Short communication

Ocean acidification and pathogen exposure modulate the immune response of the edible mussel *Mytilus chilensis*

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ABSTRACT

Ocean acidification (OA) is one of the main consequences of increasing atmospheric carbon dioxide (CO₂), impacting key biological processes of marine organisms such as development, growth and immune response. However, there are scarce studies on the influence of OA on marine invertebrates’ ability to cope with pathogens. This study evaluated the single and combined effects of OA and bacterial infection on the transcription expression of genes related to antioxidant system, antimicrobial peptides and pattern recognition receptors in the edible mussel *Mytilus chilensis*. Individuals of *M. chilensis* were exposed during 60 days at two concentrations of pCO₂ (550 and 1200 μatm) representing respectively current and future scenario of OA and were then injected with the pathogenic bacterium *Vibrio anguillarum*. Results evidenced an immunomodulation following the OA exposure with an up-regulation of *C-type Lectin* and *Mytilin B* and a down-regulation of *Myticin A* and *PGRP*. This immunomodulation pattern is partially counteracted after challenge with *V. anguillarum* with a down-regulation of the *C-type lectin* and *Mytilin B* and the up-regulation of *Myticin A*. In turn, these results evidence that pCO₂-driven OA scenarios might triggers specific immune-related genes at early stages of infection, promoting the transcription of antimicrobial peptides and pattern recognition receptors. This study provides new evidence of how the immune response of bivalves is modulated by higher CO₂ conditions in the ocean, as well one factor for the resilience of marine population upon global change scenarios.

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1. Introduction

Although the ocean has partially absorbed a significant fraction of the anthropogenic atmospheric CO₂, this has come at the expense of a significant reduction in pH, a process well known as ocean acidification (OA) [1,2]. Seawater average pH has fallen by more than 0.1 units below the preindustrial average and is predicted to decline by between 0.13 and 0.4 units by the end of the 21st century [2]. The net effect of CO₂ dissolution in seawater is an increase of hydrogent bicarbonate ion concentration and a decrease of carbonate ions [3], which has profound consequences on marine biodiversity and ecosystem functioning [4,5]. More, the decrease of carbonate ions in the ocean have been evidenced to affect several cellular and physiological process of calcifying organisms such as coral, sea urchin and mollusk as a consequence of hypercapnia and production of extra or intracellular acidosis [6–8], impacting their growth and reproductive success [9–11].

Among calcifying organism, mollusks have been shown to be particularly vulnerable to OA [12]. Different studies already demonstrated the detrimental effect of OA on mollusks’ vital processes showing a reduction in their growth, calcification rates, fertilization success [13–15], feeding behavior [16,17] and energy metabolism [18]. However, recent research also observed negative effects of OA on bivalve’s immune system, evidencing a reduction in the bacteriostatic capacity against bacteria, a modulation of the antimicrobial activity of gills [19,20] or provoking an acidosis-mediated immunosuppression [21,22] when exposed to a pH of 7.7. Here, the Chilean blue mussel *Mytilus chilensis* (Hupé, 1854) is a key economic and ecological species in Chile and mussel...
aquaculture is represented mostly by this species, which constitutes one of the lead industries in mussel production cultivated worldwide [23]. However the success of mussel aquaculture production in Chile is threatened by a wide range of microorganism [24–26] or stressors such as OA that will eventually result in a disease outbreak [27] or prevent larval settlement and successful growth in juveniles [11,28,29]. To cope with stressors, mussels and marine invertebrates in general are producing a two-component response, a specific response to the stressor and a more general response involving immune and endocrinial pathways [30]. Impacts of multi-stressors have been predicted to have additive, synergetic or antagonistic effects on marine organisms’ physiology [31]. These different effects are directly link to the amount of time between the occurrence of stressors, its intensity as well as on the organism ability to return to homeostasis before a new stressor occurs [32]. Despite these predictions, meta-data analyses are showing that most of multi-stressors studied had synergetic effects on organisms’ physiology [33]. Notably, while isolated effect on mussels’ immune system of environmental stressors or pathogens infection have been extensively studied [21,34–36], mussels’ immune response to the combination of environmental stressors such as OA and pathogen infection raises questioning on the impact of both responses involving immune and endocrinial pathways [30]. Impacts of multi-stressors have been predicted to have additive, synergetic or antagonistic effects on marine organisms’ physiology [31]. These different effects are directly link to the amount of time between the occurrence of stressors, its intensity as well as on the organism ability to return to homeostasis before a new stressor occurs [32].

