Estuarine, Coastal and Shelf Science
December 2016, Volume 182, Part B, Pages 274-282
http://dx.doi.org/10.1016/j.ecss.2016.03.011
http://archimer.ifremer.fr/doc/00321/43189/

Impact of *pCO2* on the energy, reproduction and growth of the shell of the pearl oyster *Pinctada margaritifera*

Le Moullac Gilles [1, *], Soyer Claude [1], Vidal-Dupiol Jérémie [1], Belliard Corinne [1], Fievet Julie [1], Sham Koua Manaarii [1], Lo-Yat Alain [1], Saulnier Denis [1], Gaertner-Mazouni Nabila [2], Gueguen Yannick [3]

[1] Ifremer, UMR 241 Ecosystèmes Insulaires Océaniens (EIO), Labex Corail, Centre du Pacifique, BP 49, 98719 Taravao, Tahiti, French Polynesia
[2] Université de la Polynésie Française, UMR 241 Ecosystèmes Insulaires Océaniens (EIO), Labex Corail, BP 6570, 98702 Faa'a, Tahiti, French Polynesia
[3] Ifremer, UMR 5244 IHPE, UPVD, CNRS, Université de Montpellier, CC 80, F-34095 Montpellier, France

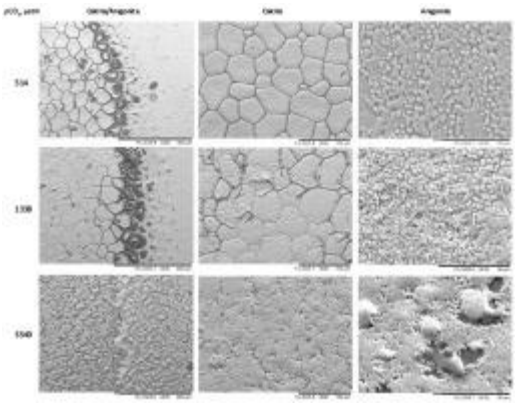
* Corresponding author : Gilles Le Moullac, Tel.: +689 40 54 60 06; fax: +689 40 54 60 99 ;
email address : Gilles.Le.Moullac@ifremer.fr

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 pCO_2 (3540, 1338 and 541µatm) 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 pCO_2 had no effect on scope for growth and gametogenesis. High pCO_2 (3540 µatm) 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 pCO_2significantly 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.

Graphical abstract

At high pCO_2 of 3540 µatm, the shell deposit rate was significantly reduced and the inside of the shell showed signs of active chemical dissolution.



Highlights

► Under high pCO_2, pearl oyster bioenergetics and reproduction were preserved. ► Shell growth was significantly reduced. ► Shell integrity was affected. ► The gene *Pmarg*-PUSP6 of the mantle cells tested showed significant down-regulation, but eight others showed no significant effect.

Keywords : Global change, Pearl oyster, Bioenergetic, Biomineralization, Pacific Ocean, French Polynesia

2. Introduction

Global climate change is a major concern caused by mainly two factors, temperature and the atmospheric 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 thecommunities 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; Fernandez-Reiriz et al. 2011; Wang et al. 2015). Nevertheless, some species are positively affected by high CO_2 such as the sea urchin *Echinometra*sp., and show strong resistance to high pCO_2 after about one year exposure (Hazanet 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 pCO_2, the scope for growth is better, promoting reproduction; this is due to better absorption efficiency and a lower ammonium excretion rate (Fernandez-Reirizet al. 2012). Lastly, the metabolic rate of the wild oyster *Saccostrea glomerata*is not impacted by low pCO_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 valuable resource in French Polynesia. Pearl culture there depends on the exploitation of a single species, the pearl oyster *P. 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-Laureauet 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-Villalbaet 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 pCO_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.

3. Material and methods

3.1 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.

3.2 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±6.5 mm and 117±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^{-1} calcein solution for 12 h, as described in Linard et al. (2011).

3.3 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 (25µm) seawater from the Vairao lagoon. The pearl oysters were placed in 500-litre tanks with controlled flow-through. Seawater was renewed at the rate of 100 L h^{-1} 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 25000 cell mL^{-1} 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_2 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).

3.4 Carbonate chemistry

Total alkalinity (TA) was measured weekly via titration with 0.01 N HCl containing 40.7 g NaCl L^{-1} 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_2 Systat package. Average pCO_2 corresponding to pH 7.4, 7.8 and 8.2 was respectively of 3540, 1338 and 514µatm. Other parameters of carbonate seawater chemistry are given in Table 1.

3.5 Bioenergetic measurements

During the last 48 hours 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 control

(Chavez-Villalbaet 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^{-1}).

Respiration rate (RR mg O_2 h^{-1}) 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 × 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^{-1}. The supernatant was removed and the pellet was washed twice with ammonium formate (37% 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:

$$AE = \frac{\%\text{OM}\mu\text{alg} - \%\text{OMbiodeposit}}{(100 - \%\text{OMbiodeposit})\text{x}\ \%\text{OM}\mu\text{alg}}$$

Ecophysiological data were converted into energetic values to define the scope for growth (SFG) for each oyster: SFG = (IR × 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_2 (Bayne and Newell 1983; Gnaiger 1983).

3.6 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.

3.7 Shell growth rate and nacremicrostructure

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 sec 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 $\mu m.d^{-1}$ (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.

