, $p_{Tukey} = 0.03$). The amount of fertilized eggs significantly increased with time ($p_{time} < 10^{-3}$) (Fig. 3). The cleavage rate was significantly

lower at lower pH_T ($p_{pH} < 10^{-3}$) (Fig. 4). The LOEC was 7.6 for embryos from progenitors from both tide pools ($p_{Dunnett} < 0.011$). Contrary to the fertilization rate, the percentage of cleaved eggs did not differ according to origin of progenitors ($p_{pH \times tide\ pool} = 0.804$) (Fig. 4).

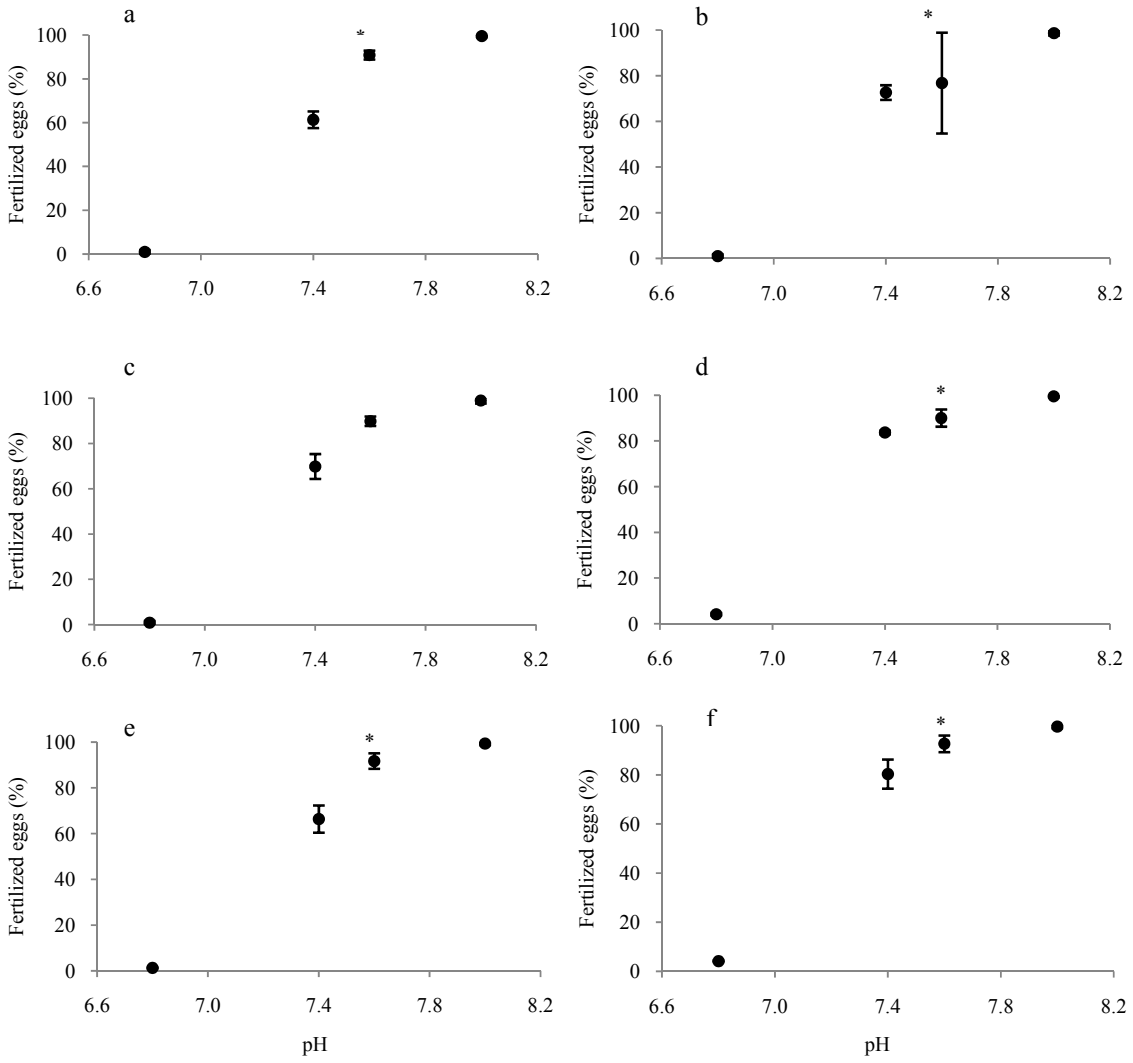

Fig. 3. Fertilization rate (%, mean ± SD, n = 3) at different nominal pH and at different time (a - b = 10 min , c - d = 60 min , e - f = 120 min) for embryos from sea urchins of tide pool 1 (a - c - e) and tide pool 2 (b - d - f). *: Mean value significantly different from control ($p_{Tukey} < 0.05$).

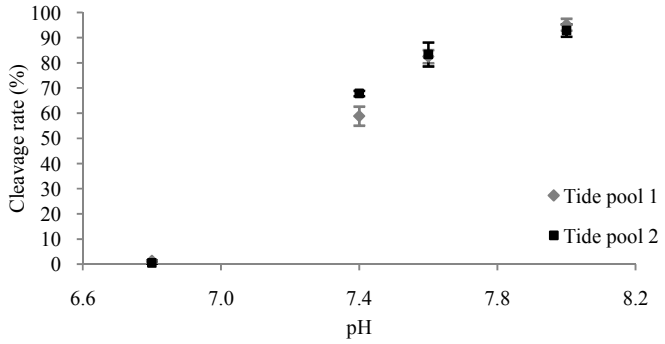

Fig. 4. Cleavage rate (%, mean ± SD, n = 3) at different nominal pH of embryos from progenitors of both tide pools. *: Mean value from both tide pools significantly different from control ($p_{Dunnett} < 0.05$).

3.3.2. Larval development

The pH_T water conditions were not significantly different between the start and the end of the experiment ($p_{ANOVA} = 0.1566$) (Table 2). Rod size was significantly smaller at lower pH_T ($p_{pH} < 10^{-3}$) (Fig. 5). The LOEC was 7.2 for larvae from genitors from both tide pools. Lower pH also significantly affected the form of pluteus larvae ($p_{pH} < 10^{-3}$) (Fig. 6), the percentage of abnormal pluteus being higher at lower pH_T. The LOEC was 7.4 and there was no difference between larvae from parents of both tide pools ($p_{tide\ pool} = 0.721$). At pH_T 6.8 no embryo developed to the pluteus stage and all larvae had an abnormal morphology (bowl shape).

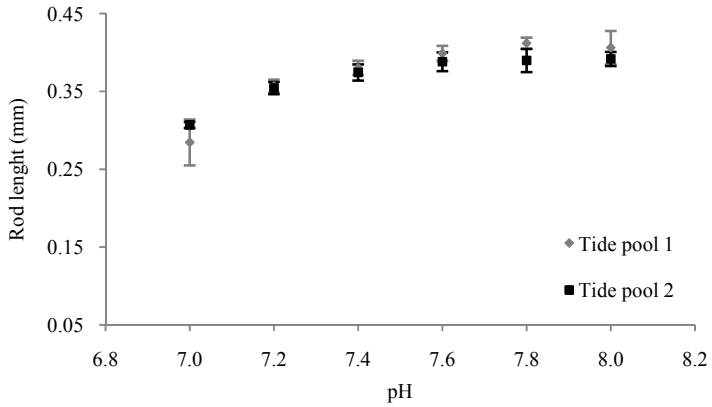

Fig. 5. Length (mean ± SD, n = 3) at different nominal pH of calcareous rod of pluteus larvae from sea urchins of both tide pools. *: Mean value from both tide pools significantly different from control ($p_{Tukey} < 0.05$).

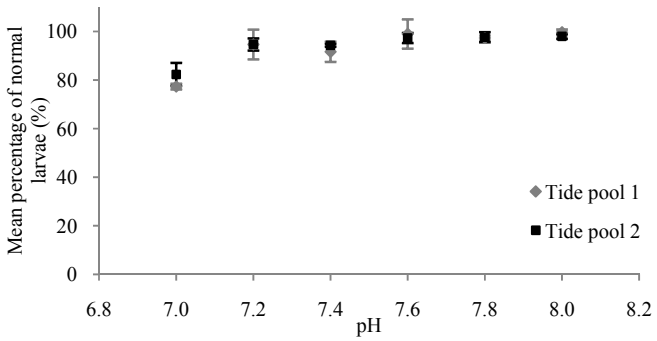

Fig. 6. Percentage (mean ± SD, n = 3) of normal pluteus larvae at different nominal pH in the offspring of sea urchins from the 2 tide pools. *: Mean value from both tide pools significantly different from control ($p_{Dunnett} < 0.05$).

Table 3. LOEC (in pH_{NIST} unit, except for *P. lividus* results where units are in pH_T) for embryonic and larval development of different species of echinoids: *P. lividus* collected from intertidal zone in temperate region, *S. neumayeri* collected from subtidal zone in Antarctic region, *P. huttoni* and *E. chloroticus* collected from subtidal zone in temperate region. Other species collected from subtidal zone in tropical region. ND: no data. NE: no pH effect observed (lowest pH tested between brackets).

Species	Fertilization rate	Cleavage rate	Larval morphology	Decreased Rod size	Reference (pH scale)
Paracentrotus lividus	7.6	7.6	7.4	7.2	Present study (pH_T)
Sterechinus neumayeri	ND	ND	ND	7.6	Clark et al. (2009) (pH_{NIST})
Pseudechinus huttoni	ND	ND	ND	NE (7.7)	Clark et al. (2009) (pH_{NIST})
Evechinus chloroticus	ND	ND	ND	7.7	Clark et al. (2009) (pH_{NIST})
Tripneustes gratilla	ND	ND	ND	7.8	Clark et al. (2009) (pH_{NIST})
	NE (7.6)	ND	ND	ND	Byrne et al (2010c) (pH_{NIST})
Centrostephanus rodgersii	NE (7.6)	ND	ND	ND	Byrne et al. (2010c) (pH_{NIST})
Heliocidaris tuberculata	7.6	ND	ND	ND	Byrne et al. (2010c) (pH_{NIST})
Heliocidaris erythrogramma	NE (7.6)	NE (7.6)	ND	ND	Byrne et al. (2009, 2010b, c) (pH_{NIST})
	7.7	ND	ND	ND	Havenhand et al. (2008) (pH_{NIST})
Hemicentrtotus pulcherrinus	6.83	6.8	7.4	7.8	Kurihara et al. (2004b), Kurihara and Shirayama (2004) (pH_{NIST})
Echinometra mathaei	7.12	ND	7.4	7.8	Kurihara et al. (2004b) (pH_{NIST})
Lytechinus pictus	ND	ND	ND	7.78	O'Donnell et al. (2010) (pH_{NIST})

4. Discussion

In the present study, the most critical phases observed during early life stages of *Paracentrotus lividus* were fertilization and cleavage, as evidenced by the lowest observed effect concentration (LOEC). The LOEC pH_T was higher (7.6) for those stages than for larval

viability (7.4) and larval size (7.2) (Table 3). This shows that the initial embryonic development of this species is sensitive to acidification levels that could be reached already in 2100, according to IPCC predictions (IPCC 2007). Gametes, zygote and early cleavage stages can be more vulnerable than cells during later ontogenetic stages as changes in surrounding pCO_2 cause a higher relative change in internal pCO_2 (Melzner et al. 2009). In line with these results, previous studies on *P. lividus* showed that an earlier exposure to acidified water (HCl) promoted more severe developmental defects than a post-hatching one (Pagano et al. 1985). Early life stages can present diverse defense mechanism against common natural stressors within a large variation range, but their vulnerability can increase while facing rapid anthropogenic environmental changes (Hamdou and Epel 2007). Furthermore, the responses of different species are diverse and highly specific (Table 3) and clear responses are hard to be established even within the same species. For instance, Havenhand et al. (2008) reported similar results to our study for the tropical species *Heliocidaris erythrogramma* where fertilization success was reduced at pH_{NIST} 7.7. However, for the same species, Byrne et al. (2010b) reported that fertilization success was not reduced at pH_{NIST} 7.6, probably due to methodological differences (see Byrne et al. 2010b) such as fertilization success measurement timing (Reuter et al. 2010). It is noteworthy that the methodology of the present study was analogous to that of Byrne et al. 2010b, i.e. the use of gamete pools and not of single female-male pairs, and that even so, at a similar pH, a reduction of fertilization success of *P. lividus* was already observed in our study. In other cases, larval development was more impaired than fertilization and cleavage stages, such as for *Hemicentrotus pulcherrinus* (Kurihara et al. 2004b, Kurihara and Shirayama 2004) and for *Echinometra mathaei* (Kurihara et al. 2004b) (Table 3). Nevertheless, concerning *P. lividus* larval development, the LOEC pH_T was near or lower than those of other species studied (Table 3). It is noteworthy that the impact in larvae development was not only a size reduction, but also an increment of abnormal larvae that will never develop further. Actually, the LOEC pH_T for the latter was higher than for the former effect. This indicates that contrary to what was observed in other sea urchins species, the impact of ocean acidification can have more severe effects than that of just a simple delay in development (see Dupont et al. 2010b for discussion). The larvae from intertidal *P. lividus* seem to be more resistant to acidification than those of species collected from subtidal sites. This suggests that sea urchins living in the stressful intertidal zone may be adapted or acclimatized to pH stress. In their study, Clark et al. (2009) observed that the Antarctic sea urchin *Sterechinus neumayeri* larvae

were the least affected by low pH compared to tropical and temperate sea urchin species. They postulate that, evolving in an environment with historically higher levels of CO_2, as a result of polar seawater temperatures and upwelling of CO_2-rich water, *S. neumayeri* would be adapted to higher CO_2 conditions and, therefore, may have greater capacity to acclimatize to lowered seawater pH. In this context, it is noteworthy that *P. lividus* is a widely distributed species, ranging from Scotland to Morocco through the whole Mediterranean, indicating that the adaptation potential of the species is very high, even if the embryo and larva development of this species appeared rather sensitive to pH in comparison with other species. The intertidal populations of *P. lividus* experience appreciable decrease of pH when compared to subtidal populations. The offspring of sea urchins from the tide pool with higher pH decrease (tide pool 2) showed a better resistance to acidification at pH_T 7.4 than that of sea urchins from the tide pool with low pH decrease (tide pool 1) in terms of fertilization rate, viz. a reduction of over 30 % compared to about 20 % for tide pool 2. A possible explanation could be a better resistance of male and/or female gametes to acidification. For instance, there could be a greater allocation of resources in reproduction by individuals subjected to lower pH. The eggs would then be stronger and more resistant to lower pH values. However, no significant differences were detected between cleavage rate of embryos according to progenitor origin. Moreover, gonad maturation also did not differ significantly between sea urchins of both tide pools. As a consequence, the better resistance to acidification of the offspring of sea urchins from tide pool 2 could be explained by a better performance of the spermatozoa. The impact of pH on the sperm of sea urchins has been widely documented by embryologists. A decrease in pH of seawater lowers the internal pH of sperm. This is accompanied by a decrease in sperm motility and in acrosome reaction (Christen et al. 1986). The regulation of internal pH of sperm is controlled by a Na^+/K^+-ATPase pump and Na^+/H^+ transporters (antiport) located on the plasma membrane of spermatozoa (Gatti and Christen 1985). In spermatozoa already subjected to pH stress in the gonads, these transmembrane proteins may be more effective or expressed in greater number. This could be due to either acclimatization (phenotypic plasticity) or natural selection. As the fertilization rate of sea urchins can be affected by sperm motility (Havenhand et al. 2008), the fertilization rate of acclimatized/selected sperm would be higher. This fact remains to be confirmed and we think that pH stressed populations offer the possibility to better understand potential gonad and gamete adaptations. Endotrophic larval growth of *P. lividus* was only affected at pH levels predicted for 2300 (IPCC 2007) or lower (Table 3) indicating the relative resistance of this

stage. No information is currently available on the effect of pH on exotrophic larval development of this nor of other sea urchin species (for a review see Dupont et al. 2010b). Further investigation should not only address this aspect, but also compare the development success of different populations, such as intertidal vs. subtidal, or with those from upwelling zones. Echinoid mechanisms of pH stress resistance and/or adaptation are currently poorly understood and little studied. Additional knowledge of the consequences of ocean acidification and the potential ability of organisms to cope with it will require such information. Applying a widespread, practical and sensitive chronic toxicology test (the use of exposed sea urchin gametes, embryos and larvae to a stress) and relevant endpoints such as the LOEC offers the opportunity to clarify one species sensitivity to lower pH during distinct life stages, allowing inter-specific comparisons. Furthermore, this type of stress-response evaluation can allow the development of monitoring tools in areas highly vulnerable to ocean acidification, such as upwelling and cold water regions, in water bodies subject to industrial acid waste and in areas where acid dependent toxicants can be present.

Acknowledgments: The authors are grateful to M. Byrne for discussion on fertilization membrane elevation and to an anonymous reviewer whose comments helped improving this manuscript. Special thank to B. Danis (Scarmarbin, Belgium), A.V. Borges (Université de Liège, Belgium), M. Bauwens, Ph. Pernet and C. Moureaux (BIOMAR, Université Libre de Bruxelles, Belgium). A.I. Catarino holds a FCT grant (SFRH/BD/27947/2006, Portugal). Ph. Dubois is a Senior Research Associate of the NFSR (Belgium). Work supported by FRFC contract 2.4532.07 and Belspo contract SD/BA/02B.

Chapter II

Sea urchin *Arbacia dufresnei* (Blainville 1825) larvae response to ocean acidification

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Sea urchin *Arbacia dufresnei* (Blainville 1825) larvae response to ocean acidification

Ana I. Catarino[1], Chantal De Ridder[1], Marcelo Gonzalez[2], Pablo Gallardo[3], Philippe Dubois[1]

[1]Laboratoire de Biologie Marine, Université Libre de Bruxelles, Belgium

[2]Instituto Antártico Chileno, Punta Arenas, Chile

[3]Universidad de Magallanes, Centro de Cultivos Marinos Bahía Laredo, Punta Arenas, Chile

Under review in Polar Biology

Abstract: Increased atmospheric CO_2 emissions are inducing changes in seawater carbon chemistry, lowering its pH, decreasing carbonate ion availability and reducing calcium carbonate saturation state. This phenomenon, known as ocean acidification, is happening at a faster rate in cold regions, i.e., polar and sub-polar waters. The larval development of the offspring from a sub-Antarctic population from the species *Arbacia dufresnei* was studied at high (8.0), medium (7.7) and low (7.4) pH waters, the two later treatments corresponding to the expected surface pH in 2100 and 2300, respectively. The results show that the offspring from sub-Antarctic populations of *A. dufresnei* are susceptible to a larval development delay at low pH, with no significant increase of abnormal forms in the tested pH. Larvae were isometric between pH treatments. Even at calcium carbonate ($CaCO_3$) saturation states (of both calcite and aragonite used as proxies of the magnesium calcite one) values lower than 1, skeleton deposition occurred. The *A. dufresnei* larvae do not seem to be sensitive to near future pH decrease. This reinforces the idea that polar and sub-polar sea urchin larvae can show resistence to acidification, emphasizing this species potential to poleward migrate and further colonize southern regions.

Keywords: Ocean acidification, sea urchin, sub-Antarctic population, *Arbacia dufresnei*, larvae

1. Introduction

Increased atmospheric CO_2 emissions are inducing changes in seawater carbon chemistry, lowering its pH, a phenomenon known as ocean acidification. The average surface seawater pH has reduced by approximately 0.1 units since the industrial revolution and future reductions are expected to be around 0.3-0.5 units by 2100 and 0.7-0.8 units or more by 2300 (Caldeira and Wickett 2003, 2005, IPCC 2007). The result is a decrease in the concentration of the carbonate ion (CO_3^{2-}) which reduces the saturation state of calcium carbonate (Ω_{CaCO3}) minerals (Feeley et al. 2004, Orr et al. 2005). These shifts in seawater chemical and pH levels are occurring at a rate not experienced by marine organisms in the last 20 or more millions of years (Turley et al. 2006, Ridgwell and Schmidt 2010), challenging the potential of species to acclimatize and/or to adapt. Ocean acidification is happening at an even faster rate in high-latitude regions such as Antarctica, as a result of a higher CO_2 solubility due to cold temperatures and because of CO_2 enriched waters supplied by an active upwelling system (Feely et al. 2004, Fabry et al. 2009). In fact, most models of ocean-carbon cycles predict that the shallowing of the $CaCO_3$ saturation horizons will be more significant and will occur earlier at higher latitudes, such as in polar and sub-polar waters (Feely et al. 2004, Orr et al. 2005, Anderson et al. 2008, Fabry et al. 2009).

These changes are believed to affect several physiological functions of marine organisms, namely growth, reproduction and even behaviour (Pörtner 2008). A reduction of calcification rates has broadly been reported and associated with a decrease of CO_3^{2-} availability and consequent low $CaCO_3$ saturation states (Fabry et al. 2009, Hofmann et al. 2010, Hofmann and Todgham 2010). Furthermore, early developmental stages of marine invertebrates can be particularly sensitive to environmental stresses, such as ocean acidification (Pörtner and Farrell 2008, Dupont et al. 2010b). Indeed, early stages of sea urchin development in lower pH seawaters have revealed vulnerabilities such as decreased fertilization and cleavage rates, a reduction of the pluteus larva size and/or a delay in their development (Kurihara and Shirayama 2004, Havenhand et al. 2008, Clark et al. 2009, Dupont et al. 2010b, Moulin et al. 2011, Sheppard Brennand et al. 2010). In fact, echinoids could be particularly vulnerable to seawater acidification due to low acid-base regulation (when compared to other organisms possessing more developed excretory, respiratory and ion regulation structures such as crustaceans and fish) (Pörtner 2008) and due to the nature of

their magnesium calcite (Ca-Mg-CO_3) skeleton, the solubility of which is similar or higher than that of aragonite (Morse and Mackenzie 1990, Morse et al. 2006, Andersson et al. 2008). Nevertheless, larvae with delayed development of the arctic-boreal sea urchin *Strongylocentrotus droebachiensis* raised at pH 7.7 were more successful in reaching metamorphosis than those raised at control pH (8.0), meaning that a slower growth rate might not be an impediment for larval development to be completed (Dupont and Thorndyke 2008). Natural selection can play an important role in defining species responses as some echinoids coming from contrasting environments present distinct larval development when reared at low pH waters, possibly due to acclimatization (due to phenotypic plasticity at an individual level) and/or adaptation (linked to genetic variability at a population level) (Clark et al. 2009, Moulin et al. 2011). Early stages of *Heliocidaris erythrogramma* seem not to be affected by a pH decrease (until 7.6), but rather by temperature (Byrne et al. 2009, 2010b). The only Antarctic species investigated in this matter, *Sterechinus neumayeri*, (Clark et al. 2009, Ericson et al. 2010) is thought to be more robust to ocean acidification than tropical and temperate subtidal species, but less than a temperate one (Clark et al. 2009, Moulin et al. 2011). Overall, the response of echinoid early life-history stages appears to be highly species-specific (Dupont and Thorndyke 2008, Dupont et al. 2010b, Moulin et al. 2011). More studies on different species will therefore help clarifying the response of polar and sub-polar sea urchin larvae in an acidified ocean.

