Facultad de Ciencias
Universidad de La Laguna

Efectos de la larga exposición a condiciones de bajo pH en las estructuras calcáreas del erizo de mar *Arbacia lixula* (Linnaeus, 1758)

Effects of long exposure to low pH conditions on calcareous structures of the sea urchin *Arbacia lixula* (Linnaeus, 1758)

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Máster en Biología Marina: Biodiversidad y Conservación

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Facultad de Ciencias
Universidad de La Laguna

JOSÉ CARLOS HERNÁNDEZ PÉREZ, profesor titular y SARA GONZÁLEZ-DELGADO contratada predoctoral

CERTIFICAN:

Que la memoria presentada por Naira Sosa Navarro, titulada "Efectos de la larga exposición a condiciones de bajo pH en las estructuras calcáreas del erizo de mar *Arbacia lixula* (Linnaeus, 1758)" ha sido realizada bajo nuestra dirección y consideramos que reúne todas las condiciones de calidad y rigor científico requeridas para optar a su presentación como Trabajo de Fin de Máster, en el Máster de Biología Marina: Biodiversidad y Conservación de la Universidad de La Laguna, curso 2020-2021.

Y para que así conste y surta los efectos oportunos, firmamos el presente certificado en San Cristóbal de La Laguna, a 1 de septiembre de 2021.

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ABSTRACT

The CO_2 emissions due to human activities have caused the oceans to absorb a larger amounts of carbon dioxide. This situation has generated chemical reactions, leading to a decrease in pH and in the concentration of carbonate ions (CO_3^{2-}) in seawater. This process is known as Ocean Acidification (OA) and has major repercussions on the physiological, ecological and behavioural functions of marine animals, especially in calcifying species, and it is considered a major threat to marine life. This study aims to determine the effects of OA on the sea urchin *Arbacia lixula* using a long-term natural experiment, the CO_2 seeps system off Fuencaliente coast in La Palma Island (Canary Islands). In this area, there are spots were the pH drops to 7.56 units during low tide and a clear pH gradient can be found on the surrounding of those emission points. We have studied the external morphometry of the skeleton and Aristotle's lantern, as well as the fracture force, the sea urchin growth, and the mineralogy across the pH gradient. Regarding the growth of *A. lixula*, we have explored different growth rings reading techniques applied on the genital plates. The most useful techniques have been Moore, 1935 and Schuhbauer, 2010; with three other combinations of techniques, that also worked fine for reading rings. It was observed that in low pH zone, sea urchins decreased in body size and spine length but showed stronger test (more resistant to fracture). There was also a slight increase in the percentage of Mg in the calcite of the test and the lantern. These unexpected results have shown how these natural experiments are essential to understand the real consequences of OA on calcareous organisms.

Key words: Ocean Acidification, *Arbacia lixula*, calcified structures, growth rings, volcanic CO_2 vent.

RESUMEN

Las emisiones de CO_2 debidas a las actividades humanas han provocado que los océanos absorban una mayor cantidad de dióxido de carbono. Esta situación ha generado reacciones químicas que han provocado una disminución del pH y de la concentración de iones carbonato (CO_3^{2-}) en el agua de mar. Este proceso se conoce como Acidificación Oceánica (AO) y tiene importantes repercusiones en las funciones fisiológicas, ecológicas y de comportamiento de los animales marinos, especialmente en las especies calcificadoras, y se considera una gran amenaza para la vida marina. Este estudio pretende determinar los efectos de la AO sobre el erizo de mar *Arbacia lixula* utilizando un experimento natural a largo plazo, el sistema de filtraciones de CO_2 frente a la costa de Fuencaliente en la isla de La Palma (Islas Canarias). En esta zona, existen puntos en los que el pH desciende hasta 7,56 unidades durante la marea baja y se puede encontrar un claro gradiente de pH en los alrededores de estos puntos de emisión. Hemos estudiado la morfometría externa del esqueleto y la linterna de Aristóteles, así como la fuerza de fractura, la mineralogía y el crecimiento del erizo de mar a través del gradiente de pH. En cuanto al crecimiento de *A. lixula*, hemos explorado diferentes técnicas de lectura de anillos de crecimiento aplicadas en las placas genitales. Las técnicas más útiles han sido Moore, 1935 y Schuhbauer, 2010; con otras tres combinaciones de técnicas e ideas, que también funcionaron bien para la lectura de anillos. Se observó que, en la zona de pH bajo, los erizos de mar disminuyeron el tamaño del cuerpo y la longitud de sus espinas pero mostraron una esqueleto más fuerte (más resistente a la fractura). También hubo un ligero aumento del porcentaje de Mg en la calcita del esqueleto y la linterna. Estos resultados inesperados han mostrado cómo estos experimentos naturales son esenciales para entender los impactos reales de la AO en los organismos calcáreos.

Palabras clave: Acidificación Oceánica, *Arbacia lixula*, estructuras calcificadas, anillos de crecimiento, afloramiento volcánico de CO_2.

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1. INTRODUCTION

The oceans act as sinks, absorbing the 27% of atmospheric CO_2 from emissions produced by human activity (fossil fuels burning, deforestation, changes in land use, etc.) (Sabine et al., 2004). CO_2 emissions are produced into the atmosphere increasingly, which leads to the oceans absorbing a large amounts of carbon dioxide. This excess of carbon dioxide absorbed is generating chemical reactions and, as a result, it produces a decrease in both pH and carbonate ion (CO_3^{2-}) concentration of seawater. This process is known as ocean acidification (OA) and because it may impair physiological, ecological and behavioural functions of marine animals, OA is considered a great threat to marine life (Feely et al., 2004; Melzner et al., 2009; Kroeker et al., 2013; IPCC, 2014; Sunday et al., 2014).

Furthermore, the decrease in CO_3^{2-}, results in a significant reduction of saturation state (Ω) of calcite and aragonite (Orr et al., 2011). Both carbonate calcium minerals are essential for many marine organisms such mollusks or echinoderms to create their calcareous structures. Therefore, when the saturation state of these minerals is below 1, the integrity of the skeleton of these calcifying marine organisms are negatively affected (McDonald et al., 2009; Rodolfo-Metalpa et al., 2011; Dubois, 2014; Collard et al., 2016).

Until recently, the effects of OA in calcifying species have been studied under laboratory experimental condition (McDonald et al., 2009; Dubois, 2014; García et al., 2018; Byrne & Hernández, 2020). However, with the used of shallow natural CO_2-enriched areas like CO_2 vents, it has shown that predicting the effect of OA is not as simple as previously thought (Byrne & Hernández, 2020). Through a natural CO_2 gradient, some calcifying species decline such as corals (Rodolfo-Metalpa et al., 2011; Fabricius et al., 2011), but many remain alive in extreme environments (Thomsen et al., 2010), and some species such as herbivorous mollusks increase (Connell et al., 2017). Even so, calcification of these organisms is affected, and calcium carbonate skeletons may dissolve when the Ω of the calcareous minerals drops, as seen in foraminifera, coccolithophores, sea urchins and mollusks at different CO_2 vents worldwide (Pettit et al., 2013; Milazzo et al., 2014; Ziveri et al., 2014; Collard et al., 2016; Duquette et al., 2017; Viotti et al., 2019).

The OA effects on echinoderms have been studied extensively (Byrne & Hernández, 2020), because of the major ecological role that these organisms play in marine ecosystems, as they are regulators of algal growth in many benthic communities (Hernández, 2017). Furthermore, they are among the organisms theoretically most susceptible to acidification, since their endoskeletons are made up by the one of the most soluble forms of calcium carbonate, high-magnesium calcite (Morse et al., 2006). Abundance, ecological and physiological studies have reported a wide variety of responses ranging from absence of echinoids to adaptation and resilience to natural CO_2 environments (Calosi et al., 2013b; Bray et al., 2014; Brinkman, 2014; Small et al., 2015; Collard et al., 2016; Lamare et al., 2016; Uthicke et al., 2016; Nogueira et al., 2017; Foo et al., 2018).

On reflection, OA will have many specific nuances and their impacts will be quite wide-ranging. This is because, the OA is associated with different factors such as low pH, Ω, and until now it is difficult to know which one or more of these factors influence sea urchins. In this sense, pH systems like CO_2 vents provide the proper place for studying sea urchin populations. In these places urchin populations may also evolve and develop resistance strategies to OA (Byrne & Hernández, 2020).

