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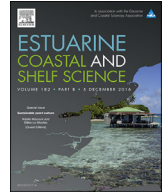
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# Impact of $p\text{CO}_2$ on the energy, reproduction and growth of the shell of the pearl oyster *Pinctada margaritifera*



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## ABSTRACT

The possible consequences of acidification on pearl farming are disruption of oyster metabolism and change in growth. In the laboratory, we studied the impact of  $p\text{CO}_2$  (3540, 1338 and 541  $\mu\text{atm}$ ) on the physiology of pearl oysters exposed for 100 days. This experiment was repeated after an interval of one year. Several physiological compartments were examined in pearl oysters: the scope for growth by measuring ingestion, assimilation and oxygen consumption, gametogenesis by means of histological observations, shell growth by measurement and observation by optical and electronic microscopy, and at molecular level by measuring the expression of nine genes of mantle cells implied in the biomineralisation process. Results from both experiments showed that high  $p\text{CO}_2$  had no effect on scope for growth and gametogenesis. High  $p\text{CO}_2$  (3540  $\mu\text{atm}$ ) significantly slowed down the shell deposit rate at the ventral side and SEM observations of the inside of the shell found signs of chemical dissolution. Of the nine examined genes high  $p\text{CO}_2$  significantly decreased the expression level of one gene (*Pmarg*-PUSP 6). This study showed that shell growth of the pearl oyster would be slowed down without threatening the species since the management of energy and reproduction functions appeared to be preserved. Further investigations should be conducted on the response of offspring to acidification.

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## 1. Introduction

Global climate change is a major concern caused by mainly two factors, temperature and the atmospheric  $\text{CO}_2$  level. In the marine world, this translates into the elevation of the temperature of the oceans and a tendency to acidification of sea water. Some marine ecosystems may suffer from this global change, including the tropical coral reef ecosystems and all the communities who live there. It is accepted that the pH in the global ocean has already fallen by 0.1 units and is likely to fall a further 0.3 units by 2050 and 0.5 units by 2100 (Caldeira and Wickett, 2005; Orr et al., 2005). Some recent studies suggest that ocean acidification would directly affect the population of calcifiers (Kurihara and Ishimatsu, 2008) and have negative impacts on invertebrate reproduction (Siikavuopio et al., 2007; Kurihara et al., 2008). The potential effects

of the decline in pH, however, on marine organisms and ecosystems are disturbances affecting growth (Berge et al., 2006), calcification (Ross et al., 2011; Gazeau et al., 2013) and metabolic rates (Thomsen and Melzner, 2010; Fernández-Reiriz et al., 2011; Wang et al., 2015). Nevertheless, some species are positively affected by high  $\text{CO}_2$  such as the sea urchin *Echinometra* sp., and show strong resistance to high  $p\text{CO}_2$  after about one year exposure (Hazan et al., 2014). Similarly the brittlestar *Amphiura filiformis* shows an increase in metabolism and calcification when exposed to pH 7.3 (Wood et al., 2008). In the mussel *Mytilus galloprovincialis* exposed to high  $p\text{CO}_2$ , the scope for growth is better, promoting reproduction; this is due to better absorption efficiency and a lower ammonium excretion rate (Fernández-Reiriz et al., 2012). Lastly, the metabolic rate of the wild oyster *Saccostrea glomerata* is not impacted by low  $p\text{CO}_2$  (Parker et al., 2012). This literature review shows that the responses of organisms can be very different. The present challenge is to understand the potential impact of acidification of the aquatic environment on the physiology of the pearl oyster, which is a

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valuable resource in French Polynesia. Pearl culture there depends on the exploitation of a single species, the pearl oyster *Pinctada margaritifera*, and relies entirely on the supply of wild juveniles collected on artificial substrates (Thomas et al., 2012). Cultured pearls are the product of grafting *P. margaritifera* and then rearing these oysters in their natural environment (Cochennec-Laureau et al., 2010). Considering that the pearl oyster *P. margaritifera* is an emblematic bivalve of the South Pacific atoll, especially French Polynesia, it is important to assess if climatic stressors impact its physiology, in terms of energy management and biomineralization process. Scope for growth (SFG) is a physiological index commonly used in the strategy of energy management (Bayne and Newell, 1983). Acquisition of energy in bivalves is described by the ingestion rate (IR), the concentration of microalgae being used as a marker (Yukihira et al., 1998) which is a saturating function of microalgae concentration in *P. margaritifera* (Le Moullac et al., 2013). Assimilation efficiency (AE) can then be derived by considering the residual organic matter content in the animal's faeces and pseudofaeces. Assimilation of organic matter by a bivalve varies according to the quantity and quality of suspended particulate matter (Saraiva et al., 2011). Energy losses involve oxygen consumption and excretion (Pouvreau et al., 2000) and are mainly related to temperature and food level (Chavez-Villalba et al., 2013). The scope for growth (SFG), resulting in energy gained or lost, is the difference between the energy acquired by feeding and that lost by respiration and excretion (Pouvreau et al., 2000). So, knowing the impact of  $p\text{CO}_2$  on energy management could help us to evaluate the threshold of risk for survival of the species.

The proper functioning of the process of biomineralisation is a challenge in terms of growing pearl oysters and pearl culture. Mantle edge cells are the headquarters of the molecular processes involved in the production of calcite and aragonite (Joubert et al., 2010; Kinoshita et al., 2011). The molecular processes that control shell growth are subject to environmental conditions. Joubert et al. (2014) have shown that the deposition rate of nacre at the ventral edge of the shells of *P. margaritifera* depends on environmental conditions and some genes are specifically regulated by the level of food whereas others are controlled by the seawater temperature. Previous studies show that acidification could deregulate the expression of some genes associated with calcification (Liu et al., 2012). The question is how these molecular deregulations will impact on the biomineralisation of shell structures.