The sea urchin *Arbacia dufresnei* can be found from Argentina (Atlantic 35 ºS) and Chile (Pacific 42ºS) to the southern tip of South America, and in the Antarctic Peninsula (southern limit 65ºS, 64ºW) (Bernasconi 1953, David et al. 2005, Mutschke and Ríos 2006). Adults are highly mobile and inhabit kelp forests and rocky shores from the surface to 300 m (Bernasconi 1953, Jara and Céspedes 1994, David et al. 2005). This species has an important role in its habitats: it regulates other species distributions, not only because of its voracious grazing behaviour, but also due to a significant carnivorous activity (Vásquez et al. 1984, Jara and Céspedes 1994, Penchaszadeh and Lawrence 1999, Zaixso 2004). However, no information is available on the impact of low pH on this species. Consequently, the goal of the present work was to increase the knowledge on the effects of ocean acidification on the early development of high latitude sea urchin larvae, in order to assess if these species can present some degree of resistance to low pH waters. In this context, we investigated the development of *A. dufresnei* larvae (before reaching its exotrophic stage) in waters with pH levels as predicted for years 2100 and 2300.

2. Material and Methods

Adult specimens of *Arbacia dufresnei* (ambital diameter exceeding 3.5 cm) were collected in the Magellan Strait at 53º37'S, 70º56'W in Punta Santa Ana/Fuerte Bulnes (Punta Arenas, Chile) in November 2008, corresponding to this species reproduction season (Bröger et al. 2004). Animals were collected at the base of a kelp forest of *Macrocystis pyrifera* by scuba diving at around 6 m depth and transported immediately to the aquaculture facilities of the *Centro de Cultivos Marinos Bahía Laredo* (Universidad de Magallanes, Punta Arenas) until further use. Seawater supplying the aquaculture centre and used in the present experiment was pumped in from the front bay.

Adults were injected with 1-1.5 ml 0.5 M KCl into their perivisceral cavity to induce spawning. Eggs were obtained from 5 females and checked to ensure that these had a normal round shape. Sperm was obtained from 5 males. Gametes of the same sex were gently mixed in order to have a homogeneous batch and to average individual variations. Eggs were suspended as a homogeneous layer in a Petri dish containing control natural seawater (filtered at 0.22 μm) and a drop of diluted sperm was added. Around 2 hours later, the presence of the fertilization membrane was checked. The fertilization rate was higher than 95 %. Four hours after insemination, replicates of around 30 embryos ml^{-1} were transferred into three 125 ml vials, filled with filtered seawater at each selected nominal pH: high 8.0, medium 7.7 and low 7.4 (3 replicates per pH). Medium and low pH were adjusted by bubbling CO_2 (AGA, Sweden) in previously highly aerated seawater. Vials were completely filled to avoid air spaces, preventing gas exchanges, and placed in a bath of running water at constant temperature (9.6ºC ± 0.48, n = 5) and salinity (30.7), corresponding to field conditions (Valdenegro and Silva 2003). Larvae were reared for 5 days in the dark in order to avoid algal development until early pluteus stage with most larvae in controls having 2 arms. One of the control replicates had a drastic pH drop at the end of the experiment (data not shown) and its results were not included in the analysis. Although the mortality was not quantified, at the time of the experiment completion there was no significant deposit of dead larvae at the bottom of experimental vessels.

Larvae were fixed in ethanol 70% (v/v). One hundred pluteus larvae were observed in each replicate using an optical microscope and the number of normal, abnormal and larvae with delayed development was recorded. The morphological criteria to evaluate larvae were

adapted from Warnau and Pagano (1994): abnormality was considered in presence of abnormal arm shape, complete abnormal shape or when embryos were unable to differentiate, i.e., arrested development at gastrula or blastula stage. Normal larvae (Brögger 2004) were distinguished into pluteus (2 and 4 arms) and prism stages.

Twenty-five larvae in each replicate were observed in a phase contrast inverted microscope and photographed using a digital camera (QImaging, Micropublisher, software Qcapture, Canada). Morphometric measurements were performed as described by Lamare and Braker (1999). The postoral arm length (PL), i.e. the linear distance between the tip of the postoral arm and its base, and the overall length (OL), that is the linear distance between the tip of the postoral arm and the ventral end of the larval body, were measured using the freeware ImageJ (NIH, USA) (see Lamare and Barker 1999 and Kurihara and Shirayama 2004 for measurements illustration).

Physicochemical parameters were measured at the beginning and at the end of the experiment: temperature, salinity and pH_{NIST} (National Institute of Standards and Technology), also known as the NBS (previous National Bureau of Standards, now NIST) scale. The temperature and pH_{NIST} were measured using a 827 pH Lab Metrohm meter (Switzerland) with a combined glass electrode (Metrohm 6.0228.010 with temperature sensor) calibrated with pH_{NIST} buffers 4 and 7 (Merck CertiPUR®, Germany). The salinity was measured using a conductivity meter pH/Cond 340i WTW (USA). The electromotive force (e.m.f.) values were further measured and applied to the calculation of the pH expressed in total scale using standard buffers of known pH, 2-aminopyridine/HCL (AMP) and tris/HCL (TRIS), (DOE 1994, Del Valls and Dickson 1998, Dickson et al. 2007). Seawater samples were collected at the beginning and end of the experiment, immediately filtered (0.22 µm) and used to determine total alkalinity (TA). This was carried out by a potentiometric titration with HCl 0.1 M using a Titrino 718 STAT Metrohm (Switzerland), and calculated using the Gran function (Gran 1952). Our measurements had a deviation of 0.65% of the standard certified material provided by Andrew G. Dickson's Oceanic Carbon Dioxide Quality Control laboratory. Aragonite and calcite saturation values (Ω_{ar} and Ω_{cal} respectively) and pCO_2 were determined from TA, pH_T, temperature and salinity data using the software CO2SYS (Pierrot et al. 2006) and by using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and from Dickson (1990) for K of SO_4.

To test differences in seawater pH in the vials between the beginning and the end of the experiment (5 days), a Repeated Measures ANOVA was done. Absolute frequencies of the different morphological types of larvae (normal, abnormal and delayed) were summed for each treatment, gathered in a contingency table and analysed using a G-Test (model II with one fixed margin and 4 degrees of freedom). The null hypothesis was the independence of variables, in this case of "larval stage" and "pH treatment". The proportion of abnormal larvae (arcsine transformed) in each treatment and analyzed using a one-way ANOVA, fixed factor "pH treatment". The PL data were analyzed using a model III nested ANOVA: fixed factor "pH treatment" and random factor "replicate vial" nested in "pH treatment". The PL data was further analyzed using a model III nested ANCOVA (factors as stated before and "OL" as the covariate). A regression analysis was done for the variables "PL" (dependent) and "OL" (independent) for each pH treatment and the homogeneity of slopes was tested using ANCOVA. The level of significance α was set at 0.05 for all tests.

3. Results and Discussion

The pH of seawater in which larvae were raised decreased between the beginning and the end of the experiment ($p_{ANOVA} < 10^{-6}$, Table 1) since water was not changed and there was an accumulation of CO_2 released by larval activity via their cellular respiration, and metabolite release. However, all 3 tested pH conditions (high, medium and low pH) remained different between each other ($p_{Tukey} < 2.9\times10^{-4}$). Furthermore, the observed differences between the mean starting pH and the final one, 0.17, 0.13 and 0.05 for high, medium and low pH, respectively, were similar to those reported in previous works where sea urchin larval development was studied (Kurihara and Shirayama 2004, Moulin et al. 2011).

Table 1. Seawater physicochemical parameters (mean ± SD) measured and calculated (CO2SYS) at the beginning and at the end (5 days) of the larval development experiment. Effective (n) was of 2 or 3, with the exception of the initial TA values (*), where sampling was done from the original seawater batch. Temperature was of 9.6ºC ± 0.48 (n = 5) and salinity was constant at 30.7.

Treatment pH	Initial Values			Final Values		
	High pH (n=2)	Medium pH (n=3)	Low pH (n=3)	High pH (n=2)	Medium pH (n=3)	Low pH (n=3)
pH_T	7.95	7.69±0.002	7.40±0.006	7.78	7.56±0.007	7.35±0.036
TA (μmol kg^{-1})	2.05*	2.05*	2.05*	2.05	2.06±0.007	2.05±0.023
DIC (μmol kg^{-1})	1.92	2.00±0.001	2.08±0.002	1.99	2.06±0.009	2.11±0.030
pCO_2 (μatm)	479	940±4.4	1882±26.2	722	1255±23.7	2062±182.3
$\Omega_{Calcite}$	2.54	1.45±0.006	0.79±0.012	1.54	0.96±0.014	0.61±0.053
$\Omega_{Aragonite}$	1.61	0.92±0.004	0.50±0.008	0.97	0.60±0.009	0.38±0.033

After 5 days most *Arbacia dufresnei* echinopluteus were at 2 arms stage and not in the 4 arms stage. The few larvae where the second pair of arms was beginning to develop were only observed in control replicates. This larval growth rate is slightly slower than that reported for *A. dufresnei* Argentinean specimens (adults collected at 42º46'S, 65º02'W) (Brögger et al. 2004).

The proportion of the different morphological categories differed according to the pH ($G = 35.66$, 4 d.f., $p = 3.40\times10^{-7}$, Table 2). Nevertheless, the proportion of abnormal larvae did not differ according to treatment ($p_{ANOVA} = 0.66$). So, lower pH induced a delay in development, but did not increase abnormality. The proportion of abnormal larvae was rather high. This could have been due to reduced oxygen in the vials. For instance, Marsh et al. (1999) reported maximum oxygen consumption rates per individual of *Sterechinus neumayeri* larvae before feeding stage of 17 pmol O_2 h^{-1} (overall length 0.35 mm). In a worst case scenario, had embryos and larvae consumed oxygen at maximal rates during 5 days and at the same larval density as in our study (30 larvae ml^{-1}) this would only have had represented a reduction of 17% of oxygen saturation, leaving 83 % O_2 saturation in experimental seawater. However, individual oxygen consumption rate increases with the age of the embryo and larvae and earlier stages uptake oxygen at lower rates (Marsh et al. 1999). Therefore, transporsing this example to our study, oxygen was most likely not restrictive. Indeed, by the end the experiment *A. dufresnei* larvae were actively swimming (personal observation) and developmental arrest probably occurred at a time where O_2 would not have been limiting (earliest stages). The most probable cause for the proportion of abnormal larvae was probably related to the natural variability of the mature state of the gametes within or among progenitors (Brögger 2005, Kino and Agatsuma 2007).

Table 2. Percentage (mean ± SD) of pluteus, prism and abnormal larvae (n = 200 for high pH, n = 300 for each of the other treatments) and postoral arm length (PL, mean ± SD, n = 50 for high pH, n = 75 for each of the other treatments) observed in each treatment. The PL data with different superscripts differed significantly ($p_{Tukey} < 0.05$).

pH treatment	High pH	Medium pH	Low pH
Pluteus (%)	66 ± 3.5	46 ± 3.5	44 ± 12.7
Prism (%)	9 ± 3.5	25 ± 6.0	21 ± 7.1
Abnormal (%)	26 ± 7.1	29 ± 5.2	35 ± 16.0
PL size (mm)	0.11^{a} ± 0.017	$0.086^{a,b}$ ± 0.025	0.067^{b} ± 0.015

Postoral arm length (PL) was significantly smaller at lower pH ($p_{ANOVA} = 0.0063$) (Table 2), with larvae exposed to the low pH significantly differing from those observed at control

pH (p_{Tukey} = 0.006). The mean PL had a ~20% size reduction when early stages were raised at medium pH, compared to those raised at control values, and ~40% when raised at low pH. This is in accordance with increasing evidence that ocean acidification slows down growth of sea urchin larvae, affecting their development rates (Dupont et al. 2010b). The overall length (OL) observed values in high pH treatments (Fig. 1) correspond to normal development sizes previously reported for *A. dufresnei* echinopluteus at 2 arms stage (0.19 - 0.24 mm) by Bernasconi (1953) and Brögger et al. (2005).

The PL was strongly related to the OL in all treatments (Fig. 1) and even though the larvae at medium and low pH were smaller than the control one, their size was always isometric as the slopes did not differ significantly for the 3 regressions (p_{pH*OL} = 0.3, Fig. 1). When taking OL into account, the pH effect on PL was no longer evident ($P_{ANCOVA\ model\ III}$ = 0.7). One of the most frequently stated impacts of reduced larval size caused by ocean acidification is a decrease of feeding activity due to reduction of arm ciliated band, i.e. the food capture apparatus (Kurihara and Shirayama 2004, Kurihara 2008, Sheppard Brennand et al. 2010). Longer arms/ciliated bands increase food uptake (Hart and Strathmann 1994, Miner 2005, Soars et al. 2009). However, as larvae are facing an isometric growth delay it is expected that their relative foraging capacity will not be globally affected. Isometric size reduction *per se* should not result in an insufficient food gathering. No studies have yet reported if or how food acquisition by echinoid larvae will respond to ocean acidification (for a review see Dupont et al. 2010b) and so it is not yet known if a smaller size can be linked to an altered feeding behaviour (e.g. due to modified ciliary activity). Furthermore, pH effects on physiological processes starting with feeding are also not yet investigated.

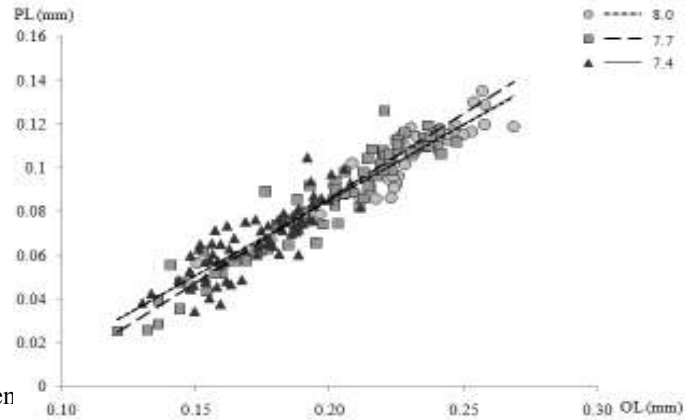

Fig. 1. Postoral arm len aents (total n = 225). Linear model equations were for high pH: y = 0.69x-0.52, R^2 = 0.86, medium pH: y = 0.77x-0.69, R^2 = 0.91 and low pH: y = 0.69x-0.53, R^2 = 0.70, all $p < 10^{-6}$. Slopes were homogeneous (p_{pH*OL} = 0.3).

Size reduction can be a result of a combination of factors, such as developmental delay and metabolic depression. Nevertheless, depending on the intensity and/or variability of the effect, it can have serious consequences by increasing vulnerability to predation and possibly reducing recruitment success (Pedrotti and Fenaux 1992, Pedrotti 1993, Balch and Scheibling 2001). However, Dupont and Thorndyke (2008) observed that larvae with delayed development of *Strongylocentrotus droebachiensis* raised at pH 7.7 were not only able to reach metamorphosis, but were actually more successful than those raised at high pH. Caution should be taken while interpreting these facts. In marine invertebrates with complex life cycles, larval experience can also affect juvenile performance due to latent effects, i.e. events that are experienced in one stage but are only manifested later in the life cycle, such as a larval size reduction or a metamorphosis delay (Pechenik 2006, Allen and Marshal 2010, Giménez 2010). Juvenile mortality in benthic marine invertebrates is known to be high and the success of the first days of the benthic life can partly depend on the history of the premetamorphic larvae (Gosselin and Qian 1997, Vaïtilingon et al. 2001).

Although existing literature often emphasizes the impact of ocean acidification on calcification, this is just one of the physiological functions that can potentially be affected (Pörtner 2008). Calcification in echinoids occurs in a calcium carbonate supersaturated closed compartment, the calcification vacuole (Dubois and Chen 1989). In addition, the carbonate (CO_3^{2-}) ion, essential for calcification and considered to be problematic due to its reduced availability caused by ocean acidification, hardly ever crosses biological membranes, entering cells via CO_2 diffusion or by bicarbonate (HCO_3^-) transport (Hofmann and Todgham 2010). In the present study, even if rod size decreased, it is noteworthy that at pCO_2 values as high as ~2000 µatm corresponding to saturation states (Ω) of both calcite and aragonite lower than 1, and therefore also of magnesium-calcite, skeleton deposition still occurred in *A. dufresnei* larvae. The same was observed in other echinoid species whose larvae were raised in very low pH (for reviews see Kurihara 2008, Dupont et al. 2010b, Moulin et al. 2011). Thus, the influence of seawater physicochemistry in calcification, if it exists, will instead be an indirect one, most likely due to low acid-base regulation and/or metabolic depression (Pörtner 2008, Hofmann and Todgham 2010).

The larval size decrease was very significant at pH 7.4. This is higher than that reported for other species and lower than that for *S. neumayeri* (7.6) (Clark et al. 2009, Moulin et al. 2011). Furthermore, larval morphology was not significantly affected at pH 7.4, a pH at which temperate and tropical species showed a higher proportion of abnormal larvae

(Kurihara et al. 2004b, Kurihara and Shirayama 2004, Moulin et al. 2011). Studies have reported that the pH in the Magellan Strait can be quite low, varying between 7.8 and 8.0 during spring (Valdenegro and Silva 2003), and these populations can be either acclimatized or adapted to already low pH conditions. So, the present results suggest that at medium pH, approximately the surface seawater pH predicted to occur by 2100, *A. dufresnei,* just as the Antarctic sea urchin *S. neumayeri* (Clark et al. 2009, Ericson et al. 2010), would not be more sensitive than other low latitude species, reinforcing the idea that polar species might not be especially at a higher risk in front of acidification. *A. dufresnei* can clearly cross the Antarctic convergence, a natural boundary for many species distributions, an indication of the resilience of its larvae (as the adult distribution limit is 300 m depth, these are unable to cross the Drake passage), and is present in the Antarctic Peninsula (Bernasconi 1953, David et al. 2005), a place considered to be a hotspot of anthropogenic induced changes (Barnes and Peck 2008). Even if the synergetistic effects of global warming and ocean acidification on the larval development of this species are unknown, we can predict that global change will promote a shift on *A. dufresnei* distribution. Should the sea surface warming continue and extend further in the water column, this invasive potential could be amplified by breaking the physiological barrier of water temperature. Furthermore, its adult opportunistic feeding behaviour can be highly competitive, promoting a potential migration of other Antarctic ecosystems besides the Antarctic Peninsula.

Acknowledgements: A. I. Catarino holds a FCT grant (SFRH/BD/27947/2006, Portugal). Ph. Dubois is a Senior Research Associate of the NFSR (Belgium). Work supported by FRFC contract 2.4532.07 and Belspo contract SD/BA/02B. The authors are grateful to the Laredo Centre personnel for technical support, to E. Newcombe and C. Cardenas (INACH) for diving support and technical advice, to C. Gimpel and G. Asencio (INACH) for assistance, to P. Gosselin for technical advice. The authors are also thankful to N. Khatri, the reviewers S. Dupont and M. Thorndyke (Sven Lovén Centre for Marine Sciences) and to an anonymous reviewer for helping improving the quality of this work.

Chapter III

Coping with ocean acidification:
adult sea urchins responses to low pH conditions

Chapter III

Coping with ocean acidification: adult sea urchins responses to low pH conditions

Ana I. Catarino[1], Laure Moulin[1], Thomas Claessens[1], Davorin Medakovic[2], Marcelo Gonzalez[3], Pablo Gallardo[4], Luc André[5], Philippe Dubois[1]

[1]Laboratoire de Biologie Marine, Université Libre de Bruxelles, Belgium; [2]The Centre for Marine Research, Ruđer Bošković Institute, Croatia; [3]Instituto Antártico Chileno, Punta Arenas, Chile; [4]Universidad de Magallanes, Centro de Cultivos Marinos Bahía Laredo, Punta Arenas, Chile; [5]Section of Petrography Mineralogy Geochemistry, Royal Museum of Central Africa, Tervuren, Belgium

Submitted to Marine Ecology Progress Series

Abstract: Surface seawater pH is expected to decrease 0.3-0.5 units by 2100 and 0.7-0.8 by 2300. We studied the effects of ocean acidification on two sea urchin species, *Paracentrotus lividus*, temperate intertidal, and *Arbacia dufresnei*, sub-Antarctic, with the aim of assessing the impact of low pH on acid-base balance, metabolic activity and spine regeneration. Field data on *P. lividus* acid-base status during spring low tide cycles were also collected. The pH of the coelomic fluid of experimental individuals decreased with seawater pH and was highly related with the amount of CO_2 present in the coelomic fluid. The later was related to the seawater pCO_2 and to an individual surface diffusion factor. The coelomic fluid seemed to present a buffer capacity higher than that of surrounding seawater, which was most likely not related with skeleton passive dissolution (no differences in coelomic fluid Mg/Ca ratios were recorded among treatments). Field data showed that during low tide, individuals can experience seawater pH variation of 0.4 units in a 4 h period. Nevertheless, their coelomic fluid pH remained stable during that time. Feeding activity, RNA/DNA ratio (measured in body wall and gonads) and carbonic anhydrase activity did not differ from control values in individuals submitted to lower pH treatments. Likewise, calcification of regenerating spines was not affected. The ability of these species to cope with pH ranges within ocean acidification prediction (minimum pH tested 7.4) can be linked to acclimatization/adaptation to pH fluctuations or low regimes already experienced by these sea urchins in their natural environment.