Sea urchin skeletons are of fundamental importance in defining the organism shape, providing protection against currents, waves and predators (Asnaghi et al., 2019). Consequently, any deterioration of the skeleton would have considerable consequences on the sea urchin fitness. One of the key factors determining the skeletal structure and composition of echinoderms is the diet (Shirayama and Thornton, 2005; Asnaghi et al., 2014). In addition, sea urchins present allometries with respect to Aristotle's lantern and their test, whose changes may be related to food availability (Pérez, 2018). Thus, when food availability is low, sea urchins use the energy obtained to enlarge their jaws, instead of increasing the size of their test, strengthening the jaws help them to obtain enough food (Black, 1984; Levitan, 1991; Pérez, 2018). So, studying the relationship between the jaw and the test size can give us some information about the diet of the sea urchins.

Echinoderms living in natural CO_2 system where Ω are below 1 have shown some unexpected responses. For example, *Arbacia lixula*, *Evechinus chloroticus*, *Paracentrotus lividus*, and *Echinometra* sp. can survive or even outperform in natural low pH conditions (Calosi et al., 2013a; Bray et al., 2014; Brinkman, 2014; Collard et al., 2016; Uthicke et al., 2016). In the case of *Echinometra* sp. the diet has been very important in determining its performance under OA (Uthicke et al., 2016).

Arbacia lixula is an echinoderm that live along the shores of the tropical Atlantic, including Brazil, the African coast, the Macaronesian archipelagos and the Mediterranean (Benedetti-Cecchi et al., 1998; Palacín et al., 1998; Guidetti et al., 2003; Guidetti and Dulcic, 2007; Hereu et al., 2012; Hernández et al., 2013). Laboratory studies have reported that *A. lixula* is resilient to future OA, especially with moderate temperature increases (Wangensteen et al., 2013, Gianguzza et al., 2014; Visconti et al., 2017; Garcia et al., 2018) with the exception that larval morphology may be affected (Wangensteen et al., 2013; Visconti et al., 2017). This species shows a range of different responses in different CO_2 vents, from their abundance being negatively related to increased seawater CO_2 (Hall-Spencer et al., 2008; Kroeker et al., 2013) to their density not being affected because of its resilience to the acidified conditions (Calosi et al., 2013; Bray et al., 2014). Although Bray et al. (2014) reported that the distribution of *A. lixula* is unaffected by a low pH environment, also indicated that acidification causes test dissolution in *A. lixula*. Regarding reproductive aspects, Foo et al. (2018) have reported that the size of the jelly coats of *A. lixula* organisms present at a vent site shows a plastic response, which could facilitate the maintenance of gamete function and thus fertilization success in a low pH ocean.

These previous results make *A. lixula* an interesting species to study. In this study, we aimed to use *A. lixula*, present in the acidified system of La Palma Island (Canary Islands), to determine organism level effects due to a lifelong exposure to low pHs. This study comprises: (1) morphological measures and mechanical properties of the sea urchin's skeletal structures (test, spines, and Aristotle's lantern); (2) estimation of the growth rate of the sea urchins by reading the growth rings from genital plates, as well as; (3) X-Ray diffraction estimations of the skeletal composition of the carbonate structures.

2. METHODS

2.1. Study areas and sea urchin collection

Sampling was conducted on the CO_2 vent system of Fuencaliente, in the southeast coast of La Palma Island (Canary Island) (Figure 1). Here, the natural CO_2 emission is generated by a continuous brackish water discharge from aquifer which is being acidified by magmatic emissions of CO_2 gas. The chemical properties create a natural gradient of pH, alkalinity and saturation state (Ω) of calcite and aragonite, especially at low tides (González-Delgado et al., 2021). Although there are no traces of the presence of volcanic elements such as methane or sulfates, there is an extra supply of different elements such as Mg that comes from groundwater (González-Delgado et al., 2021).

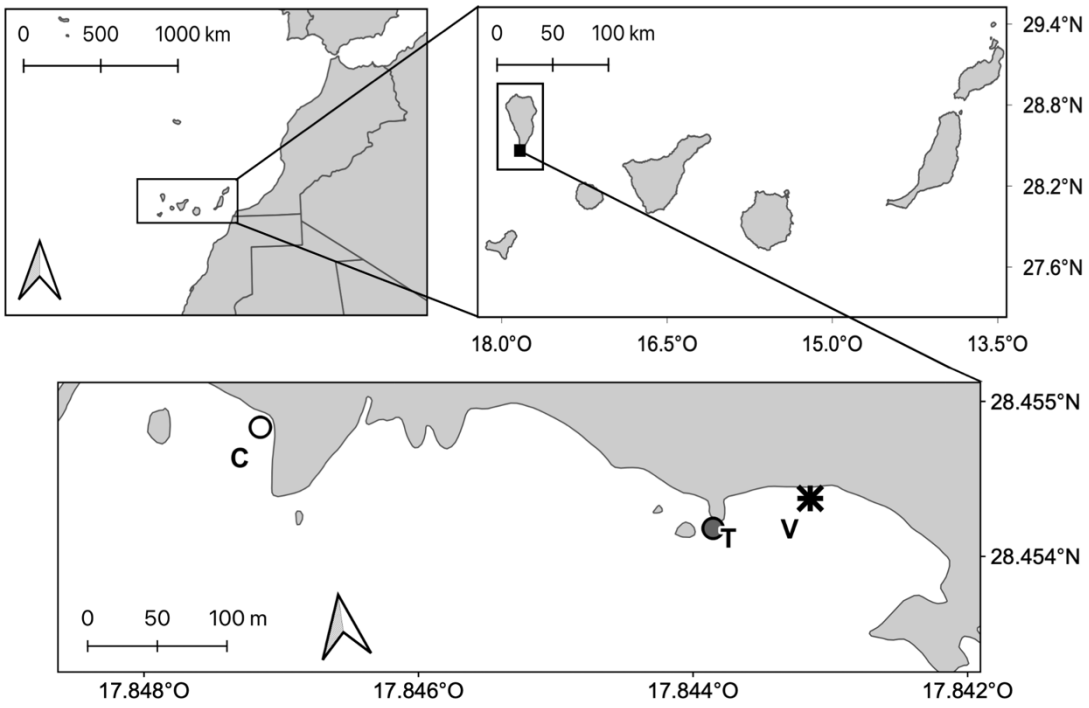


Figure 1. The Canary Islands, and the location of the acidified system of Punta de Fuencaliente in La Palma Island, with the three sampling points: "Control" (C), "Transition" (T) and "Vent" (V). (https://grafcan.es/, last access: 02 June 2021).

In April of 2017, a total of 108 individual ("Control" = 34, "Transition" = 31, "Vent" = 43) of the sea urchins *Arbacia lixula* were randomly collected by snorkeling at three different sites following the natural gradient between 2 and 5 m depth. The three collecting sites were characterized by different pH level (C: "Control" = pH 8.1; T: "Transition" = pH 7.8; V: "Vent" = pH 7.6) (Table 1).

Table 1. Chemical measures of the studied sites at low and high tides (TA = Total Alkalinity, cal = calcite, ara = aragonite).

	Low Tide				High Tide			
	pH	**TA (µmol/kg)**	**Ωcal**	**Ωara**	**pH**	**TA (µmol/kg)**	**Ωcal**	**Ωara**
Control	8.14	2573.98	6.11	3.98	8.11	2559.17	5.76	3.76
Transition	7.81±0.01	2783.85±11	3.55±0.06	2.32±0.04	8.15±0.01	2501.37±8	6.03±0.10	3.93±0.07
Vent	7.56±0.02	3252.43±42	2.48±0.07	1.62±0.04	7.95±0.07	2728.37±11	4.57±0.58	2.98±0.38

2.2. Morphological measurements

The *Arbacia lixula* specimens were transported to the laboratory and weighted (to the nearest 0.01 g). Test diameter (with and without spines) and height of each sea urchin were measured along three diameters per urchin using a digital caliper (precision: ± 0.01 mm) to reduce measurement error. Additionally, length and thickness of 5 different randomly spines were also measured for each specimen. Then, sea urchins were dissected and cleaned of internal organs. The calcified structures, test, spines, and Aristotle's lantern were stored for analyzes described below.