3.8 Gene expression in mantle

At the end of the 100 days exposure at different pCO_2, 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 withTRIZOL®Reagent (Life Technologies) according to the manufacturer's recommendations. RNA was quantified with a NanoDrop®ND-1000 spectrophotometer (NanoDrop®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® 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 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 reactionswere 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: ratio = $2^{-[\Delta Ct\ sample-\Delta Ct\ calibrator]} = 2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Here the ΔCt calibrator represented the mean of the ΔCt values obtained for all tested genes in all conditions.

3.9 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 pCO_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 pCO_2 level. Post hoc comparison was done with the unilateral test of Dunnet using the lowest pCO_2 levels as a control for comparison with the highest one. α was set at 0.05 for all analyses. Impact of pCO_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 Dunnet using the lowest pCO_2 levels as a control for comparison with the highest one.α was set at 0.05 for all analyses.

4. Results

4.1 Bioenergetics

The 100-day exposure to various pCO_2 levels did not induce modifications of the bioenergetics descriptors. 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 (Figure 1a, 1b, 1c). 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).

4.2 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 pCO_2 level on gametogenesis was analysed for three gonadic stages and the two experimental series were grouped (Figure 2). With this data set, the Chi-square test did not show any significant effect of the pCO_2 level on the gametogenic process (chi2 = 4.81, p=0.31).

4.3 Shell integrity, growth and microstructural organisation

The external side of the shells whitened during the 100 days of exposure to acidification. Figure 3 shows the three lots of pearl oysters grouped by level of pCO_2 exposure. We observed a gradient in the bleaching of the outer surface of the shell depending on the level of exposure to pCO_2. The shells of pearl oysters exposed to pCO_2 of 3540 atm had blanched considerably, and those exposed to $pCO_2$1338 atm were an intermediate shade (pCO_2 514 µatm).

Shell deposition rate (SDR) measurement was done with calcein marking in order to analyse the effect of environmental pCO_2level on shell growth after one year of experimentation. Two-way ANOVA showed a significant effect of pCO_2level 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 (Figure 4a). At microstructural scale, MEB observation and measurement showed that the thickness of aragonite tablets did not change with pCO_2 level. The mean value of aragonite thickness tablets remained an average size of 0.4 nm (Figure 4b).

Observation of the growing edge of nacre lining the shells showed that shells exposed to acidified conditions (pCO_2 of 1338 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_2. Oysters from the control (pCO_2 514 µatm) had nacre that showed a distinct boundary between the fully formed and developing nacre tablets (Figure 5a), the organic matrix was intact (Figure 5b) and growth of nacre also showed a clear wave-like pattern and nacre tablets forming within an extensive organic matrix (Figure 5c). Inspection of nacre

from oysters held at pCO_2 of 1338 µatm still showed a distinct boundary between calcite and aragonite (Figure 5d), although the organic matrix disappeared in places (Figure 5e) and the wave-like pattern became anarchic (Figure 5f). AtpCO_2 of 3540 µatm the boundary was visible (Figure 5g), but the organic matrix disappeared completely (Figure 5h), the aragonite tablets also disappeared leaving space for a kind of nacre where the shelves were merged (Figure 5i).

4.4 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 pCO_2 level compared with the lowest (Table 5, Figure 6).

5. 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 pCO_2 level. We acclimatised pearl oysters for 100 days at three levels of pCO_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 this 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 tested nine.

5.1 pCO_2 did not influence energy management and reproduction

The other main result of this study was that high pCO_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 (Fernandez-Reiriz et al. 2012). The same group (Fernandez-Reiriz et al. 2011) revealed a consistent effect of high pCO_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 have shown that the cellular process is not affected by pCO_2. Often, when stress or a nutritional problem occurs, the germinal process will stop at the level of 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_2 increase at energy management level implying hence reproduction.

5.2 pCO_2 influences shell integrity, growth rate and molecular process

A high pCO_2 level acted on *P margaritifera*'s shell and biomineralisation process. First, the external side of the shell was highly blanched at pCO_2 of 3540 µatm. Bleaching of the external side of the shell is the consequence of periostracum dissolution, 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 pCO_2 of 3540 µatm, 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 pCO_2 of 1338 µatm, 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 $pCO_2$of 3540 µatm 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 pCO_2. The expression of the *Pmarg*-PUSP6 gene decreased about five fold under the effect of the higher pCO_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 is 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 pCO_2.

6. 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 pCO_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 edu*lis that pCO_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.

7. Acknowledgment

This study is a part of the research project ''Management and adaptation of pearl culture in French Polynesia in the context of global change: an environmental, economic and social approach'' (POLYPERL) funded by the French National Research Agency within the framework of theprogram AGROBIOSHERE (ANR-11-AGRO-006). The authors greatly acknowledge the electron microscopy platform of the University of French Polynesia and its personnel. The authors wish to thank the staff of the laboratory of studies and environmental monitoring (LESE) for his help in the realization of chemical measurements of sea water.

References

Bayne, B.L., Hawkins, A.J.S., Navarro, E., 1987. Feeding and digestion by the mussel *Mytilus edulis* L. (Bivalvia: Mollusca) in mixtures of silt and algal cells at low concentrations. Journal of Experimental Marine Biology and Ecology 111, 1-22.

Bayne, B.L., Newell, R.C., 1983. Physiological energetics of marine molluscs. In: Saleuddin, A.S.M., Wilbur, K.M. (Eds.), The Mollusca, Vol 4. Academic Press, New York, NY, pp. 407–515.