We studied the impact of acidification (pH 7.8 and 7.4) compared with an actual pH of 8.2 on the physiology of pearl oysters at a bioenergetic level by measuring metabolic flux, reproduction and shell biomineralisation at microscopic, microstructural and molecular levels. The effects of these changes will be studied across the organism (individuals, population) by means of an experimental approach designed to simulate environmental conditions.

## 2. Material and methods

### 2.1. Ethical statement

The authorisation (No. 542) for pearl oysters' translocation from Takaroa atoll (14°26'59.12"S, 144°58'19.91"W, Tuamotu Archipelago, French Polynesia) to the lagoon of Vairao (Ifremer marine concession No. 8120/MLD: 17°48'26.0"S, 149°18'14.4"W, Tahiti, French Polynesia) was issued by the Ministry of Marine Resources on 2 February 2012. After collection from Takaroa atoll, 400 pearl oysters of an average height of 80 mm were packed in isothermal boxes for shipment (by air). Upon their arrival at Vairao, pearl oysters were immersed for 30 min in a hyper-saline water bath

(Salinity 120) following the prophylactic recommendations supplied with the transfer authorisation. Then, the pearl oysters were stored in the lagoon of Vairao for four months to enable complete physiological recovery and to avoid any bias caused by the shipment and/or the hyper-saline water treatment. This study did not involve protected or endangered species.

### 2.2. Experimental design

Three different pH levels, 8.2, 7.8 and 7.4, were maintained for 100 days by means of the experimental system described below. This experiment was conducted twice, first from 24 May 2012 and then, after the interval of one year, from 17 June 2013, with different pearl oysters of the same set. The individuals used had an average height of  $98.1 \pm 6.5$  mm and  $117 \pm 12.6$  mm for the first and the second experiment, respectively. In total, and for each experiment, 60 oysters were randomly distributed in the three tanks. In order to measure the impact of each treatment on the oysters' growth rate their shells were marked with calcein (Sigma Aldrich, France) one day before the beginning of each experiment. The stain powder was dissolved over 12 h at 24 °C in filtered seawater (0.1 µm) with a magnetic stirrer. Pearl oyster shells were marked by immersion of the pearl oysters in 150 mg L<sup>-1</sup> calcein solution for 12 h, as described in Linard et al. (2011).

### 2.3. Rearing system and pH control

The rearing system was set up in an experimental bivalve hatchery operated by Ifremer in Vairao, Tahiti, French Polynesia. The facility is supplied with filtered (25 µm) seawater from the Vairao lagoon. The pearl oysters were placed in 500-L tanks with controlled flow-through. Seawater was renewed at the rate of 100 L h<sup>-1</sup> in all the experiments. The pearl oysters were fed with microalgae *Isochrysis galbana* (T-Iso) supplied continuously by means of Blackstone dosing pumps (Hanna). A constant concentration of 25,000 cell mL<sup>-1</sup> was maintained throughout the experiments. Temperature and algae concentration were controlled continuously by a fluorescent probe (Seapoint Sensor Inc.) and a temperature sensor (PT 100). The pH was manipulated in flow-through tanks by bubbling CO<sub>2</sub> until the target pH was reached. This was operated by pH electrodes and temperature sensors connected to a pH-stat system (Dennerle) that continuously monitored pH (calibrated to the NIST scale).

### 2.4. Carbonate chemistry

Total alkalinity (TA) was measured weekly via titration with 0.01 N HCl containing 40.7 g NaCl L<sup>-1</sup> and using a titrator (Schott Titroline Easy). Parameters of carbonate seawater chemistry were calculated from pH, mean TA, temperature, and salinity with the free access CO<sub>2</sub> Systat package. Average  $p\text{CO}_2$  corresponding to pH 7.4, 7.8 and 8.2 was respectively of 3540, 1338 and 514 µatm. Other parameters of carbonate seawater chemistry are given in Table 1.

### 2.5. Bioenergetic measurements

During the last 48 h of each treatment, the pearl oysters were placed on biodeposit collectors to quantify the assimilation of organic matter. Once the exposures were finished, four oysters from each treatment were transferred to the ecophysiological measurement system (EMS), where they were individually placed in a metabolic chamber to monitor clearance rate and oxygen consumption. The EMS consists of five open-flow chambers. For each treatment, each of the four oysters was placed, successively, in one of the chambers and the fifth chamber remained empty for use as a

**Table 1**  
Water parameters calculated from samples taken from all experimental trays across the experimental period. Measurements were made once a week. The carbonate parameters were calculated using CO2sys software.

pH	Temperature (°C)	Salinity	Alcalinity (μmol/kg)	pCO <sub>2</sub> (μatm)	Ωca.	Ωar.
7.4	26.1 ± 0.6	35	2768 ± 231	3540 ± 402	1.32 ± 0.20	0.88 ± 0.13
7.8	26.2 ± 0.7	35	2753 ± 77	1338 ± 172	3.03 ± 0.26	2.01 ± 0.17
8.2	26.1 ± 0.7	35	2673 ± 198	514 ± 67	5.74 ± 0.57	3.80 ± 0.37

control (Chavez-Villalba et al., 2013). The experimental conditions applied during the adaptation period were replicated in the EMS during measurements.

Ingestion rate, an indicator of feeding activity, is defined as the quantity of microalgae cleared per unit of time. Ingestion rate (IR) was estimated by means of fluorescence measurements and calculated as:  $IR = V(C1 - C2)$ , where C1 is the fluorescence level of the control chamber, C2 is the fluorescence of the experimental chamber containing an oyster, and V is the constant water flow rate (10 L h<sup>-1</sup>).

Respiration rate (RR mg O<sub>2</sub> h<sup>-1</sup>) was calculated from differences in oxygen concentration between the control and experimental chambers where by  $RR = V(O1 - O2)$ , where O1 is the oxygen concentration in the control chamber, O2 is the oxygen concentration in the experimental chamber, and V is the water flow rate.