Keywords*:* Ocean Acidification, sea urchin, acid-base balance, metabolism, spine regeneration

1. Introduction

Increased carbon dioxide (CO_2) sequestration by seawater is leading to a process known as ocean acidification, i.e. to a drop in pH, carbonate ion (CO_3^{2-}) availability and saturation state of calcium carbonate minerals (Ω_{CaCO3}) (Feely et al. 2004, Orr et al. 2005). Since the industrial revolution, oceans surface pH has been reduced by approximately 0.1 units and in the near future it is expected to decrease further 0.3-0.5 units by 2100 and 0.7-0.8 by 2300 (Caldeira and Wickett 2003, IPCC 2007). These seawater chemical and pH shifts are occurring at a rate not experienced by marine organisms in the last 20 or more million years (Turley et al. 2006, Kump et al. 2009), challenging the potential of species to acclimatize and/or to adapt. This phenomenon has been associated with a reduction of marine organisms calcification rates (Royal Society 2005, Fabry et al. 2008), even though responses varied in magnitude (Kroeker et al. 2010). Metabolic processes, reproduction and survival can also be at risk (Pörtner 2008, Melzner et al. 2009, Hofmann et al. 2010, Hofmann and Todgham 2010). Furthermore, organisms may be at an even greater risk if effects of ocean acidification and ocean temperature increase are synergistic.

Some habitats are naturally enriched in CO_2 and present intermittent or permanent lower pH conditions such as upwelling zones (Feely et al. 2008), coastal and estuarine areas (Wootton et al. 2008, Feeley et al. 2010, Thomsen et al. 2010, Yu et al. 2011), the deep-sea (Park 1966, Millero 1996), volcanic carbon dioxide vents (Hall-Spencer et al. 2008), the intertidal zone (Truchot and Duhamel-Jouve 1980, Morris and Taylor 1983, Denny and Gaines 2007, Moulin et al. 2011) and cold water regions (Valdenegro and Silva 2003, Yamamoto-Kawai et al. 2009). Numerous marine organisms are able to cope with a wide range of ambient CO_2 conditions, most likely due to adaptive features and thanks to their phenotypic plasticity (Pörtner 2008, Hofmann and Todgham 2010). However, extreme and/or prolonged exposure can lead to severe hypercapnia, metabolic depression, shell dissolution and reproduction impairment (Hall-Spencer et al. 2008, Pörtner 2008, Hofmann and Todgham 2010).

Although many echinoderms are present in CO_2 enriched habitats, they are generally considered to be particularly vulnerable to seawater acidification due to their probable low acid-base regulation (Miles et al. 2007, Pörtner 2008) and to the nature of their magnesium calcite (Ca-Mg-CO3) skeleton, whose solubility is similar or higher than that of aragonite

(Morse and Mackenzie 1990, Andersson et al. 2008, Kurihara 2008, Sewell and Hofmann 2011). However, this last prediction which is only based on the solubility of the mineral form, should be considered with care, as it does not account for biogenic calcification processes (Pörtner 2008, Kroeker et al. 2010).

The impact of lower seawater pH in sea urchin larvae is known to promote a delay in their development, an increment in skeleton malformations (Kurihara 2008, Clark et al. 2009, Dupont et al. 2010b, Ericson et al. 2010, Moulin et al. 2011) and an increment of post-metamorphic abnormalities (Byrne et al. 2010a). Echinoids coming from contrasting environments seem to present distinct larval development responses to low pH waters, possibly due to acclimatization and/or adaptation (Clark et al. 2009, Moulin et al. 2011, Ericson et al. 2010). Moreover, adult long-term exposure to lower pH waters can affect gonad growth (Siikavuopio et al. 2007, Kurihara 2008). Brooding species were also suggested to be more vulnerable to ocean acidification (Sewell and Hofmann 2011).

In adult echinoderms, the reported effects of ocean acidification appear very diverse with some with some apparently conflicting results. In brittle stars the metabolic state was increased at low pH (Wood et al. 2008, 2010, 2011). Mean feeding and relative growth rates of an intertidal starfish, *Pisaster ochraceus*, increased when individuals were reared in high CO_2 conditions, even if calcified mass decreased at low pH and temperature (Gooding et al. 2009). Calcification and arm regeneration rates increased at low pH in *Amphiura filiformis* (at the cost of muscle wastage), but it decreased in *Ophiura ophiura* and *Ophiocten sericeum* (Wood et al. 2008, 2010, 2011). These effects can be unbalanced by temperature or not, depending on the concerned species (Wood et al. 2010, 2011). In aquaculture systems, the growth of sea urchin *Paracentrotus lividus* was affected when individuals were exposed to high CO_2 levels, i.e. to pH between 7.1-7.7 (Grosjean et al. 1996, 1998). Likewise, the growth and survival of sea urchins *Hemicentrotus pulcherrinus* and *Echinometra mathaei* were affected when raised for five months at $[CO_2]$=560 ppm (Shirayama and Thornton 2005). Conversely, another study reported that survival of adult *H. pulcherrinus* was not affected after being reared eight months at 1000 ppm, a longer and higher CO_2 exposure (Kurihara unpublished quoted in Kurihara 2008 and reviewed by Dupont et al. 2010b). In a field study near a CO_2 volcanic vents, Hall-Spencer et al. (2008) showed that the abundance of *Arbacia lixula* and *Paracentrotus lividus* was only significantly reduced in waters with a pH below 7.5-7.4. Furthermore, Ries et al. (2009) observed a positive and a negative net calcification rate according to aragonite saturation state in the sea urchins *Arbacia punctulata*

and *Eucidaris tribuloides*, respectively. Moreover, Catarino et al. (Chapter 5) proposed that deep sea Antarctic cidaroid spines present adaptive morphological and chemical characteristics that are advantageous when exposed to waters with a low magnesium calcite saturation state.

The diversity of effects of ocean acidification on echinoderms could be linked to contrasting abilities in acid-base balance regulation and subsequentment metabolic effects (Pörtner 2008, Melzner et al. 2009). However, the latter are poorly investigated. Therefore, we assessed the responses of two sea urchins species living in contrasting environments, i.e. the temperate intertidal *Paracentrotus lividus* and the Subantarctic subtidal *Arbacia dufresnei*. We measured the effects of low pH on the acid-base balance, the metabolic activity and the spine regeneration (used as a short term growth measurement).

2. Methods

2.1 Experimental setup and procedures.

The *Paracentrotus lividus* individuals were collected during low tide on a temperate European rocky coast in France, while *Arbacia dufresnei* were collected by scuba diving at the base of a kelp forest of *Macrocystis pyrifera* (6 m depth) from a sub-Antarctic Chilean population (Table 1). Adults were then transported to the experimental facilities until further use.

Table 1. Experimental setup for both species *Paracentrotus lividus* and *Arbacia dufresnei*.

Species	***Paracentrotus lividus***	***Arbacia dufresnei***
Experiments Location/Period	BIOMAR, ULB, Brussels, Belgium - May 2008	Centro de Cultivos Marinos Bahía Laredo, Punta Arenas, Chile - December 2008
Circuit, water renewal rate	Closed, 50% $week^{-1}$	Open, ~300 % day^{-1}
Acclimation period	2 weeks	1 week
Experimental period	4 weeks (28 days)	3 weeks (21 days)
Individuals per aquarium	14-19	15
Diameter (mm)	20-35	20-35
Regenerating spines per individual	12	12
Food supply	Artificial(ZeiglerTM, USA)	Natural (*Macrocystis pyrifera)*

The experimental setup differed for both tested species due to different available facilities, but general procedures were similar. After the acclimation period (Table 1), the shaft of 12 ambital primary spines (per individual) were cut 2-3 mm from the base (milled

ring) using dissection scissors. Spines were then left to be regenerated (on the sea urchins) for a few weeks in control and low pH seawaters (Table 1). The pH was manipulated by bubbling CO_2 supplied by Airliquide, (France) in Belgium and AGA (Sweden) in Chile. Each aquarium had a 60 L capacity and the ones dedicated to low seawater pH were equipped with a pH electrode connected to a controller (Aquastar, IKS ComputerSysteme GmbH, Karlsbad, Germany) which regulated CO_2 supply by plugging an electronic valve. The *Paracentrotus lividus* feeding rate was quantified by monitoring the daily amount of consumed artificial sea urchin food pellets (Zeigler^{TM}, USA). The tested nominal pH in the case of *P. lividus* were 7.8, 7.5 and 7.4 and the case of *Arbacia dufresnei* 8.0, 7.7 and 7.4. There were two aquaria per treatment with the exception of the 7.8 *P. lividus*, where one aquaria was lost during the experimental procedures. Actual values in the experimental aquaria are reported on Table 2.

Table 2. Seawater physicochemical parameters during experiments: mean ± SD (n). During the *Paracentrotus lividus* experiment the temperature was of 14.6 ± 0.54 ºC (193) and the salinity 33.4 ± 0.08 (193), while in the *Arbacia dufresnei* one it was 9.3 ± 1.01 ºC (100) and 30.7 ± 0.05 (100), respectively. The pH_T stands for pH measured in total scale, TA for total alkalinity, DIC for dissolved inorganic carbon, pCO_2 for partial pressure of carbon dioxide and Ω for saturation state.

	Paracentrotus lividus				
Nominal pH	**7.8**		**7.5**	**7.4**	
pH_T	7.77±0.0.091 (23)	7.55±0.068 (23)	7.53±0.121 (23)	7.39±0.124 (23)	7.39±0.103 (23)
Mg/Ca	4.63±0.098 (7)	4.65±0.131 (7)	4.80±0.094 (7)	4.74±0.036 (7)	4.81±0.050 (7)
TA (mmol kg^{-1})	2.82±0.464 (10)	2.60±0.200 (10)	2.22±0.222 (10)	2.61±0.059 (10)	2.44±0.109 (10)
DIC (mmol kg^{-1})	2.72	2.57	2.20	2.64	2.47
pCO_2 (µatm)	1031.8	1632.9	1462.7	2410.3	2253.5
HCO_3^- (µmol kg^{-1})	2570.2	2450.9	2096.6	2502.9	2340.0
CO_3^{2-} (µmol kg^{-1})	106.0	60.9	49.8	43.1	40.2
$\Omega_{Calcite}$	2.55	1.47	1.20	1.04	0.97
$\Omega_{Mg\text{-}Calcite}$	2.35	1.24	1.03	1.04	0.90
$\Omega_{Aragonite}$	1.63	0.94	0.77	0.66	0.62
	Arbacia dufresnei					
Nominal pH	**8.0**		**7.7**		**7.4**	
pH_T	8.02±0.021 (16)	8.02±0.026 (16)	7.79±0.097 (22)	7.72 ±0.102 (22)	7.48 ±0.073 (22)	7.45 ±0.203 (22)
Mg/Ca	4.66±0.055 (5)	4.72±0.073 (5)	4.69±0.065 (5)	4.69±0.050 (5)	4.67±0.033 (5)	4.71±0.049 (5)
TA (mmol kg^{-1})	2.06±0.005 (5)	2.05±0.006 (5)	2.06±0.019 (5)	2.06±0.010 (5)	2.06±0.013 (5)	2.05±0.005 (5)
DIC (mmol kg^{-1})	1.92	1.92	2.00	2.01	2.09	2.08
pCO_2 (µatm)	394.9	394.1	707.8	839.1	1505.2	1609.1
HCO_3^- (µmol kg^{-1})	1804.7	1801.2	1904.8	1921.9	1983.9	1979.3
CO_3^{2-} (µmol kg^{-1})	100.6	100.4	62.5	53.7	31.9	29.7
$\Omega_{Calcite}$	2.46	2.45	1.53	1.31	0.78	0.73
$\Omega_{Mg\text{-}Calcite}$	2.34	1.87	1.51	1.22	0.74	0.53
$\Omega_{Aragonite}$	1.55	1.54	0.96	0.82	0.49	0.46

The highest pH achieved in the *P. lividus* experiment was 7.8 due to the seawater origin and to experimental conditions. The seawater pH significantly differed according to pH treatment, but pH in replicate aquaria did not ($p_{t\text{-}test} < 0.002$ for *P. lividus* experiment and $p_{t\text{-}test} < 10^{-3}$ for *A. dufresnei*). Coelomic fluid collection and individuals dissections took place within the 3 last days of each experiment.

2.2 Seawater physicochemical parameters of the experimental setup

Salinity was measured using a conductivity meter pH/Cond 340 i WTW (USA). The temperature, pH_{NIST} (National Institute of Standards and Technology, previously known as National Bureau of Standards, NBS) and the electromotive force (e.m.f) were measured using a 827 pH Lab Metrohm meter (Switzerland) with a combined glass electrode (Metrohm 6.0228.010 with temperature sensor) calibrated with pH_{NIST} buffers 4 and 7 (Merck CertiPUR®, Germany). The e.m.f values were applied to the calculation of the pH expressed in total scale (pH_T) using standard buffers of known pH, 2-aminopyridine/HCL (AMP) and tris/HCL (TRIS), (DOE 1994, Del Valls and Dickson 1998, Dickson et al. 2007). Collected seawater samples were immediately filtered (0.22 µm, Millipore, USA) in order to determine total alkalinity (TA) and magnesium to calcium ratio (Mg/Ca expressed in mol/mol). The TA was measured by means of a potentiometric titration with HCl 0.1M using a Titrino 718 STAT Metrohm, and calculated using the Gran function (Gran 1952). Our measurements had a deviation of 0.65 % of the standard certified material provided by Andrew G. Dickson's Oceanic Carbon Dioxide Quality Control Laboratory (USA). In order to measure Mg^{2+} and Ca^{2+} concentrations, seawater samples were diluted 20 times in MilliQ water (Millipore) acidified (10 %) with HNO_3 65% (Suprapur® Merck, Germany) prior to analyses and were later analysed with an Iris Advantage (Thermo Jarrell Ash, USA) Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). The calibration was achieved using artificial multi-elemental solutions made from certified mono-elemental solutions (Merck, Germany) and seawater certified reference materials (CRM) for quality check (High Purity Standards, USA). Results for the CRM were always within ± 10% of the certified values. Dissolved inorganic carbon (DIC), carbon dioxide partial pressure (*p*CO2), seawater bicarbonate, $[HOC_3^-]$, and carbonate concentrations, $[CO_3^{2-}]$, and aragonite and calcite saturation state values ($\Omega_{Calcite}$ and $\Omega_{Aragonite}$), were determined from TA, pH_T, temperature and salinity data using the software CO2SYS (Pierrot et al. 2006) and by using the

dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and the K_{SO4} dissociation constant given by Dickson (1990) (Table 2). The saturation state of the Mg-calcite ($\Omega_{Mg\text{-}calcite}$) was calculated using all the data attained for the carbonate system and the seawater Mg^{2+}, Ca^{2+} and CO_3^{2-} ions stoichiometric activity coefficients (Millero 2001, Morse et al. 2006, Andersson et al. 2008). The magnesium concentration measured in regenerated spines (see below) was used to obtain the stoichiometric saturation (K) by means of the "biogenic curve of Plummer and Mackenzie", also known as the "minimally prepared curve" (Plummer and Mackenzie 1974, Morse and Mackenzie 1990, Andersson et al. 2008) (Table 2).

2.3 Field data

Observations were done in two tide pools, in Aber, Crozon peninsula (48º140N; 04º270W, southern Brittany, France), during spring low tidal cycles in February and April 2009. Intertidal adult individuals occurred in tide pools and showed a sedentary behaviour in self burrowed holes (confirmed by tagging experiments, data not shown). They are partially protected from wave action and never get emersed during low tide. The physicochemical parameters of two tide pools temperature, salinity and pH_{NIST} were measured every half an hour starting at pool individualization (ebbtide) until its cover (rising tide) during two night and two day low tides using methods previously described. Seawater samples were collected at the beginning and end of each low tide and immediately filtered (0.22 μm) in order to determine TA. The seawater parameters such as DIC, $pCO2$, $\Omega_{Calcite}$ and $\Omega_{Aragonite}$ were calculated as previously described (Table 3). The coelomic fluid pH_{NIST} of 3-5 individuals was measured (procedure as follows) at ebbtide and at rising tide. Every individual was used only one time.

2.4 Coelomic fluid analysis

In both experiments and in field sea urchins, the coelomic fluid (CF) of each individual was collected through the peristomial membrane using an insulin syringe (Myjector 0.5 ml, Terumo, Japan). In the case of *Paracentrotus lividus* the labelled samples were stored on ice until further analysis (on collection day). The CF pH_{NIST} (in the impossibility of making the pH total scale calibration) and CO_2 partial pressure (pCO_2) were measured using a MHRA

Blood Gas Analyser ABL™ 700 Series (Radiometer Medical ApS, Denmark) at the Erasmus Hospital (Hôpital Erasme, Université Libre de Bruxelles, Belgium). For *Arbacia dufresnei*, the CF pH was immediately measured after collection using a microelectrode (Metrohm 6.0228.100) whose calibration and e.m.f. values conversion to pH in total scale were done as previously described. In addition, CF samples were acidified with HNO_3 65% (Suprapur® Merck), stored until ICP-AES analysis of Mg and Ca as previously described. Finally, in both species, the difference between the CF pH and the seawater mean one (from each treatment) was calculated. The CF pH_{NIST} of field *P. lividus* individuals was immediately measured at ebbtide and at rising tide using a microelectrode (Metrohm 6.0228.100).

Table 3. Seawater physicochemical parameters of the 3 studied tide pools and of the open sea (OS; beginning of each tide cycle). The T stands for temperature, pH_{NIST} for pH measured in NIST scale, TA for total alkalinity (mmol kg^{-1}), DIC for dissolved inorganic carbon (mmol kg^{-1}), pCO_2 for partial pressure of carbon dioxide (μatm) and Ω for saturation state.

Date	Time	Cycle	Pool	Salinity	T (ºC)	pH_{NIST}	TA	DIC	pCO_2	$\Omega_{Calcite}$	$\Omega_{Aragonite}$
08-02-2009	19:40	Night	OS	34.4	7.6	8.08	2.29	2.16	476.48	2.55	1.61
08-02-2009	23:27	Night	1	34.4	7.2	8.01	2.31	2.20	571.44	2.19	1.39
08-02-2009	23:24	Night	2	34.4	7.2	7.84	2.31	2.26	873.67	1.53	0.97
10-02-2009	10:34	Day	OS	34.3	7.6	8.11	2.29	2.14	440.53	2.70	1.71
10-02-2009	13:31	Day	1	34.3	7.6	8.31	2.24	2.01	253.81	3.90	2.47
10-02-2009	13:28	Day	2	34.2	8.5	8.48	2.26	1.93	160.38	5.48	3.47
25-04-2009	22:15	Night	OS	35.0	11.7	8.14	2.33	2.14	425.31	3.32	2.12
25-04-2009	01:51	Night	2	35.0	10.8	7.79	2.30	2.25	1015.13	1.55	0.99
25-04-2009	02:00	Night	3	35.0	10.6	7.68	2.33	2.31	1345.18	1.23	0.79
27-04-2009	10:52	Day	OS	34.7	11.2	8.01	2.32	2.19	592.41	2.49	1.59
27-04-2009	14:46	Day	2	34.6	12.2	8.00	2.18	2.06	577.24	2.36	1.51
27-04-2009	14:50	Day	3	34.6	12.3	8.15	2.10	1.92	374.98	3.07	1.96

In order to determine the coelomic fluid buffer capacity of *Paracentrotus lividus*, a titration was performed on 0.5 ml samples using HCl 0.1 N and calculations were done using the Gran function (Gran 1952). Aquarium seawater samples and standard seawater certified material (by Andrew G. Dickson's Oceanic Carbon Dioxide Quality Control Laboratory) were titrated using the same method. Seawater certified material measurements had a deviation of 1.6 % of the original batch value.

2.5 RNA to DNA ratio analysis

At the end of the experiments, samples of the body wall and gonads of both species were collected, frozen in liquid nitrogen and then stored at -80º C until RNA/DNA determinations. Nucleic acid concentrations were determined using a one dye (ethidium bromide)/one-enzyme (RNase), 96-well microplate microplate fluorometric assay based on the protocol described by Catarino et al. (2008). Fluorescence was read using a Spectrofluor Plus microplate reader from Tecan© (Switzerland). Excitation and emission wavelengths were 365 and 590 nm, respectively. The RNA fluorescence was calculated by subtracting the DNA fluorescence reading from the total nucleic acid value. Sample nucleic acid concentrations were estimated by comparing fluorescence readings with those obtained from standard curves. Residual fluorescence (evaluated before the study by using DNase (D-4263, Sigma-Aldrich) was considered to be negligible. The RNA/DNA ratios were determined for each sample and expressed as µg RNA mg^{-1} dry weight sample divided by µg DNA mg^{-1} dry weight sample.

2.6 Carbonic anhydrase activity

Samples from *Arbacia dufresnei* body wall were collected after dissection, frozen in liquid nitrogen and then stored at -80º C until further analysis. They were then added with Triton X-100, to permeabilize cell membranes, and centrifuged at 4º C for 10 min (6000 rcf). Carbonic anhydrase (CA) activity was investigated using Maren's phenol red colorimetric test (Maren 1960). The reaction vessel was maintained under 4º C in an ice bath. All reagents were kept under a CO_2 constant flow (80 mmHg). The reagents were inserted into the reagent vessel as followed: 200 µl phenol red indicator, 150 µl MilliQ water, the enzyme source, i.e. the body wall samples and the run started with the final addition of 50 µl of the carbonate buffer (0.3 M Na_2CO_3, 0.206 M $NaHCO_3$). The enzyme CA catalyzed the reaction of CO_2 with H_2O, resulting in the formation of HCO_3^- and H^+. The proton production was monitored by means of measuring the reaction time elapsed between buffer addition and the indicator colour change from red to yellow. The CA activity (A) was calculated using the uncatalysed reaction time in seconds (t_0) and the catalysed reaction time (t_{enz}, s) through the following equation: $A = (t_0/t_{enz}) - 1$. One unit (U) of CA activity was defined as the enzyme activity

required in the final assay volume to halve the uncatalysed reaction time ($t_0 = 2t_{enz}$). Only values above the detection limit (DL) were used (DL is defined as the mean value of t_0 plus or minus 3 times its standard deviation). The CA activity was expressed by protein sample content (U μg^{-1}), measured by the Bradford (1976) method.

2.7 Regenerated spines

The regenerated spines were detached from the test using a scalpel. They were cleaned from associated soft tissues in 2 M NaOH (pro analysis, Merck), then successively rinsed 3 times with MilliQ water and finally air dried for more than 12 h. Spines were observed in an optical microscope, photographed and their regenerated length measured using the freeware Image J. 1.33 u (Wayne Rasband, National Institutes of Health, USA).

The magnesium content of a pool of 3 regenerates coming from each individual was measured by flame atomic spectrometry (F-AAS) and expressed as μg^{-1} dry weight (DW) as reported by Hermans et al. (2010). The accuracy was checked using the CCH-1 certified reference material (Govindaraju 1994; European Commission, Institute for Reference Materials and Measurements, Belgium). Each analysis was done in triplicate and reproducibility was always below 10% residual standard deviation for experimental samples and below 3% for the certified reference material. Analysis of the certified reference material were always within 10% of the certified value. The magnesium content of spines was used to calculate the magnesium calcite saturation state ($\Omega_{Mg\text{-}calcite}$) as previously described.