The Aristotle's lantern of *A. lixula* was dissected out. Soft tissue was removed by immersion in 5% sodium hypochlorite for 24 h and the lantern elements were thoroughly rinsed with distilled water and air dried. One jaw (demi-pyramid) was selected from each lantern to measure using a digital caliper (precision: ± 0.01 mm).

2.3. Fracture force

To measure the fracture force of whole *A. lixula* tests, a compression test was applied to the sea urchin. The sea urchins from each site (N = 25) were crushed using a measured as the maximum force (kilogram-force, kgF) that the test could tolerate before to fracture. The mechanical tests were conducted using a 7t digital compression testing machine (Model MUE-1E, Salmer-Pacam) with a load cell fitted to the precise control over the force application. The speed of the tester was set at 5 mm * min^{-1}. Following Collard et al. (2016) indications the spines at the ambitus were manually removed to avoid any interference of the spines on the measurement of the test strength.

2.4. Testing growth ring reading methods

To study the growth of *Arbacia lixula* using growth rings we first tested different protocols (Moore, 1935; Nichols et al., 1985; Gebauer and Moreno, 1995; Schuhbauer, 2010; Ouréns et al., 2013) (Table 2), which were based on an initial cleaning of the genital plates by polishing their surface with sandpaper of different grits (1.000 grit and 800 grit). We used one or two genital plates of each individual to carry out different techniques, since sometimes in the sanding process the plates broke, and we had to use another one. Then, some of the plates were dipped in different products, such as acetone, 70% alcohol, xylol, sodium hypochlorite or methylene blue. Finally, the plates were observed under light transmitted microscope.

Table 2. Growth rings detection techniques that were tried in *Arbacia lixula*.

Technique	Methodology used
Ouréns et al., 2013	1. Hand-polished the plates with water-sandpaper of 600-1.000 grit. 2. Polish the interior face in order to facilitate homogenous polishing of the external face, where the readings were performed. 3. The plates are usually immersed in xylol when they are read, in order to see the rings more clearly. This carcinogenic compound was replaced with body oil, as it has the same effect. 4. The plates were examined under a binocular microscope with cold light from an epiluminator.
Moore, 1935	1. The genital plates were sanded with fine sandpaper (grain 1.000) until the outer surface was removed. 2. The plate was damped with alcohol and examined from time to time during the grinding. 3. The plate was transferred for a few minutes to absolute alcohol. 4. Then transferred to xylol and mounted face downwards on a slide with Canada balsam.

	5. When dry the plate could then be examined. In our case, the plates were not mounted in Canada balsam. Once they had been transferred to xylol for 10 minutes, they were first observed under a microscope without oil and later with a drop of body oil.
Gebauer and Moreno, 1995	1. The genital plates were treated with 39% sodium hypochlorite for bleach the plates to facilitate their reading. 2. The plates were sanded with water-based sandpaper and observed immersed in xylol under a stereoscopic microscope with reflected light. In our case, we replaced the xylol for body oil, following the methodology of Ouréns et al., 2013.
Schuhbauer, 2010	1. The urchins were cut in half, with two-thirds cut transversally and the rest longitudinally. Each individual was then cleaned and dried (either in air or in a moderate oven). 2. Once dry, the genital plates were dissected and placed in a 14% NaOCl solution for 48h to bleach the plate. 3. They were rinsed in a water bath to remove NaOCl. 4. At least one of the five genital plates of each individual was sanded with fine sandpaper (800 grit). 5. The plates were read in 96% ethyl alcohol under reflected light using a stereomicroscope (Olympus SZX12). In our case, we do not cut the urchins in half, we separate a genital plate directly from the whole urchin for analysis.

Nichols et al., 1985	1. The four non-madreporic genital plates were treated with sodium hypochlorite. 2. They were dried and then individually sanded with fine sandpaper. In our case, we only use one non-madreporic genital plate from each individual. For the observation of the growth rings in the microscope we use a drop of body oil, to facilitate the observation.

In addition, we also test other methodologies that were the combinations of different techniques or ideas that we came up with during the experimental trials. Below there is a list of techniques used and their methodology (Table 3).

Table 3. Combination of different growth ring detection techniques in *Arbacia lixula*.

Technique nº	Methodology
1	1. One of the genital plates was sanded with 1.000 grit sandpaper and cleaned with water to remove dust. 2. The plate was placed in a petri dish and a few drops of xylol were added for 40 minutes under a fume hood. 3. After 40 min the xylol evaporated and the genital plate was observed under a microscope with a drop of body oil.

2	1. The genital plate was sanded with 1.000 grit sandpaper and cleaned with water to remove dust. 2. The plate was moistened in alcohol 70% to facilitate the sanding process. 3. Then the plate was observed in the microscope immersed in alcohol 70%.
3	1. One of the genital plates was sanded with 1.000 grit sandpaper and cleaned with water to remove dust. 2. The cleaned plate was heated with a lighter for a few seconds. 3. The genital plate was placed in a petri dish and a few drops of xylol were added for 25 minutes under a fume hood. 4. Then the plate was rinsed with ethanol 70˚. 5. The genital plate was observed under a microscope. 6. But as the growth rings could not be seen, the plate was observed again with a drop of body oil.
4	1. The genital plate was sanded with 1.000 grit sandpaper and cleaned with water to remove dust. 2. Finally, the genital plate was observed under a microscope. 3. As the growth rings could not be seen, the genital plate was observed again with a drop of body oil.
5	1. One of the genital plates was sanded with 800 grit sandpaper and cleaned with water to remove dust. 2. The plate was immersed in sodium hypochlorite for 20 minutes to remove organic matter and bleach the plates to facilitate their reading.

	3. After that, the genital plate was observed under a microscope. 4. As the growth rings could not be seen, the genital plate was observed again with a drop of body oil.
6	1. The genital plate was placed in 70˚ ethanol for a few seconds. 2. Then plate was sanded with 1.000 grit sandpaper. 3. The plate was placed in a petri dish and a few drops of xylol were added for 10 minutes under a fume hood. 4. Finally, the plate was observed under a microscope with a drop of body oil.
7	1. The genital plate was immersed in acetone for 24 hours. 2. After that, the plate was sanded with 1.000 grit sandpaper. 3. We observed the plate under a microscope. 4. As the growth rings could not be seen, the genital plate was observed again with a drop of body oil.
8	1. The plate was immersed in acetone for 20 minutes. 2. Then we sanded the genital plate with 1.000 grit sandpaper. 3. Finally, the plate was observed under a microscope. 4. But as the growth zones could not be seen, the genital plate was observed again with a drop of body oil.

9	**Methylene blue staining:** 1. One of the genital plates was sanded with 1.000 grit sandpaper and cleaned with water to remove dust. 2. The genital plate was immersed for 2 minutes in an eppendorf tube with methylene blue. 3. The plate was then washed with water for a few seconds. 4. The plate was dried with absorbent paper and observed under a microscope. Subsequently, as growth rings were not seen, the plate was again observed with a drop of body oil.

Below there is a table with the rest of the techniques collected, which could not be carried out and their corresponding description (Table 4).

Table 4. Growth rings detection techniques that were not tested.

Technique	Methodology
Jensen, 1969	1. A genital plate is dissected, and the interior is carefully cleaned with watchmaker's forceps and a soft brush. This is most easily done in alcohol. 2. The interior of the plate is heated by holding it over an alcohol flame for 1/2 -2 minutes, or until the plates start to turn dark brown. 3. After cooling, the plate is transferred to xylol and then the centre of the plates can be seen, surrounded by alternating dark (summer) and light (winter) growth zones. If some of the growth zones are not clear after xylol treatment, the plates can be placed in methyl benzoate for approximately one hour, after which the growth zones should be visible.

	4. After rinsing with alcohol, the treated samples can be stored dry or in 70% alcohol. When re-monitoring is desired later, the growth zones can be clearly seen as soon as the plates are placed in xylol (or methyl benzoate).
Deutler's, 1926	1. Clean the plates of organic matter by boiling in sodium hypochlorite solution (Eau de Javelle). 2. Wash in detergent. 3. Dehydrating in ethanol. 4. Embedding in balsam. 5. Grind and polish. 6. Finally, clearing for examination of the plates in a terpineol-methyl benzoate mixture.
Durham, 1955	He attempted differentiation of the growth zones by different techniques, including grinding, staining, acid etching and photographing in transmitted and reflected light.
Birkeland and Chia, 1971	1. The genital plates were dried at 75 to 85˚ after sanding the surface. 2. Then the plates were rinsed in xylol.
Sumich and McCauley, 1973	1. The genital plates were dried at 75 to 85˚ after cleaning them with sodium hypochlorite, commercial bleach. 2. Finally, the plates were rinsed in xylol.
Pearse and Pearse, 1975	1. The genital plates were cleaned with hypochlorite. 2. Then the hypochlorite cleaned plates were charred in a muffle furnace at 300˚C for 10 minutes.