To compare ingestion and respiration rates, it was necessary to correct for differences in specimen weight. Values of the ecophysiological activities were converted to a standard animal basis (1 g, dry weight) by using the formula  $Ys = (Ws/We)b \times Ye$ , where Ys is the physiological activity of a standard oyster, Ws is the dry weight of a standard oyster (1 g), We is the dry weight of the specimen, Ye is the measured physiological activity, and b is the allometric coefficient of a given activity. The average b allometric coefficients were 0.66 for ingestion rate and 0.75 for oxygen consumption rate (Savina and Pouvreau, 2004).

Assimilation efficiency (AE) of organic matter was assessed by analysing microalgae, faeces and pseudofaeces according to Conover's method (1966). The pearl oysters were laid out in a collector, in which the deposits were collected on a 10-μm sieve. Biodeposits were centrifuged for 15 min at 4500 t min<sup>-1</sup>. The supernatant was removed and the pellet was washed twice with ammonium formate (37g L<sup>-1</sup> in distilled water). The pellet was then put in a pre-weighed aluminium cup to be dried at 60 °C for 48 h before being burnt at 450 °C for 4 h. Microalgae OM was obtained by the centrifugation of 5 L of the microalgae mixture and treatment of the pellet according to the same procedure as for the biodeposits. The absorption efficiency (AE) was then calculated according to the following equation:

$$AE = \frac{\%OM_{\mu\text{alg}} - \%OM_{\text{biodeposit}}}{(100 - \%OM_{\text{biodeposit}}) \times \%OM_{\mu\text{alg}}}$$

Ecophysiological data were converted into energetic values to define the scope for growth (SFG) for each oyster:  $SFG = (IR \times AE) - RR$ , where IR is the ingestion rate, AE is the assimilation efficiency, and RR is oxygen consumption. We used 20.3 J for 1 mg of particulate organic matter (Bayne et al., 1987) and 14.1 J for 1 mg O<sub>2</sub> (Bayne and Newell, 1983; Gnaiger, 1983).

## 2.6. Gonad histological analysis

Gonad development stages were determined by histological methods and samples were classified according to the different categories of gonadic tissues previously described in Teaniniuraitemoana et al. (2014). In order to simplify analysis, certain stages were grouped according to the dynamic of

gametogenesis. Gonads in early, intermediate and mature stages were grouped in one group, and gonads in regression and undetermined constituted the two other groups.

## 2.7. Shell growth rate and nacre microstructure

To investigate shell growth, the shells were sawn with a 'Swap Top' Trim Saw machine (Inland, Middlesex, UK), which included a diamond Trim Saw Blade (Thin Cut) IC-40961. Shell edges were then polished for 5 s with various grades of water sandpaper sheets. The shell sections were then examined under a Leitz Dialux 22 compound fluorescence microscope equipped with a I3-filter block and an optical micrometer. Shell growth was measured by evaluating the thickness of deposits at the ventral side of the shell and the calcein marks with an optical micrometer (Linard et al., 2011). Shell deposit rate (SDR) was calculated by dividing the thickness of deposits by the time which had elapsed since the marking. SDR was expressed in μm.d<sup>-1</sup> (Linard et al., 2011; Joubert et al., 2014).

Electron Microscopy was performed on the electron microscopy platform (Université de la Polynésie Française). The structure of the shell deposit was observed by scanning electron microscopy (SEM) with a Hitachi Analytical Table Top SEM TM3030. The aragonitic tablets and the growing edge of nacre lining the shells were examined. Before observation, the sawn shells were treated by formic acid (1%), sonicated and dried. Observations relied on pictures, taken at the internal side of the shell (magnification 9000, accelerating voltage 15 KV). The thickness of aragonitic tablets was measured with post-acquisition image tools.

## 2.8. Gene expression in mantle

At the end of the 100 days exposure at different pCO<sub>2</sub>, mantle samples from each pearl oyster were withdrawn and grouped randomly in fours, thus constituting five pools to limit the variability of individual responses for gene expression. Total RNA was extracted from each sample with TRIZOL<sup>®</sup> Reagent (Life Technologies) according to the manufacturer's recommendations. RNA was quantified with a NanoDrop<sup>®</sup> ND-1000 spectrophotometer (NanoDrop<sup>®</sup> Technologies Inc.); 3000 ng of total RNA were treated for each sample with DNase (Ambion) to degrade any potentially contaminating DNA in the samples. First-strand cDNA was synthesised from 500 ng of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche), using 2 μL of anchored-oligo(dT) and 1 μL of random hexamer primers. The expression levels of nine genes were analysed by quantitative RT-PCR analysis with a set of forward and reverse primers (Table 2). Three genes, commonly used as reference genes for comparisons of gene expression data, were chosen because of their ubiquitous and constitutive expression pattern: 18S rRNA gene (Larsen et al., 2005), GAPDH (Dheilly et al., 2011) and SAGE. Quantitative-RT-PCR amplifications were carried out on a Stratagene MX3000P (Agilent Technologies), using 12.5 μL of Brilliant II SYBR<sup>®</sup> Green QPCR Master Mix (Stratagene) with 400 nM of each primer and 10 μL of 1:100 cDNA template. The following amplification protocol was used: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and extension at 72 °C for

**Table 2**

Set of forward and reverse primers used for the gene expression analysis (\*SRA accession number of EST library published in Joubert et al., 2010).