In order to determine if regenerated spines presented corrosion signs, 2 cleaned specimens per condition were coated with gold and observed in a scanning electron microscope (SEM - JEOL JSM 6100, Japan) with secondary electron image acquisition by the software 3.0 SemAfore Jeol 1993-1997 (J. Rimppi Oy, Finland).

2.8 Data statistical analysis

The pH in the different aquaria were compared using a repeated measures ANOVA (using “time” and “aquarium” as factors) followed by a paired t-test procedure using a Bonferroni correction in order to determine if aquaria would be similar among treatments and different from the others. Feeding rate of *Paracentrotus lividus* was studied using a repeated

measures nested ANOVA with "pH" as a fixed factor, "aquarium" as a random factor nested in "pH" and "time" as the repeated factor.

The following dependent variables were compared between treatments using a nested ANOVA procedure: RNA/DNA ratio, carbonic anhydrase activity, Mg/Ca in coelomic fluid, regenerate length and its magnesium content. The factors used were "pH" (fixed) and "aquarium" (random, nested in "pH"). For RNA/DNA gonads analysis a further "sex" (fixed) factor was tested. Data was ln transformed whenever necessary.

The weight of the pooled regenerates (used in magnesium content measurements) was further analysed with a multiple regression using a forward stepwise procedure with the following independent variables: seawater pH in each aquaria (mean value of the whole experiment), spine height, spine base diameter and sea urchin diameter.

Coelomic fluid pH from field *P. lividus* individuals was analysed using a t-test procedure in order to understand if pH differed between the beginning and the end of the tidal cycle.

The coelomic fluid pH was tested against seawater pH in the previous 24 h in each aquarium. Furthermore, the carbon dioxide content of *P. lividus* experimental individuals was further analysed using a forward stepwise procedure with the following independent variables: seawater pH in the previous 24 h, pCO_2 calculated in seawater in the previous 24 h, sea urchin diameter and the square value of sea urchin diameter.

Significant level was set at 0.05. All presented R^2 of regression analysis correspond to the adjusted R^2.

3. Results

3.1 Feeding rate

The feeding rate of *Paracentrotus lividus* did not differ between treatments ($F = 0.922$, $p_{ANOVA} = 0.487$) (Table 4).

3.2 Coelomic fluid parameters

Regression analysis showed that pH of the coelomic fluid (CF) of experimental individuals, *Paracentrotus lividus* and *Arbacia dufresnei*, decreases with seawater pH measured in the previous 24h prior to CF collection ($p_{Regression} < 1x10^{-3}$, $R^2 > 0.2$) (Fig. 1).

The pH CF of *P. lividus* also decreased significantly with the amount of CO_2 present on coelomic fluid ($p_{Regression} < 10^{-6}$, $R^2 = 0.4$) (Fig. 1). A forward stepwise multiple regression analysis showed that the amount of CO_2 in *P. lividus* CF is related with the seawater pCO_2 24 h prior to CF collection and to the individual size factor (the square value of the diameter) ($p < 10^{-6}$, $R^2 = 0.4$) (Table 5). The magnesium to calcium ratio in *A. dufresnei* coelomic fluid did not differ between treatments ($F = 1.2$, $df = 2$, $p_{ANOVA} = 0.41$) (Table 4). The pH_{CF} of *P. lividus* field individuals did not differ between the beginning and the end of the tidal cycles ($p_{t\text{-}test} > 0.09$) for both the night and day cycles ($p_{t\text{-}test} < 0.03$), except in the tide pool 2 (April) individuals. The differences in the mean CF pH_{NIST} between the end and the beginning of the low tide cycle according to the differences of the pH_{NIST} in the respective tide pool seawater are presented in Fig. 2. The buffer capacity of *P. lividus* individuals was of 5.03 ± 0.367 mmol kg^{-1}, n = 4. The total alkalinity of their aquarium seawater was of 3.31 ± 0.044 mmol Kg^{-1} (n = 3).

Table 4. Variables measured in *Paracentrotus lividus* and *Arbacia dufresnei* specimens submitted to different pH treatments, mean ± SD (n), and ANOVA results (α=0.05). BW stands for body wall, F for females, M for males, CA for carbonic anhydrase and CF stands for coelomic fluid.

Parencentrotus lividus	**7.4**	**7.5**	**7.8**	**F**	**p**
Feeding rate	0.80 ± 0.220 (34)	0.63 ± 0.141 (37)	0.77 ± 0.134 (14)	0.922	0.487
RNA/DNA BW	0.088 ± 0.0500 (22)	0.071 ± 0.0481 (30)	0.059 ± 0.0400 (11)	1.602	0.199
RNA/DNA Gonads F	1.16 ± 0.160 (25)	1.70 ± 1.463 (28)	2.26 ± 1.742 (10)	1.741	0.315
RNA/DNA Gonads M	0.23 ± 0.053 (6)	0.39 ± 0.099 (7)	0.22 (2)		
Regenerate height (mm)	1.91 ± 0.616 (209)	2.15 ± 0.743 (193)	2.47 ± 0.785 (79)	0.104	0.910
Regenerate Mg mol %	3.18 ± 0.503 (27)	3.21 ± 0.543 (30)	3.06 ± 0.374 (11)	0.273	0.785
Arbacia dufresnei	**7.4**	**7.7**	**8.0**	**F**	**p**
RNA/DNA BW	0.299 ± 0.1481 (27)	0.328 ± 0.1460 (27)	0.310 ± 0.2020 (30)	0.148	0.871
RNA/DNA Gonads F	3.99 ± 2.824 (12)	3.89 ± 2.022 (18)	4.72 ± 1.344 (8)	0.355	0.727
RNA/DNA Gonads M	0.20 ± 0.134 (10)	0.29 ± 0.225 (13)	0.20 ± 0.186 (15)		
CA activity (U μg^{-1}) x 10^{-3}	3.986 ± 1.2413 (17)	3.575 ± 0.9726 (22)	4.181 ± 1.1932 (23)	2.108	0.265
Mg/Ca CF	4.54 ± 0.120 (28)	4.56 ± 0.118 (28)	4.58 ± 0.148 (28)	1.200	0.414
Regenerate height (mm)	1.46 ± 0.250 (306)	1.58 ± 0.260 (298)	1.54 ± 0.283 (282)	1.480	0.357
Regenerate Mg mol %	4.07 ± 1.276 (26)	3.99 ± 0.895 (28)	4.21 ± 0.946 (28)	0.314	0.761

3.3 Metabolic parameters.

The RNA/DNA ratios measured in the body wall did not differ between treatments in both species ($p_{ANOVA} > 0.2$) (Table 4). Gonads RNA/DNA ratios also did not differ between

treatments ($p_{ANOVA} > 0.3$), but they differed between sex ($p_{ANOVA} < 1.4x10^{-3}$), females having higher ratios than males (Table 4). Carbonic anhydrase activity did not differ between treatments in *Arbacia dufresnei* body wall samples ($p_{ANOVA} > 0.20$).

3.4 Regenerated spines

The height of regenerated spines did not differ between pH treatments ($p_{ANOVA} > 0.36$) in both species (Table 4). Their magnesium content also did not differ significantly ($p_{ANOVA} > 0.76$) (Table 3). Regenerated spines observed in SEM revealed no sign of corrosion (Fig. 3). The *Paracentrotus lividus* spine regenerate weight was linked with spine regenerate height, its diameter and sea urchin diameter ($p_{Regression} < 10^{-6}$, $R^2 = 0.46$) (Table 5), but not with seawater pH. The same was valid for *Arbacia dufresnei* except for the sea urchin diameter was which had no significant effects ($p_{Regression} < 4x10^{-5}$, $R^2 = 0.21$) (Table 5). The SEM results are shown on Fig. 3.

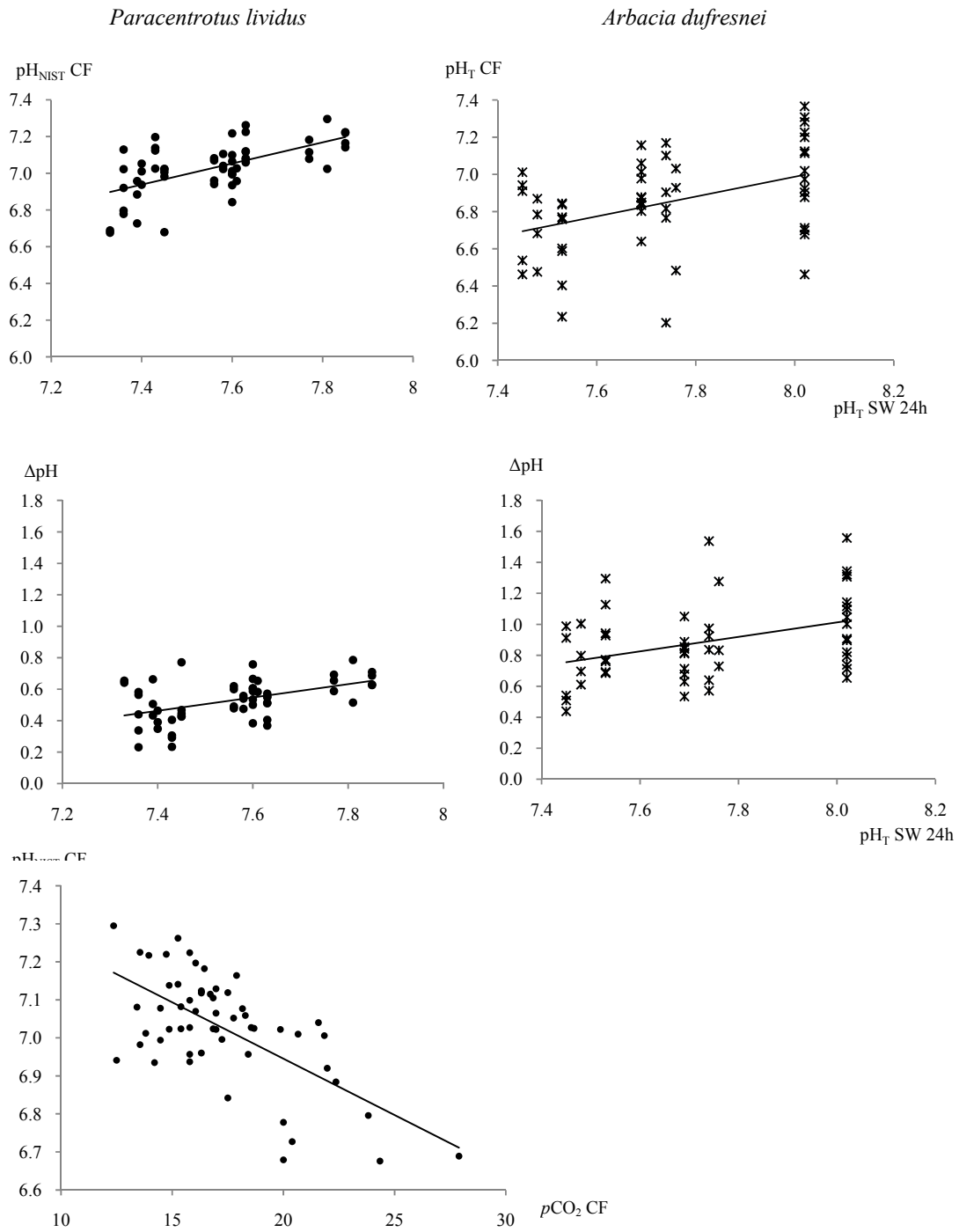

Fig. 1. Coelomic fluid parameters measured in sea urchins *Paracentrotus lividus* (a, c, e) and *Arbacia dufresnei* (b, d) submitted to experimental pH. a, b) Coelomic fluid (CF) pH measured in the coelomic fluid according to experimental aquaria pH measured in the previous 24 h. c, d) The difference between the pH CF and the experimental aquaria pH measured in the previous 24 h (ΔpH) according to experimental pH. e) Coelomic fluid pH according to pCO_2 CF (10^{-3} atm). Regression curves: a) $y = 0.58\ x + 2.7$, $R^2 = 0.36$; b) $y = 0.53\ x + 2.7$, $R^2 = 0.17$; e) $y = 0.030\ x + 7.5$, $R^2 = 0.42$. All regressions $p < 4.5 \times 10^{-3}$.

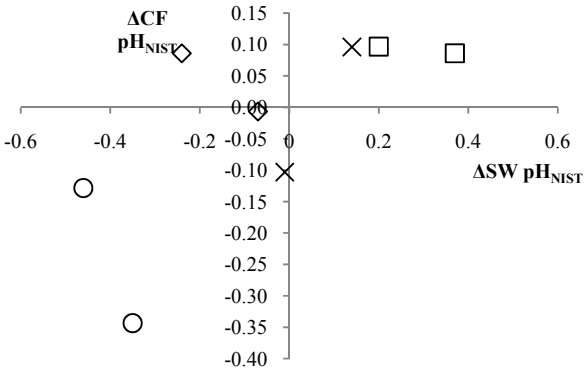

Fig 2. Difference in the mean coelomic fluid (CF) pH_{NIST} between the end and the beginning of the low tide cycle (ΔCF pH_{NIST}) according to the differences of the pH_{NIST} in the respective tide pool seawater (SW) during the same period (ΔSW pH_{NIST}). □ - Day cycle, February; ◇ - night cycle, February; × - day cycle, April; ○ - night cycle, April.

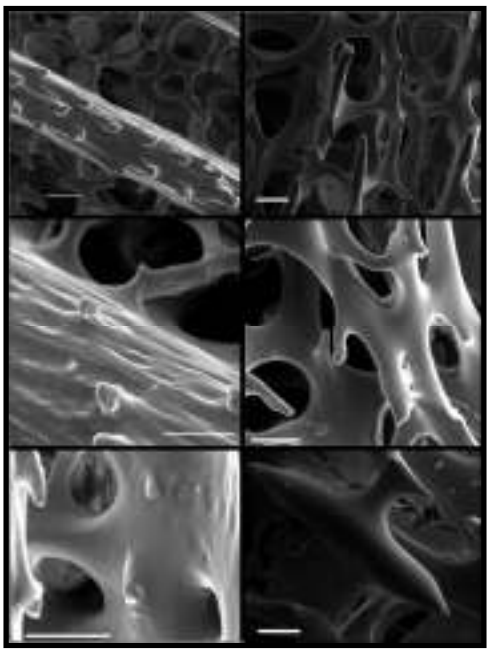

Fig. 3. SEM images of regenerate spines from *Paracentrotus lividus* (a, pH 7.8; d, pH 7.5; e, pH 7.4) and *Arbacia dufresnei* (b, pH 8.0; c, pH 7.7; f, pH 7.4) individuals submitted experimentally to control and lower pH seawater. White bars correspond to 10 µm.

Table 5. Results of forward stepwise multiple regression analysis (Beta: standardized regression coefficient, SE: standard error). Model 1: dependent variable was pCO_2 in the coelomic fluid of *Paracentrotus lividus*, $p < 10^{-6}$, SE = 1.8, $R^2 = 0.4$, n = 56. Model 2: dependent variable was regenerate weight of *P. lividus*, $p < 10^{-6}$, SE = 0.13, $R^2 = 0.5$, n = 62. Model 3: dependent variable was regenerate weight of *Arbacia dufresnei*, $p < 4x10^{-5}$, SE = 0.04, $R^2 = 0.2$, n = 81.

Model	Independent variables	Beta	SE	t	p
1	pCO_2 seawater 24 h	0.63	0.10	6.2	10^{-6}
1	Squared sea urchin diameter	-0.20	0.10	-1.9	0.06
2	Regenerate height	0.56	0.10	5.4	10^{-6}
2	Sea urchin diameter	-0.26	0.11	-2.4	0.02
2	Regenerate base diameter	0.28	0.12	2.4	0.02
3	Regenerate height	0.44	0.10	4.4	$3x10^{-5}$
3	Regenerate base diameter	0.17	0.10	1.8	0.08

4. Discussion

The coelomic fluid pH (pH_{CF}) of both *Paracentrotus lividus* and *Arbacia dufresnei* decreased significantly with the pH measured in the seawater in the previous 24 h prior to CF collection. This acidosis was caused by the accumulation of CO_2 in the coelomic fluid, as shown by the significant correlation of pH_{CF} and coelomic fluid pCO_2 content. The carbon dioxide accumulated was strongly dependent on the pCO_2 of the seawater but also on a surface diffusion factor (given by the square value of the diameter). This accumulation of coelomic fluid CO_2 was expected, due to slow elimination of CO_2 which depends on diffusion gradients for gas exchange with seawater (Farmanfarmaian 1966). Regular echinoids have tube feet (external extensions of the water vascular system) specialized in gas exchange with larger stem than adhesive tube feet (Shick 1983, Lawrence 1987). In the absence of other structures that would increase external convection, gas exchange is highly dependent on sea urchin surface (Shick 1983). However, the coelomic fluid pH is not only dependent on its CO_2 content. Interestingly, the difference between the pH_{CF} and the seawater pH decreased with the latter, indicating that the coelomic fluid owns a certain buffer capacity or that the respiratory activity decreases with pH. The latter can be ruled out as in several echinoderms oxygen uptake was shown to be increased at low pH (Wood et al. 2008, 2010, 2011), including in *P. lividus* (Chapter IV). Furthermore, the buffer capacity in the coelomic fluid of *P. lividus* measured in this study exceeded that of seawater alkalinity by 52 %. The coelomic fluid buffer capacity was not related with skeleton dissolution as previously proposed for sea urchins (Spicer 1995, Miles et al. 2007), since the coelomic fluid Mg content (given by the Mg to Ca ratio) of *A. dufresnei* sea urchins submitted to lower pH treatments did not differ from the control ones. In a companion study, Mg/Ca ratio in coelomic fluid did not differ of control values in *P. lividus* individuals exposed to low seawater pH (Chapter IV). Likewise, Burnett et al. (2002) also did not observe skeleton dissolution in sea urchin individuals during respiratory acidosis (neither magnesium nor calcium concentrations increments were recorded). In mussels, a shell dissolution buffer mechanism had also been previously proposed, but no Mg^{2+} or Ca^{2+} accumulation was verified in their haemolymph when exposed to lower pH seawaters (Thomsen et al. 2010 and references therein). Thus, passive skeleton or shell dissolution does not seem like a widespread mechanism to buffer physiological fluids of marine invertebrates by contributing with HCO_3^- ions. In fact, coelomic fluid is primarily buffered by the carbon dioxide-

bicarbonate system (Farmanfarmain 1966, Shick 1983), but Shick (1983) hypothesised that this could be further reinforced by its protein content, which can be around 0.2-0.5 mg ml^{-1} (Holland et al. 1967, Burnett et al. 2002). Even if buffer capacity of such protein concentrations is considered to be very low (Heisler 1986, Harrison et al. 1990, Miles et al. 2007), other contributions to the buffer capacity of invertebrates physiological fluids cannot be discarded, such as the presence of organic and inorganic phosphates, succinate, lactate, ammonia and other acid or bases produced metabolically or exchanged against strong ions (Harrison et al. 1990, Truchot 1988, Ali and Nakamura 2000). The complete nature of the buffer capacity of the coelomic fluid still remains unclear, but unknown components can play an important role in this function. Extracellular pH depression can lead to metabolic disturbances (Pörtner 2008). Whereas intracellular pH is strongly regulated, the extracellular one typically remains uncompensated in some marine invertebrates (Reipschläger and Pörtner 1996, Pörtner 2008). Therefore, the possibility to buffer this extracellular pH, even partially, can offer protection against mild hypercapnia and possible induced metabolic changes.

In field specimens of *P. lividus* although seawater pH could descend or increase c.a. 0.4 units within 3-4 h of low tide, the coelomic fluid pH never changed as much, most likely due to a slow CO_2 diffusion rate into the animal and to the possible buffer capacity of the coelomic fluid. In fact, despite the fact that seawater pH varied, most coelomic fluid pH measurements did not differ from the beginning to the end of the tidal cycle. Similarly, Spicer (1995) also did not find any acid-base and ionic disturbances in the coelomic fluid of the sea urchin *Psammechinus miliaris* when field individuals experienced a night low tide cycle for about 5 h 30 min. Spicer (1995) attributed this fact to a slow metabolism and to a certain ability of the coelomic fluid to buffer extreme fluctuations that intertidal sea urchins might be submitted to. In our study, coelomic fluid pH values only differed in one specific pool in April, both in the day and night cycles, even if this did not correspond to the highest pool pH variations, or any other measured seawater parameter (Table 3). This disparity was most likely due to natural coelomic fluid pH variation or to interference of an unmeasured factor that could have affected sea urchin coelomic fluid carbon dioxide accumulation. For instance, in case of surrounding seawater deficient circulation during low tide, it is probable that the sea urchins experience a further CO_2 accumulation in their immediate surrounding area, making it difficult to eliminate the coelomic fluid one, as observed in aquaculture conditions (Grosjean et al. 1996, 1998).

The index of condition tested, the RNA to DNA ratio, both from body wall and gonads, did not differed significantly from the control values, revealing no metabolic activity differences both in body wall and gonads. These indices have been previously used with success as stress, growth and metabolic condition in field and laboratory research, including sea urchins (Watts and Lawrence 1984, Frantzis et al. 1992, Liyana-Pathirana et al. 2002, Dahlhoff 2004). Somatic growth is not always depressed when individuals are exposed to low pH conditions. The *Pisaster ochraceus* starfish growth was promoted when exposed to lower pH, by a mechanism not yet identified (Gooding et al. 2009), while in mussels it was not affected (Thomsen et al. 2010).

Feeding rate in *Paracentrotus lividus* was studied and no significant differences were observed between treatments. Furthermore, gonads, an important nutrient reservoir in sea urchins (Giese et al. 1959), also allow comparison of the nutritional status of sea urchin individuals using RNA/DNA ratios (Watts and Lawrence 1984, Frantzis et al. 1992, Liyana-Pathirana et al. 2002), as feeding rate *per se* could not indicate efficiency in nutrient absorption (Lawrence 1987). The fact that the RNA/DNA ratios observed in gonads did not differ between pH treatments such as the *P. lividus* feeding rates suggest that digestive functions and nutrient transportation are possibly not affected in case of mild coelomic fluid hypercapnia, within the studied pH range (minimum seawater pH = 7.4). Conversely, *Strongylocentrotus droebachiensis* feed intake and gonad growth were affected, but individuals were submitted to a much lower experimental pH (6.98) (Siikavuopio et al. 2007), indicating a possible metabolic disruption when individuals are exposed to more extreme hypercapnia states.