	3. Both sides of the plates were polished, either before or after carbonization, on 600 grit sandpaper, to remove spine bosses of the column and the inner layer of the callus that generally darkened the areas. 4. For ease of handling and viewing, the genital plates were permanently mounted in a xylol-based mounting medium.
Sime and Cranmer, 1985	1. The four non-madreporic genital plates was gently ground onto the outer surface with fine sandpaper. The rings are most easily seen when the outer surface is barely abraded. 2. The development of clear rings can be assessed during the sanding process by spotting with alcohol from time to time and briefly examining them under a low power reflected light microscope. 3. When distinguishable rings were obtained, the inner surface was sanded until the plates are very thin. 4. The prepared plates were placed in an equal mixture of terpineol and methyl benzoate as a cleaning agent. 5. The examination can be performed by reflected or transmitted light.

2.5. X-ray analysis

At the end of the fracture force analyses, the sea urchin tests were used to mineralogy analysis and measure the thickness test. To prepare calcified structures soft tissues were removed by immersion in 10% sodium hypochlorite for 24 h and were rinsed generously with distilled water and air dried. Cleaned calcified structures were ground to produce fine powders with an agate mortar and pestle. In total 50 samples were examined, including test plates ($N_{control}$=6, $N_{Transition}$=5, N_{vent}=6), primary spines ($N_{control}$=4, $N_{Transition}$=6, N_{vent}=5) and jaws ($N_{control}$=6, $N_{Transition}$=6, N_{vent}=6).

$MgCO_3$ was determined by Panalytical Empyrean with a Cu X-ray source and a linear detector model PIXcel. The diffractograms obtained were analysed using Highscore+ software and the ICDD (International Centre for Diffraction Data) PDF4+ crystallographic database. The Mg percentages were estimated from the information provided by the phase identification of the samples. Numerous entries of calcite and magnesian calcites with different Mg percentages were obtained and the one that best matched each diffractogram was selected in each case.

2.6. Data analysis

- For external morphometry: Samples were ordinated using the multivariate matrix of external morphometric variables (sea urchin weight, test diameter and height, spines length and thickness) by mean of a Principal Coordinates Ordination (PCO). The variables test height and urchin weight were not analyzed due to present collinearity with the test diameter, which better represents the sea urchin's size. For comparison purposes a Permutational analysis of variance (PERMANOVA) was performed using the distance-based similarity matrix and a design with the factor "Sites", with three levels of variation ("Control", "Transition" and "Vent").

- For size frequency distribution and Aristotle's lantern morphometry: Test size frequency distributions were compared between sites though a Kolmogorov-Smirnov two-sample test using Bonferroni correction procedures (Siegel and Castella, 1988). Model II allometric regressions were calculated for test diameter *versus* jaw length for different sites (Ebert & Russell, 1994). Finally, the PRIMER-e DOMDIS procedure (Clarke et al., 2014) was used for comparing the slopes of the obtained regressions.

- For fracture force: The fracture force of whole *A. lixula tests* were analysed using Permutational analysis of covariance (PERMANCOVA). The design of the analysis presented a fixed factor "Site", with three levels of variation ("Control", "Transition" and "Vent") and the test diameter of the specimens selected was included as a covariate. In addition, box-plots were obtained for these analyses.

- For mineralogy: To assess the effect of site on %Mg in calcite, calcite and carbonates, further one-way PERMANOVA analysis were performed using also the factor "Sites" with its three levels of variation ("Control", "Transition" and "Vent"). Because the $\%MgCO_3$ differs between calcified structures, the demi-pyramid, test and spine were statistically analysed separately (Weber et al., 1969; Duquette et al., 2018).

All statistical analyses were carried out using Euclidean distance of raw data and 4999 permutations of the appropriate exchangeable units and untransformed data in PRIMER 6 & PERMANOVA + v. 1.0.1 software. Significant terms in the full models were examined using *a posteriori* pairwise comparisons by permutations.

3. RESULTS

3.1. External morphometric of the skeletal elements

There was difference in external morphometric between the sea urchins collected at different study sites (Table 5A). In external morphometric variables, the "Vent" differs from the "Control" and "Transition" sites (Table 5B). The best variables that explain these differences were the diameter and thickness of the spines (Figure 2).

Table 5. PERMANOVA for the morphological measures of the calcareous structures of *Arbacia lixula* (A) and Pairwise comparisons by permutations for the morphological measures of their calcareous structures (B) of the factor "Site" with three levels: "Control", "Transition" and "Vent".

(A)	df	SS	MS	Pseudo-F	P(perm)
Site	2	0.155	7.73E-2	7.198	**0.001**
Res	105	1.129	1.0748E-2		
Total	107	1.283			
(B)	Control-Transition	Control-Vent		Transition-Vent	
t	1.325	3.477		2.618	
P(perm)	0.175	**0.001**		**0.004**	

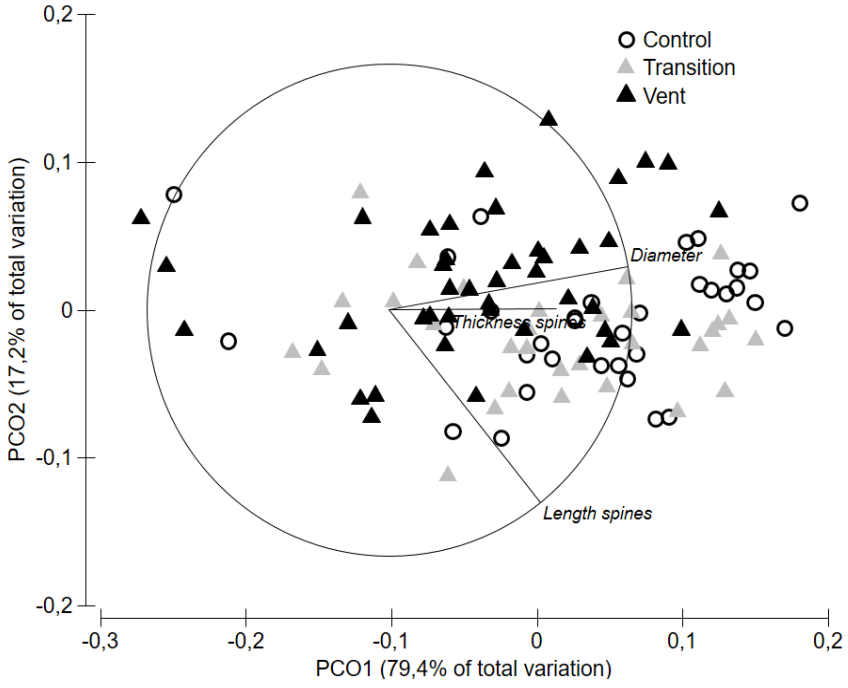


Figure 2. PCO of samples using the external morphometric variables (diameter, length, and thickness spines) between the three levels "Control", "Transition" and "Vent".

3.2. Test size frequency distribution and Aristotle´s lantern morphometric

Diameter frequency distributions by Kolmogorov-Smirnov showed variations between the "Vent" and other sites ($K\text{-}S_{control\text{-}transition}$: 0.23, $p>0.05$; $K\text{-}S_{control\text{-}vent}$: 0.37, $p<0.05$; $K\text{-}S_{transition\text{-}vent}$: 0.19, $p>0.05$; Figure 3).

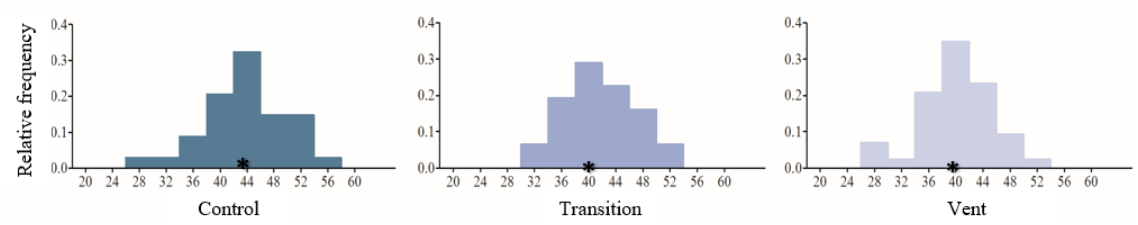


Figure 3. Frequency distributions of test diameter in each site ("Control", "Transition" and "Vent"). The asterisk shown the median of each distribution.