Berge, J.A., Bjerkeng, B., Pettersen, O., Schaanning, T., Oxnevad, S., 2006. Effects of increased seawater concentration of CO_2 on growth of the bivalve *Mytilus edulis* L. Chemosphere 62, 681-687.

Bishop, C.D., Watt, S.A., 1994. Two-stage recovery of gametogenic activity following starvation in *Lytechinusvariegatus* Lamarck (Echinodermata: Echinoidea). Journal of Experimental Marine Biology and Ecology 177, 27-36.

Caldeira, K., Wickett, M.E., 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. Journal of Geophysical Research 110, C09S04, doi:10.1029/2004JC002671.

Chavez-Villalba, J., Soyez, C., Aurentz, H., Le Moullac, G., 2013. Physiological responses of female and male black-lip pearl oysters (*Pinctadamargaritifera*) to different temperatures and concentrations of food. Aquatic Living Resources 26, 263–271. Publisher's official version : http://dx.doi.org/10.1051/alr/2013059 , Open Access version : http://archimer.ifremer.fr/doc/00157/26855/.

Cochennec-Laureau, N., Montagnani, C., Saulnier, D., Fougerouse, A., Levy, P., Lo, C., 2010. A histological examination of grafting success in pearl oyster *Pinctadamargaritifera* in French Polynesia. Aquatic Living Resources 23, 131-140. Publisher's official version: http://dx.doi.org/10.1051/alr/2010006.

Conover, R., 1966. Assimilation of organic matter by zooplankton. Limnology and Oceanography 11:338-345.

Dheilly, N., Lelong, C., Huvet, A., Favrel, P., 2011. Development of a Pacific oyster (*Crassostrea gigas*) 31,918-feature microarray: identification of reference genes and tissue-enriched expression patterns. BMC Genomics 2011, 12:468 doi:10.1186/1471-2164-12-468.

Fernández-Reiriz, M.J., Range, P., Álvarez -Salgado, X.A., Esponisa, J., Labarta, U., 2012.Tolerance of juvenile *Mytilus galloprovencialis* to experimental seawater acidification. Marine Ecology Progress Series 454, 65-74.

Fernández-Reiriz, M.J., Range, P., Álvarez-Salgado, X.A., Labarta, U., 2011. Physiological energetics of juvenile clams *Ruditapes decussatus* in a high CO_2 coastal ocean. Marine Ecology Progress Series 433, 97-105.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A., Martin, S., Pörtner, H.-O., Ross, P.M., 2013. Impacts of ocean acidification on marine shelled molluscs. Marine Biology 160, 2207-2245.doi10.1007/s00227-013-2219-3.

Gnaiger, E.,1983. Heat dissipation and energetic efficiency in animal anoxibiosis. Economy contra power. Journal of Experimental Zoology 228, 471-490.

Hazan, Y., Wangensteen, O.S., Fine, M., 2014. Tough as a rock-boring urchin: adult *Echinometra* sp. EE from the Red Sea show high resistance to ocean acidification over long-term exposures. Marine Biology 161, 2531-2545, doi: 10.1007/s00227-014-2525-4.

Joubert, C., Linard, C., Le Moullac, G., Soyez, C., Saulnier, D., Teaniniuraitemoana, V., Ky, C.L., Gueguen, Y., 2014.Temperature and food influence shell growth and mantle gene expression of shell matrix proteins in the pearl oyster *Pinctada margaritifera*. PLoS ONE 9(8),e103944. doi:10.1371/journal.pone.0103944.

Joubert, C., Piquemal, D., Marie, B., Manchon, L., Pierrat, F., Zanella-Cleon, I., Cochennec-Laureau, N., Gueguen, Y., Montagnani, C., 2010. Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. Bmc Genomics 11, 1-13. Publisher's official version : http://dx.doi.org/10.1186/1471-2164-11-613.

Kinoshita, S., Wang, N., Inoue, H., Maeyama, K., Okamoto, K., Nagai, K., Kondo, H., Hirono, I., Asakawa, S., Watabe, S., 2011.Deep sequencing of ESTs from nacreous and prismatic layer producing tissues and a screen for novel shell formation-related genes in the pearl oyster. PLoS ONE 6(6),e21238. doi:10.1371/journal.pone.0021238.

Kurihara, H., Asai, T., Kato, S., Ishimatsu, A., 2008. Effects of elevated pCO_2 on early development in the mussel *Mytilus galloprovincialis*. Aquatic Biology 4,225-233. doi: 10.3354/ab00109.

Kurihara, H., Ishimatsu, A., 2008.Effects of high CO2 seawater on the copepod (*Acartia tsuensis*) through all life stages and subsequent generations. Marine Pollution Bulletin 56, 1086-1090.doi: 10.1016/j.marpolbul.2008.03.023.

Larsen, J.B., Frischer, M.E., Rasmussen, L.J., Hansen, B.W., 2005. Single-step nested multiplex PCR to differentiate between various bivalve larvae. Marine Biology 146, 1119–1129.

Le Moullac, G., Soyez, C., Sham-Koua, M., Levy, P., Moriceau, J., Vonau, V., Maihota, M., Cochard, J.C., 2013. Feeding the pearl oyster *Pinctada margaritifera* during reproductive conditioning. Aquaculture Research 44, 404-411.

Linard, C., Gueguen, Y., Moriceau, J., Soyez, C., Hui, B., Raoux, A., Cuif, J.-P., Cochard, J.-C., Le Pennec, M., Le Moullac, G., 2011. Calcein staining of calcified structures in pearl oyster *Pinctada margaritifera* and the effect of food resource level on shell growth. Aquaculture 313, 149-155. Publisher's official version: http://dx.doi.org/10.1016/j.aquaculture.2011.01.008.