The aim of the present study was to investigate the combine effects of OA and bacterial infection on the expression of immune-related genes in M. chilensis. Mussels were exposed during 60 days to both current and futuristic pH level and were then challenged with the pathogenic bacterium Vibrio anguillarum. Herein, the expression of nine immune-related genes (PGRP, C-type Lectin, Myticin A, Mytilin B, Defensin, HSP70, HSP90, Ferritin, Catalase) were evaluated in gills of M. chilensis at 0, 8 and 16 h post-infection. The present study highlights the importance of multi-stressors studies to gain a better understanding of the immune response in bivalves facing ocean acidification scenarios.

2. Materials and methods

2.1. Biological material

Adult individuals of M. edulis chilensis (Hupe, 1854) (~70 mm shell size) were collected from culture ropes (5 m depth) at a mussel farming center located in Vilupulli, Chiloé (~42°35′35″S; 73°47′18″W) during July 2015. After collection, mussels were transported in chilled conditions to the Marine Biology Station of the University of Concepción and acclimatized in tanks (30 cm x 40 cm) with filtered seawater (0.1 μm, pH = 7.9 ± 0.01, T = 11.1 ± 0.01 °C, and salinity = ~30 psu) and constant aeration during 2 months. Throughout this acclimation period, mussels were fed daily with a phytoplankton suspension (~40 μg cell mL⁻¹, Phytoplankon-S).

2.2. Experimental set-up

A total of twenty-four mussels were divided equally in four tanks (n = 6 mussels per tank). Two of these tanks correspond to the OA control condition with a pCO₂ level in sea water reared at 550 μatm corresponding to the average level in mussel farming area (unpublished data by MUSELS Research Center). In the other two tanks (n = 12 mussels in total, n = 6 per tank) sea-water was reared to a pCO₂ level of 1200 μatm which corresponds to the worst case scenarios (RCP8.5) predicted for 2100 [37]. Mussels were exposed to these two different levels of pCO₂ during two months.

2.2.1. Seawater acidification system and measurements

To obtain the two different levels of pCO₂, dry air with pure CO₂ was blended to each target concentration using mass flow controllers (MFCs) for both air and CO₂. To achieve pCO₂ levels, the experimental system was implemented by MUSELS Research Center at the Experimental Marine Station, Universidad de Concepción. For each experimental tanks, temperature, pH and salinity were monitored every day while total alkalinity was measured every 10 days (Table 1). The pH was measured potentiometrically in 25-ml at 25.0 °C using a Metrohm 713 pH meter (input resistance >1013Ohm, 0.1 mV sensitivity, and nominal resolution 0.001 pH units) and a glass combined double junction Ag/AgCl electrode (Metrohm model 6.0219.100) calibrated with 8.089 Tris buffer 25 °C. pH values are reported on the total hydrogen ion scale. Temperature and salinity were measured using an Oakton SALT 6 + handheld salinity meter with probe. Total alkalinity was measured using the method of Haraldsson [38]. The pH, total alkalinity, phosphate, dissolved silicate, and hydrographic data were used to calculate the remaining carbonate system parameters and the saturation stage of Omega Aragonite using CO2SYS software set with Mehrbach solubility constants readjusted by Dickson [39].

2.2.2. Bacterial challenge

After two month exposed with two levels of pCO₂ (550 and 1200 μatm), mussels were challenged with 100 μl of Vibrio anguillarum directly injected in the adductor muscle. V. anguillarum was obtained from Laboratory of Biotechnology and Aquaculture Genomics and grown in Luria-Bertani (LB) at 20 °C for 48 h. Three individuals for each pCO₂ condition, chosen randomly in the two tanks, were collected at 0, 8 and 16 h post infection. Additionally, at 70, a control of injection was performed with three individuals from each pCO₂ condition that were injected with 100 μl of the LB medium without bacteria. After collection, gills of each individual were sampled and stored in RNA later (Ambion, USA) at −80 °C until RNA extraction.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 100 mg of gill tissue using 1 ml of Trizol Reagent (Invitrogen, Life Technologies, USA) Briefly, gills were homogenized and lysed in a Retsch MM 200 Mixer Mill (Retsch Inc., Düsseldorf, Germany) at 20 Hz for 5 min and the standard protocol for RNA extraction using Trizol Reagent was carried out following the manufacturer’s instructions. The phases were separated with 100% chloroform and nucleic acid was precipitated with 100% isopropanol previously cooled at −20 °C. Subsequently, total RNA was washed using 75% ethanol cooled to −20 °C and then treated with DNAses (DNase I, Thermo

