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Short communication

Insight into the messenger role of reactive oxygen intermediates in immunostimulated hemocytes from the scallop *Argopecten purpuratus*

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ABSTRACT

Reactive oxygen intermediates (ROI) are metabolites produced by aerobic cells which have been linked to oxidative stress. Evidence reported in vertebrates indicates that ROI can also act as messengers in a variety of cellular signaling pathways, including those involved in innate immunity. In a recent study, an inhibitor of NF-kB transcription factors was identified in the scallop *Argopecten purpuratus*, and its functional characterization suggested that it may regulate the expression of the big defensin antimicrobial peptide *ApBD1*. In order to give new insights into the messenger role of ROI in the immune response of bivalve mollusks, the effect of ROI production on gene transcription of *ApBD1* was assessed in *A. purpuratus*. The results showed that 48 h-cultured hemocytes were able to display phagocytic activity and ROI production in response to the β-glucan zymosan. The immune stimulation also induced the transcription of *ApBD1*, which was upregulated in cultured hemocytes. After neutralizing the ROI produced by the stimulated hemocytes with the antioxidant trolox, the transcription of *ApBD1* was reduced near to base levels. The results suggest a potential messenger role of intracellular ROI on the regulation of *ApBD1* transcription during the immune response of scallops.

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1. Introduction

The immune response of marine mollusks has been widely studied with a focus on the identification and characterization of genes homologous with vertebrates (Little et al., 2005). However, many processes remain unknown, and a systemic notion of the interaction between molecular components is required for a functional understanding of mollusk immune response. Among aquacultured mollusk species, the scallop *Argopecten purpuratus* represents one of the most important species in northern Chile (López et al., 2000). During the last decade, hatchery-reared larvae of scallops have suffered mass mortality events associated among others factors to the Gram-negative bacterium *Vibrio splendidus* (Rojas et al., 2015). Therefore, a deeper understanding of the immune response mechanisms in this species is essential for the improvement of management strategies in aquaculture.

Like all invertebrates, the immune response of mollusks depends exclusively on innate immune mechanisms, mediated by cellular and humoral components. Marine mollusk immunity has been reviewed in detail (Song et al., 2015; Bachère et al., 2015), and can be summarized in three main stages carried out through circulating and infiltrating immunocompetent cells, the hemocytes. These cells mediate (i) the recognition of non-self molecules by soluble and membrane receptors, (ii) the consecutive activation of intracellular signaling cascades and (iii) the triggering of cellular and humoral responses, such as phagocytosis and the expression of antimicrobial peptides, which are key effectors of antimicrobial immune response (Canesi et al., 2002). Antimicrobial peptides (AMPs) are present in virtually all organisms, displaying diverse roles in immunity (reviewed in (Bulet et al., 2004)). To date, two AMPs have been characterized from scallops; a fragment of the histone H2A and a big defensin. The big defensin displays antimicrobial properties and it is upregulated during immune response (Song et al., 2015), and as with many other AMPs, it appears to be regulated by a putative NF-kB pathway (Oyanedel et al., 2016).

Reactive oxygen intermediates (ROI) are metabolites

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constitutively produced mainly in the mitochondria as a side reaction of the respiratory chain. They can be induced in response to damage associated molecular patterns (DAMPs) such as pollutants and ionizing radiation (Bartosz, 2009; Lodovici and Bigagli, 2011), and in response to microorganism associated molecular patterns (MAMPs) during the phagocytic process (Flannagan et al., 2012; Donaghy et al., 2014). Evidence reported in higher vertebrates suggests that ROI can also act as messengers regulating a variety of signaling molecules, including transcription factors, G protein-coupled receptors, phosphatases and proteases that affect several cellular processes (Forman et al., 2010). Despite the fact that hemocytes produce ROI as part of their base metabolism as well as in response to immune stimuli, the multiple intracellular sources of ROI are poorly described in marine mollusks (Donaghy et al., 2014). Moreover, to date there is no information on the potential role of ROI as an immune messenger in invertebrates.