Gene	GenBank Accession number	Forward primer	Reverse primer
<i>Pmarg</i> -PIF 177	HE610401	5'-AGATTGAGGGCATAGCATGG-3'	5'-TGAGGCCGACTTCTTGG-3'
<i>Pmarg</i> -Pearlin	DQ665305	5'-TACCGGCTGTGTGCTACTG-3'	5'-CACAGGGTGAATATCTGGAACC-3'
<i>Pmarg</i> -MRNP34	HQ625028	5'-GTATGATGGGAGGCTTTGGA-3'	5'-TTGTGCGTACAGCTGAGGAG-3'
<i>Pmarg</i> -MSI60	SRX022139*	5'-TCAAGAGCAATGGTGTAGG-3'	5'-GCAGAGCCCTTCAATAGACC-3'
<i>Pmarg</i> -Shematrin 9	ABO92761	5'-TGGTGGCGTAAGTACAGGTG-3'	5'-GGAACCTAAGGCACGTCAC-3'
<i>Pmarg</i> -Prismalin 14	HE610393	5'-CCGATACTCCCTATCTACAATCG-3'	5'-CCTCCATAACCGAAAATTGG-3'
<i>Pmarg</i> -PUSP6	SRX022139*	5'-TTCATTTTGGTGGTATGGAATG-3'	5'-CCGTTCCACCTCCGTTAC-3'
<i>Pmarg</i> -Aspein	SRX022139*	5'-TGGAGGTGGAGGTATCGTTC-3'	5'-ACACCTGATACCTGCTTGG-3'
<i>Pmarg</i> -Nacrein A1	HQ654770	5'-CTCCATGCACAGACATGACC-3'	5'-GCCAGTAATACGGACCTTGG-3'

1 min. Lastly, to verify the specificity of the product, a melting curve analysis was performed from 55 to 95 °C increasing by 0.5 °C. All q-RT-PCR reactions were duplicated. The comparative Ct (threshold cycle) method was used to analyse the expression levels of the candidate genes. The relative expression ratio of each analysed cDNA was based on the delta-delta method normalised with three reference genes for comparing the relative expression results, which is defined as:  $\text{ratio} = 2^{-[\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{calibrator}}]} = 2^{-\Delta\Delta\text{Ct}}$  (Livak and Schmittgen, 2001). Here the  $\Delta\text{Ct}$  calibrator represented the mean of the  $\Delta\text{Ct}$  values obtained for all tested genes in all conditions.

### 2.9. Statistical analysis

Normality of data distribution and homogeneity of variance were tested with the Shapiro-Wilk test and the Bartlett test, respectively. Respiration rate data followed the conditions of application of parametric tests, but Ingestion rate and SFG data were subjected to the Box Cox transformation to satisfy these conditions. AE was analysed by using arcsine square root AE/100 values. Comparison of energy values and SDR after 100 days of exposure to different  $p\text{CO}_2$  levels was done by using a two-way ANOVA where factors were the experimental series (one-year interval between the two experiments) and the  $p\text{CO}_2$  level. Post hoc comparison was done with the unilateral test of Dunnett using the lowest  $p\text{CO}_2$  levels as a control for comparison with the highest one.  $\alpha$  was set at 0.05 for all analyses. Impact of  $p\text{CO}_2$  levels on gametogenesis was analysed with the Chi-square test. The expression values of the nine candidate genes met the condition for parametric ANOVA after normalization with the BoxCox transformation. Post hoc comparison was done with the unilateral test of Dunnett using the lowest  $p\text{CO}_2$  levels as a control for comparison with the highest one  $\alpha$  was set at 0.05 for all analyses.

## 3. Results

### 3.1. Bioenergetics

The 100-day exposure to various  $p\text{CO}_2$  levels did not induce modifications of the bioenergetics descriptors (Table 3). The

assimilation efficiency (AE;  $F = 0.134$ ,  $p = 0.87$ ), ingestion rate (IR;  $F = 0.22$ ,  $p = 0.8$ ), respiration rate (RR;  $F = 1.35$ ,  $p = 0.26$ ) and SFG ( $F = 0.02$ ,  $p = 0.98$ ) were not significantly changed by any of the treatments in either of the two experiments (Fig. 1a–c). However, the comparison between the two experiments highlights that all bioenergetics descriptors increased significantly with oyster size; AE ( $F = 59.86$ ,  $p < 0.0001$ ), IR ( $F = 7.14$ ,  $p = 0.01$ ), RR ( $F = 7.56$ ,  $p = 0.01$ ) and SFG ( $F = 12.82$ ,  $p = 0.001$ ).

### 3.2. Reproduction

It was necessary to do groupings since the sample sizes for certain classes was insufficient to meet the conditions for the application of the Chi-square test. Therefore, the impact of  $p\text{CO}_2$  level on gametogenesis was analysed for three gonadic stages and the two experimental series were grouped (Fig. 2). With this data set, the Chi-square test did not show any significant effect of the  $p\text{CO}_2$  level on the gametogenic process ( $\chi^2 = 4.81$ ,  $p = 0.31$ ).

### 3.3. Shell integrity, growth and microstructural organisation

The external side of the shells whitened during the 100 days of exposure to acidification. Fig. 3 shows the three lots of pearl oysters grouped by level of  $p\text{CO}_2$  exposure. We observed a gradient in the bleaching of the outer surface of the shell depending on the level of exposure to  $p\text{CO}_2$ . The shells of pearl oysters exposed to  $p\text{CO}_2$  of 3540 atm had blanched considerably, and those exposed to  $p\text{CO}_2$  of 1338 atm were an intermediate shade.

Shell deposition rate (SDR) measurement was done with calcein marking in order to analyse the effect of environmental  $p\text{CO}_2$  level on shell growth. Two-way ANOVA showed a significant effect of  $p\text{CO}_2$  level on SDR ( $F = 3.208$ ,  $p = 0.045$ ) but also an age effect ( $F = 61.11$ ,  $p < 0.0001$ ). The unilateral test of Dunnett highlighted that the SDR decreased significantly between the control treatment and pH 7.4 (Fig. 4a). At microstructural scale, MEB observation and measurement showed that the thickness of aragonite tablets did not change with  $p\text{CO}_2$  level. The mean value of aragonite thickness tablets remained an average size of 0.4 nm (Fig. 4b).