Allometric regressions of model II between the jaw length (J) and test diameter (D), transformed using a natural logarithms, in the populations are shown in Figure 4. These correlations were positive for all cases, confirming the general dependence between test diameter and jaw length. When comparing the slopes of these regressions by PRIMER-e DOMDIS procedure at all sampled sites, not significant variations were observed (R=0.022; p=0.108). However, when making two-by-two comparisons, individuals from "Control" site were significantly different from those from the "Vent" site (R=0.058; p=0.033) (Table 6). As slight allometry can be observed in the "Vent" when compared to the "Control" individuals, with smaller jaws for the same diameters (Figure 4).

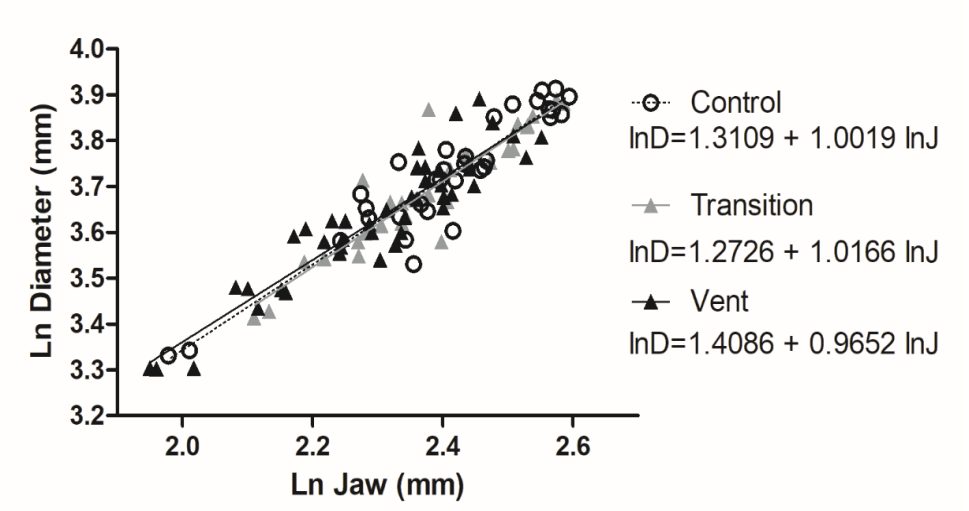


Figure 4. Model II allometric regressions test diameter vs jaw using natural logarithm (Ln).

Table 6. Two-by-two comparison of the slopes of the obtained regressions by PRIMER-s DOMDIS procedure between "Sites" ("Control", "Transition" and "Vent").

Groups	Statistic	Level %
Control-Transition	-0.006	49.8
Control-Vent	**0.058**	**3.3**
Transition-Vent	0.006	32.7

3.3. Fracture force

There was difference in fracture force of test between sea urchins of different sites (Table 7A). Pairwise comparison show a significant different between "Control" and both "Transition" and "Vent" sites (Table 7B), being the sea urchins from acidified site stronger than the control site (Figure 5).

Table 7. Permutational ANCOVA for the fracture force of the Arbacia lixula test with "Diam" (test diameter) as covariate (A) and pairwise comparisons by permutations for the fracture force of their test (B) of the factor "Site" with three levels: "Control", "Transition" and "Vent".

(A)	df	SS	MS	Pseudo-F	P(perm)
Diam	1	1715.4	1715.4	59.973	**0.001**
Site	2	208.33	104.16	3.642	**0.032**
DiamxSite	2	15.846	7.923	0.277	0.755
Res	87	2488.5	28.603		
Total	92	4428.1			
(B)	Control-Transition	Control-Vent		Transition-Vent	
t	2.635	2.181		0.043	
P (perm)	**0.011**	**0.036**		0.988	

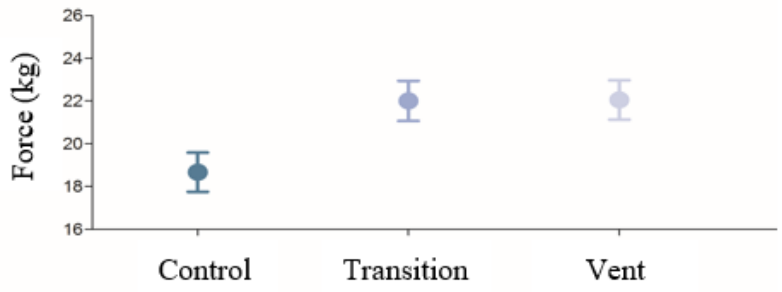
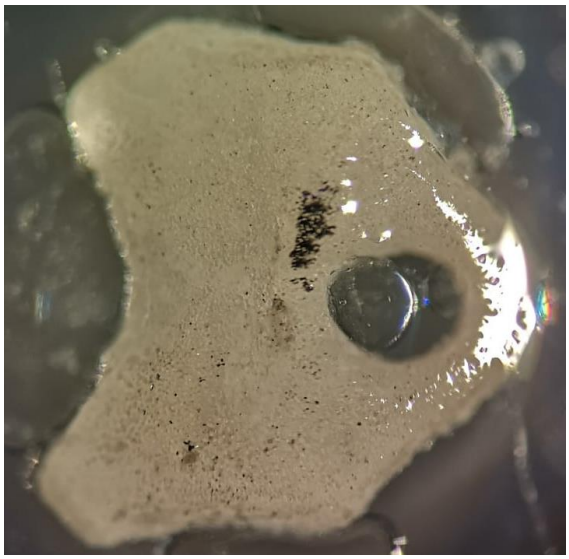


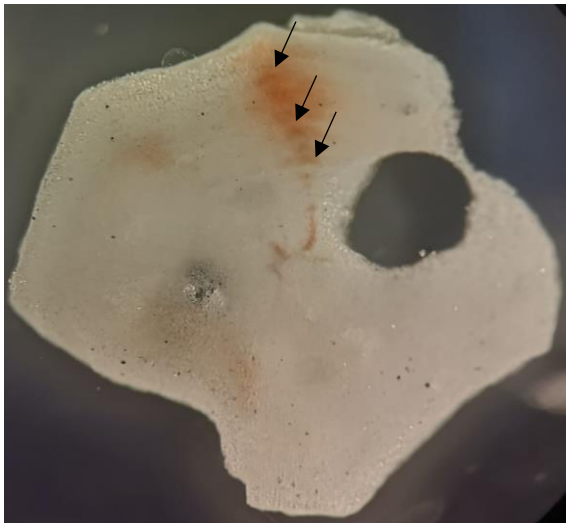
Figure 5. Average and deviation of the fracture strength (force in Kg) of each site ("Control", "Transition" and "Vent").

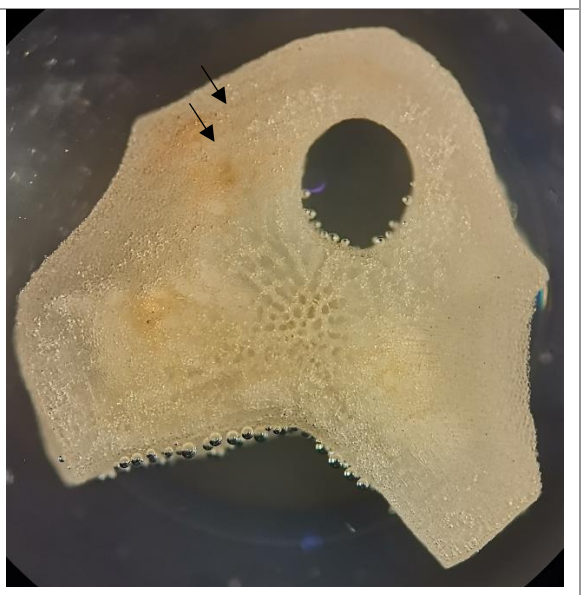
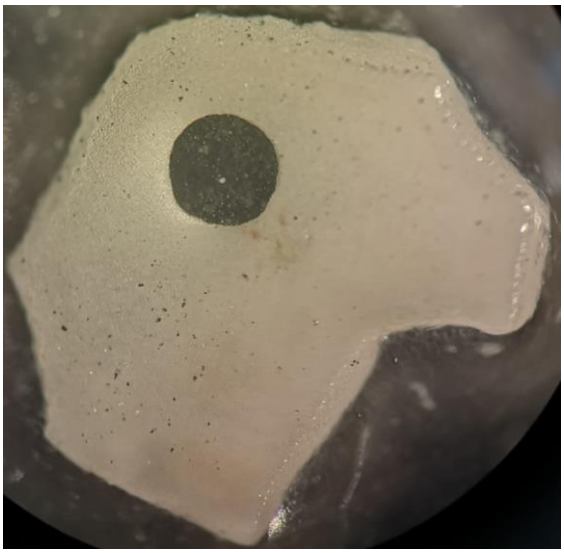
3.4. Growth/age estimation