Considering that the main strategies of the innate immune response in mollusks involves both, ROI production and antimicrobial peptide expression derived from signaling events, the aim of this study is to investigate the effect of intracellular ROI production on the expression of the big defensin antimicrobial peptide in the scallop *Argopecten purpuratus*. Results obtained in this study constitute, to the best of our knowledge, the first evidence of a positive association between the ROI produced during phagocytosis and the expression of an antimicrobial peptide in mollusks.

2. Material and methods

2.1. Scallop procurement and maintenance

US National Research Council guidelines for the care and use of laboratory animals were strictly followed during this research (National Research Council, 2011). Adult scallops were collected from the Central Culture Center at the Universidad Católica del Norte (UCN), in Coquimbo, transported to the Experimental Laboratory of Aquaculture Curauma, (PUCV) and placed in a 60 L sea water tank connected to a water pump with a UV system (SERA), a biological filter (SERA) and a chiller field control circuit (HAQOS). The water was maintained at 17 °C and seawater was replaced 1:10 every day. The scallops were fed daily with the microalgae *Isochrysis galbana* and *Tetraselmis suecica* until use.

2.2. Hemocyte primary cultures: immune stimulation and ROI scavenger

The hemolymph was collected from the adductor muscle of the scallops using a cold syringe coupled to an 18G needle. Hemolymph was pooled in polypropylene tubes in ice and the total hemocyte number was determined using a hemocytometer. Hemolymph was centrifuged at 800 × *g* for 5 min at 4 °C. Hemocyte pellets were resuspended in modified L15 medium with Glutamine (Thermofisher Scientific), supplemented with 10% FBS, 50% 0.22 μm filtered scallop plasma, 500 mOsm NaCl, penicillin (100 U/mL) and Streptomycin (50 μg/mL). Cell number counts were adjusted to 4×10^6 cell/mL, 1 mL was added to 12-well culture plates and then incubated for 3 h at 17 °C. After adhesion, the cells were washed with sterile sea water (SSW) and fresh medium was added. Cell viability was evaluated using trypan blue staining. Cultured hemocytes were incubated at 17 °C for 48 h before the experiments. For cytological analyses, hemocytes were fixed in 4% paraformaldehyde at pH 7 for 10 min and stained with May-Grunwald-Giemsa. Unfixed hemocytes were incubated with 0.4% Trypan blue and examined by phase-contrast microscopy. Samples were examined under a Leica DM5000B microscope equipped with a Leica DFC450C digital camera. Fixed hemocytes were also stained with Alexa Fluor 488 Phalloidin (Thermofisher Scientific) for actin filaments and to-pro iodide (Thermofisher Scientific) for nuclear staining. Confocal images were obtained with a Leica 40 × 1.25 Oil HCX PL APO CS lens (Leica Microsystems). For the immune stimulation, a stock of 1×10^8 particles/mL of Zymosan A from *Saccharomyces cerevisiae* (Sigma) was prepared in SSW, and 10 particles of zymosan per hemocyte were added to the culture wells in fresh medium and incubated at different time points. For the phagocytosis assay, the hemocytes were incubated for 30 min with zymosan to allow complete engulfment of the particles and phagocytic vesicle formation. For ROI neutralization, 10 μM of the scavenger trolox® (SIGMA) was added to the 4×10^6 cells 3 h before zymosan treatment, as determined by previous essays in our lab. Trolox is a cell permeable compound that works as a chemical analogue of vitamin E, acting as a potent antioxidant due to its free radical scavenging activity (Hamad et al., 2010). The cells were then incubated at 17 °C until (i) paraformaldehyde fixation, (ii) RNA extraction or (iii) ROI detection. All conditions were performed in triplicate. ROI accumulation was detected by 2′,7′ −dichlorofluorescin diacetate DCFDA Cellular Reactive Oxygen Species Detection Assay (Abcam) following the manufacturer's protocol. Fluorescence was detected (EX: 495 nm/EM: 529 nm) on a microplate reader (Thermo Appliskan) and by confocal analysis with a Leica 40 × 1.25 Oil HCX PL APO CS lens (Leica Microsystems).