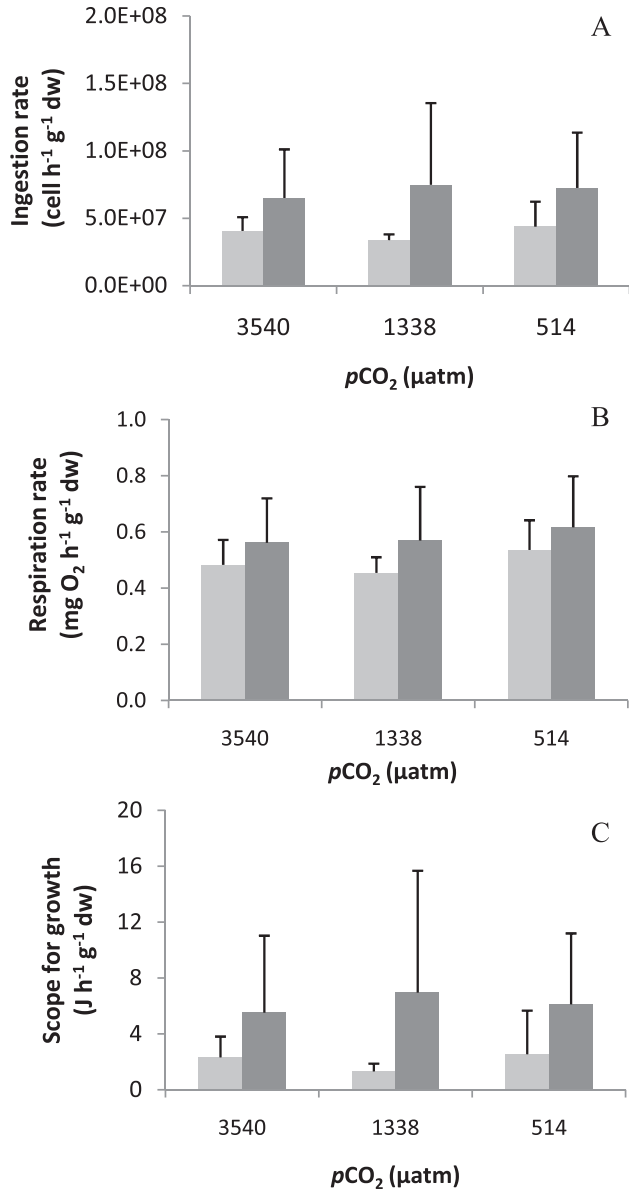
Observation of the growing edge of nacre lining the shells showed that shells exposed to acidified conditions ( $p\text{CO}_2$  of 1338

**Table 3**Two-way ANOVA results for bioenergetic values of 100 day exposure to the  $p\text{CO}_2$  level in two separated experiments at one year interval (absorption efficiency (AE), ingestion rate (IR), respiration rate (RR), scope for growth (SFG)).

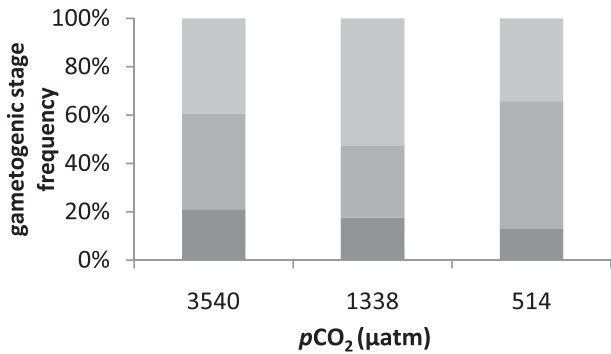
Sources of variation	ddl	AE (arcsinsqr)		IR (Box Cox)		RR		SFG (Box Cox)	
		F	p	F	p	F	p	F	p
$p\text{CO}_2$	2	0.13	0.87	0.22	0.80	1.35	0.26	0.02	0.98
Experiment	1	59.86	<b>&lt;0.0001</b>	7.14	<b>0.01</b>	7.56	<b>0.01</b>	12.82	<b>0.001</b>
$p\text{CO}_2 \times \text{experiment}$	2	1.32	0.27	0.13	0.88	0.13	0.88	0.35	0.70

The p values under 0.05 are in bold.

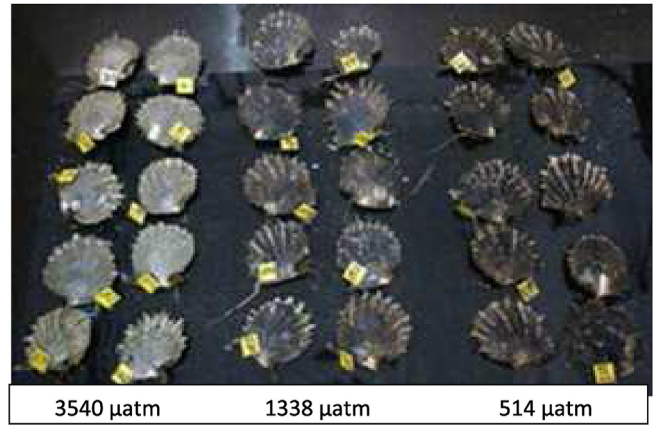




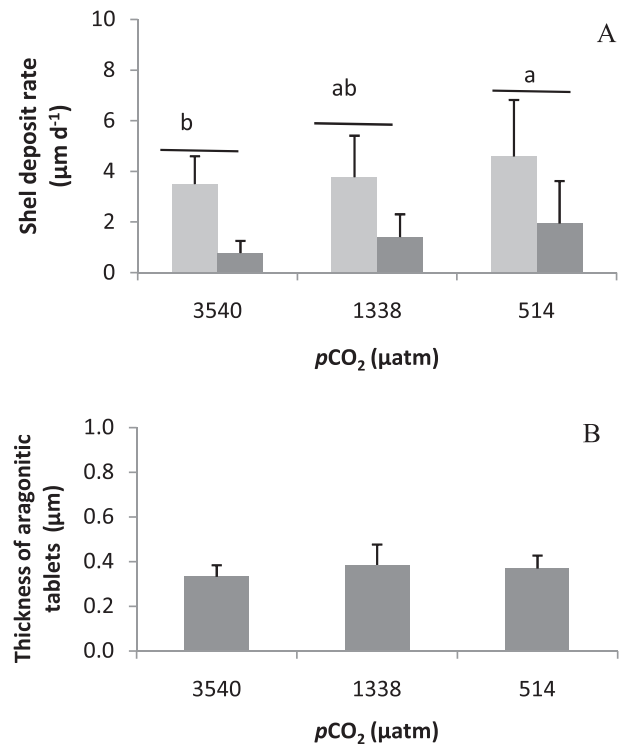
**Fig. 1.** Bioenergetic behavior after one hundred days exposure to pCO<sub>2</sub> level at one year interval (1st experiment (grey), 2nd experiment (dark grey)); (A) ingestion rate (IR), (B) respiration rate (RR) (C) scope for growth (SFG) of the black-lip pearl oyster *Pinctada margaritifera*. Means are presented with standard error (12 < n < 16).