Liu, W., Huang, X., Lin, J., He, M., 2012. Seawater acidification and elevated temperature affect gene expression patterns of the pearl oyster *Pinctada fucata*. PLoS ONE 7(3),e33679. doi:10.1371/ journal.pone.0033679.

Livak, K.J., Schmittgen, T.D., 2001.Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25,402-408.

McClintock, J.B., Angus, R.A., McDonald, M.R., Amsler, C.D., Catledge, S.A., Vohra, Y.K., 2009. Rapid dissolution of shells of weakly calcified Antarctic benthic macroorganisms indicates high vulnerability to ocean acidification. Antarctic Science 21, 449-456.

Mao Che, L., Le Campion-Alsumard, T., Boury-Esnault, N., Payri, C., Golubic, S., Bézac, C., 1996. Biodegradation of shells of the black pearl oyster, *Pinctada margaritifera* var. cumingii, by microborers and sponges of French Polynesia. Marine Biology 126, 509-519.

Marie, B., Joubert, C., Tayale, A., Zanella-Cleon, I., Belliard, C., Piquemal, D., Cochennec-Laureau, N., Marin, F., Gueguen, Y., Montagnani, C., 2012. Different secretory repertoires control the biomineralization processes of prism and nacre deposition of the pearl oyster shell. Proceedings of the National Academy of Sciences of the United States of America 109(51), 20986-20991. Publisher's official version: http://dx.doi.org/10.1073/pnas.1210552109.

Melzner, F., Stange, P., Trübenbach, K., Thomsen, J., Casties, I., Panknin, U., Gorb, S.N., Gutowska, M.A.,2011. Food supply and seawater pCO2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. Plos One 6, e24223. doi:10.1371/journalpone.0024223.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms.Nature 437(7059), 681-686.

Parker, L.M., Ross, P.M., O'Connor, W.A., Borysko, L., Raftos, D.A., Pörtner, H.-O., 2012. Adult exposure influences offspring response to ocean acidification in oysters. Global Change Biology 18,82-92. doi: 10.1111/j.1365-2486.2011.02520.x.

Pouvreau, S., Tiapari, J., Gangnery, A., Lagarde, F., Garnier, M., Teissier, H., Haumani, G., Buestel, D., Bodoy, A., 2000. Growth of the black-lip pearl oyster, *Pinctada margaritifera*, in suspended culture under hydrobiological conditions of Takapoto lagoon (French Polynesia). Aquaculture 184,133-154.

Ross, P.M., Parker, L., O'Connor, W.A., Bailey, E.A., 2011. The impact of ocean acidification on reproduction, early development and settlement of marine organisms. Water 3, 1005-1030.

Saraiva, S., van der Meer, J., Kooijman, S.A.L.M., Sousa, T., 2011. Modelling feeding processes in bivalves: a mechanistic approach. Ecological Modelling 222, 514-523.

Savina, M., Pouvreau, S., 2004. A comparative ecophysiological study of two infaunal filter-feeding bivalves: *Paphia rhomboides* and *Glycymeris glycymeris*. Aquaculture 239, 289-306. Publisher's official version : http://dx.doi.org/10.1016/j.aquaculture.2004.05.029.

Siikavuopio, S.I., Mortensen, A, Dale, T., Foss, A., 2007. Effects of carbon dioxide exposure on feed intake and gonad growth in green sea urchin, *Strongylocentrotus droebachiensis*. Aquaculture 266,97-101.

Teaniniuraitemoana, V., Huvet, A., Levy, P., Klopp, C., Lhuillier, E., Gaertner-Mazouni, N., Gueguen, Y., Le Moullac, G., 2014. Gonad transcriptome analysis of pearl oyster *Pinctada margaritifera*: identification of potential sex differentiation and sex determining genes. BMC Genomics 15, 1-20. Publisher's official version : http://dx.doi.org/10.1186/1471-2164-15-491.

Thomas, Y., Garen, P., Bennett, A., Le Pennec, M., Clavier, J., 2012. Multi-scale distribution and dynamics of bivalve larvae in a deep atoll lagoon (Ahe, French Polynesia). Marine Pollution Bulletin 65, 453-462. Publisher's official version : http://dx.doi.org/10.1016/j.marpolbul.2011.12.028.

Thomsen, J., Melzner, F., 2010. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Marine Biology 157, 2667-2676. doi:10.1007/s00227-010-1527-0.

464 Wang, Y., Li, L., Hu, M., Lu, W., 2015. Physiological energetics of the thick shell mussel *Mytilus*
465 *coruscus* exposed to seawater acidification and thermal stress. Science of the Total Environment
466 514,261-272. doi: 10.1016/j.scitotenv.2015.01.092.

467 Welladsen, H.M., Southgate, P.C., Heimann, K., 2010. The effects of exposure to near-future levels of
468 ocean acidification on shell characteristics of *Pinctada fucata* (Bivalvia: Pteriidae). Molluscan
469 Research 30,125-130.

470 Wood, H. L., Spicer, J. I., Widdicombe, S., 2008. Ocean acidification may increase calcification rates,
471 but at a cost. Proceedings of the Royal Society B: Biological Sciences 275(1644), 1767-1773.
472 doi:10.1098/rspb.2008.0343.