2.3. Total RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from cultured hemocytes under the different conditions using TRIzol® reagent in accordance with manufacturer's instructions (Thermo Scientific). RNA was then treated with DNAse I (Thermo Scientific) for 15 min at room temperature and inactivated by heat for 10 min at 65 °C. Quantification and quality of the total RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies) and 1% agarose gel electrophoresis, respectively. First strand synthesis was carried out from 1 μg of total RNA using 500 ng oligo-(dT)12–18 (Thermo Scientific), 1 mM dNTPs (Promega), 25 U Rnasin (Promega) and 200 U M-MLV reverse transcriptase in reverse transcriptase buffer (Promega) following the manufacturer's protocol. For RT-qPCR analyses, the 10 μl-volume reaction consisted of 1 × Brilliant II SYBR Green QPCR master mix (Stratagene), 0.3 μM of each primer and 1 μL of cDNA diluted to 1:5 in sterile ultra-pure water. Primers were for the big defensin (GenBank no. KU499992): *ApBD1f*: 5′-TGGCAACAGCGGATGGTGT-3′; *ApBD1r:* 5′-AACGCCTAAGTTCCCACCTCG-3′ and for the β-actin (GenBank no. ES469330): *ApAct-f*: 5′-CACTGCTCTTGCTCCACAAAC-3′: *ApAct-r*: 5′-GAAGGTGGACAGAGATGCCAA-3′. RT-qPCR assays were performed in triplicate in a Biorad C1000 Touch Thermocycler CFX96, and primer pair efficiencies (E) were calculated from the given slopes in the BioRad CFX software according to the equation: $E = 10^{[-1/\text{slope}]}$. Samples were submitted to an initial denaturation step of 10 min at 95 °C followed by an amplification of the target cDNA (40 cycles of denaturation at 95 °C for 5 s, annealing at 56 °C for 5 s and extension time at 60 °C for 15 s) and fluorescence detection. After an initial 10 s denaturation step at 95 °C, a melting curve was obtained from a start temperature of 65 °C to a final temperature of 95 °C, with an increase of 0.06 °C/s. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Calculations of means, standard deviations and statistical analysis using the Kruskal-Wallis test for expression analysis were carried out using GraphPad Prism software version 6.01 (significant value: $P < 0.05$).

3. Results

3.1. Establishment of primary cultures and cytological analyses of A. purpuratus hemocytes

Immunocompetent primary cultures of hemocytes were first established in order to obtain an *in vitro* model of immunostimulation. The results showed that after the initial seeding of hemocytes, spontaneous cellular aggregation occurred and dense clumps of cells adhered to the plate. After 3 h, cells started to migrate from the clumps and spread along the surface (Fig. 1A). Clumps were progressively disrupted between 24 and 48 h, leading to the appearance of two well-differentiated morphological subtypes of (i) rounded and small cells named blast-like cells and (ii) spread-pseudopod forming fibroblast-like cells (Fig. 1A). The latter ended up forming an extensive interconnected network of hemocytes, showing formation of 2–3 pseudopods on each cell. The extensive networks formed by the pseudopod projections were linked to the actin filaments observed by fluorescent staining at 48 h of cell culture. (Fig. 1B). The hemocytes displayed an endoplasm (en) containing the nucleus and organelles, and an ectoplasm (ec) surrounding the cytoplasm and extending into the pseudopods, observed by May grunwald-Giemsa staining (Fig. 1C). Phagocytic capacity was determined using 48 h-cultured hemocytes challenged with zymosan particles. After 30 min of contact, numerous intracellular phagocytic vacuoles were observed in fixed hemocytes, compared to the control group (Fig. 1D–E). Phagocytosis of alive bacteria was also observed by 48 h-cultured hemocytes (Supplementary data).

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3.2. Immune stimulation with zymosan induces ROI production and the gene expression of the big defensin in 48 h-cultured hemocytes

Cultured hemocytes showed total disaggregation of the cell clumps, morphological differentiation and phagocytic activity at 48 h. Thus, this was the chosen time point for the assessment of ROI production by hemocytes challenged with zymosan particles. Non-induced hemocytes and zymosan-stimulated hemocytes were incubated with the redox probe DCFDA and live cells were observed under confocal microscopy. Confocal images showed that the phagocytic activity of zymosan particles was accompanied by an increase in ROI production, observed as an intensification of intracellular fluorescence compared to the control group after 15 min of incubation (Fig. 1F–G). In parallel, the transcription of *ApBD1* was assessed from non-induced hemocytes and zymosan-stimulated hemocytes using RT-qPCR at different time points (Fig. 2A).