**Fig. 2.** Effect of the pCO<sub>2</sub> level on gametogenesis of pearl oyster exposed for one hundred days (38 < n < 41).

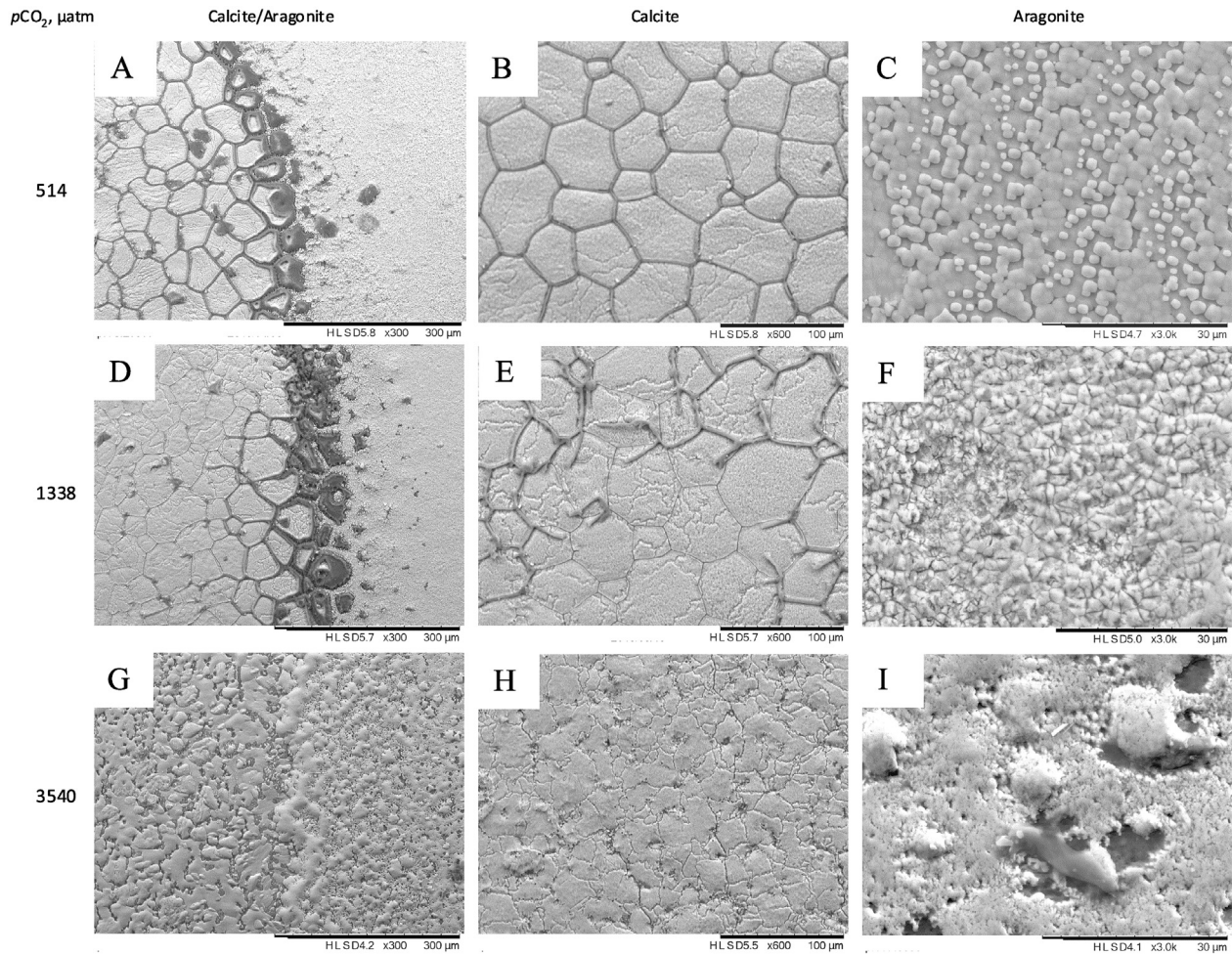


**Fig. 3.** Effect of the pCO<sub>2</sub> level on bleaching of external side of shells of pearl oyster exposed for one hundred days.



**Fig. 4.** Effect of the pCO<sub>2</sub> level on shell growth (A) shell deposit rate, (1st experiment (grey), 2nd experiment (dark grey)), (B) thickness of aragonite tablet, 2nd experiment. (n = 8–9).

and 3540 μatm) showed signs of malformation and/or dissolution compared with controls. This study found notable differences in the appearance of the growing edge of the nacreous layer of *P. margaritifera* kept at high pCO<sub>2</sub>. Oysters from the control (pCO<sub>2</sub> 514 μatm) had nacre that showed a distinct boundary between the fully formed and developing nacre tablets (Fig. 5a), the organic matrix was intact (Fig. 5b) and growth of nacre also showed a clear wave-like pattern and nacre tablets forming within an extensive organic matrix (Fig. 5c). Inspection of nacre from oysters held at pCO<sub>2</sub> of 1338 μatm still showed a distinct boundary between calcite and aragonite (Fig. 5d), although the organic matrix disappeared in places (Fig. 5e) and the wave-like pattern became anarchic (Fig. 5f). At pCO<sub>2</sub> of 3540 μatm the boundary was visible (Fig. 5g), but the



**Fig. 5.** SEM of growing edge of the nacre layer within shells of *Pinctada margaritifera* (A, D, G), calcite formation (B, E, H), aragonite (C, F, I) after one hundred days exposure to  $p\text{CO}_2$  level (514, 1338, 3540  $\mu\text{atm}$ ).

organic matrix disappeared completely (Fig. 5h), the aragonite tablets also disappeared leaving space for a kind of nacre where the shelves were merged (Fig. 5i).