473 Yukihira, H., Klumpp, D.W., Lucas, J.S., 1998. Effects of body size on suspension feeding and energy
474 budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima*. Marine Ecology Progress Series
475 170,120-130.

476 Zhang, H., Shin P.K.S., Cheung, S.G., 2015. Physiological responses and scope for growth upon
477 medium-term exposure to the combined effects of ocean acidification and temperature in a subtidal
478 scavenger *Nassarius conoidalis*. Marine Environmental Research 106, 51-60.

479

Figure 1: Bioenergetic behavior after one hundred days exposure to pCO_2 level at one year interval (1^{srt} experiment (grey), 2^{nd} 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).

Figure 2: Effect of the pCO_2 level on gametogenesis of pearl oyster exposed for one hundred days (38 < n < 41).

Figure 3: Effect of the pCO_2 level on bleaching of external side of shells of pearl oyster exposed for one hundred days.

Figure 4: Effect of the pCO_2 level on shell growth (A) shell deposit rate, (1^{srt} experiment (grey), 2^{nd} experiment (dark grey), (B) thickness of aragonite tablet, 2^{nd} experiment. (n= 8-9**).**

Figure 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 pCO_2 level (514, 1338, 3540 µatm).

Figure 6: Effect of the pCO_2 level on mantle gene expression in pearl oyster exposed one hundred days shell deposit rate, (1^{srt} experiment (grey), 2^{nd} 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) N*Pmarg*-Nacrein A1(n= 5**).**

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 sofware.

pH	Temperature (°C)	Salinity	Alcalinity (µmol/kg)	pCO_2 (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

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, 2001)

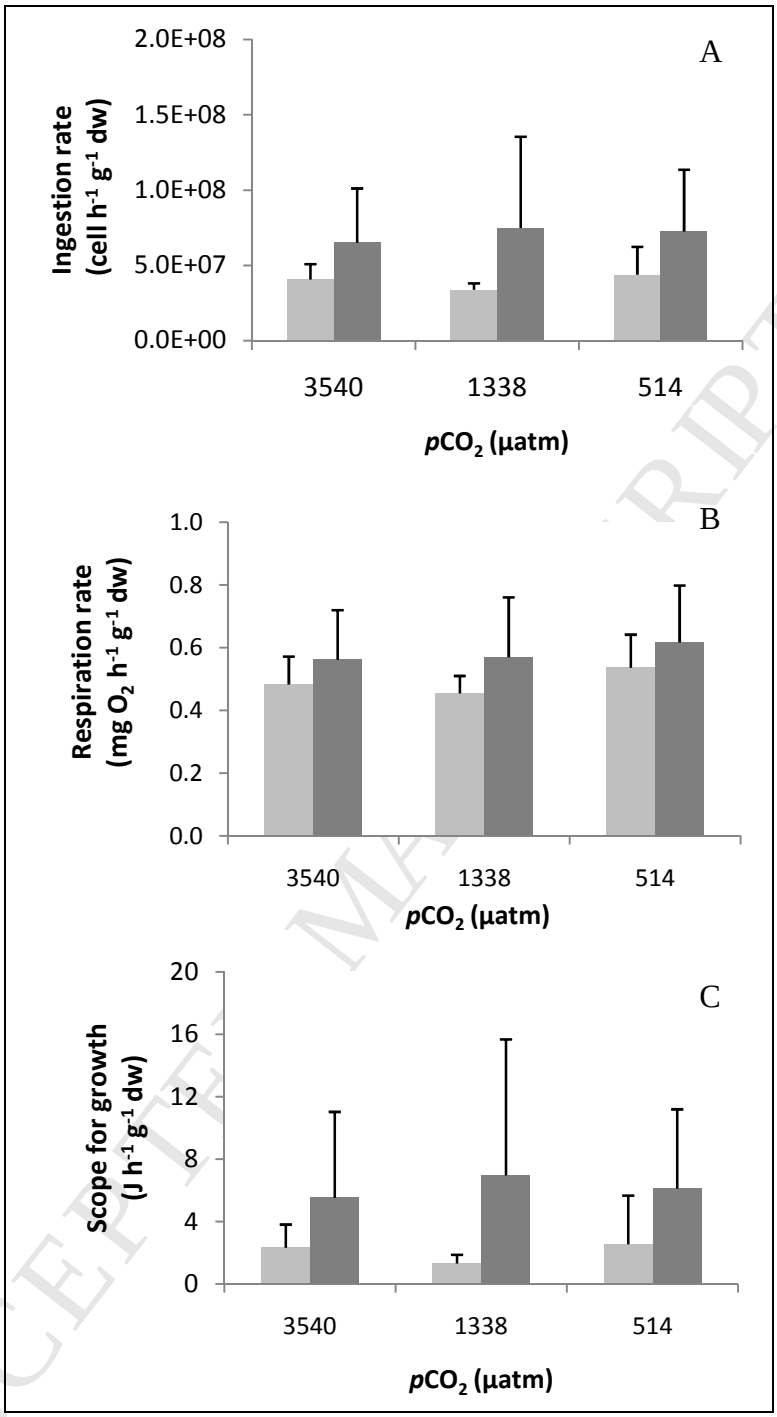
Gene	GenBank Accession Number	Forward primer	Reverse primer
***Pmarg*-PIF 177**	HE610401	5'-AGATTGAGGGCATAGCATGG-3'	5'-TGAGGCCGACTTTCTTGG-3'
***Pmarg*-Pearlin**	DQ665305	5'-TACCGGCTGTGTTGCTACTG-3'	5'-CACAGGGTGTAATATCTGGAACC-3'
***Pmarg*-MRNP34**	HQ625028	5'-GTATGATGGGAGGCTTTGGA-3'	5'-TTGTGCGTACAGCTGAGGAG-3'
***Pmarg*-MSI60**	SRX022139*	5'-TCAAGAGCAATGGTGCTAGG-3'	5'-GCAGAGCCCTTCAATAGACC-3'
***Pmarg*-Shematrin 9**	ABO92761	5'-TGGTGGCGTAAGTACAGGTG-3'	5'-GGAAACTAAGGCACGTCCAC-3'
***Pmarg*-Prismalin 14**	HE610393	5'-CCGATACTTCCCTATCTACAATCG-3'	5'-CCTCCATAACCGAAAATTGG-3'
***Pmarg*-PUSP6**	SRX022139*	5'-TTCATTTTGGTGGTTATGGAATG-3'	5'-CCGTTTCCACCTCCGTTAC-3'
***Pmarg*-Aspein**	SRX022139*	5'-TGGAGGTGGAGGTATCGTTC-3'	5'-ACACCTGATACCCTGCTTGG-3'
***Pmarg*-Nacrein A1**	HQ654770	5'-CTCCATGCACAGACATGACC-3'	5'-GCCAGTAATACGGACCTTGG-3'