The results showed a significant increase in the transcription levels of *ApBD1* at every time point compared to non-induced hemocytes, with the highest level detected at 6 h post-stimulation ($P < 0.01$). These results show that 48 h-cultured hemocytes maintain the ability to set up a cellular and humoral immune response after induction with zymosan.

3.3. Effect of the neutralization of ROI on ApBD1 expression in zymosan-stimulated hemocytes

To evaluate the effect of ROI production on the expression of *ApBD1*, the accumulation of ROI on zymosan-stimulated hemocytes was neutralized by pre-incubation of the cells with the vitamin E analog, trolox. The results showed that zymosan-stimulated hemocytes were able to significantly increase ROI production, quantified by means of the relative fluorescent units emitted by the redox probe DCFDA (Fig. 2B). A maximum 3-fold change in relative fluorescence was registered at 15 min of induction compared to the control conditions. In contrast, a significant decrease of the 15 min-peak in ROI production was observed when zymosan-stimulated hemocytes were pre-treated with trolox (Fig. 2B). Gene transcription of the big defensin was determined for each experimental condition at 6 h post-stimulation using RT-qPCR (Fig. 2C). The results showed the upregulation of *ApBD1* in zymosan-stimulated hemocytes and a significant decrease of *ApBD1* on stimulated cells pre-treated with the ROI scavenger. Thus, expression of the big defensin was reduced near to base levels by neutralization of the ROI accumulation.

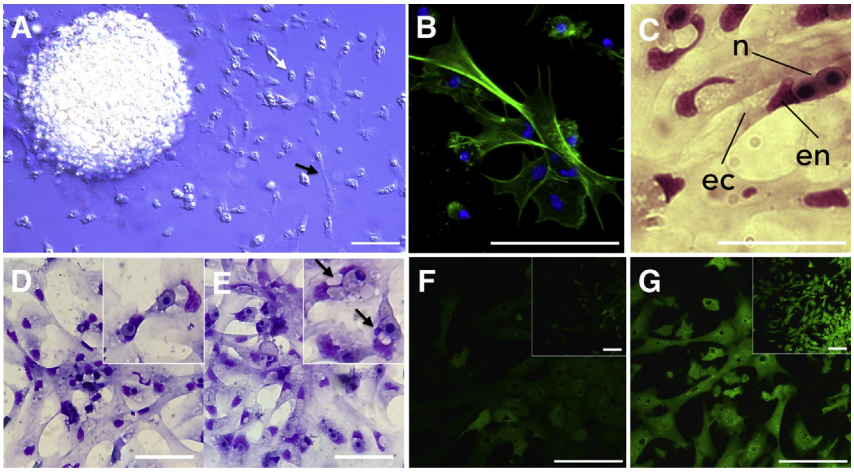

Fig. 1. Immunocompetent primary cultures of *A. purpuratus* hemocytes. **A**. Hemocyte clump and cellular migration after 24 h of incubation observed by contrast phase microscopy. White arrow, blast-like cells. Black arrow, fibroblast-like cells. **B**. Adherent 48 h-cultured hemocytes showing pseudopod projections of actin filaments by phalloidin-fluorescent staining (green) and nuclear staining (blue) by confocal microscopy. **C**. Adherent 48 h-hemocytes showing a central region containing the nucleus (n) and organelles contained in the endoplasm (en), surrounded by ectoplasm (as determined by (Hamad et al., 2010)). **D** and **E**. Phagocytosis of zymosan particles by 48 h-cultured hemocytes. Control hemocytes (**D**) and zymosan-stimulated hemocytes (**E**) fixed in 4% paraformaldehyde and stained with May grunwald-Giemsa at 30 min post-challenge. Black arrows indicate phagocytic vesicles inside hemocytes. **F** and **G**. ROI production from control hemocytes **(F)** and zymosan-induced hemocytes **(G)** detected by DCFDA and fluorescence detection by confocal microscopy. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