### 3.4. Mantle gene expression

Among the nine candidate genes tested, the expression of eight of them was not modulated by the experimental treatments. The one affected was the *Pmarg*-PUSP6 gene. Its expression significantly decreased at the highest  $p\text{CO}_2$  level compared with the lowest (Table 4, Fig. 6).

## 4. Discussion

Bivalve growth is known to be strongly influenced by environmental conditions such as food supply and water temperature. The aim of this study was simultaneously to evaluate *P. margaritifera* bioenergetics and biomineralisation ability depending on  $p\text{CO}_2$  level. We acclimatised pearl oysters for 100 days at three levels of  $p\text{CO}_2$  in two identical experiments with a one-year interval between them. We determined that acidification does not impact on the pearl oyster at the energy management level; hence gametogenesis is not affected. Our observations showed that the shell growth slowdown could result from active chemical dissolution of shell and/or from a deregulation of some genes since we found that

the functioning of one of them altered amongst the nine tested.

### 4.1. $p\text{CO}_2$ did not influence energy management and reproduction

The other main result of this study was that high  $p\text{CO}_2$  did not change bioenergetics in *P. margaritifera* exposed for 100 days. This has already been found for the mussel *Mytilus galloprovincialis*, for which the acidified seawater did not change clearance, ingestion and respiration rates. However, SFG increases significantly under more efficient assimilation of organic matter (Fernández-Reiriz et al., 2012). The same group (Fernández-Reiriz et al., 2011) revealed a consistent effect of high  $p\text{CO}_2$  as shown by the reduction of ingestion rate by 60% in the clam *Ruditapes decussatus* and slow growth as a result of acidification. Other studies have shown no effect of low pH on SFG in the mussel *Mytilus coruscus* exposed for 14 days (Wang et al., 2015). Similarly, in the gastropod *Nassarius conoidalis*, 31-day exposure to acidification has no effect on the energetics of the species (Zhang et al., 2015). These results show that bivalves can adapt immediately without suffering any impact on their physiology or in the longer term with an impact on growth. For *P. margaritifera*, in the short and medium term, our results showed no impact on energy management and gametogenesis. Histological observations of gonads have shown that the cellular process is not affected by  $p\text{CO}_2$ . Often, when stress or a nutritional problem occurs, the germinal process will stop at the level of

**Table 4**

Significance level of ANOVA test of calcifying genes expression level according to pCO<sub>2</sub> level in two separated experiments at one year interval.

Sources of variation	Pmarg-PIF177		Pmarg-Nacrein		Pmarg-PUSP6		Pmarg-Pearlin		Pmarg-MRNP34		Pmarg-MSI60		Pmarg-Shematrin9 9		Pmarg-Prismalin14		Pmarg-Aspein	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p	F	p
pCO <sub>2</sub>	1.16	0.34	0.63	0.54	4.62	<b>0.02</b>	0.56	0.58	1.95	0.17	1.73	0.21	2.10	0.16	1.45	0.26	0.49	0.62
Experiment	14.29	<b>0.001</b>	7.06	<b>0.02</b>	0.535	0.47	0.002	0.97	2.94	0.10	38.63	<b>0.0001</b>	14.07	<b>0.001</b>	2.84	0.11	7.35	<b>0.01</b>
pCO <sub>2</sub> × experiment	1.45	0.26	1.65	0.22	0.55	0.59	2.65	0.10	2.08	0.15	2.23	0.14	1.39	0.28	1.37	0.28	0.094	0.91

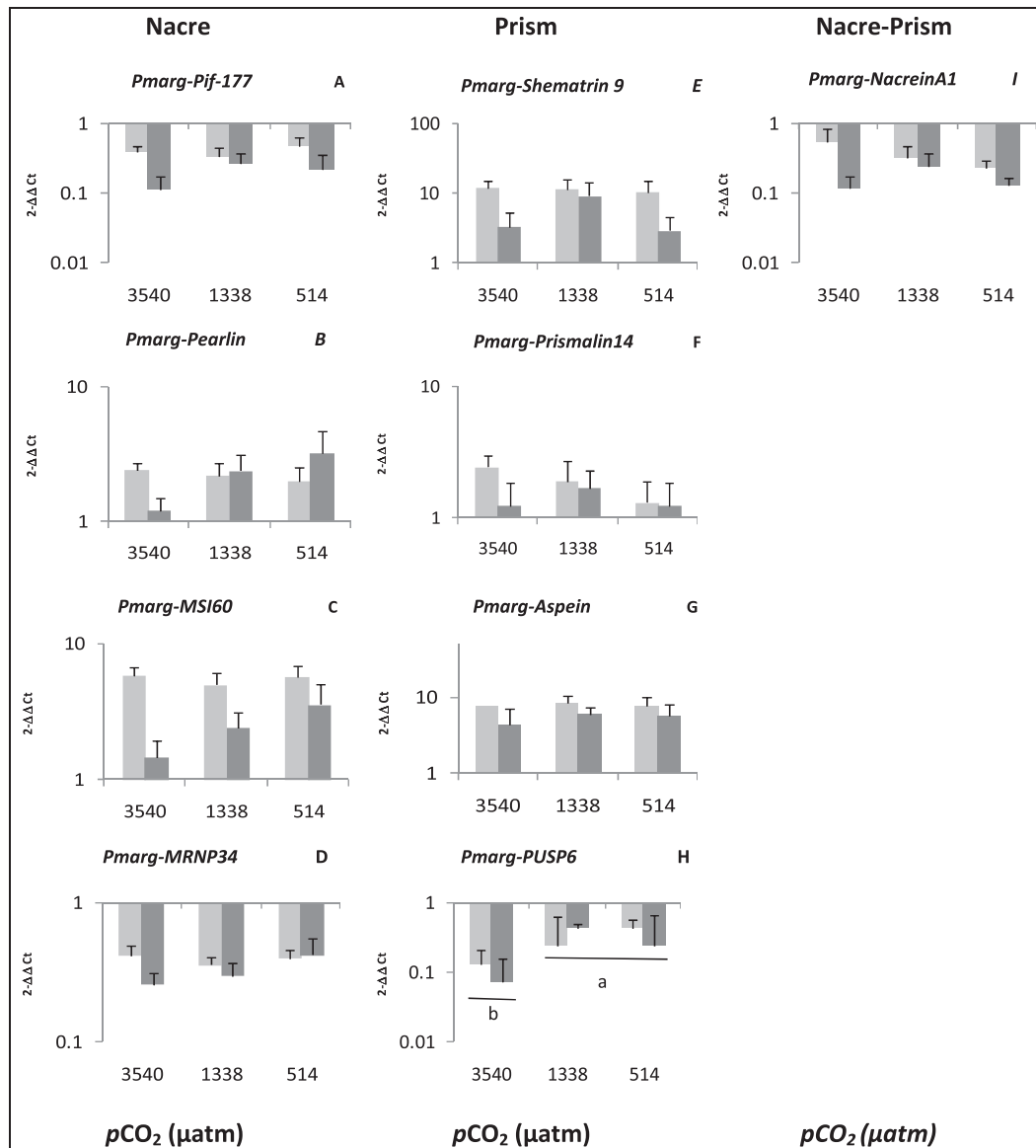
The p values under 0.05 are in bold.