A compilation of growth ring detection techniques has been used with sea urchin's calcareous structures (mainly genital plates). Of all the techniques tested, only some of them were satisfactory and some growth rings were observed. Below there is a table with the different techniques that were carried out and their corresponding images (Table 8). The techniques with the best results have been Moore, 1935 and Schuhbauer, 2010, as the growth rings have been observed more clearly.

Table 8. Results of the genital plates observation for the detection of growth rings. Technique from table 2.

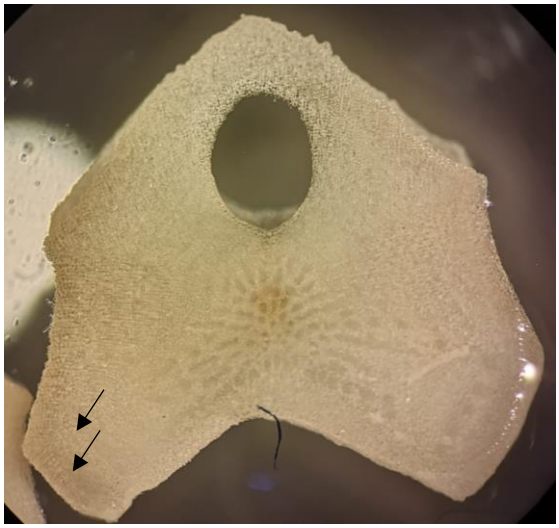
Technique from	
Ouréns et al., 2013	
In the development of this technique, the growth rings could not be observed.	

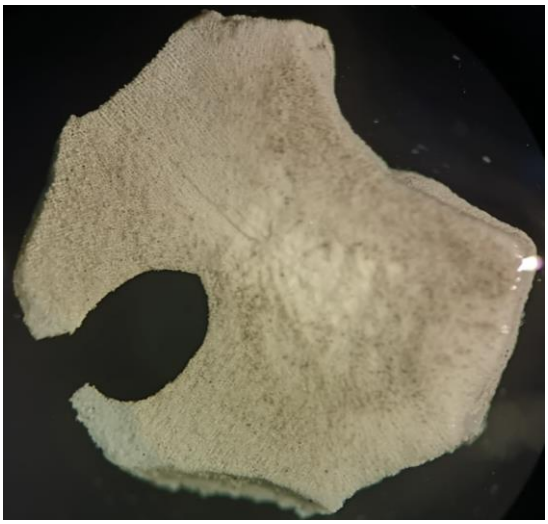
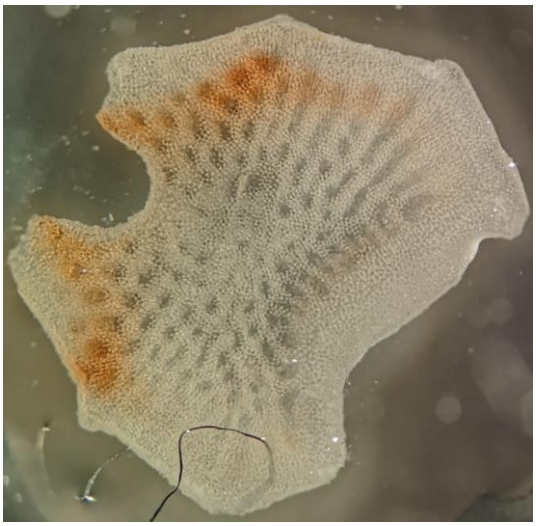
Moore, 1935	
With this technique, we could see some growth rings, between 2 and 3, which would be equivalent to about 2 or 3 years. The growth rings are diffuse, they are not all over the edge of the genital plates equally, but this could be a good method to implement in a future growth study.	
Gebauer and Moreno, 1995	
With this technique, we did not observe any growth zones.	

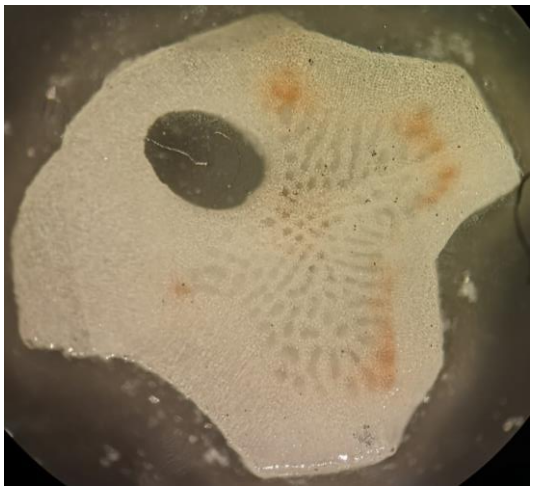
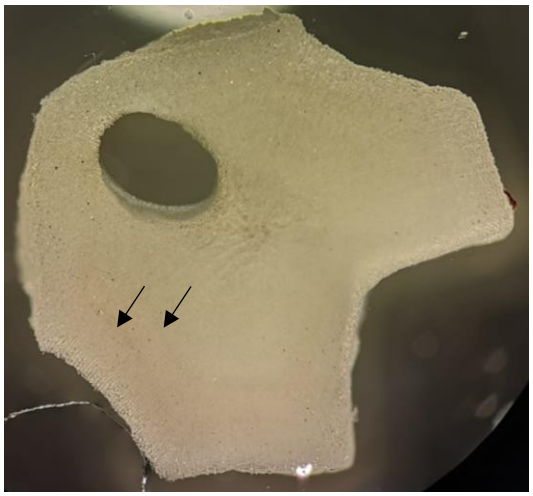
Schuhbauer, 2010	
In this case, we could see some growth rings, which are a little bit diffuse, they are not all over the edge of the genital plates equally, but this could be a useful method to implement in a future study.	
Nichols et al., 1985	
In this case, we did not observe any growth rings.	

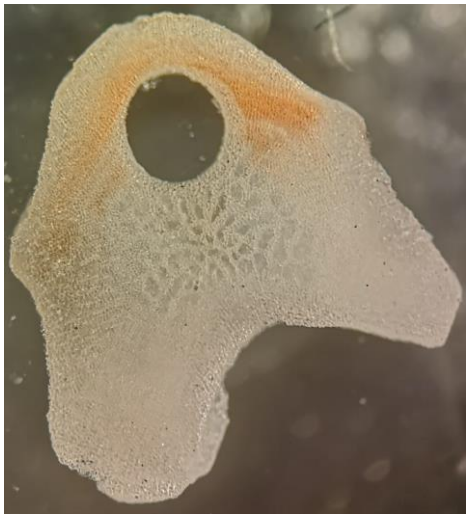
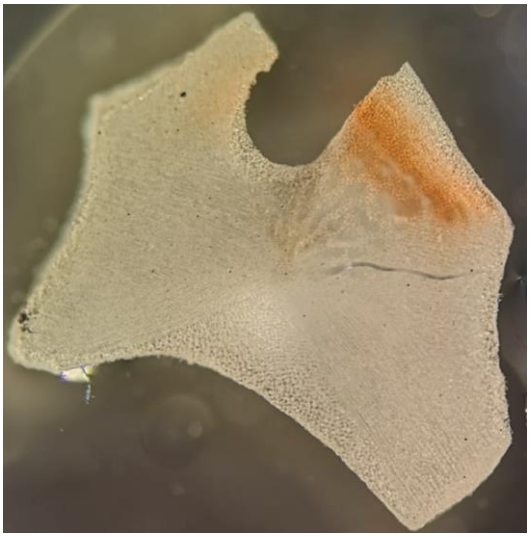
In the case of the combined techniques or ideas that we came up with during the experimental trials, we also obtained useful results in some of them, where we could observe some growth rings. Below there is a table with the number of the techniques, which were described above in the methods section (Table 3), and their corresponding images (Table 9). The combinations of techniques or ideas with the best results were technique number 1, 2 and 6. However, the most satisfactory was the technique number 6, as the growth rings were observed most clearly.