The carbonic anhydrase (CA) activity measured in *A. dufresnei* body wall did not present any difference between treatments, although the coelomic fluid pH decreased in low pH treatments. The wide distribution of this enzyme in echinoderm tissues suggests that it has a role in calcification but it also facilitates CO_2 transport and participates in respiration processes (Heatfield 1970, Chen and Lawrence 1986, Chen and Lawrence 1987, Mitsunaga et al. 1986, Donachy et al. 1990, Livingston et al. 2006). However, CA activity did not present any differences in individuals submitted to low pH treatments, indicating no sign of metabolic depletion or increased respiratory processes.

Neither the spine regeneration rate, given by regenerate height nor their magnesium content differed significantly between treatments for both species. Also, regenerate weight (a measure of skeleton structure density) was not correlated with aquaria seawater pH, but with

regenerate height and spine diameter, as previous seen for sea urchins spine regeneration (Ebert 1988). We can therefore conclude that calcification in spine regeneration was not affected by pH treatment and it occurred at magnesium calcite saturation states lower than 1 (Table 2). Actually, calcification in echinoids occurs in a confined compartment supersaturated towards calcium carbonate (Smith 1990, Dubois and Chen 1989, Dubois and Ameye 2001). The confinement of this compartment allows a tight control if its ionic composition and pH, which can strongly differ from seawater conditions. Besides, the carbonate (CO_3^{2-}) ion, essential for calcification and considered to be problematic due to its availability decrease caused by ocean acidification, hardly ever crosses biological membranes, entering cells via CO_2 diffusion or by bicarbonate (HCO_3^-) transport (Hofmann and Todgham 2010). If existing, the influence of seawater pH and physicochemistry in calcification will rather be an indirect one most likely owing to low acid-base regulation and/or metabolic depression (Pörtner 2008, Hofmann and Todgham 2010). Moreover, the skeleton of echinoderms is internal, being embedded in the dermal connective tissue which is itself separated from the seawater and coelomic fluid by, respectively, the epidermis and the mesothelium. Indeed, the SEM pictures revealed no signs of regenerate corrosion in lower pH treatments, thus lower saturation state of magnesium calcite.

Additionally, the nutrition status of sea urchins has an influence on calcification rates of the different skeletal components and resource allocation differs between starved and fed individuals (Lewis et al. 1990). In our study, sea urchins were well fed and their feeding rate was not affected by pH treatment, which most likely had a positive impact on their health and allowed calcification rates to be maintained. Spines are vital structures related to defence and food gathering and their rapid healing and repair is an adaptive feature (Ebert 1988, Edwards and Ebert 1991). It is therefore expected that resources are intensively allocated to regenerating spines and even in starved individuals, spine regeneration promptly takes place (Edwards and Ebert 1991). Moreover, it is known that spine damage can promote higher calcification rates in both spines and other skeletal structures, such as the test plates (Edwards and Ebert 1991), a feature also seen in other organisms where shell deposition was higher during shell repair than during normal growth (Palmer 1983). Thus, sea urchins seem to present a high degree of phenotypic plasticity that allows them to deal with environmental stresses, including pH reductions within ocean acidification predictions.

Hypometabolic organisms like echinoderms are expected to be less able to cope with ocean acidification effects, precisely because they lack ion-regulatory machinery that could

protect body fluids against hypercapnia (Pörtner 2008, Melzner et al. 2009). Conversely, many echinoderms are present and seem to be acclimatized and/or adapted to extreme and fluctuating pH environments such as estuaries, intertidal, upwelling and cold water areas, showing some capacity to tolerate environmental pH stresses. Ocean acidification effects on larval echinoderms seem to be highly species specific and tolerance to pH can further depend on their life history (Clark et al. 2009, Byrne 2010, Dupont et al. 2010b, Moulin et al. 2011) as species are associated with different strategies to face environmental stressors and disturbances. The studied temperate *P. lividus* specimens had an intertidal origin and can undergo a 0.4 pH unit change within a 3-4 h period. These fluctuations can be expected to be higher during summer time, when algal density and greater air temperatures can further influence the pool pH (Denny and Gaines 2007). The Subantarctic *A. dufresnei* studied population subsists in a low pH environment. The Magellan Strait surface pH can be quite low during spring (7.5/7.8) due high freshwater inputs from the surrounding fjords (Valdenegro and Silva 2003). Besides, cold water environments, due to thermodynamic constrains, present a higher CO_2 dissolution and thus a lower surface pH (Orr et al. 2005). Both species seem to present some degree of coelomic fluid buffer capacity which possibly contributes to physiological maintenance and therefore low seawater pH did not promote metabolic depression during this study. Additionally, calcification rate of regenerate spines was not affected. We propose that the capacity to resist low pH within the range predicted for 2100-2300 could be linked to the origin of the studied individuals. Likewise, the blue mussel seems to perform differently towards ocean acidification depending on their origin and calcification rates of Kield fjord individuals were not strongly depressed by hypercapnia exposure as previously reported to North Sea specimens (Thomsen et al. 2010). Thus, there seems to be scope for possible acclimatization of natural populations to low pH effects. However, further environmental stressors, such as global warming and consequent climate change, can narrow individual and population tolerance windows. Therefore, studies encompassing several environmental potential stresses (acidification, temperature, salinity, hydrodynamism among others) are needed.

Acknowledgements: A. I. Catarino holds a FCT grant (SFRH/BD/27947/2006, Portugal). Ph. Dubois is a Senior Research Associate of the NFSR (Belgium). Work supported by FRFC contract 2.4532.07 and Belspo contract SD/BA/02B. The authors are grateful to Prof. Fred T. Mackenzie for his help on the Mg-calcite saturation state calculation and to Dr. E. De Bruyn and his team for allowing us to employ their blood gas

analyser at the Eramus Hospital (ULB, Belgium). The authors would also like to thank the Laredo Center personnel for technical support, Dr. E. Newcombe and C. Cardenas (CEQUA) for diving support, Ph. Pernet, M. Bauwens (ULB), C. Gimpel, G. Asencio, T. Figueroa (INACH) and Dr. B. Hamer and A. Borcic (Ruđer Bošković Institute) for assistance. The authors are also grateful to Prof. Ph. Grosjean (UMons), Prof. A. Borges (ULG), Prof. N. Heisler (H-U Berlin), Dr. J. Hermans, C. Moureux and Dr. J. Harley (ULB) for scientific and technical advice. Statistical advice was kindly given by Prof. J. Rohlf (Stony Brook University), Prof. P. Doncaster (University of Southampton) and Prof. J. H. McDonald (University of Delaware).

Chapter IV

Acid-base balance and metabolic response of the sea urchin *Paracentrotus lividus* to different seawater pH and temperatures

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Acid-base balance and metabolic response of the sea urchin *Paracentrotus lividus* to different seawater pH and temperatures

Ana I. Catarino, Mathieu Bauwens, Philippe Dubois

Laboratoire de Biologie Marine, Université Libre de Bruxelles, Belgium

In preparation

Abstract: The uptake of anthropogenic CO_2 emissions by the ocean results in a decrease of its surface pH, a phenomenon known as ocean acidification. In the near future pH is expected to decrease 0.3-0.5 and 0.7-0.8 units by 2100 and 2300, respectively. We investigated the response to low pH of an intertidal sea urchin species, *Paracentrotus lividus*, submitted to control (8.0) and low pH (7.7 and 7.4) at two different temperatures (10º C and 16º C, 19 days). The relation between the coelomic fluid acid-base status, the RNA/DNA ratio of gonads and the individual oxygen uptake were studied. The coelomic fluid pH decreased with the aquarium seawater, independently of temperature, but this explained only 13% of the pH variation. The coelomic fluid showed though a partial buffer capacity that was not related to skeleton dissolution ($[Mg^{2+}]$ and $[Ca^{2+}]$ did not differ between pH treatments). There was an interaction between temperature and pH on the oxygen uptake (V_{O2}) which was increased at pH 7.7 and 7.4 at 10ºC in comparison with controls, but not at 16ºC, indicating an upregulation of the metabolism at low temperature and pH. However, gonad RNA/DNA ratios did not differ according to pH and temperature treatments, indicating that even if maintenance of physiological activities has an elevated metabolic cost when individuals are exposed to stress, they are not directly affected during short term exposure. However, long term studies are need in order to verify if gonad production/growth will be affected.

Keywords: Ocean acidification, temperature, *Paracentrotus lividus*, acid-base balance, oxygen uptake, RNA/DNA

1. Introduction

Since the industrial revolution ocean surface pH has been reduced by approximately 0.1 units due to seawater uptake of anthropogenic CO_2 emissions, a phenomenon known as ocean acidification. In the near future, pH is expected to decrease 0.3-0.5 units by 2100 and 0.7-0.8 by 2300 (Caldeira and Wickett 2003, 2005, IPCC 2007). The metabolism of marine organisms can be depressed when organisms are exposed to high CO_2 conditions (hypercapnia) (Pörtner 2008, Melzner et al. 2009) and processes such as reproduction, calcification and growth are vulnerable to acidification (Doney et al. 2009, Fabry et al. 2008, Pörtner 2008, Melzner et al. 2009, Hofmann et al. 2010, Hofmann and Todgham 2010). However, there seems to be an increasing body of evidence that even closely related species and/or different life stages respond differently to seawater pH levels within ocean acidification predictions (Melzner et al. 2009, Ries et al. 2009, Dupont et al. 2010b).

The maintenance of extracellular pH is considered to be crucial in protecting individuals against hypercapnia induced disturbances (Heisler 1989, Seibel and Walsh 2003, Pörtner 2008). One of the main acid-base regulation mechanisms is associated with active ion transport achieved across specialized epithelia such as gills, renal or digestive tissues, and therefore also with osmorregulation (Whiteley et al. 2001, Seibel and Walsh 2003, Pane and Barry 2007, Melzner et al. 2009). Consequently, hypometabolic and osmoconformer organisms should be less able to cope with ocean acidification effects, precisely because they lack ion-regulatory machinery that could protect physiological fluids against hypercapnia (Pane and Barry 2007, Pörtner 2008, Melzner et al. 2009). Low capacity of acid-base regulation is a possible explanation for the elevated sensitivity of some marine invertebrates to ocean acidification as studies have shown that a decrease of extracellular pH can be linked with metabolic depression (Reipschläger and Pörtner 1996, Michaelidis et al. 2005, Pörtner 2008). Metabolic suppression is usually completed by limiting the energy directed to costly cellular processes such as protein synthesis, which results in decreased growth and reproduction (Guppy and Withers 1999, Seibel and Walsh 2003).

Echinoderms are abundant marine benthic invertebrates, widely distributed in a variety of habitats and that play important key roles in their ecosystems. Adults have a poor ability to regulate ion concentration in their extracellular fluids (Stickle and Diehl 1987 and references therein) and they are considered to be hypometabolic (Melzner et al. 2009) as they have low respiratory rates due to their undeveloped muscular system and subsequent reduced

locomotory activity (Lawrence and Lane 1982, Shick 1983). Their oxygen uptake is mostly dependent on the nutritional state, size and ambient temperature, but also on oxygen tension, seasonality, salinity and pH (e.g. Hiestand 1940, Farmanfarmain 1966, McPherson 1968, Sabourin and Stickle 1981, Lawrence and Lane 1982, Brockington and Clarke 2001, Talbot and Lawrence 2002, Siikavuopio and Mortensen 2008, Wood et al. 2010, 2011). The ionic composition of the coelomic fluid is similar to that of seawater, but there are, however, some species whose physiological fluids can be hyperosmotic (Binyon 1966, Ferguson 1990) and there is evidence that limited ionic regulation is possible in some fluid compartments (Binyon 1966, Bishop et al. 1994, Vidolin et al. 2007). The coelomic fluid pH is usually 0.5-1.5 units lower than seawater most likely as a result of CO_2 retention (slow diffusion rate) and due to accumulation of acidic metabolites (Farmanfarmaian 1966, Shick 1983). However, it has been hypothesized that the coelomic fluid have a slightly higher buffer capacity than seawater (Binyon 1966, Shick 1983). Notwithstanding, coelomic fluid pH decreased with seawater pH, indicating either a very low or only partial compensation ability (Miles et al. 2007). These low ion regulation abilities suggests that adult echinoderms should be severely impacted by ocean acidification.

Actually, a variety of responses of adult echinoderms to environmental hypercapnia have been observed and they seem highly species specific. Calcification and/or regeneration of calcified structures was enhanced in a few species submitted to low pH (Wood et al. 2008, Ries et al. 2009), while in others it was depressed (Gooding et al. 2009, Ries et al. 2009, Wood et al. 2010, 2011). Similarly, effects of low pH on growth differed, both increased and decreased growth rates being reported (Grosjean et al. 1996, 1998, Shirayama and Thornton 2005, Gooding et al. 2009). Feeding rates were reported to be depressed (Siikavuopio et al. 2007). Metabolism and/or oxygen uptake, were enhanced at low pH, indicating a higher energetic cost of other function maintenance (Wood et al. 2010, 2011). Finally, temperature had a synergistic effect with pH on calcification, growth and oxygen uptake (Gooding et al. 2009, Wood et al. 2010, 2011).

In order to better understand if the metabolic responses of echinoderms were related to their acid-base status, we studied the response of an intertidal sea urchin species, *Paracentrotus lividus*, submitted to low pH at two different temperatures. The pH were chosen based on near future predictions for ocean acidification, i.e. a decrease of ca. 0.3 and 0.6 units, and the two temperatures (10ºC and 16ºC) were within the range experienced by this species in the field (Boudouresque and Verlaque 2001). The coelomic fluid of *P. lividus*

individuals was characterized and the individual oxygen uptake and the RNA/DNA ratio of gonads were studied as metabolism proxies.

2. Methods

2.1 Experimental setup and procedures

The *Paracentrotus lividus* individuals were collected by the end of February 2010, during low tide from a temperate European rocky coast in Telgruc-Sur-Mer, Crozon, France. Adults (mean diameter of 36 ± 3.2 mm) were then transported to the laboratory and maintained in aerated seawater until further use (Marine Laboratory of the Brussels University, ULB, Belgium). Experiments took place between March and April 2010. Ten sea urchins per aquaria were submitted to 3 different pH (8.0 - control, 7.7 and 7.4) and to 2 different temperatures (10º C and 16º C) for 19 days. There were always 2 replicates (aquaria) per condition (fully crossed design). Low seawater pH were obtained by bubbling CO_2 supplied by Airliquide (France) through electrovalves regulated by a pH controller (Aquastar, IKS ComputerSysteme GmbH, Karlsbad, Germany). All aquaria were supplied with ambient air bubbles and they were kept inside a temperature controlled room. Aquaria had a 60 L capacity, their water was filtered using semi-dried water pumps (EHEIM, Germany) and seawater renewal was of 50 % per week. Individuals were fed artificial sea urchin food (Zeigler^{TM}, USA) *ad libitum*.

2.2 Seawater physicochemical parameters

Salinity was measured using a conductivity meter pH/Cond 340i WTW (USA). The temperature, pH_{NIST} (National Institute of Standards and Technology, previously known as National Bureau of Standards, NBS) and the electromotive force (e.m.f) were measured using a 827 pH Lab Metrohm meter (Switzerland) with a combined glass electrode (Metrohm 6.0228.010 with temperature sensor) calibrated with pH_{NIST} buffers 4 and 7 (Merck CertiPUR®, Germany). The e.m.f values were applied on the calculation of the pH expressed in total scale (pH_T) using standard buffers of known pH, 2-aminopyridine/HCL (AMP) and tris/HCL (TRIS), (DOE 1994, Del Valls and Dickson 1998, Dickson et al. 2007). All reported pH are expressed in seawater scale. Sea water samples were collected and

immediately filtered (0.22 µm, Millipore, USA) in order to determine total alkalinity (TA) and magnesium to calcium ratio. The TA was measured by means of a potentiometric titration with HCl 0.1 M using a Titrino 718 STAT Metrohm, and calculated using the Gran function (Gran 1952). Our measurements had a deviation of 0.09 % of the standard certified material provided by Andrew G. Dickson's Oceanic Carbon Dioxide Quality Control Laboratory (USA). For Mg^{2+} and Ca^{2+} concentrations, used to calculate the magnesium to calcium ratio (Mg/Ca expressed in mol/mol), seawater samples were diluted 20 times in MilliQ water (Millipore) acidified (10%) with HNO_3 65% (Suprapur® Merck, Germany) prior to analyses and were further analysed with an Iris Advantage (Thermo Jarrell Ash, USA) Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). The calibration was achieved using artificial multi-elemental solutions made from certified mono-elemental solutions (Merck, Germany) and using seawater certified reference materials (CRM) for quality check (High Purity Standards, USA). Results for the CRM were always within ± 10% of the certified values. Dissolved inorganic carbon (DIC), carbon dioxide partial pressure (*p*CO2), aragonite and calcite saturation state values ($\Omega_{Calcite}$ and $\Omega_{Aragonite}$) were determined from TA, pH_T, temperature and salinity data using the software CO2SYS (Pierrot et al. 2006) and by using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and the K_{SO4} dissociation constant given by Dickson (1990).

2.3 Coelomic fluid

The coelomic fluid (CF) of each individual was collected at the end of the experimental period (16 days), through the peristomial membrane using an insulin syringe (Myjector 0.5 ml, Terumo, Japan). Its e.m.f values were immediately measured using a microelectrode (Metrohm 6.0228.100) whose calibration and conversion to pH in total scale were done as previously described. In addition, CF samples were acidified with HNO_3 65 % (Suprapur® Merck), stored at 4º C until ICP-AES analysis and the ion concentrations were measured. Finally, the ΔpH, i.e. the difference between the CF pH_T and the seawater pH_T, was calculated using the mean seawater pH_T.

2.4 RNA to DNA ratio

At the end of the experiments, samples of gonads were collected, frozen in liquid nitrogen and stored at - 80º C until RNA/DNA determinations. Nucleic acid concentrations were determined using a one dye (ethidium bromide)/one-enzyme (RNase), 96-well microplate microplate fluorometric assay based on protocols described by Caldarone et al. (2001) and Belchier et al. (2004) and as reported by Catarino et al. (2008). Samples were analysed using two replicates to determine total nucleic acid concentration and two for DNA determination after RNA digestion by RNase (R-6513, Sigma-Aldrich) for 30 min at 37º C and 30 min to cool down until room temperature. Ethidium bromide was added to each well, and standard curves were established for each plate using known amounts of 18S- and 28S-rRNA from calf liver (R-0889, Sigma-Aldrich) and ultrapure, highly polymerized calf thymus DNA (D-4764, Sigma-Aldrich). Fluorescence was read using a Spectrofluor Plus microplate reader from Tecan© (Switzerland). Excitation and emission wavelengths were 365 and 590 nm, respectively. The RNA fluorescence was calculated by subtracting the DNA fluorescence reading from the total nucleic acids value. Sample nucleic acid concentrations were estimated by comparing fluorescence readings with those obtained from standard curves. Residual fluorescence (evaluated before the study by using DNase (D-4263, Sigma-Aldrich) was considered to be negligible. The RNA/DNA ratios were determined for each sample and expressed as µg RNA/mg dry weight sample divided by µg DNA/mg dry weight sample.

2.5 Oxygen uptake

The oxygen uptake was determined for each individual at the 16th day of the experiment in a sealed respirometer cylindrical chamber made of transparent plexiglas, using seawater from the individual aquarium under controlled stirring. Optode oxygen sensors (PreSens, Germany) were attached inside the chamber and oxygen concentration was measured using a luminescence signal directed and read by means of fiber-optics (Fibox 3, PreSens, calibration and salinity corrections were done following manual instructions). Signal is not dependent on flow rate and does not consume oxygen, in opposition to the traditional oxygen electrodes. Measurements were done in temperature controlled rooms. Oxygen saturation was measured every 10 minutes for one hour, and never fell under 80 % saturation. Oxygen uptake rate

(V_{O_2}) was calculated by computing the slope of the linear regression ($R^2 > 0.9$) of seawater oxygen content against time. This value was multiplied by water volume and subtracted with the average value for blank incubations.

2.6 Statistical analysis

Coelomic fluid pH (pH_{CF}) was studied using regression analysis, first computed at each temperature. The slopes obtained at each temperature were compared using a t-test (Zar 1999). Taking into account that slopes did not differ between temperatures a new regression analysis was performed for pH_{CF}.

The magnesium and calcium concentrations as well as the Mg/Ca ratio in each aquarium were compared using an ANOVA: aquarium (random factor) and temperature (fixed factor). In the coelomic fluid these parameters were compared using a model III nested ANOVA model (Doncaster and Davey 2007). Factors were pH (fixed), temperature (fixed) and aquarium (random, nested in both pH and temperature).

The RNA/DNA data were ln transformed and analyzed using model III nested ANOVA. Factors were pH (fixed), temperature (fixed), aquarium (random, nested in both pH and temperature) and sex (fixed). The oxygen uptake, by sea urchins was tested using the same ANOVA model and factors. As no difference were found between sex ($p_{ANOVA} = 0.17$), this factor was renewed and a new ANOVA was computed without this factor.

The level of significance was set at 0.05 for all tests.

The relationship between biological responses were studied for both sexes at the two different temperatures using the matrix of Pearson's correlation coefficient.

3. Results

3.1 Seawater

Seawater parameters during the experiment are presented on Table 1.

The magnesium and calcium concentrations in seawater did not differ between aquaria of both temperatures ($p_{ANOVA} > 0.1$) and were globally of 58.4 ± 3.06 and 13.1 ± 0.80 mM Kg^{-1} (mean ± standard deviation, n = 24), respectively. The Mg/Ca ratio also did not differ between aquaria ($p_{ANOVA} = 0.1$) and was 4.47 ± 0.085 (mean ± standard deviation, n = 24).

Table 1. Seawater parameters during the experiment (mean ± SD, n). pH in seawater (SW) in total scale.