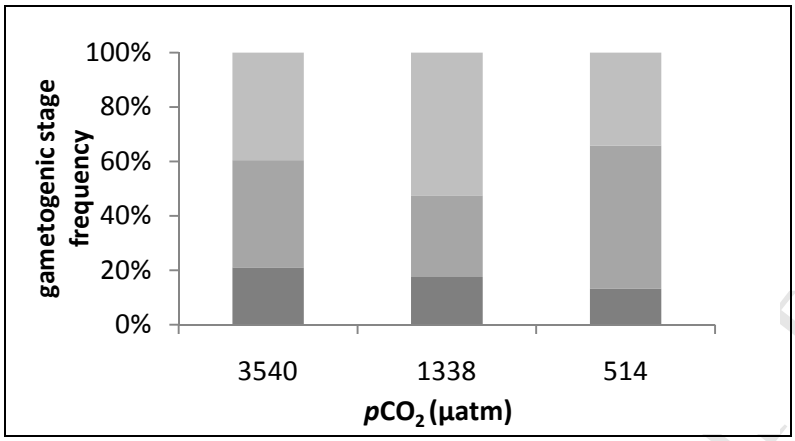
Table 3: Two-way ANOVA results for bioenergetic values of 100 day exposure to the pCO_2 level in two separated experiments at one year interval (absorption efficiency (AE), ingestion rate (IR), respiration rate (RR), scope for growth (SFG).

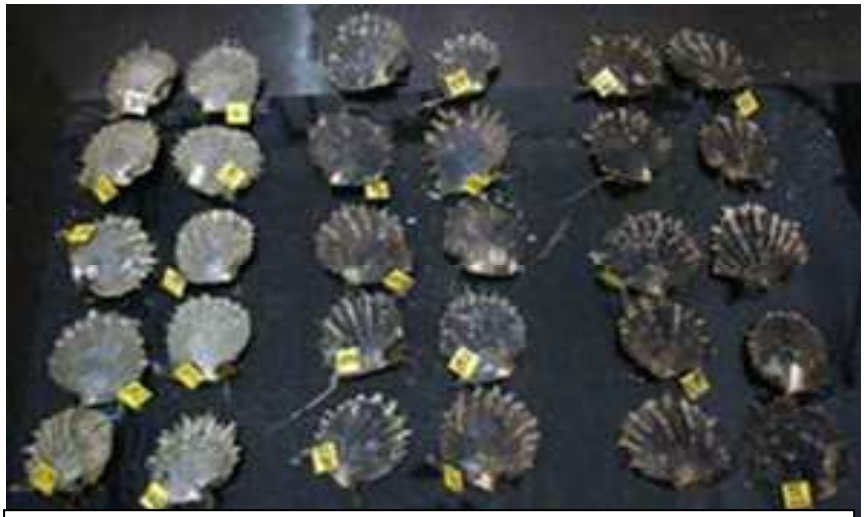
Sources of variation	**ddl**	**AE* (arcsinsqr)**		**IR (Box Cox)**		**RR**		**SFG (Box Cox)**	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
pCO_2	2	0.13	0.87	0.22	0.80	1.35	0.26	0.02	0.98
Experiment	1	59.86	**<0.0001**	7.14	**0.01**	7.56	**0.01**	12.82	**0.001**
pCO_2 x Experiment	2	1.32	0.27	0.13	0.88	0.13	0.88	0.35	0.70

Table 4: Significance level of ANOVA test of calcifying genes expression level according to pCO_2 level in two separated experiments at one year interval.