Table 9. Results of the combined techniques and ideas for the detection of growth rings. Technique from table 3.

Technique nº	
1	
In this case, we could see some growth rings, which are a little bit diffuse. They are not all over the edge of the genital plates equally, but this could be a useful method to implement in a future study.	
2	
With this technique, we could see some growth rings, which are a little bit diffuse, and they are not all over the edge of the genital plates equally, but this could be a good method to implement in a future study.	

3	
With this methodology, we did not observe any growth zones.	
4	
In this case, we could not observe any growth rings.	

5	
With this technique, we did not observe any growth rings.	
6	
In this case, we could see some growth rings, between 2 and 3, which would be equivalent to about 2 or 3 years. This growth rings are a little bit diffuse, and they are not all over the edge of the genital plates equally, but this could be a good method to implement in a future study.	

7	
With this methodology, we could not find any growth rings.	
8	
In this case, we did not find any growth zones.	

9	
With this methodology, we did not find any growth rings.	

3.5. Mineralogy of the skeletal elements by X-ray diffraction analysis

The percentage of $MgCO_3$ in the calcified structures not differed among sites (Table 10). However, there was a slight increase of the % of Mg in calcite in the sea urchins of "Vent" site in the test and lantern (Table 11).

Table 10. PERMANOVAs of %Mg and carbonates from the calcareous skeletal elements of *Arbacia lixula* (lantern, spine and test), using X-Ray diffraction of the factor Site" with three levels: "Control", "Transition" and "Vent".

%Mg					
Lantern					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	0,0018214	0,00091072	20,202	0,221
Res	15	0,0067622	0,00045081		
Total	17	0,0085836			
Spine					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	4.27E-02	2.13E-02	0,75	0,7406
Res	12	3.41E-01	2.84E-02		
Total	14	3,84E-05			
Test					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	0,0013022	0,00065108	13,545	0,2935

Res	14	0,0067294	0,00048067		
Total	16	0,0080315			
Carbonates					
Lantern					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	0,011821	0,0059107	35,709	0,0183
Res	15	0,024829	0,0016553		
Total	17	0,03665			
Spine					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	4.27E-02	2.13E-02	0,75	0,7316
Res	12	3.41E-01	2.84E-02		
Total	14	3,84E-05			
Test					
Source	df	SS	MS	Pseudo-f	P (perm)
Site	2	0,0013022	0,00065108	13,545	0,2985
Res	14	0,0067294	0,00048067		
Total	16	0,0080315			

Table 11. Composition of %Mg and carbonates from the calcareous skeletal elements of *Arbacia lixula* (lantern, spine and test), using X-Ray diffraction on each "Site": "Control", "Transition" and "Vent".

	Test		**Spine**		**Lantern**	
	%Mg	Carbonates	%Mg	Carbonates	%Mg	Carbonates
Control	8.1	99.65	6.4	100	7.5	99.93
Transition	7	99.94	6.3	98.88	8	99.92
Vent	**9.2**	99.27	6.3	98.92	**9.8**	99.98

5. DISCUSSION

The results of this study highlight the changes at the organismal level in *Arbacia lixula* due to lifetime exposure to the natural acidification of CO_2 vent of Fuencaliente (La Palma Island). With respect to morphometry, individuals of *A. lixula* living under natural acidification on the Fuencaliente coast are smaller and have thinner spines. That is, the morphometric variables studied in *A. lixula* from the Vent (pH 7.6) differ significantly from individuals from the Control (pH 8.1) and Transition (pH 7.8) sites. Furthermore, these differences are mainly based on diameter, which was the variable that best represents the size of the sea urchin, and thickness spines. In term of test size frequency, there were differences between the "Vent" and the other studied sites, with sea urchins from the acidified zone being slightly smaller in the test size. In addition, Aristotle's lantern allometry observations show that the jaws of *A. lixula* living under natural acidification in Fuencaliente are smaller with respect to their diameter. On the other hand, and in discordance with other authors, the fracture force required to break the sea urchin test was higher at 7.6 pH units, compared to control specimens. With respect to the growth study, the most useful techniques were Moore, 1935 and Schuhbauer, 2010; with three other combinations of techniques and ideas, that also worked (techniques 1, 2 and 6). Finally, mineralogical data show that there were no significant differences in calcified structures in terms of %MgCO3 between acidified and non-acidified sites.

A similar reduction in mean test size of individuals exposed to extreme pH conditions has been observe in *Psammechinus miliaris*, who also showed structural dissolution of its magnesian calcite test (Miles et al., 2007). Acidified conditions also affect *Diadema africanum* which showed damage to their external skeletal structure, such us dissolved spines and more fragile test which make them more vulnerable to predation (Rodriguez et al., 2017). Shirayama and Thornton (2005), as well as Byrne et al. (2014), have also reported that acidification affects the test plate thickness in *Hemicentrotus pulcherrimus*, *Echinometra mathaei* and T*ripneustes gratilla*, respectively. However, Asnaghi et al. (2013; 2014) found no effect on the test diameter or thickness in *Paracentrotus lividus* exposed to low pH values (pH_T 7.7). In contrast, differences in test size and robustness were dependent on food source, with growth of urchins fed with *Corallina elongata* being significantly greater than urchins fed with other non-calcareous species (Asnaghi et al., 2013; 2014). Therefore, to unravel the effects of acidification in

the near future, we must take into account the diet of organisms. Furthermore, differences between species of sea urchins are an example of how OA exerts different adaptive pressures on different species (Rodriguez et al., 2017).

The spines are the skeletal elements most vulnerable to OA due to their exposed position, but their regeneration abilities can ensure some maintenance under extreme conditions (Dubois, 2014). This regeneration capacity indicates the importance of these calcareous structures for sea urchins, as it is involved, for example, in their motility and their ability to protect themselves against predators. However, the thinner spines of *A. lixula* living at pH 7.6 indicate that individuals living under constantly acidifying conditions are unable to fully compensate for the high perturbation of protons that dissolve the calcareous structures. Thinner and more brittle spines at pH 7.4 and 7.7 have also been observed in other sea urchin species, such as *Lytechinus variegatus* (Emerson et al., 2017), *Tripneustes ventricosus* (Holtman et al., 2013), *Strongylocentrotus droebachiensis* (Dery et al., 2017) and *Eucidaris tribuloides* (Ries, 2011). Although there are still other much more resistant species, where no changes have been seen in their spines, such as *Prionocidaris baculosa* (Dery et al., 2017).

In CO_2 seeps, it is known that *A. lixula* and *P. lividus* can regulate the acid–base balance of their coelomic fluid (Calosi et al., 2013a; Collard et al., 2016; Migliaccio et al., 2019), although this is expected to have an energetic cost, inducing changes in resource allocation (Dubois, 2014). This energetic cost may explain the decrease in test size and spine thickness of *A. lixula* at CO_2 vent of La Palma.

The lantern is an indispensable feeding tool for sea urchins to grasp and chew food and hold onto the substrate (De Ridder and Lawrence, 1982). Therefore, a morphological modification of this may be an indicator of the effect on the sea urchin's welfare in the face of environmental changes (Brothers and McClintock, 2015). The relationship between jaw length and test diameter is associated with food availability. Sea urchins in habitats containing limited food possess relatively larger jaws than conspecifics in habitats with abundant food (Ebert, 1980; Black et al., 1984; Pederson and Johnson, 2007). This allometric relationship is also different between species (Contreras and Castilla, 1987; Hagen, 2008). The functional significance of jaw size is related to the adaptation of Aristotle's lantern for increased feeding capacity in resource-limited

habitats, such as barrens (Black et al., 1984; Pederson and Johnson, 2007), or for durophagous capability (Hagen, 2008). In our work, the jaws of *A. lixula* living at pH 7.6 are slightly smaller with respect to their diameter. No previous studies have observed the effect of OA on the allometric relationship of Aristotle's lantern and test size of sea urchins subjected to long-term natural or artificial acidification. Only Asnaghi et al. (2013) observed that the change in jaw size was due to a change in their diet and not with the pH treatments (exposure to low pHs) performed during the experiments.