<table>
<thead>
<tr>
<th>CO₂ System parameters</th>
<th>Experimental treatments (μatm pCO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>550</td>
</tr>
<tr>
<td>pH at 25 °C (pH units)</td>
<td>7.784 ± 0.056</td>
</tr>
<tr>
<td>pH in situ (pH units)</td>
<td>7.991 ± 0.038</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>11.00 ± 1</td>
</tr>
<tr>
<td>Salinity (psu)</td>
<td>29.50 ± 0.43</td>
</tr>
<tr>
<td>TA (μmol Kg⁻¹)</td>
<td>2004.8 ± 172</td>
</tr>
<tr>
<td>pCO₂ in situ (μatm)</td>
<td>414.1 ± 97.4</td>
</tr>
<tr>
<td>[CO₂] in situ (μatm Kg⁻¹)</td>
<td>97.5 ± 5.4</td>
</tr>
<tr>
<td>Tca</td>
<td>2.40 ± 0.13</td>
</tr>
<tr>
<td>Tdar</td>
<td>1.51 ± 0.08</td>
</tr>
</tbody>
</table>

Table 1: Sea water parameters (mean ± SD) used during the experimental period. pH in situ correspond to pH values at ambient temperature. TA: Total alkalinity, Tca: Calcite saturation, Tdar: Aragonite saturation.
Scientific, Wilmington, USA) to eliminate genomic DNA contamination. The RNA concentration and purity was measured in a ND-1000 spectrophotometer (Nanodrop Technologies, Thermo Scientific), and its integrity was visualized by electrophoresis in 1.2% MOPS-agarose gel stained with 0.001% ethidium bromide. Finally, the purified RNA was stored at -80 °C for later use. For cDNA synthesis, 200 ng of total RNA were retrotranscribed for each sample, using the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific) in accordance with the manufacturer’s instructions.

2.4. Selection and standardization of candidate genes

Nine candidate genes involved in different biological processes were selected, some of which had previously been reported to present differential responses to global stress (HSP90, HSP70), oxidative stress (Ferritin, Catalase) and pathogens (PRGP, Myticin A, Mytilin B, C-type Lectin and Defensin). The ?-tubulin gene was used as an endogenous control. Primers were designed with the commercial software Geneious v. 5.1.7 (Biomatters Ltd. Auckland, New Zealand). Genes, functions and primers are described in Table 2. Subsequently, PCR reactions were conducted in 25 L of total volume containing 1 x PCR buffer (Fermentas), 0.2 ?g/µL of BSA, 1.5 mM of MgCl2, 500 nM of sense and anti-sense primers, 0.06 U/µL of Taq Polymerase (Fermentas) and 2 µL of mussel cDNA as template. The PCR product was visualized by electrophoresis in 1.2% agarose gel stained with 0.001% ethidium bromure.

2.5. Analysis of gene expression with quantitative PCR

Quantitative PCR was carried out to evaluate the transcription expression using the 2-ΔΔCt with 3 replicates per time post infection for each treatment. Target genes were normalized with the selected endogenous control, ?-tubulin which was the most stable gene [40]. Runs were made with the Maxima SYBR-Green/Rox qPCR Master Mix 2X kit (Fermentas, Thermo Scientific, USA) according to the manufacturer’s instructions in a StepOne Plus Mastercycler (Applied Biosystemsw, Life Technologies, USA). The calibration curve for these runs consisted of five serial dilutions to a 200 ng of cDNA stock with a serial factor of 1:5, which were amplified with primers for each target gene. ΔCt values of these genes were introduced into the bioinformatic tool NormFinder and stability analysis was conducted.

2.6. Statistical analysis

Data were exported from Microsoft Excel (v. 2011, Microsoftw, USA), in which data per sample and biological groups (experimental and control) were reordered and the RQ value was manually calculated using the ΔΔCt formula [41]. Subsequently, the data were exported to Statistica software (v. 8.0, Statsoftw, Tulsa, USA), in which the distribution of the data and significant differences between treatments were evaluated by two-way ANOVA tests for parametrically distributed data, followed by Student-Newman-Keuls (SNK) test when appropriate. A value of p < 0.05 was established for significant differences among groups.