differentiation of germinal stem cells (Bishop and Watt, 1994; Le Moullac et al., 2013). Gametogenesis of pearl oysters subject to acidification of their environment is not changed.

This suggests that *P margaritifera* appears to be resistant at short term to pCO<sub>2</sub> increase at energy management level implying hence reproduction.

4.2. pCO<sub>2</sub> influences shell integrity, growth rate and molecular process

A high pCO<sub>2</sub> level acted on *P. margaritifera*'s shell and bio-mineralisation process. First, the external side of the shell was highly blanched at pCO<sub>2</sub> of 3540 μatm. Bleaching of the external side of the shell is the consequence of periostracum dissolution,



**Fig. 6.** Effect of the pCO<sub>2</sub> level on mantle gene expression in pearl oyster exposed one hundred days shell deposit rate, (1st experiment (grey), 2nd experiment (dark grey)) (a) *Pmarg-Pif-177*, (b) *Pmarg-Pearlin*, (c) *Pmarg-MSI 60*, (d) *Pmarg-MRNP34*, (e) *Pmarg-Shematrin 9*, (f) *Pmarg-Prismalin 14*, (g) *Pmarg-Aspein*, (h) *Pmarg-PUSP6*, (i) *NPmarg-Nacrein A1*(n = 5).



and hence could render pearl oysters more sensitive to shell parasites. Parasitism of the shell of *P. margaritifera* results in biodegradation by shell-boring organisms, especially sponges, and the shells become brittle (Mao Che et al., 1996).

At high  $p\text{CO}_2$  of 3540  $\mu\text{atm}$ , the shell deposit rate was reduced and the inside of the shell showed signs of active chemical dissolution. Aragonite and calcite were unstructured, appearing as fade. This appearance coincided with the total absence of the organic matrix. At  $p\text{CO}_2$  of 1338  $\mu\text{atm}$ , part of the organic matrix remained intact while in other part, the organic matrix was lacking. Therefore, the structural integrity of the shells of *P. margaritifera* seems compromised as has already been shown in tropical as well as in arctic bivalves (McClintock et al., 2009; Welladsen et al., 2010).

In parallel, high  $p\text{CO}_2$  of 3540  $\mu\text{atm}$  reduced the expression level of certain genes implied in biomineralisation. One gene (*Pmarg-PUSP6*) coding for a matrix protein among the nine tested showed down-regulation with high  $p\text{CO}_2$ . The expression of the *Pmarg-PUSP6* gene decreased about five fold under the effect of the higher  $p\text{CO}_2$  compared with controls. *P. fucata* seems more sensitive to low pH, since aspein and nacrein expression decreased by 30 and 60% at pH 8.1 and 7.7 respectively after short-term exposure (Liu et al., 2012), whereas expression of these genes (*Pmarg-Aspein* and *Pmarg-Nacrein A1*) in *P. margaritifera* did not change in the present study; that could be an adaptive response after an eventual acute response. Little is known of *Pmarg-PUSP6* activity; this gene expressed in the mantle edge and in the mantle pallium is involved in the production of calcite (Marie et al., 2012). This study details the role of *Pmarg-PUSP6* as a direct link can be made with shell growth, whose expression is regulated by  $p\text{CO}_2$ .

## 5. Conclusions

However, is this a purely chemical reaction or a coping strategy that favours the vital processes? In our study, energy management was not changed under  $p\text{CO}_2$  treatment, and reproduction was apparently maintained, although biomineralisation and the integrity of the shells were affected, giving weight to the hypothesis of Melzner et al. (2011) who observed in the mussel *Mytilus edulis* that  $p\text{CO}_2$  decreased shell length growth, suggested that energy is allocated to more vital processes (somatic mass maintenance) instead of shell conservation.

It would be useful to determine the impact of acidification on ability of gametes for fertilisation and finally on the development of larvae. The shells of larvae, primarily formed of aragonite, could be weakened by low pH, which may alter development until fixation, which is crucial for the bivalves. Changes in *Pmarg-PUSP6* expression suggest that other genes are deregulated; only a differential transcriptomic approach could analyse more accurately the effect and consequence of acidification.

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