10ºC - Nominal pH	7.4		7.7		8.0	
pH_{SW}	7.40 ± 0.022 (19)	7.40 ± 0.042 (19)	7.67 ± 0.042 (19)	7.69 ± 0.016 (19)	8.00 ± 0.059 (17)	8.01 ± 0.041 (17)
Temperature (ºC)	10.4 ± 0.47 (17)	10.3 ± 0.34 (17)	10.7 ± 0.29 (17)	10.5 ± 0.31 (17)	10.4 ± 0.53 (17)	10.4 ± 0.53 (17)
Salinity	32.3 ± 0.19 (17)	32.2 ± 0.09 (17)	32.2 ± 0.14 (17)	32. ± 0.14 (17)	32.2 ± 0.18 (17)	32.1 ± 0.10 (17)
TA (mmol kg^{-1})	2.68 ± 0.096 (7)	2.69 ± 0.082 (7)	2.66 ± 0.055 (7)	2.68 ± 0.065 (7)	2.66 ± 0.062 (7)	2.68 ± 0.048 (7)
DIC (mmol kg^{-1})	2.73	2.74	2.61	2.63	2.30	2.51
pCO_2 (µatm)	2328	2376	1213	1172	529	525
$\Omega_{Calcite}$	0.93	0.91	1.67	1.73	3.31	3.36
$\Omega_{Aragonite}$	0.59	0.58	1.06	1.10	2.10	2.12
16ºC - Nominal pH	7.4		7.7		8.0	
pH_{SW}	7.40 ± 0.025 (18)	7.39 ± 0.020 (18)	7.67 ± 0.027 (18)	7.70 ± 0.027 (18)	7.92 ± 0.063 (16)	7.90 ± 0.069 (16)
Temperature (ºC)	16.0 ± 0.28 (16)	15.8 ± 0.25 (16)	16.1 ± 0.24 (16)	16.0 ± 0.27 (16)	16.0 ± 0.42 (16)	15.9 ± 0.40 (16)
Salinity	32.8 ± 0.21 (16)	32.5 ± 0.18 (16)	32.6 ± 0.19 (16)	32.6 ± 0.24 (16)	32.6 ± 0.20 (16)	32.5 ± 0.19 (16)
TA (µmol kg^{-1})	2.56 ± 0.146 (6)	2.54 ± 0.129 (6)	2.45 ± 0.126 (5)	2.34 ± 0.129 (6)	2.39 ± 0.121 (7)	2.40 ± 0.244 (7)
DIC (µmol kg^{-1})	2585	2566	2383	2259	2224	2243
pCO_2 (µatm)	2330	2364	1135	1012	583	618
$\Omega_{Calcite}$	1.09	1.04	1.91	1.91	3.10	2.97
$\Omega_{Aragonite}$	0.70	0.67	1.22	1.22	1.98	1.90

3.2 Coelomic fluid

For each temperature the coelomic fluid pH (pH_{CF}) decreased with the aquarium seawater pH (pH_{SW}), but showed a large range of variation: $p_{Regression\ 10ºC} = 1.85x10^{-3}$, $R^2 = 0.21$ and $p_{Regression\ 16ºC} = 0.038$, $R^2 = 0.082$. As both slopes did not differ (t-value = 1.47 < $t_{0.05(2),89} = 1.987$) a single analysis was done using the data from both temperatures: $p_{Regression} = 2.5x10^{-4}$ and $R^2 = 0.13$ (Fig. 1).

The Mg/Ca ratio was higher in the coelomic fluid of sea urchins at 16ºC (4.6 ± 0.18, n = 59) treatments than at 10ºC (4.4 ± 0.13, n = 56) ($p_{ANOVA} = 2.1x10^{-5}$), but did not differ between pH treatments ($p_{ANOVA} = 0.24$). The magnesium and calcium concentrations followed the same trend and were also higher at 16ºC than at 10ºC ($p_{ANOVA} < 8.0x10^{-4}$), but did not differ between pH treatments ($p_{ANOVA} > 0.19$). The [Mg^{2+}] were 45.6 ± 2.86 mM Kg^{-1} (mean ± standard deviation, n = 59) at 16ºC and 40.2 ± 4.85 mM Kg^{-1} (mean ± standard deviation, n = 56) at 10ºC, while for [Ca^{2+}] they were 9.7 ± 0.64 mM Kg^{-1} (mean ± standard deviation, n = 59) and 9.2 ± 1.06 mM Kg^{-1} (mean ± standard deviation, n = 56), respectively.

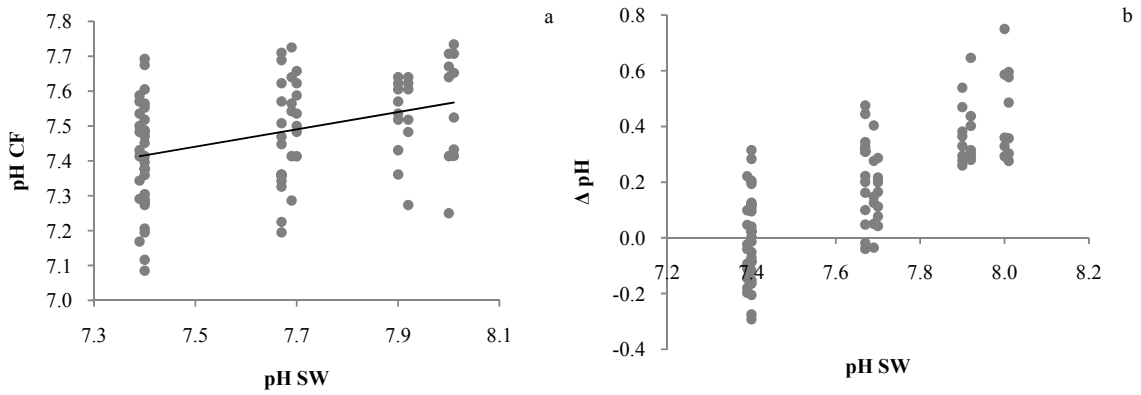

Fig. 1. a) Coelomic fluid pH (pH CF) according to the aquarium seawater pH (pH SW), y = 0.3 x + 5.6, $p_{Regression} = 2.5x10^{-4}$ and $R^2 = 0.13$. b) Delta pH (Δ pH) according to the pH SW.

3.3 RNA to DNA ratio

Gonads RNA/DNA ratio did not differ between pH treatments ($p_{ANOVA} = 0.72$) nor between temperatures ($p_{ANOVA} = 0.69$), but they differed between sex ($p_{ANOVA} = 4x10^{-6}$), females having higher ratios than males. The mean female gonad ratio was 2.98 ± 1.23 (mean ± standard deviation, n = 54) and the male one was 0.21 ± 0.17 (mean ± standard deviation, n = 52).

3.4 Oxygen uptake

The oxygen uptake (V_{O2}) of the sea urchins differed according to pH ($p_{ANOVA} = 2.9x10^{-2}$) and to temperature ($p_{ANOVA} = 4.0x10^{-3}$) as well as their interaction ($p_{ANOVA} = 3.1x10^{-2}$), but not according to sex ($p_{ANOVA} = 0.17$) (Fig. 2). The highest V_{O2} differing from control values was found in individuals submitted to pH 7.7 and 7.4 at 10ºC ($p_{Tukey} = 3.48x10^{-2}$ and $4.86x10^{-2}$, respectively) (Fig. 2). The V_{O2} values did not differ according to pH treatment at 16ºC and were similar to those of the control at 10ºC ($p_{ANOVA} > 6.7x10^{-2}$).

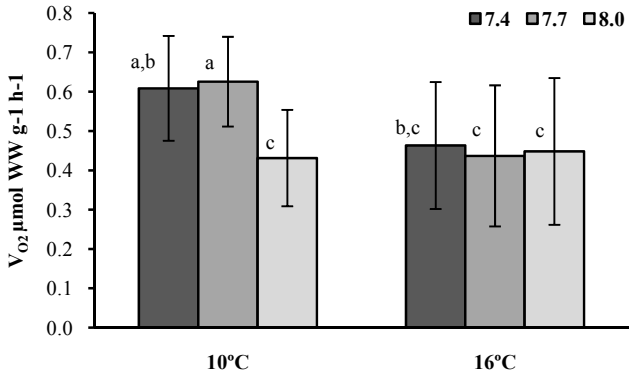

Fig. 2. Mean rates of oxygen uptake (± standard deviation) at the different pH and temperature treatments. Bars sharing the same superscript did not differ significantly ($\alpha = 0.05$).

3.5 Matrix of Pearson's correlation coefficient

The biological responses differed between males and females and also slightly with temperature (Table 2). The RNA/DNA (gonads) response of the females was the least related response with the other variables. The pH of the coelomic fluid (pH_{CF}) was in negatively related with oxygen uptake (V_{O2}) in females, especially at 16ºC. In males both oxygen uptake (V_{O2}) and RNA/DNA (in gonads) were related.

4. Discussion

The coelomic fluid pH (pH_{CF}) of the sea urchin *Paracentrotus lividus* decreased with the aquarium seawater pH (pH_{SW}), independently of temperature, but this relation only explained 13 % of the variation, showing that other more important factors were influencing it. Echinoids are basically aerobic organisms, but they have simultaneous an aerobic and anaerobic metabolism under normoxic conditions, due to the low oxygen diffusion into internal tissues (Ellington 1982, Shick 1983, Bookbinder and Shick 1986). The accumulation of organic acidic metabolites, such as lactate, malate or others might have influenced the pH decrease (Bookbinder and Shick 1986). The delta pH (ΔpH) was lower at lower pH_{SW} and in the lowest tested pH (7.4) this relation was even negative in some cases, indicating that some individuals can maintain coelomic fluid pH higher than the seawater one. The coelomic fluid pH seems to be compensated in cases of seawater moderate hypercapnia. Its buffer capacity was shown to be higher than that of seawater through titration methods on the sea urchin *P. lividus* (Chapter 3) and on other sea urchin species (Gellhorn 1927, Sarch 1932). The

coelomic fluid of echinoderms is primarily buffered by the carbon dioxide-bicarbonate system (Farmanfarmain 1966, Shick 1983, Miles et al. 2007), but this could be further reinforced by its protein content (0.2-0.5 mg ml^{-1} according to Holland et al. 1967 and Burnett et al. 2002), as hypothesised by Shick (1983), or by other N-containing molecules (Gellhorn 1927). This was further reinforced by a study where the buffer capacity of the coelomic fluid was associated with increased protein concentrations due to ovary growth (Bookbinder and Shick 1986). Even if buffer capacity of such protein concentrations is considered to be low (Heisler 1986, Harrison et al. 1990), the presence of organic and inorganic phosphates, succinate, lactate, ammonia and other acid or bases produced metabolically and exchanged against strong ions cannot be discarded (Harrison et al. 1990, Truchot 1988, Ali and Nakamura 2000). Our results showed no difference in the magnesium or calcium concentrations of the coelomic fluid, and therefore of the Mg/Ca ratio, between pH treatments. Therefore, the nature of the buffer capacity of the coelomic fluid did not seem to be related with increased passive skeleton dissolution, a possible source of HCO_3^-, at lower pH treatments as previously proposed for sea urchins (Spicer 1995, Miles et al. 2007).

Table 2. Relationship between biological responses studied for both sexes at the two different tested temperatures given by the Pearson correlation matrix.

	Females			Males		
10ºC	pH_{CF}	V_{O2}	RNA/DNA	pH_{CF}	V_{O2}	RNA/DNA
pH_{CF}	1.00			1.00		
V_{O2}	-0.24	1.00		-0.09	1.00	
RNA/DNA	0.13	-0.10	1.00	-0.20	0.25	1.00
16ºC	pH_{CF}	V_{O2}	RNA/DNA	pH_{CF}	V_{O2}	RNA/DNA
pH_{CF}	1.00			1.00		
V_{O2}	-0.60	1.00		0.13	1.00	
RNA/DNA	0.14	-0.03	1.00	-0.06	0.24	1.00

The Mg/Ca ratio was bigger in the coelomic fluid of sea urchins at 16º C treatments than at 10º C as were the coelomic fluid concentrations of Ca^{2+} and Mg^{2+}. Additionally, ion concentrations were lower than those found in seawater, whose values did not differ between aquaria and temperature. Similarly, Vidolin et al. (2007) observed a lower coelomic fluid [Mg^{2+}] of two sea urchins, *Lytechinus variegatus* and *Arbaxia lixula*, when compared with ambient seawater. In fact, despite the fact that sea urchin fluids are globally isosmotic with seawater, the specific ion concentrations are known to vary not only within body compartments, but also with the external environment, allowing internal ion gradients are to be established (Binyon 1966, Diehl 1986, Stikle and Diehl 1987, Ferguson 1990, Bishop et

al. 1994). The fact that at different temperatures ion concentrations varied was most likely related with increased activity of membrane transporters, as it is known to change with temperature (Dowben 1971).

Ion regulation, even though limited, is species specific (Binyon 1966) and gains more importance in sea urchins inhabiting coastal or shallow water environments where salinity fluctuations occur (Vidolin et al. 2007). This ability provides individuals with the possibility to resist to osmotic stresses within a limited range (Himmelman et al. 1984, Vidolin et al. 2007). In intertidal environments, pH changes are often related with salinity ones (Truchot and Duhamel-Jouve 1980, Morris and Taylor 1983). The ability to buffer such fluctuations, even if partially, can be an adaptive feature that allows organisms to cope with environmental stresses. For instance, this ability seems to be highly enhanced in crustaceans living in more variable habitats (coastal and/intertidal) and that possess highly improved osmorregulation skills than those which live in more stable environments (Whiteley et al. 2001, Dissanayake et al. 2010). However, little is known about such abilities in echinoderms. Intertidal *P. lividus* specimens, for instance, while exposed to low tidal cycles, might not experience coelomic fluid pH changes, even when seawater pH changes as much as 0.4 units (Chapter 3). Echinoderms own features that enable them to deal with such situations. For instance, the gastrointestinal epithelium possesses antiporters able to promote proton, ion and dissolved organic material exchanges with the external milieu (Bamford 1982, Ahearn and Franco 1991, Zhuang et al. 1995). Also, the high density of mitochondria in the intestinal rectum of sea urchins is an indication of its important role in transepithelial transport and was linked with ionic regulation (Santos-Gouvea and Freire 2007).

There was an interaction between temperature and pH treatments on the oxygen uptake (V_{O2}) of *P. lividus*. At lower pH (7.4 and 7.7) and at 10º C the oxygen consumption was higher than in the other treatments (Fig. 2). In echinoderms, the V_{O2} is known to change with temperature (Lawrence and Lane 1982, Shick 1983) and pH (Hiestand 1940, Wood et al. 2010, 2011). Interestingly, in our results, the oxygen uptake did not differ between control values from the two tested temperatures. Actually, 10º C and 16º C are temperatures experienced in the field during spring time, by the sea urchins *P. lividus* (Boudouresque and Verlaque 2001). Furthermore, in the present study sea urchins were most likely acclimated to experimental temperatures. Ulbricht and Pritchard (1972) showed that in other echinoid species, when acclimated in the laboratory, temperature changes within their tolerance windows did not always led to a higher oxygen consumption. These authors also discussed

the fact that metabolic activity in an intertidal species, *Strongylocentrotus droebachiensis*, could be less temperature dependent than in other non-intertidal ones. Physiological state, namely the feeding activity of sea urchins, can also have an impact on temperature acclimation and consequent variations of metabolic rates (given by oxygen consumption) (McPherson 1968, Lawrence and Lane 1982). In the present study, individuals were fed *ad libitum* an artificial formula especially made for rearing sea urchins in aquaculture (Zeigler^{TM}), which was therefore highly nutritive. Even if nutrition state seemed optimal, at the lowest temperature and pH the metabolism was upregulated, indicating a response to an increased energetic demand. Likewise, ophiuroids showed an increase of oxygen uptake when individuals were submitted to lower pH treatments within values predicted for ocean acidification in the near future (Wood et al. 2010, 2011). This energy was most likely used in the maintenance of a normal physiological steady-state. Therefore, on a first stage, this energetic demand did not affect directly other functions such as gonadal growth. Similarly, *P. lividus* did not show any difference on RNA/DNA ratio between treatments and temperatures. Under normoxic environmental conditions, the gonads of echinoids have a large anaerobic metabolic component (Ellington 1982, Bookbinder and Shick 1986) and so gonad metabolism might not be affected by hypercapnia exposure.

Lawrence and Lane (1982) reported that ovaries have a higher energetic demand than testis which can represent a higher nutrient drain for the individuals, a fact seen in our results with RNA/DNA being higher in females. The RNA/DNA ratio is a reliable indicator of gonadal production (Liyana-Pathirana et al. 2002) and it is known that gonads have both a reproductive and a nutrient storage function (Lawrence and Lane 1982, Hughes et al. 2006). Gonad production depends on food uptake, ingestion rate, reproductive cycle, season and temperature (Moore 1966, Lawrence and Lane 1982, Liyana-Pathirana et al. 2002, Hughes et al. 2006, Siikavuopio and Mortensen 2008). Some of these factors are deeply related and their relative contribution can be hard to distinguish. Furthermore, female gonads can even be slightly bigger in some echinoid species, but no differences in feeding rate between sexes have been reported (Lawrence and Lane 1982, Schäfer et al. 2011), implying a considerable complexity of metabolic pathways.

Even though sea urchin coelomic fluid owned a certain buffer capacity, the Pearson correlation coefficient showed a negative relation between the females pH_{CF} and the individual oxygen uptake (V_{O2}), a measure of energy production, not related directly with the RNA/DNA ratios. So, although ovary production, which can be supported by 76-92 % of

anaerobic metabolism (Bookbinder and Shick 1986), was not affected by hypercapnia exposure, the metabolic energetic pathways from which it can be dependent, such as nutrient allocation, might have been. In case these pathways depend more directly on extracellular pH, then the higher energetic demand of female individuals might have had an indirect impact on aerobic metabolism. This difference was not statistically significant in the V_{O2} analysis, most likely due to the short term exposure which might not have allowed enough time for the differences to be enhanced. On the longer term, gonadal production/growth can be depleted for some species. For instance, long term exposition to pH 7.8 led to a decrease in gonadal development and fecundity of the sea urchin *Hemicentrotus pulcherrimus* (Kurihara unpublished cited in Kurihara 2008 and reviewed by Dupont et al. 2010). Also, sea urchins submitted to severe hypercapnia, for a couple of months, had their gonadal growth affected due to impairment of feeding ability and nutrient conversion efficiency (Siikavuopio et al. 2007). If the energetic demand of the entire organism is kept at abnormal levels for a long period of time or if this is submitted to a more severe pH stress, then it is probable that the individual health will be ultimately impaired, especially if nutrient pathways are altered. Since gonad production varies seasonally, these expected differences might be attenuated during some periods of the year.

In conclusion, the fact that the *P. lividus* pH_{CF} is not mainly ruled by the seawater pH implies that other parameters are, such as metabolic end products released in the coelomic fluid. These, together with a small protein content, can enhance the buffer capacity of the coelomic fluid, which although existing is still considered to be low. However, neither pH_{CF} nor the ΔpH were dependent on temperature. Gonadal production was not affected by temperature, neither was V_{O2} at control treatments. These facts are most likely related with an acclimation ability of the sea urchin *P. lividus*. This sea urchin presents strategies that allow it to inhabit coastal areas where stress (a parameter that limits production) and disturbance (parameter that causes destruction of biomass) are frequent (Lawrence 1990). Its thermal tolerance window is broad and it can be exposed to winter temperatures as low as 4º C and summer ones as high as 28º C, which suggests a large phenotypic plasticity. The possibility to cope with intertidal seawater parameters fluctuations can be due to a buffer ability of the coelomic fluid, even if limited, as well as to the skill to selectively control ion concentrations and/or their uptake. Buffer capacity, osmoregulation and excretion are physiological activities that are intimately related and that could contribute to the maintenance of the metabolic activities of these sea urchins. On the other hand, the

metabolism of *P. lividus* was upregulated at lower pH and temperatures. However, only in females the V_{O2} seemed to relate with pH_{CF}. This indicates that a complex energetic pathway might be behind total individual production. Thus in order to better understand acclimation processes of intertidal species such as *P. lividus*, it will be necessary to submitted individuals to hypercapnia on a long term, simultaneously exploring their thermal tolerance window.

Acknowledgements: A. I. Catarino holds a FCT grant (SFRH/BD/27947/2006, Portugal). Ph. Dubois is a Senior Research Associate of the NFSR (Belgium). Work supported by FRFC contract 2.4532.07 and Belspo contract SD/BA/02B. The authors are grateful to A. Borges (ULG), for TRIS-AMP buffers supply, and to T. Dupont, S.M. Zoudi, Ph. Pernet and L. Geerts (ULB) for technical support. Further acknowledgements N. Dahkani (MRAC) for IPC-AES analysis, C.P. Doncaster (University of Southampton) for statistical advice and C. Moureaux, C. De Ridder, Prof. Jangoux (ULB) and Prof. Heisler (H-U Berlin) for theoretical advice.

Chapter V

Antarctic cidaroid spines and ocean acidification:
lessons from the deep

Chapter V

Antarctic cidaroid spines and ocean acidification: lessons from the deep

Ana I. Catarino[1], Virginie Guibourt[1], Claire Moureaux[1], Chantal De Ridder[1], Philippe Compère[2], Philippe Dubois[1]

[1]Laboratoire de Biologie Marine, Université Libre de Bruxelles, Belgium

[2]Département des sciences et gestion de l'environnement, Université de Liège, Belgium

In preparation

Abstract: Ocean acidification is caused by an increase in seawater pCO_2 which is leading to changes in the oceanic carbonate system and pH. As a result, calcium carbonate saturation horizon is shallowing, especially at high latitudes. Sea urchins can be particularly vulnerable due to the nature of their high-magnesium calcite skeleton, since the solubility of Mg-calcites is either similar or greater than that of aragonite. Cidaroid urchins have particular spines covered by a polycrystalline cortex which becomes exposed to seawater when mature as they are no longer covered by an epidermis like the spines of other sea urchins. Despite these characteristics, numerous species live below aragonite saturation horizon, especially at high latitudes. We investigated the morphology and the magnesium content of *Ctenocidaris speciosa* spines collected at different depths from the Weddell Sea. The cortex of *C. speciosa* presents a thicker inner cortex layer and a lower Mg^{2+} content below the aragonite saturation horizon and at lower calcite saturation states. We suggest that the naked cortex (not covered by the epidermis) of cidaroid species seems able to resist to low calcium carbonate saturation state, most likely thanks to the important organic matrix that surrounds the crystallites of the cortex.