3. Results

3.1. Effect of OA on the expression of immune-related genes

After exposure to elevated pCO2, significant and contrasting differences in the expression of Pattern Recognition Receptors were observed, with a down-regulation of PRGP and an up-regulation of the C-type Lectin (Fig. 1). Likewise, antimicrobial peptides have

Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank accession number</th>
<th>Molecular function</th>
<th>Primers (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRGP</td>
<td>MGC04209 (Mytibase)</td>
<td>Pattern recognition receptor</td>
<td>TGGCAACTGAAAGATGCTGGACTGGA</td>
<td>Nuñez-Acuña et al., 2013</td>
</tr>
<tr>
<td>C-type Lectin</td>
<td>AY676351</td>
<td>Pattern Recognition Receptors</td>
<td>CGGAAATGACGGGTCTTGTGAT</td>
<td>Détrée et al., 2016</td>
</tr>
<tr>
<td>Myticin A</td>
<td>AF162334</td>
<td>Antimicrobial peptide</td>
<td>CGGGAAATGTCCATCCCCC</td>
<td>Nuñez-Acuña et al., 2013</td>
</tr>
<tr>
<td>Mytilin B</td>
<td>HQ693567</td>
<td>Antimicrobial peptide</td>
<td>TGAGCCGACAGGCGTTGTG</td>
<td>Nuñez-Acuña et al., 2012</td>
</tr>
<tr>
<td>Defensin</td>
<td>HQ693570</td>
<td>Antimicrobial peptide</td>
<td>TGGGGAACGGCTCCACGCCA</td>
<td>Nuñez-Acuña et al., 2013</td>
</tr>
<tr>
<td>HSP70</td>
<td>HQ693569</td>
<td>Chaperone/response to stress</td>
<td>TGAGCTGATCAGGTAACCC</td>
<td>Nuñez-Acuña et al., 2012</td>
</tr>
<tr>
<td>HSP90</td>
<td>HQ693565</td>
<td>Chaperone/response to stress</td>
<td>TGAGCTGATCAGGTAACCC</td>
<td>Nuñez-Acuña et al., 2012</td>
</tr>
<tr>
<td>Ferritin</td>
<td>HQ693568</td>
<td>Response to oxidative stress</td>
<td>TGAGCTGATCAGGTAACCC</td>
<td>Nuñez-Acuña et al., 2012</td>
</tr>
<tr>
<td>Catalase</td>
<td>HQ693566</td>
<td>Response to oxidative stress</td>
<td>TGAGCTGATCAGGTAACCC</td>
<td>Nuñez-Acuña et al., 2012</td>
</tr>
</tbody>
</table>
distinct pattern of expression upon OA with an up-regulation of Mytilin B, a down-regulation of Defensin while no effects are observed for Myticin A. Among the four genes involved in a response to stress (HSP90, HSP70, Ferritin, Catalase), the expression of the HSP90 is the only one that differs after exposure to elevated pCO2, with a significant down-regulation observed.

3.2. Combine effects of OA exposure and V. anguillarum challenge

Following the 60 days of OA exposure, a bacterial challenge was performed by injecting M. chilensis with the pathogenic bacterium V. anguillarum. The expression of candidates’ genes was measured at 0, 8 and 16 hpi (hours post-injection) and a two-way ANOVA was performed to determine interactions between stressors and significant differences (Table 3). Results showed a global effect of OA on the expression of immune-related genes during the challenge (Fig. 2) with a different kinetic of expression observed under OA exposure compared to the experimental control. For instance, the expression of PGRP is down-regulated at 8 and 16 hpi under control pCO2 level while under elevated pCO2 level, a down-regulation is observed at 8 hpi followed by a rise, back to basal level at 16 hpi. Similarly, the expression of C-type Lectin is slightly down-regulated at only 8 hpi under control pCO2 condition and its expression is gradually down-regulated under OA stress with a near null expression at 16 hpi (Fig. 2A). Interestingly, the expression of antimicrobial peptides seems to be highly modulated by OA, with adverse responses against pathogenic bacteria between control and OA condition. While Myticin A is down-regulated after injection of V. anguillarum in control condition, its expression is up-regulated under OA. Similarly, upon infection, the expression of Mytilin B is down-regulated in OA condition while up-regulated in control condition. Finally, no effects are observed upon infection on the expression of Defensin after OA exposure while a down-regulation is observed in the control condition (Fig. 2B). In the case of stress-related genes, no variation of expression of HSP70, HSP90, Ferritin and Catalase are observed upon infection under OA stress, whereas a down-regulation of HSP70 and 90 is observed under control pCO2 condition (Fig. 2C). It is worth noting that the expression results observed for both pCO2 levels do not correspond to a response to injection as the expression of each selected gene is not statistically different in individuals not injected and in individuals injected only with LB at T0 (Fig. S1).