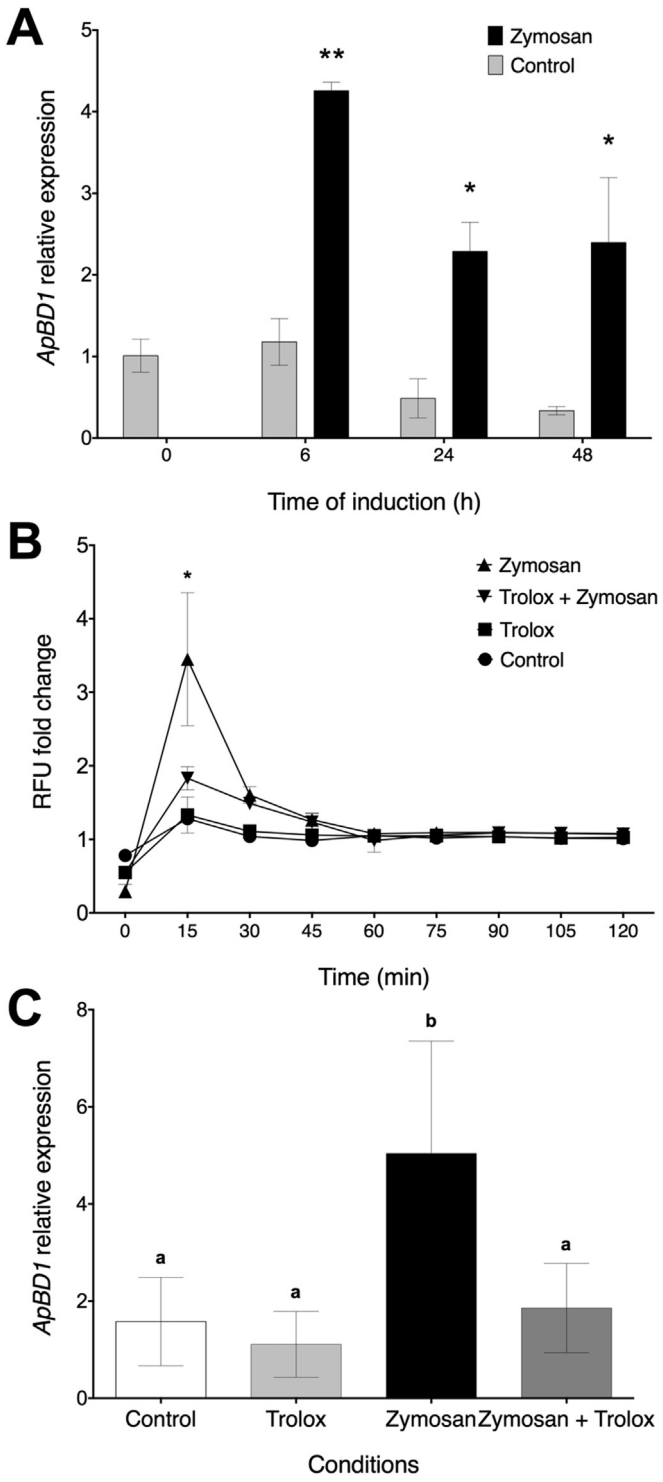

Fig. 2. ROI neutralization in zymosan-stimulated hemocytes by trolox leading to reduced gene transcription of *ApBD1 in vitro*. **A**. Relative transcription levels of *ApBD1* in 48 h-cultured hemocytes induced with zymosan at different time points obtained by RT-qPCR. The scallop β-actin gene used as reference gene. Results are expressed as mean values ± SD from triplicates for each condition. Significant differences indicated by asterisks (*$P < 0.05$, **$P < 0.01$). **B**. ROI detection by CDFDA and fluorescence detection on microplate from cultured hemocytes. ROI levels expressed as the fold change in fluorescence emission of DCFDA related to the preceding measurement point. **C**. Relative expression levels of *ApBD1* in cultured hemocytes were obtained by RT-qPCR from non-stimulated cells (white bar), trolox pre-treated cells (light gray bar), zymosan-stimulated cells (black bar) and trolox pre-treated and zymosan-stimulated cells (dark gray bar). The scallop β-actin gene used as reference gene. Results expressed as mean values ± SD from triplicates for each condition. Significant differences between conditions indicated by different lowercase letters ($P < 0.05$).