Keywords: Ocean Acidification, cidaroid spines, cortex, stereom, magnesium, calcium carbonate saturation state

1. Introduction

Anthropogenic carbon dioxide (CO_2) emissions to the atmosphere are being uptaken by the oceans at a rate unprecedented in the last 650,000 years (Siegenthaler et al. 2005, Lüthi et al. 2008). This phenomenon has led to what is referred to as ocean acidification, i.e. a modification in the seawater carbonate equilibrium resulting in reduced pH and carbonate ion concentration, which in turn decreases the saturation state of calcium carbonate minerals (Kleypas et al. 1999, Feeley et al. 2004, Orr et al. 2005, Morse et al. 2006). Since preindustrial times, the average surface seawater pH has already been reduced by approximately 0.1 units and a further decrease of 0.3-0.4 pH units is expected by the end of the 21st century if atmospheric CO_2 emissions reach concentrations around 800 ppm (Caldeira and Wickett, 2003, 2005, IPCC 2007). Subsequently, more soluble mineral forms than calcite, such as aragonite and magnesium calcite (Mg-calcite) whose solubility increases with Mg^{2+} content (Morse and Mackenzie 1990, Morse et al. 2006, Andersson et al. 2008, Zeebe and Wolf-Gladrow 2001), may become undersaturated in the surface ocean within the 21st century, especially at the poles (Feely et al. 2004, Caldeira and Wickett 2005, Orr et al. 2005, Andersson et al. 2008).

Several studies revealed that ocean acidification could be a threat to marine organisms by impairing a number of physiological functions, in particular to calcifying metazoa (Fabry et al. 2008, Pörtner 2008). In most cases, calcification rates of shell-forming organisms have been shown to decreased with lower pH and calcium carbonate saturation state (Smith and Buddemeier 1992, Kleypas et al. 1999, Gazeau et al. 2007, De Bodt et al. 2008, Marubini et al. 2008). However, in other cases, calcification rates either appeared unaffected by lower pH waters, like with cephalopods (Gutowska et al. 2008) or even increased as reported for ophiuroids (Wood et al. 2008) and cirripedes (McDonald et al. 2009).

Sea urchins and other echinoderms have a well-developed high-magnesium calcite endoskeleton whose precursor is a transient amorphous calcium carbonate (ACC) phase (Politi et al. 2004), a $CaCO_3$ form 30 times more soluble than calcite (Brečević and Nielsen 1989, Politi et al. 2004). In fact, they are considered to be particularly vulnerable to ocean acidification effects (Kurihara 2008, Dupont et al. 2010b, Sewell and Hofmann 2010). Interestingly, many sea urchin species reach depths under the saturation horizon of aragonite, and most likely of Mg-calcite (Feely et al. 2004, David et al. 2005, Orr et al. 2005), even

though their skeleton structures can be thinner and less calcified (Sewell and Hofmann 2010). The impact of lower seawater pH (corresponding to realistic near future predictions) in sea urchin larvae is known to promote a delay in their development and an increment of skeleton malformations (e.g.: Kurihara and Shirayama 2004, Clark et al. 2009, Dupont et al. 2010b, Moulin et al. 2011). In what concerns adults, aquaculture studies suggest that exposure to low pH can impair *Paracentrotus lividus* growth (Grosjean et al. 1996, 1998). In their experimental study, Shirayama and Thornton (2005) showed that the growth and survival of sea urchins *Hemicentrotus pulcherrinus* and *Echinometra mathaei* were affected when they were raised for five months in high CO_2 (560 ppm). In contrast, it was reported that survival of adult *H. pulcherrinus* was not affected after an eight month exposure to a higher CO_2 concentration (1000 ppm) (Kurihara unpublished cited in Kurihara 2008 and reviewed by Dupont et al. 2010b). Also, Hall-Spencer et al. (2008) showed in a field study that the abundance of *Arbacia lixula* and *Paracentrotus lividus* was significantly reduced in the proximity of CO_2 volcanic vents only at pH below 7.5-7.4. Furthermore, Ries et al. (2009) observed a positive and a negative net calcification rate to aragonite saturation state in the sea urchins *Arbacia punctulata* and *Eucidaris tribuloides*, respectively. Therefore, it is clear that diverse responses to ocean acidification might be expected within the Echinoidea class.

Sea urchin spines are essential structures that play an important role in locomotion, defense, food gathering and inter and intra-specific interactions. Furthermore, they possess the ability to regenerate once broken or removed. The cidaroid primary spines have particular differences when compared with those of other sea urchins ("euechinoids"). First, they are characterized by the presence of a monocrystalline stereom surrounded by a polycrystalline cortex (Märkel et al. 1971). At the same time, an epithelium covers the shaft only until the cortex has been deposited and when the spine becomes mature the epithelium disappears (Prouho 1885, Märkel and Röser 1983), leaving the cortex exposed to environmental physical and chemical conditions. Subsequently, the shaft becomes heavily colonized by epibionts making these sea urchins islands of biodiversity, especially in deep sea where muddy substrate predominates just as in the Southern Ocean (> 60º S) (Hétérier et al. 2008). As most models of ocean-carbon cycle predict that the shallowing of the $CaCO_3$ saturation horizons due to increased anthropogenic CO_2 emissions will be more important at higher latitudes (Feely et al. 2004, Orr et al. 2005, Anderson et al. 2008), the effects of acidification on the cidaroid spines will be relevant to the ecology of this region. Therefore, we investigated the possible morphological and chemical adaptations of cidaroid spines from

field specimens collected in deep-sea. In our study, the spine morphology and chemical composition of *Ctenocidaris speciosa* collected at different depths in the Weddell Sea (Antarctica) was studied in order to evaluate possible differences according to aragonite saturation states, using these values as proxies for Mg-calcite.

2. Methods

2.1 Field samples and data

Eleven primary aboral and ten oral spines from the species *Ctenocidaris speciosa* were studied. They were provided by the Paris Natural History Museum (France) and Laboratory of Marine Biology (Brussels University, Belgium) (Table 1). The specimens were dragged in the Weddell Sea (Antarctica) during EPOS 3 (in 1989) and ANT XV/3 (in 1998) campaigns (research ship R/V Polarsten, Alfred Wegener Institute for Polar and Marine Research, Germany). The sampling depths were 237 m, 602 m and 810 m for EPOS 3 and 1286-1681 m for ANT XV/3 (Table 1). All spines (from 1 individual from 237 m, 2 for 810 m and 3 for 602 m) from EPOS 3 were stored dried, while those (from 3 individuals) coming from the ANT XV/3 campaign were stored in ethanol.

Table 1. Origin of sampled specimens (Weddell sea, Antarctica) from the Paris Natural History Museum (MNHN), France, and the Brussels University (ULB), Belgium.

Expedition	Date of collection	Depth (m)	Latitude	Longitude	Museum reference	No. aboral spines	No. oral spines
EPOS 3	18/01/1989	237	60º37.6'S	46º58.1'W	MNHN EcEs 9310	3	2
EPOS 3	03/02/1989	602	74º39.9'S	29º31.3'W	MNHN EcEs 9308	2	3
EPOS 3	04/02/1989	810	75º32.4'S	29º53.0'W	MNHN EcEs 9235	3	2
ANT XV/3	04/02/1998	1286-1681	73º28.05'S-73º28.04'S	22º30.0'W-22º40.05'W	ULB	3	3

Total dissolved inorganic carbon (DIC) and total alkanity (TA) data from the sampling sites were obtained using the data base from the WOCE Southern Ocean Atlas (Orsi and Whitworth 2004). Therefore, it was possible to calculate the saturation state values (Ω) of calcite and aragonite using the software CO2SYS (Pierrot et al. 2006) and by using the dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987) and K_{SO4} by Dickson (1990). It is not possible to estimate the saturation state of Mg-calcite,

since there is no correction for the stoichiometric saturation (K) at pressures different from the atmospheric one, i.e. at different depths (Andersson et al. 2008).

2.2 Specimen analysis

Spines were sectioned at the shaft base and cleaned with a solution of 3.3% v/v NaClO aqueous solution (Loda, Belgium). They were mounted on aluminum stubs in positions parallel and perpendicular to the long axis of the spine and coated with gold (JFC-1100E ion sputter, JEOL, Japan). They were observed in a scanning electron microscope (SEM - JEOL JSM 6100, Japan) with secondary electron image acquisition by the software 3.0 SemAfore Jeol 1993-1997 (J. Rimppi Oy, Finland) (Moureaux et al. 2010). The thickness of the different cortical layers was measured on spine cross sections using the software Image J. 1.33u (Wayne Rasband, National Institutes of Health, USA).

In order to quantify the magnesium content on different spine layers, energy dispersive X-ray analysis (EDX) was carried out on cross sections. Segments of the shaft were cut and embedded in polyester resin (MI 42, Mida Composites, Belgium) with 2% hardener-MEC. Cross sections of around 6 mm were cut from each one with a low speed saw (Isomet, 11-1180, Buehler, USA). The sections surface was polished using wet sandpapers of decreasing granulometry (P80; P150; P400; P600) and finally mirror polished with a non-aqueous 1 μm diamond suspension (1PS-1MIC, ESCIL, France). The polished slices were carbon-coated (rotary evaporator Balzers MED-010, Liechtenstein). Dispersive X-ray analysis spectra were obtained in an environmental SEM (XL30 ESEM-FEG-FEI Philips, The Netherlands) operating at 10kV and at a working distance of 10 mm. The analyses were repeated three times for each region on each spine. Analyses performed on mineral zones of about 1-10 μm^2 in size with a normalized acquisition time of 100 s. The standartless quantitative analysis software calculated the elemental composition in weight percent and atomic percent using an automatic background subtraction function and a ZAF correction matrix. The composition was thereafter corrected by subtraction of the carbon-coating evaluated on the carbon-coated aluminium stub. The $MgCO_3$ molecular percentage was calculated from the elemental composition in atomic percent (Moureaux et al. 2010).

2.3 Data analysis

Data analysis were done using ANOVA tests, followed by the mean multiple comparison Tukey test whenever necessary, using SYSTAT 9 software (Systat Software Inc., USA). The significance level (α) was set at 0.05. Values expressed as proportions were arcsine transformed prior to the analysis in order to achieve data normality. Graphs were plotted using untransformed data. The following dependent variables were studied using a nested model III ANOVA (Zar 1996) with the fixed factor "depth" and the random factor "spine" nested in "depth": total cortex thickness, outer cortex thickness, inner cortex thickness, $MgCO_3$ proportion in the outer cortex, $MgCO_3$ proportion in the inner cortex, $MgCO_3$ proportion in the central stereom.

3. Results

Calculated carbonate parameters for the collection stations are presented on Table 2.

Table 2. Seawater physicochemical parameters calculated for the sampling stations. TA stands for total alkalinity, DIC for dissolved inorganic carbon and Ω for saturation state, pH_T in total scale.

Sampling depth (m)	237	602	810	1286-1681
Depth (m)	200	600	800	1400
Salinity	34.5	34.5	34.7	34.7
Temperature (ºC)	-1.0	0.0	0.5	0.2
TA (mmol kg^{-1})	2.34	2.35	2.35	2.35
DIC (mmol kg^{-1})	2.26	2.27	2.27	2.26
pH_T	8.01	7.98	7.97	7.97
***p*CO_2 (µatm)**	520.2	536.7	549.7	510.6
[CO_3^{2-}] (µmol kg^{-1})	72.9	73.4	73.1	75.3
$\Omega_{calcite}$	1.68	1.55	1.48	1.35
$\Omega_{aragonite}$	1.06	0.98	0.94	0.86

All the *Ctenocidaris speciosa* aboral spines presented a well developed cortex and an internal stereom core (Figure 1a). In samples collected at 237 m and 602 m the cortex was made of two layers, an outer (oc) and an inner (ic) one, while in samples from deeper waters the outer layer was missing (Figure 1).

The total thickness of the cortex of both types of spines did not differ according to depth ($p_{ANOVA} > 0.07$). The thickness of the outer cortex in aboral spines did not differ between 602

m and 237 m (p_{ANOVA} = 0.29), whereas the thickness of the inner cortex was significantly higher in deeper specimens (p_{ANOVA} = 0.012) (Figure 2). The magnesium concentration of the outer cortex did not decreased significantly with depth (p_{ANOVA} = 0.1), whilst the one from the inner one did (p_{ANOVA} = 10^{-6}). The concentration in the stereom did not differ significantly (p_{ANOVA} = 0.5) (Figure 3). Oral spines did not present systematically this cortex separation. Nevertheless the outer cortex was only visible above 602m in two spines. The thickness of the inner cortex in oral spines was significantly higher in deeper waters (p_{ANOVA} = 0.04) (Figure 2).

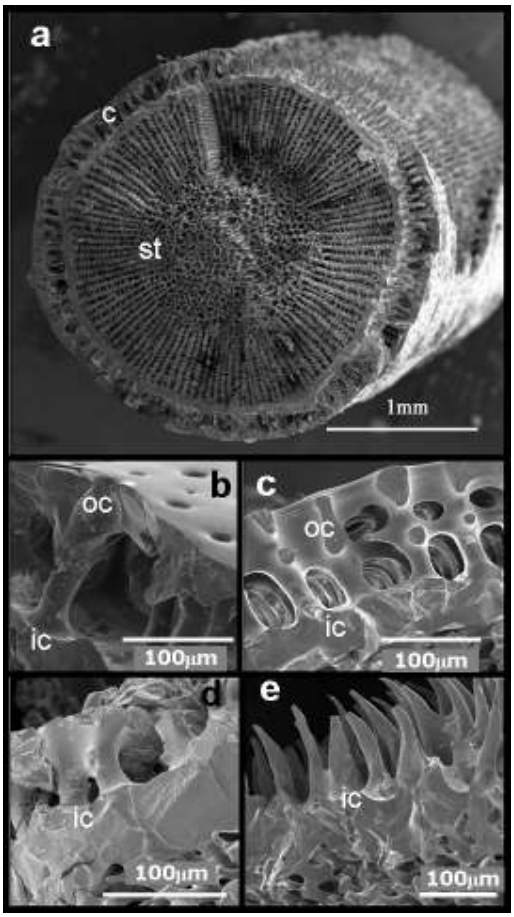

Fig. 1. SEM images of transverse sections of *Ctenocidaris speciosa* aboral spines: (a) entire cross-section of a spine from a specimen collected at 237 m (c- cortex, st-stereom); (b, c, d, e) details of the cortex in spines from specimens collected at 237 m, 602 m, 810 m and 1236-1681 m respectively (oc- outer cortex, ic- inner cortex).

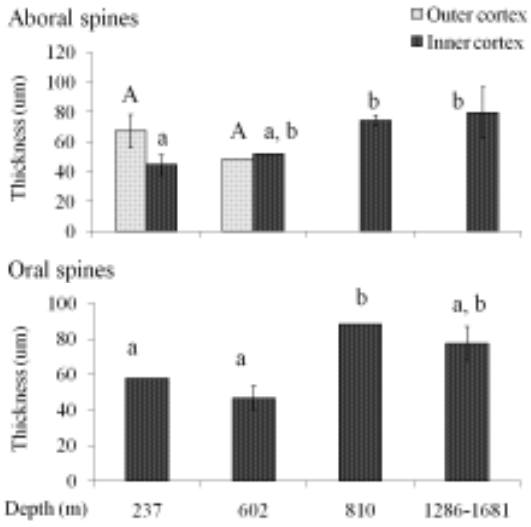

Fig. 2. Thickness (mean ± SD, n = 2 or 3) of the outer and inner cortex layers of *Ctenocidaris speciosa* aboral and oral spines according to depth. Bars sharing the same superscript did not differ significantly (α = 0.05).

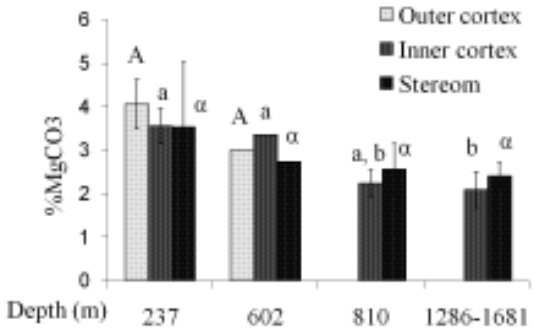

Fig. 3. $MgCO_3$ content in mole % (mean ± SD, n = 2 or 3) of *Ctenocidaris speciosa* aboral spines layers at different depths. Bars sharing the same superscript did not differ significantly (α = 0.05).

4. Discussion

The mature spines of *Ctenocidaris speciosa*, collected from the Weddell Sea (Antarctica), presented differences both in their morphology and chemical composition according to depth. We observed the occurrence of an outer cortex differing from an inner one in all aboral spines and in some oral spines from individuals collected at or above 600 m. This morphological feature had already been reported (Fell 1976 cited by David et al. 2009), but not yet related with depth distribution. The total thickness of the cortex did not differ with depth, most likely thanks to the inner layer thickness increase, compensating the lack of

the outer one after 600 m. We propose that these morphological differences can be related to the individual growth rate. Actually, it is known that deep sea urchins growth is slower than that of their congeners from the upper continental slope or from shallow waters (Gage et al. 1986). Thus, the presence of an outer cortex may be associated with the ability of a faster calcification rate. Slower growth rate in deeper specimens may be linked to both a lower magnesium calcite saturation state, which is depth associated, and/or to a reduced food availability, in turn also correlated with depth.

Lower [Mg^{2+}] were measured in the cortex of *C. speciosa* collected at higher depths. Similarly, Dissard et al. (2010) mentioned the possibility of a pressure effect responsible for Mg^{2+} content variations along depth in some foraminifera species. Also, Lowenstam (1972) reported an inverse correlation between depth and $MgCO_3$ concentration in the skeleton of the Pacific sea cucumber *Elpidia glacialis*, distinct from any temperature effect. Similarly, our observations cannot be attributed to usual environmental factors affecting skeletal magnesium content, i.e. Mg/Ca ratios. Indeed, the seawater Mg/Ca ratio is highly conservative all over the ocean and reported temperatures and salinity for the Weddell Sea at depths between 200-1500 m do not present changes that could explain the variation in spine cortical magnesium content, thus in Mg/Ca ratio (Marshall 1979, Antonov et al. 2006, Locarnini et al. 2006). Actually, both temperature and salinity slightly increase from 200 m to higher depths. However, it is known that the calcium carbonate saturation state decreases with depth (Zeebe and Wolf-Gladrow 2001). Precisely how the calcium carbonate saturation state (Ω_{CaCO3}) influences the skeletal Mg/Ca is currently unknown. Lower $\Omega_{Mg\text{-}calcite}$ could decrease calcification rates, which would give more time to exchange the calcium ions for the magnesium "impurity", as proposed by Weber (1973), decreasing magnesium content. However, recent experimental data showed that the growth rate by itself does not influence the skeletal Mg/Ca ratio in echinoderms (Borremans et al. 2009, Hermans et al. 2010). A higher saturation state is thought to favor the precipitation of the amorphous calcium carbonate (ACC), which in turn favors the incorporation of magnesium into calcium carbonate, including after crystallization into calcite (Raz et al. 2000, Loste et al. 2003). The ACC is known to be the initial mineral deposited during sea urchin skeleton formation (Politi et al. 2004). So, we suggest that the Ω_{CaCO3} may influence the Mg/Ca ratio, i.e. the magnesium content, of *C. speciosa* cortex by increasing ACC formation during initial steps of calcification.

It is noteworthy, however, that the skeletal magnesium content differed significantly according to depth only in *C. speciosa* spines cortex, a polycrystalline structure, and not in the central stereom, monocrystalline. Also, the cortex is richer in organic matrix (Märkel et al. 1971), in comparison with the very delicate organic matrix which pervades the stereom (accounting for less than 0.1% w/w of the skeleton) (Dubois 1991, Ameye et al. 2001). It has been recently shown that the sea urchin organic matrix composition affects the Mg/Ca ratio in calcium carbonate precipitation (Hermans et al. 2011). So, it can also be suggested that the nature of the organic matrix synthesized by the sea urchin may change according to depth, playing a role in their magnesium composition, although this idea need yet to be tested. Accordingly, Thalmann et al. (2001) reported for instance that the presence of the protein otoconin 90 in the organic matrix of the mammal inner ear calcite components, the otoconia, has a protection effect against a low pH environment, i.e., the endolymph. An additional factor that can protect the cortex is the biofilm that covers any exposed underwater surface including spines (David et al. 2009). Whatever the actual mechanism, the lower Mg^{2+} content in the cortex of deeper *C. speciosa* is an advantageous feature as it reduces the solubility, compensating the potential $\Omega_{Mg\text{-}calcite}$ decrease, of the cortex once the epithelium degenerates and the spine becomes directly exposed to ambient seawater.

Actually, from the 21 known cidaroid Antarctic species, 16 can currently be found at depths below 750 m (David et al. 2005), thus below the aragonite and presumably of the Mg-calcite saturation horizon (Orr et al. 2005), even if their skeleton structures are possibly less calcified (Sewell and Hofmann 2010). Whether this fact demonstrates a strong adaptation potential of cidaroid species to lower saturation state conditions by cidaroid species remains yet to be determined. For instance little is known about their early deep sea colonization. Also, it is unknown how geological events where ocean acidification was observed, such as the Paleocene-Eocene Thermal Maximum (PETM), affected deep sea cidaroid fauna. Current global changes are occurring at a faster rate than events such as PETM (Ridgwell and Schmidt 2010). In fact, the aragonite saturation horizon in the Southern Ocean could be found at around 730 m depth in 1994 and is predicted to reach surface waters by 2100 (Orr et al. 2005). Cidaroid sea urchins can have a long life expectancy and whether they will be or not be able to cope, i.e. to adjust thanks to their phenotypic plasticity, with the speed at which ocean acidification promotes biogeochemical changes in their environment is unknown. Nonetheless, we have seen for instance that the cortex provides stereom protection towards adverse conditions, minimizing the risk of spine exposure to more corrosive waters.

Acknowledgements : A. I. Catarino is holder of a FCT grant (SFRH/BD/27947/2000; Portugal). Ph. Dubois is a Senior Research Associate of the NFSR (Belgium). Work supported by FRFC contract 2.4532.07 and Belspo contract BIANZO II-2 SD/BA/02B. Special acknowledgements for specimens access to N. Ameziane, Muséum National d'Histoire Naturelle (Paris) and to B. David, K. Andreas, A. Smith and D. Néraudeau for enlighten on cidaroid phylogenetics.

General Discussion and Perspectives

GENERAL DISCUSSION

I - SEA URCHINS IN AN ACIDIFYING OCEAN: COPING WITH CHANGE?