Principal component analysis revealed gene expression patterns for two principal components (PC1 and PC2), which accounted for 57.4% of gene expression variability (Fig. 3). At T0, PC1 showed the main separation between control and OA exposed individuals whereas PC2 revealed this separation at 16 hpi and both PC1 and PC2 at 8 hpi. These results confirm that gene expression seems to be related to the exposition to OA with different pattern observed in the different time point post-infection (Fig. 3).

4. Discussion

Changing ocean conditions are directly impacting biological processes in marine organisms [42]. A growing body of evidence has shown that OA represents a threat to ecologically and economically important mollusks, disrupting their energy balance by impacting processes associated with high energetic cost such as growth, development, immunity and disturbing energy metabolism by, among other, causing shifts in metabolic pathways [18]. However, in the environment, stressors are not isolated and it has long been recognized that the impact of one stressor can be modified by others [43] and that there is a need to study their synergistic effects [44–46]. In this study, we investigated the combined effects of OA and pathogenic infection using V. anguillarum as a driving force for infectious diseases. The expression of 9 immune-related genes such as PGRP, C-type Lectin, Myticin A, Mytilin B, Defensin, HSP70 and 90, Ferritin and Catalase were therefore investigated in the gills of M. chilensis individuals exposed to OA and to both OA and V. anguillarum.

Among the nine immune-related genes studied, five were differentially expressed in response to OA of which two pattern recognition receptors (PRR), two antimicrobial peptides and the HSP90. PRRs such as PGRP and C-type Lectin are immune membrane-bound or cytosolic proteins capable of recognizing a large number of stimuli and to trigger a cascade of reaction leading to the production of antimicrobial peptides (AMPs) or inflammatory proteins in the case of PGRP or to endocytosis or opsonization in the case of the C-type lectin [47]. In this study, these two PRRs are evidencing opposite pattern under elevated pCO2 with the up-regulation of C-type lectin when the PGRP is down-regulated. Similarly, Mytilin B and Defensin are presenting opposite pattern. These antimicrobial peptides are produced as precursor molecules processed to active compounds within the hemocytes [48] and have been shown to decrease their mRNA expression under short-term bacterial infection, while increasing it when facing short-term physical stress (Shell filling, and heat shock) [36,49]. Our study shows more contrasting results of the expression of these two antimicrobial peptides under OA suggesting a shift in immunomodulation dynamics. Results on Defensin and C-type Lectin are congruent with the one observed in corals and oyster under OA [50,51] but seem to be in contradiction with in vitro results of Hernroth and co-authors who observed a global diminution of bacteriostatic activity [20].

When combining the effects of OA and bacterium infection, antimicrobial peptides production seems to be strongly affected with counteracting patterns observed in the expression of both Myticin A and Mytilin B upon infection while the expression of PRR is globally diminishing at 8 hpi. These results suggest that upon a combination of OA stress and bacterial infection, individuals of M. chilensis are displaying a different immunomodulation pattern than the one observed previously with a short-term down-
Fig. 2. Transcripts relative expression levels for each candidate gene of *Mytilus chilensis* after 8 and 16 h post-injection with *V. anguillarum* under two nominal pCO$_2$ levels. Each group of gene represents a specific function: pattern recognition receptors (A), antimicrobial peptides (B) and response to stress (C). In the rectangles below each figure are the results of the SNK statistical test, > or <: significantly superior or inferior respectively, NS: No Significant. For each condition n = 3.
regulation of PRRs and a counteracting expression pattern of AMPs. Although most of the studies associated with combinations of multiple-stressors observed synergetic effects of stresses on marine organisms’ physiology [32], here we hypothesize that a combination of OA stress and short-term *V. anguillarum* infection may rather have partial antagonistic effects on *M. chilensis* immunity and physiology. Altogether our results suggest that under a combination of OA and bacterial infection, mussels’ physiology and global homeostasis are affected, leading to the necessity of modeling the expression of immune receptors and antimicrobial peptides. Furthermore, results on AMPs allow the assumption that a combination of elevated pCO$_2$ and bacterial infection may have antagonistic effects which may be on account of the characteristic of multi-stressors exposure in our study with a short and asynchronical infection with *V. anguillarum*. However, our results do not allow to determine if OA as a first stressors increase either the susceptibility or the tolerance (known as cross-protection) of mussels toward *Vibrio*’s infection. To elucidate this last point and gain better understanding on the effects of these stressors on aquaculture species and production, further studies associating the expression of immune-related genes with energy budget are needed and this, applied to synchronically and long-term exposure to both stressors. Further studies are required to understand how the bacteria physiology and the pathogenic mechanism displayed during the infection are modulated by high levels of pCO$_2$ that in turn can modify the innate immune response of marine invertebrates.

**Acknowledgements**

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.08.047.

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