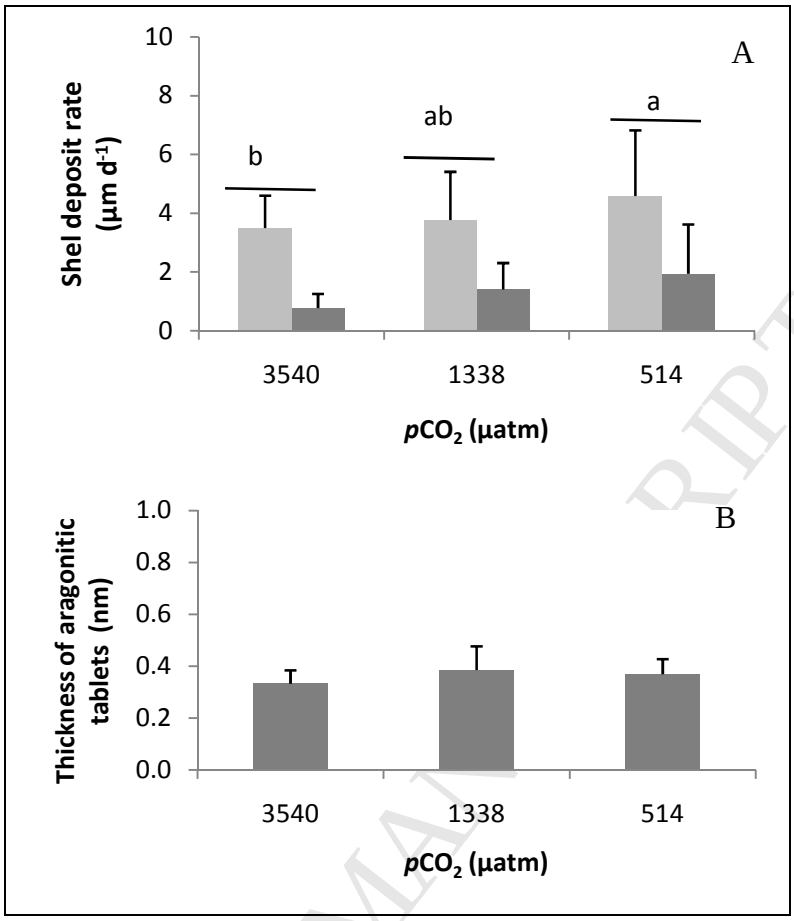
Sources of variation	***Pmarg-PIF177***		***Pmarg-Nacrein***		***Pmarg-PUSP6***		***Pmarg-Pearlin***		***Pmarg-MRNP34***		***Pmarg-MSI60***		***Pmarg-Shematrin9***		***Pmarg-Prismalin14***		***Pmarg-Aspein***	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	*F*	*p*
pCO₂	1.16	*0.34*	0.63	*0.54*	4.62	***0.02***	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*	***0.001***	*7.06*	***0.02***	*0.535*	*0.47*	*0.002*	*0.97*	*2.94*	*0.10*	*38.63*	***0.0001***	*14.07*	***0.001***	*2.84*	*0.11*	*7.35*	***0.01***
pCO₂x 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*