1. EARLY STAGES

Stages in the life of an organism can present distinct vulnerabilities towards environmental stressors (Pörtner and Farrell 2008). In the present work, we observed that both Atlantic *Paracentrotus lividus* and *Arbacia dufresnei* larvae responded to ocean acidification more promptly than adults, with some effects appearing at pH 7.6-7.4. This is linked to body size and volume, to morphology and metabolism. Fertilization success and cleavage rate in *P. lividus* were more affected than larval development. This contrasts with the impact of low pH reported in other sea urchins species (Dupont et al. 2010b). Also, the percentage of *P. lividus* abnormal larvae was increased at pH 7.4 whereas this number in *A. dufresnei* experiments did not differ (at the tested range). *A. dufresnei*, however, already inhabits areas (Magellan Strait) where spring pH can be quite low (Valdenegro and Silva 2003) and this might explain its lower vulnerability in this aspect. Furthermore, Martin et al. (2011) reported a lower vulnerability towards ocean acidification in a population of *P. lividus* from the Mediterranean than that presented in the current study. The contrasting results clearly emphasize the disparity in species but also populations vulnerabilities to ocean acidification. Indeed, there are considerable genetic differences between populations from the Mediterranean and Atlantic basins due to the geographical barrier of the Strait of Gibraltar that restricts gene flow (Duran et al. 2004, Calderón et al. 2008). Interestingly, the Mediterranean populations presented a higher degree of genetic variability, a fact that can be linked to the higher degree of hypercapnia resistance. In fact, it has been demonstrated that closely related sea urchin species inhabiting contrasting environments, intertidal and deep sea, present genome divergences due to negative (purifying) and positive (directional) selection (Oliver et al. 2010). Indeed, these authors reported a clear evidence that genes expressed in adult ovaries and during larval development undergo significant negative selection showing the important role of developmental constrains in species divergences. Therefore, we propose, along with Clark et al. (2009) and Yu et al. (2011), that species from areas where sea urchins already experience low pH conditions could be better

acclimatized/adapted to such conditions and, therefore, be less vulnerable to ocean acidification effects.

The main and most consistent effect of ocean acidification on sea urchin larvae of several species seems to be a reduction of their size (Kurihara and Shirayama 2004, Kurihara 2008, Sheppard Brennand et al. 2010, Dupont et al. 2010b, O'Donnell et al. 2010, Yu et al. 2011, Chapter 1 and 2). The mechanism responsible for this size reduction has not been determined yet, but it could result from a combination of factors including developmental delay or reduced growth due to depressed metabolism. *A. dufresnei* smaller larvae (lowest pH 7.4) were isometric to those in control pH. Similarly, Martin et al. (2011) showed that larval isometry was kept in *P. lividus* larvae submitted to lower pH and that their calcification rates, that were apparently lower in lower pH, were in fact dependent on larval size. Also, only at very low pH (7.25-7.0) the metabolism of these larvae was affected. Delaying development or an altering developmental path are usual coping responses in front of environmental stresses (Hamdoun and Epel 2007). Exotrophic echinoplutei, for instance, are able to change body form and developmental trajectory whenever there is less food availabe (McEdward and Miner 2001), revealing a high phenotypic plasticity. So, the main answer of sea urchin larvae to ocean acidification appears to be a "regular" reaction to environmental stress, able to maintain the general trajectory of normal, even if delayed, development.

Population recruitment is highly dependent on larval supply which is in turn conditioned by factors such as hydrodynamics, temperature, salinity, predation and food availability (Balch and Scheibling 2001, McEdward and Miner 2001). A slower development will imply that larvae will be exposed during a larger period of time to adverse conditions, increasing the risk of predation or accumulation of stresses that can have a synergistic effect, such as temperature. In the closely related species co-occurring in the same geographic area, *Strongylocentrotus droebachiensis* and *Strongylocentrotus purpuratus*, recruitment success is higher in the former (Ebert 1983). This seems to be related to the shorter period of larval development of this species. However, sea urchin recruitment is a highly variable event, both temporally and spatially, and in some years a number of populations might not even recruit at all (Ebert 1983, Balch and Scheibling 2001). Larval supply is in fact largely determined by large scale water movement (e.g. currents, upwelling) and competent larvae can delay metamorphosis until a suitable substrate for settlement is found (Balch and Scheibling 2001). This means that a longer larval life is not directly disadvantageous.

Furthermore, larval experience can affect juvenile performance due to latent effects, i.e. events that are experienced in one stage but are only manifested later in the life cycle (Pechenik 2006, Allen and Marshal 2010, Giménez 2010). This was the case of the sea urchin, *Heliocidaris erythrogramma* (lecithotrophic larvae), where larval exposure to low pH (7.6 the lowest) resulted in increased abnormal forms in juveniles (Byrne et al. 2010a). Conversely, Dupont and Thorndyke (2008) reported that delayed larvae of *Strongylocentrotus droebachiensis* raised at pH 7.7 were not only able to reach metamorphosis, but were actually more successful than those raised at control pH (8.0), although the juveniles were smaller. Smaller juvenile size of sea urchin can, for instance, be preferred by predators (Scheibling and Robinson 2008) and a slower growth rate increases mortality due to prolonged predation exposure (Rowley 1990). Furthermore, it has been seen for other organisms that smaller size at metamorphosis can have repercussions later in life. For example, it was associated with slower growth rates of juvenile forms, with longer time to reach reproductive maturity, smaller body size at reproductive maturity and reduced fecundity (see review in Pechenik et al. 2010).

The majority of echinoids broadcast their gametes, but there are also some brooding species, mostly occurring in Antarctic and deep-sea regions (Mercier and Hamel 2009). Although no experimental studies have yet evaluated the vulnerability of brooding sea urchins, these species are considered to be at risk. Sewell and Hofmann (2011) hypothesized that the shoaling of the saturation horizon of aragonite and calcite can have negative effects on the distribution of the brooding Antarctic species. These assumption should be considered with care, as many of the analyzed species already maintain healthy populations at depths under the saturation horizons of calcium carbonate materials.

Even though larval stages respond more promptly than later stages to low pH effects, sea urchin endotrophic larvae do not seem to be as vulnerable as first expected and proposed by many authors. However, very few studies were dedicated to endotrophic larvae and metamorphosis. In the current state of knowledge, it is not probable that within ocean acidification predictions for the next 100 years a 0.3-0.4 pH reduction will decrease drastically larval survival directly by promoting an elevated number of abnormalities and therefore reducing larval supply and recruitment. The most critical effect is a slower development rate that can imply some survival reduction due to an increase of planktonik phase and consequent exposure to other adverse environmental factors. But taking into account that many species already present a high level of plasticity in larval development,

this fact might not be critical in determining recruitment success. Finally, species from varied origins also seem to cope differently with low pH effects which means that there is scope for acclimatization and possibly for adaptation.

2. ADULTS

Adult sea urchins studied in this work presented interesting responses to low pH/calcium carbonate saturation state seawaters. Coelomic fluid pH of *Paracentrotus lividus* and of *Arbacia dufresnei* decreased slightly with seawater pH. Until 7.4, the lowest experimentally tested pH in this work (Chapter 3 and 4), this decrease was partially buffered in the coelomic fluid which owns a buffer capacity higher than that of seawater (Chapter 3, Gellhorn 1927, Sarch 1932). This buffer capacity does not compensate totally for an extracellular pH decrease, and is in fact considered to be low (Heisler 1986), but it can be a first step allowing to maintain metabolic functions that depend on extracellular pH, and thus reduce the energetic demand of intracellular pH maintenance.

Sea urchins are able to produce magnesium calcite skeleton even in undersaturated waters, such as in the experimental study of *P. lividus* and *A. dufresnei* spine regeneration (Chapter 3 and 4). Other species, such as *Ctenocidaris speciosa*, are found at depths where seawater is permanently undersaturated (Chapter 5). It is not surprising that calcification does not seem to be directly impacted by ocean acidification since it occurs in a confined compartment. Also, the skeleton of echinoderms is internal, with the exception of fully grown cidaroid spines. When mature, they no longer have their stereom protected by an epidermis, but by a polycrystalline cortex layer which is directly exposed to seawater. Thus, the influence of seawater physicochemistry in calcification, when existing, will rather be an indirect one, most likely due to low acid-base regulation and/or metabolic depression (Pörtner 2008, Hofmann and Todgham 2010). It has been, however, reported a slower growth and/or calcification rate in some sea urchins species (Shirayama and Thornton 2005, Ries et al. 2009), including *P. lividus* reared in aquaculture systems (Grosjaen et al. 1996, 1998). In fact, the pH and elevated saturation states at the calcification site are kept thanks to meditated and active membrane transport of ions and proton elimination, i.e. Cl^--HCO_3^--ATPase, Na^+/K^+-ATPase pump and Na^+/H^+ transporters (Mitsunaga et al. 1987a, b). If the energy availability to maintain such processes is reduced due to metabolic impairment or if the energetic need to eliminate H^+ against a much stronger gradient become too demanding, it is

probable that calcification rates could decrease as well as growth. This situation will most likely only occur in extreme and/or chronic hypercapnia exposure since energetic resources are usually allocated to calcifying structures, such as in the case of spine regeneration (Edwards and Ebert 1991).

Several metabolic parameters were measured in *P. lividus* and in *A. dufresnei* in order to understand if low pH exposure would induce metabolic depletion. The RNA/DNA (protein production proxy) in body wall and gonads and the carbonic anhydrase activity in the body wall from individuals exposed to lower pH for 3-4 weeks did not differ significantly from high pH treatments. The RNA/DNA in body wall showed that somatic growth of both species was not affected. Accordingly, the fact that no differences in the activity of the carbonic anhydrase enzyme in the body wall were observed showed no sign of metabolic depletion or increased respiratory processes of *A. dufresnei*. The RNA/DNA index also did not show gonad production depletion. However, gonad production was reported to be affected by long term hypercapnia exposure (pH 7.8, 9 months *Hemicentrotus pulcherrimus* in Kurihara unpublished cited in Kurihara 2008 and reviewed by Dupont et al. 2010b) and severe medium term exposure (pH 6.98, 56 days, *Strongylocentrotus droebachiensis* in Siikavuopio et al. 2007). In the last case this was most likely due to diminished food consumption of sea urchins submitted to such conditions. In our work, *P. lividus* food intake was not affected by low pH exposure (7.4). Qualitative observations also did not reveal any alterations on the feeding pattern of *A. dufresnei* (personnel observation). Gonads are essentially anaerobic, having a high CO_2 content. Therefore, mild hypercapnia exposure within ocean acidification predictions, especially if extracellular fluids are able to partially compensate for a pH decrease and thus guarantee nutrient allocation, will not directly affect gonad production. Likewise, the field *P. lividus* individuals from tide pools where sea urchins experience different pH fluctuation regimes did not present any difference in gonad maturity stage (Chapter 1). However, it is possible that prolonged and/or extreme exposure to hypercapnia requires more energy for the maintenance of normal physiological functions and that this could have been the case of *H. pulcherrimus* and *S. droebachiensis* reduction in gonad production. The mentioned sea urchins are free-spawners, i.e. they rely on a r-strategy to broadcast their gametes and maintain their progeny. This means that even if there is a lower investment on gonad growth, spawning will likely take place (Mercier and Hamel 2009) and a substantial larvae production may still be expected.

So far most of the ocean acidification effects observed in sea urchins and other adult echinoderms have been sublethal ones (Dupont et al. 2010b). Even though some effects may appear in longer term experiments and/or in more vulnerable species, adult stages appear to be quite resistant to future pH changes and sea urchin survival does not appear to be at immediate risk.

3. SEA URCHINS AND GLOBAL CHANGE: INTERACTION BETWEEN TEMPERATURE AND pH STRESSES

Ocean acidification is not the only ongoing phenomenon due to anthropogenic CO_2 emissions. Organisms are also facing a temperature increase in surface waters due to global warming. The main concern of hypercapnia exposure of organisms, including sea urchins, is the narrowing of thermal tolerance windows (Pörtner 2008). When early stages of *Strongylocentrotus franciscanus* larvae reared in CO_2 enriched waters were exposed to acute temperature stress during one hour, the ability to mount physiological response was reduced (O'Donnell et al. 2008). Negative effects of extreme temperatures in fertilization and embryonic development might not undergo any synergistic effects with pH. In fact, specimens of *Heliocidares erythrogramma* were affected by temperature, but not pH (lowest pH 7.6, Byrne et al. 2009).

However, a temperature increment can increase sea urchin larval growth and development rates (Emlet et al. 1987). Actually, in *Tripneustes gratilla* specimens reared at slightly higher temperatures (within the predictions of global change) the negative pH effects on echinoplutei development were attenuated (lowest pH 7.6; Sheppard Brennand et al. 2010). Likewise, in adult *P. lividus* the oxygen uptake (V_{O2}) at 16ºC, was not affected by pH while it was increased in answer to low pH and low temperature (10ºC), suggesting that a temperature increase can attenuate the energetic demand to mitigate hypercapnia effects (Chapter 4). Accordingly, in a mesocosm experiment Hale et al. (2011) reported that echinoderm abundance is enhanced at higher temperatures and pH. Small temperature increments might actually have a positive effect on larval development and adult metabolism compensating negative pH effects.

Responses to global change, i.e. to a temperature increase and a pH decrease, can be highly variable. Both parameters will vary greatly locally and seasonally. If changes take enough time, some species can acclimatize or even adapt to environmental shifts. Others, if

owning the potential and/or the opportunity to change their distribution, can migrate or occupy new habitats, depending on their degree of mobility or dispersal aptitude. Overall, physiological constrains on performance and ecological patterns will surely interact. In case of geographical or bathymetrical shifts, invasive species can seriously affect the community functioning. For instance, a temperature increase in the Southern Ocean might allow an expansion of the species *A. dufresnei* distribution and its voracious feeding behavior might allow it to compete with local sea urchins and other grazing species.

4. COMMUNITIES: INFERRING RESPONSES

Echinoid populations density is greatly dependent on recruitment events (Ebert 1983). Virtually nothing is known on how ocean acidification will affect this process, even though it is likely that shifts on larval development could have some negative consequences. Post metamorphic sea urchins have a predominant role in shaping communities (Lawrence and Sammarco 1982). Regular sea urchins consume mainly non-motile food items such as algae, soft body organisms, but also hard encrusting algae and coral or bivalves. Furthermore, drifting material can also be consumed (De Ridder and Lawrence 1982), as in the case of *Paracentrotus lividus*. Algal biomass, for instance, can be significantly decreased by grazing activity of sea urchins. Sea urchins can show food preferences in the field, that will also condition local algal composition, but their diet choices will depend on food availability. Under extreme grazing pressures, only a few species of seaweeds will survive, especially opportunistic, toxic or distasteful ones (see Lawrence and Sammarco 1982 for review). Grazing activity is not only limited to algae and sea urchin will also consume epibenthic fauna which can affect settlement events, including in coral communities. Relief of pressure by some urchin species resulted in a community shift from coralline algae and encrusting ectoprocts to fleshy algae, sponges, tunicates and erect ectoprocts (Lawrence and Sammarco 1982). Both *Paracentrotus lividus* and *Arbacia dufresnei* are species whose grazing activity deeply affects algal assemblages (Jara and Céspedes 1994, Boudouresque and Verlaque 2001, Privitera et al. 2008). *A. dufresnei*, however, does not only consume algae, but more than 50% of their gut content can include food items from animal origin, such as barnacles, juvenile bivalves, polychaetes among others (Vásquez et al. 1984, Penchaszadeh and Lawrence 1999). Sea urchins can furthermore be important bioeroders in coral reefs (Mokady et al. 1996, Tribollet and Golubic 2011), but echinoids can also interfere in local

communities behind trophic relations. For instance, the burrows that species like *P. lividus* form in rocky intertidal pools can function as additional microhabitat heterogeneity and providing refuge for other species (Lawrence and Sammarco 1982, Schoppe and Werding 1996).

Adult population maintenance is conditioned by food availability, predation, competition, wave energy or anthropogenic action, among others (Lawrence and Sammarco 1982). One can speculate that if some species of sea urchins present adaptive features towards others in face of ocean acidification, they will also have competitive advantages. Ultimately, their distribution patterns can even change. Moreover, in some ecosystems great community shifts are predictable. Due to the expected vulnerability of some calcified organisms, fleshy seaweeds will most likely increase their algal cover when compared to encrusting calcareous species (Russell et al. 2009). An increase in carbon dioxide availability enhances the photosynthetic activity, promoting a positive response in seaweed production and growth (Connell and Russell 2010, Jiang et al. 2010). This pattern was already observed in the vicinity of carbon dioxide vents in the Mediterranean Sea. In fact, not only the density of calcareous algae was diminished, but also that of grazers such as mollusks and the sea urchins *P. lividus* and *Arbacia lixula*, all calcifying organisms. However, the density of the sea urchins species was only affected at pH bellow 7.4 (Hall-Spencer et al. 2008). Also, at moderate pH decrease (7.8) some calcareous algae can grow in the surroundings of CO_2 vents and only at very low pH will their distribution be affected (Porzio et al. 2011). Sea urchin grazing activity can also be relevant in kelp recruitment control. In fact, this can be negatively affected in low pH seawaters, since turf seaweeds cover competes for space with these species, a fact that is aggravated by the absence of grazing activity (Connell and Russell 2010).

In fact it seems complex to speculate about the fate of communities where sea urchins are present. If some echinoid species do present adaptive advantages and if populations will be able to acclimatize to new pH regimes, their grazing activity might help controlling seaweeds population growth. In such scenario, seasonal food will not be limiting and this energetic input might allow echinoid populations to be kept in a good health. However, in areas where the present sea urchin species are more vulnerable to hypercapnia exposure or where recruitment will be seriously impaired, local communities might suffer an ecological shift. For instance, when sea urchins with significant grazing activity are removed of a certain area, the distribution and abundance of erect algae increase, including in kelp forests

(e.g. Bulleri et al. 1999, Harrold and Pearse 1987, Andrew and Byrne 2001, Boudouresque and Verlaque 2001, Scheibling and Hatcher 2001, Ling et al. 2010). Higher algal canopy can increase the local habitat complexity, increasing recruitment refugees for marine organisms such as fish. Also, areas where echinoid grazing activity was restricted, the abundance of sponges, hydroids, tunicates, anemones, among others, increased (Harrold and Pearse 1987). However, sea urchin grazing activity is not always extreme as sea urchins populations are controlled by predation and disease, and it can actually improve species diversity and abundance in communities. In fact, grazing helps controlling fast growing and space competitive species, such as ascidians, and the creation of bare spaces can improve colonization opportunities for other species (Harrold and Pearse 1987, Andrew and Byrne 2001, Boudouresque and Verlaque 2001). Sea urchin browsing, an intermittent activity due to local absence of individuals, introduces a heterogeneity factor of space available for algal colonization that can lead to a substantial increase in the number of "co-existing" species in rocky areas (Paine and Vadas 1969). Therefore, a decline in sea urchin populations can have, on a more global scale, a negative impact on some communities.

II - CONCLUSIONS AND PERSPECTIVES

Ocean acidification is an ongoing phenomenon that together with global warming is expected to have a profound impact in marine organisms and communities. On a first stage the scientific community focused their attention on calcifiers, since seawater carbon chemistry changes were expected to deeply affect these organisms. Gradually, it became evident that other metabolic and physiological aspects of the organisms could also be impaired. Curiously, some species seem to be less vulnerable to hypercapnia stresses which can be related to the presence of adaptive features that allow them to deal with lower pH waters. Others live currently in enriched CO_2 environments. Although in these last 10 years knowledge on ocean acidification effects increased exponentially, there are still many aspects that remain unsolved, especially in what concerns (phenotypic) plasticity and possible adaptations that allow individuals to deal with hypercapnia conditions. Under the realistic scenario of a decrease of ca. 0.4 units of pH by 2100, sea urchins, and echinoderms in general, appear to be robust for most studied processes. Even thought, this general response can depend on different parameters such as exposure time, pH level tested, the process and

the life stage considered, our results show that there is scope for echinoids to cope with ocean acidification.

It is therefore interesting to study deeper the possible mechanisms of acclimatization of species with contrasting ecologies and to understand what makes some less vulnerable than others. Studies should therefore focus on designing experiments where individuals from one same species are tested, but from populations from contrasting environments. For instance, species such as *Paracentrotus lividus* and *Arbacia dufresnei*, which have a broad geographic distribution and can be found in varied habitats, offer an opportunity to understand how local conditions can influence the responses to chronic or acute hypercapnia. Also, translocation experiments can further help clarifying this issue. Long term studies will illustrate if hypercapnia exposure effects will worsen the individual responses or if acclimation processes will take place. These studies should address problematics such as how the nutrition status of individuals affects their responses to hypercapnia, how much energy does it cost to an individual to maintain its normal functioning and how much does the coelomic buffer capacity actually contributes to maintain intercellular pH. These studies should take into account the possible synergistic effects of multiple stressors such as pH, temperature and hydrodynamic forces.

Besides the physiological responses, gene expression studies can add new information and be useful in the understanding of the expressed phenotypes. Microarrays (the study of mRNA produced by cells) and proteomics (expressed proteins) are used in ecotoxicology studies or in ecological studies of tolerance to extreme habitats. Some studies have already looked at gene expression in sea urchin larvae (e.g. O'Donnell et al. 2008, Todgham and Hofmann 2009, O'Donnell et al. 2010, Martin et al. 2011), but nothing is yet known in adult sea urchins. It is important to recognize which processes are exactly being affected and which ones are up or down regulated.

If there is scope for acclimate/acclimatize to low pH conditions, there might as well be for adaptation. This is a hard matter to analyze, since sea urchins have long life cycles and to follow successive generations is a time consuming task. Comparison of genomic sequences of divergent species can help to understand which adaptive processes are taking place (Oliver et al. 2010). The goal is to understand the actual genetic variability present in certain species and/or populations from contrasting environments and in which conditions adaptations can in fact exist and persist. Clarifying if genetic diversity of some populations is the key behind

their plasticity while responding to environmental stresses will allow the identification of potential robust species.

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Annex

Other collaborations that took place during the development of the current project:

Collard M, Bonnet S, Catarino AI, Dubois Ph (in prep) Ocean acidification effects on the physiology and adhesion properties of the starfish *Asterias rubens*

Ingels J, Brandt A, Catarino AI, David B, De Broyer C, De Ridder C, Dubois P, Gooday A, Martin P, Pasotti F, Robert H, Vanreusel A. (submitted to Global Change Biology) Possible effects of global environmental changes on Antarctic benthos

Kaiser S, Brandão SN, Brix S, Barnes DKA, Bowden D, Ingels J, Leese F, Linse K, Schiaparelli S, Arango C, Bax N, Blazewicz-Paszkowycz M, Brandt A, Catarino AI, David B, De Ridder C, Dubois Ph, Ellingsen KE, Glover A, Griffiths HJ, Gutt J, Halanych K, Havermans C, Held C, Janussen D, Lörz AN, Pearce D, Riehl T, Rose A, Sands CJ, Membrives AS, Schüller M, Strugnell J, Vanreusel A, Veit-Köhler G, Wilson N, Yasuhara M (submittd to Biological reviews) Pattern, process and vulnerability of Southern Ocean benthos - a decadal leap in knowledge and understanding