4. Discussion

The results obtained in this study suggest that ROI produced during the phagocytic process of hemocytes could promote the expression of an AMP, implying the existence of possible crosstalk between the two major defense strategies in scallops against pathogenic invasion, namely ROI and AMP production. In this study, primary cultures of *A. purpuratus* hemocytes were shown to respond to an immune stimulation with phagocytic activity and ROI production, but also with the gene overexpression of an immune effector. The transcription of the big defensin gene was considered as a control of immunostimulation due to its well-known upregulation after an immune challenge in bivalve mollusks (Zhao et al., 2007; Rosa et al., 2011; Wang et al., 2014). The big defensin also displayed potent antibacterial activity, suggesting its participation in immune defense mechanisms (Zhao et al., 2007). Notably, after the neutralization of the ROI produced in zymosan-stimulated hemocytes by pre-treatment with an ROI scavenger, the down-regulation of the transcription of the big defensin was observed near to base levels. Therefore, ROI produced by the phagocytic process of hemocytes appears to promote the expression of this antimicrobial peptide. The fact that ROI may also be produced in response to DAMPs, highlights the importance of evaluating the effect of damage signals in the regulation of antimicrobial peptide expression.

An essential tool for gaining new insights into the immune response mechanisms in mollusks is the availability of well-characterized *in vitro* model systems. To date, no immortalized cell lines of mollusk hemocytes are available, and several previous studies have successfully developed primary cultures of mollusk hemocytes to evaluate an immune response (Estrada et al., 2013; Astuya et al., 2015). The adhesion and morphological progression observed for scallop cultured hemocytes has only been reported in the scallop *Nodipecten subnodosus* (Estrada et al., 2013). Nevertheless, in our study the supplementation of the media with filtered plasma was essential for the successful culture of *A. purpuratus* hemocytes. The presence of many soluble plasmatic proteins in bivalves such as lectins and antioxidants is well described (Wang et al., 2012), and they may be involved in the differentiation, cellular communication and immunocompetence of these cells.

The oxygen scavenging activity of trolox was chosen to diminish the amount of intracellular ROI present in stimulated hemocytes, and function as a neutralizing end-product targeting strategy (Cho et al., 2010). This ROI neutralization approach was chosen over the addition of exogenous oxygen intermediates because the purpose was to assess the effect of intracellular ROI during an immune response and not an exogenous and prolonged stress-like stimulus. It was considered that hemocytes of marine bivalves produce moderate levels of ROI compared with vertebrate models (Donaghy et al., 2014), while also taking into account the existence of highly contradictory results on the effect of exogenously added ROI in the activation of NF-kB (de Oliveira-Marques et al., 2007). The results found in this study showed that trolox was an effective neutralizing agent of ROI on zymosan-stimulated cells. Interestingly, a current view proposes that ROI can play a role in cellular signaling and metabolism, modifying target proteins and/or changing the redox state (Bartosz, 2009). Different mechanisms for the messenger role of ROI have been proposed, such as the oxidation of specific residues of NF-κB dimers which prevent the binding of the transcription factor to DNA (Schieven et al., 1993), the modulation of certain types of tyrosine phosphatases (Reth, 2002), and the oxidation of

inhibitors of NF-κB molecules (IκBs) which may cause the proteosomal degradation and release of active NF-κB dimers that translocate into the nucleus (Takada et al., 2003). A previous study in our laboratory postulates a putative NF-κB signaling pathway as a regulator of the big defensin transcription in *A. purpuratus* (Oyanedel et al., 2016). It is therefore tempting to speculate that the promoting effect of ROI over big defensin transcription may be due to the interaction of ROI with a member of the protein family of IκBs, resulting in its degradation and the release of NF-kB dimers. Further studies in this area should consider the characterization of (i) NOX and DUOX homologue genes in *A. purpuratus* for deeper comprehension of the molecular machinery for ROI production and (ii) other components of the NF-κB signaling pathway. A proteomic approach to evaluate the effect of ROI neutralization of the big defensin phenotype should also be performed to confirm the functional role of this proposed effect during the immune response. This first evidence will help to deepen the knowledge on the role of ROI as messengers in mollusks.

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