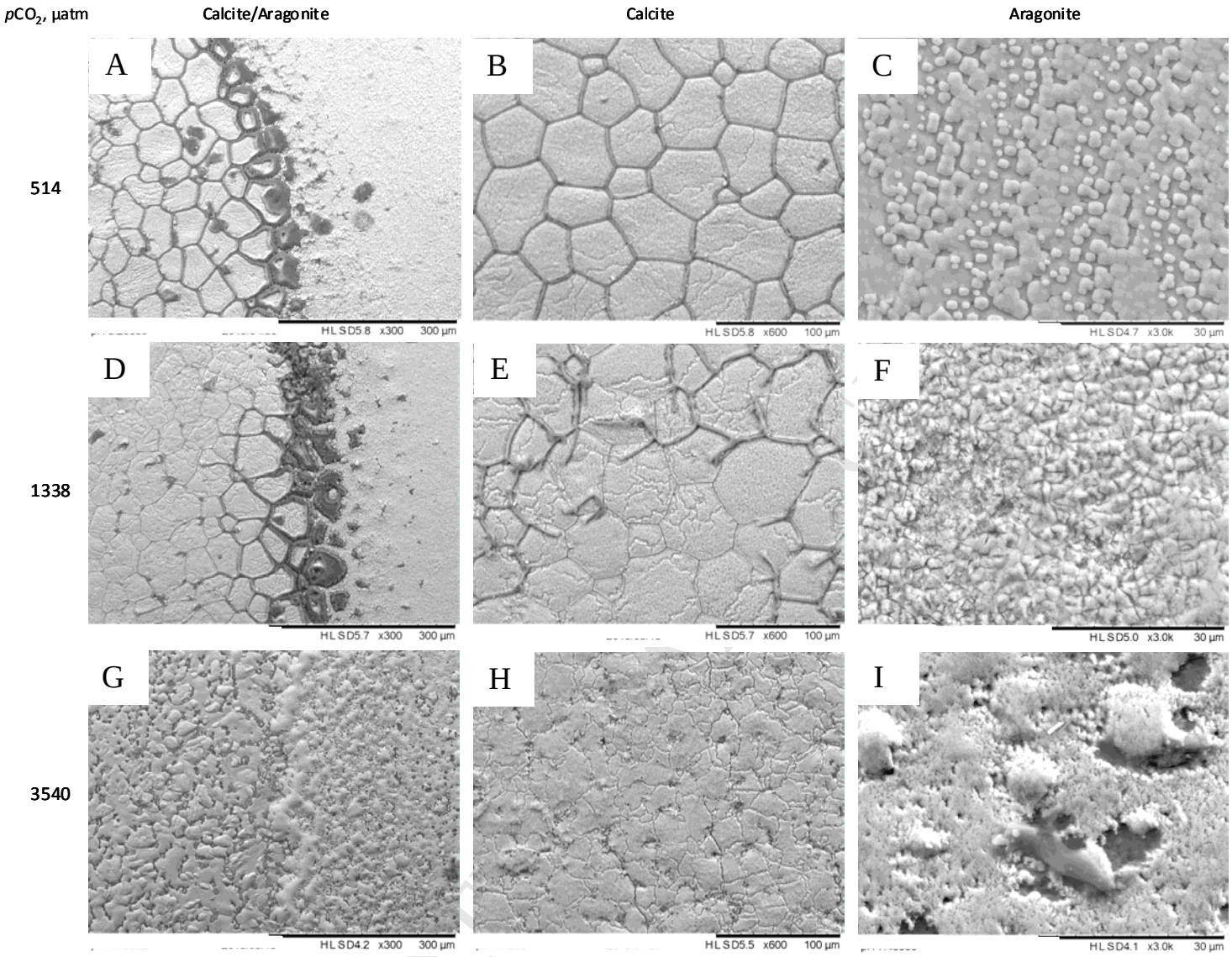


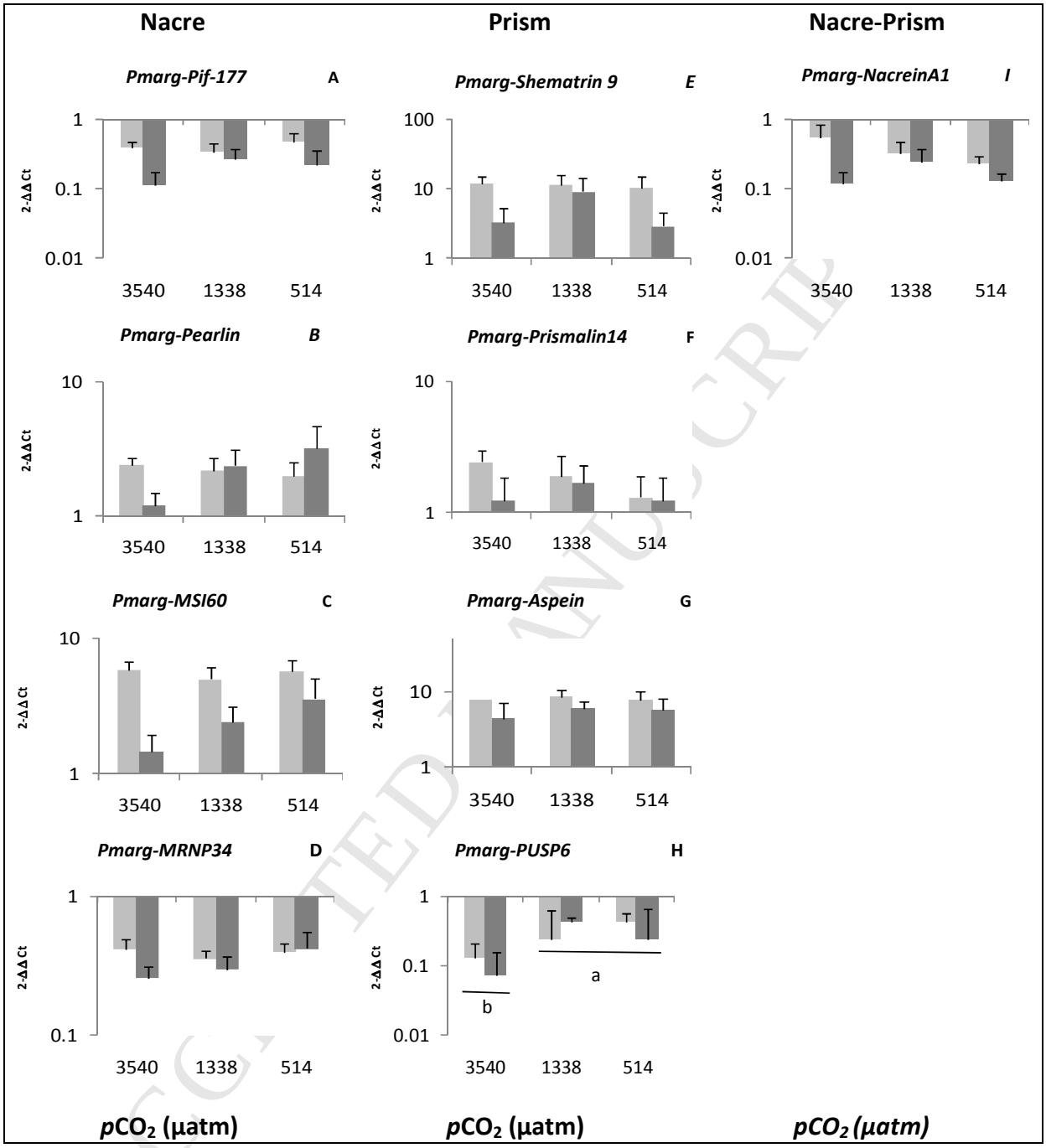




3540 µatm	1338 µatm	514 µatm






Nacre
Prism
Nacre-Prism
Pmarg-Pif-177
A
Pmarg-Shematrin 9
E
Pmarg-NacreinA1
I
Pmarg-Pearlin
B
Pmarg-Prismalin14
F
Pmarg-MSI60
C
Pmarg-Aspein
G
Pmarg-MRNP34
D
Pmarg-PUSP6
H
2-ΔΔCt
3540
1338
514
a
b
pCO2 (µatm)
pCO2 (µatm)
pCO2 (µatm)