Individuals of *A. lixula* living under natural acidification at Fuencaliente seeps may have modified their test-lantern relationship to compensate for the change in their diet. It is important to highlight that in the acidified system of La Palma, the calcareous communities are replaced by fleshy macroalgal and soft invertebrate communities (González-Delgado et al., in prep). Therefore, it is very likely that the change in the benthic community composition has affected the feeding habits of this sea urchin species and therefore, indirectly, the allometric relationship of jaw-test diameter. In our case, it seems that smaller jaws in relation to their test size are enough to feed upon soft macroalgae and invertebrate communities, and that urchins are not food limited.

Unexpectedly, in this study, we have found stronger *A. lixula* tests on acidified sites compared to control. In other words, at 7.6 pH the sea urchins have stronger test. These results are not in accordance with those obtained by other authors. Most studies where sea urchins have been exposed to low pHs, in a relatively short period of time (months), have obtain significantly lower fracture strength or test thinness, as in the case of *Tripneustes* spp. (Byrne et al., 2014; Dery et al., 2017), *P. lividus* (Shirayama and Thornton, 2005; Asnaghi et al., 2013; Rodriguez et al., 2017*), L. variegatus* (Emerson et al., 2017) and *D. africanum* (Rodriguez et al., 2017). This discordance may be due to an adaptation of the sea urchins living at vents. For example, research conducted in intertidal pools exposed to natural acidification at the Vulcano CO_2 vent (Italy) did not observe effects on the test robustness on *P. lividus* living there (Collard et al., 2016). In the case of *Arbacia lixula* from CO_2 vent of La Palma, they had a long exposure to low pHs, which is estimated to be 2-3 year according to their size distribution (Figure 3) and the previous estimates of the growth curve by sizes of *A. lixula* (Miguel-Barrera, 2018). So, an important question remains unanswered, are the sea urchin's living at vents growing slower? If they do so, their test may be stronger because of this, the sutures between plates

are less plastic than fast growing individuals which would make their test stronger (Ellers et al., 1998; Hernández et al., 2010). Therefore, sea urchins living under acidification in natural environments, may experience reduced growth rates and show modified resource allocation (Edwards and Ebert, 1991). This leads to increased maintenance of the skeleton and thickening, as was shown for sea urchins living in wave exposed environments compared with those living in protected areas (Ebert, 1975). So, urchins living at vents would have stronger test due to a slower growth rate. However, the jaw-test diameter allometric results have shown that the sea urchins living at vents are not food limited so they have enough energy to have normal growth rates. Therefore, stronger test could be a consequence of accelerating metabolism to compensate internal pHs (Calosi et al., 2013a), which may promote the precipitation of calcite.

Despite all the twenty-one techniques tested and collected in our study to estimate the growth or age of *A. lixula* living in the vent of La Palma, the most useful ones have been Moore, 1935 and Schuhbauer, 2010. In addition, from the combination of techniques and ideas, three of them also worked (technique 1, 2 and 6). Therefore, if we consider the efficiency of the techniques used, we can say that the best technique was Schuhbauer, 2010 as it has a relatively simple procedure in which it is not necessary to use products that are harmful to humans, such as xylol. Furthermore, from the combinations of techniques or ideas, the most efficient one was number 2, as its methodology is very simple, and it is not necessary to use products that are harmful to humans. So, it would be interesting to repeat these methods in a future study to estimate growth. The formation of growth zones in echinoid skeletal plates is mainly the result of structural features that lead to the formation of opaque (fast) and translucent (slow) growth zones (Pearse and Pearse, 1975). The formation of growth zones in sea urchin skeletal structures and their interpretation to determine age structure and growth rates is a technique that has been applied to the study of numerous marine echinoderm species (Moore, 1935; Gage and Tyler, 1985; Gage, 1991; Gage, 1992; Ebert & Russell, 1992; Gebauer and Moreno, 1995; Schuhbauer et al., 2010; Fagerli et al., 2015). Of these skeletal structures, genital plate are the zones most used and validated (directly and indirectly) for age determination in sea urchins (e.g. Moore, 1935; Dix, 1972; Walker, 1981; Gage, 1992; Gebauer and Moreno, 1995; Agastsuma and Nakata, 2004; Ouréns et al., 2013). These plates are present at metamorphosis and remain throughout the life cycle of the individual (Gordon, 1926; Arrau, 1958; Dix, 1972). The formation of growth zones has been associated with

seasonal variability in food availability, temperature and water salinity (Moore, 1935; Ebert, 1968; Gage, 1992; Agastsuma and Nakata, 2004; Narvaez et al., 2016) and, in some cases, with reproductive activity (Pearse and Pearse, 1975). It is therefore ideal for estimating the growth of sea urchins living under natural acidification. Even so, age determination by reading growth rings is not completely accurate, ranging from 90% to 73% correct determination (Gebauer and Moreno, 1995; Schuhbauer et al., 2010; Balboa, 2018) and more accurate techniques using chemical tagging have already been developed (Ebert, 2020). Therefore, caution should be exercised as the use of inaccurate age determination may result in an incorrect estimation of growth, affecting the results of age-based models and/or sea urchin size (Balboa, 2018).

In our study, the % $MgCO_3$ in calcified structures of *A. lixula* did not differ between acidified and non-acidified sites. High-magnesium calcite is the main calcium carbonate mineral formed by adult sea urchins and one of the most soluble forms of calcite (Morse et al., 2006). Considering this, it is the Mg content that increases the solubility of Mg-calcite, and therefore, that causes many echinoderms species to show structural dissolution under acidification (Miles et al., 2007; Asnaghi et al., 2014) Even so, sea urchins use their physiology in order to adapt to different environmental conditions, which means that the Mg content of calcite alone is not a valid indicator for assessing the effects of ocean acidification (Dubois, 2014), because these effects will vary between species. This is because sea urchins exhibit a range of ion precipitation and calcification rate responses when exposed to low pH environments, which varies between populations and even between species (Ries, 2011; Byrne et al., 2013; Courtney et al., 2013; Pespeni et al., 2013).

Overall, the effect of OA on the sea urchin skeleton may compromise its protective roles (Byrne & Hernández, 2020). However, changes to the three-dimensional organization of the skeletal pores and trabeculae as well as the elasticity of plate ligaments, are also likely to have impacts on organismal function beyond the skeleton itself (Ellers et al., 1998; Byrne & Hernández, 2020). Hence, the properties of the test of sea urchins living in naturally acidified environments, such as *A. lixula*, need to be further investigated in order to know at what level the effect of OA is also modifying its relationships with other organisms and the ecosystems functioning.

6. CONCLUSIONS

1. The chemical characteristics of the CO_2 vent of La Palma are low pH, decreased carbonate ion concentration (CO_3^{2-}) and low levels of aragonite and calcite. Therefore, fine-scale studies are needed to understand the total repercussion of cean Acidification on the organisms and communities at a local scale. The presence of *Arbacia lixula* populations across the pH gradients generated by the CO_2 vents makes it as a good model species.

2. Individuals of *Arbacia lixula* from the CO_2 vent of Fuencaliente are smaller and have thinner spines. In CO_2 vents, this sea urchin species can efficiently regulate the acid-balance of their coelomic fluid but with an energetic cost which may explain the decrease in body size and spine length of *A. lixula* at CO_2 vent of La Palma.

3. The jaws of *Arbacia lixula* from the Vent site are slightly smaller with respect to their size. This could be due to a change in their diet because of the contrasting benthic community found at the Vent site compared with the Control site, which is mainly composed by fleshy algae and soft invertebrates.

4. Sea urchins test from the acidified environments have been found to be stronger than those from the control areas. Although a slower growth rate may be the cause of this contrasting results the growth rings of collected individuals could not be studied yet.

5. Regarding the growth of *Arbacia lixula*, of all the techniques tested, the best technique is Schuhbauer, 2010 as it has a simple procedure in which it is not necessary to use products that are harmful to humans, such as xylol. Furthermore, from the combinations of techniques or ideas, the most efficient one is number 2, as its methodology is also very simple, and the products used are not harmful to humans.

6. The percentage of $MgCO_3$ in the calcified structures not differed among sites.

7. ACKNOWLEDGEMENTS

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