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Out of the deep-sea into a land-based aquarium environment: Investigating innate immunity in the hydrothermal vent mussel *Bathymodiolus azoricus*

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Abstract: Deep-sea hydrothermal vents are considered as some of the most adverse environments in the world, yet the animals dwelling around the vent sites exhibit high productivity and therefore must cope with unusual levels of heavy metals, pH, temperature, CO_2 and sulfide, in addition to environmental microbes. In an attempt to understand the physiological reactions of animals normally set to endure extreme conditions we investigated molecular indicators of acclimation processes during which the mussel *Bathymodiolus azoricus* was maintained over long periods of time under laboratory conditions. Even in the absence of the characteristic high hydrostatic pressure found at deep-sea vent sites and without methane and/or sulfide supplementation, vent mussels seem to endure well aquarium conditions. The presence and induction of a Rel Homology Domain containing gene was investigated along with the induction of the antibacterial gene mytilin following experimental infections carried out with the marine bacteria *Vibrio parahaemolyticus*. It was then concluded that vent mussels are capable of performing typical immune gene transcription activity as their shallow water congeners. The mussel's viability was determined during stimulations achieved with the hyperbaric chamber IPOCAMP, by comparing the expression levels of, among other genes, the metal-responsive Metallothionein (MT) gene at 170 bars. Our results suggest that *Bathymodiolus azoricus* maybe used in experimental studies as an indicator species, for assessing immunity, toxicity or metal stress and the effect of hydrostatic pressure.

Keywords: Bathymodiolus azoricus ● Innate immunity ● Gene expression ● IPOCAMP ● Physiological adaptation ● Hydrothermal vent

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Introduction

The hydrothermal vent mussel *Bathymodiolus azoricus* (Von Cosel & Comtet in Von Cosel, Comtet & Krylova, 1999) is commonly found in dense aggregates around vents at the Mid Atlantic Ridge, south of the Azores islands. Other Bathymodiolid species are also found in large populations associated with deep-sea hydrothermal vents or cold-water sulfide/hydrocarbon seeps distributed in the Atlantic, Pacific and Indian oceans, demonstrating the functional adaptability of the genus to these extreme environments (Gustafson et al., 1998; Von Cosel et al., 1999). The presence of both chemoautotrophic and methanotrophic bacterial symbionts in specialized epithelial cells of *Bathymodiolus azoricus* gill tissues is believed to provide substantial nutritional advantage to the mussel, allowing it to obtain energy from both sulfide and methane at the vent sites (Distel et al., 1995; Fiala-Médioni et al., 2002; Duperron et al., 2006).

During the course of acclimation, under laboratory conditions and at atmospheric pressure, the number of endosymbiont bacteria is frequently reduced, until apparent total disappearance from gill tissue of mussels maintained in aquaria for at least 2-3 weeks in plain seawater. Nonetheless, there is evidence indicating that some endosymbiont bacteria remain in gill tissues, after months in seawater aquaria supplemented with methane and sulfide at atmospheric pressure conditions (Dando et al., unpublished results). Consequently, *B. azoricus* long-term maintenance at atmospheric pressure may constitute a useful model to study yet uncharacterized molecular relationships under which the regulation of gene transcription may be affected by aquaria conditions and "sea-level" pressure. It may also reveal new physiological responses of animals sustaining immunological and metal experimental challenges in the laboratory and thus provide new approaches to assess the effect of natural micro-organisms and metal toxicity at the vent natural environments. Vent mussels retrieved from 850-m depth endure well experimental acclimatization conditions even in the absence of hydrostatic pressure and their adaptation, to prolonged aquaria conditions, may be evaluated by physiological parameters including the transcriptional activity of immune genes (Bettencourt et al., 2007 & 2008). However, experiments conducted under hydrostatic pressure, using the hyperbaric chamber IPOCAMP (Shillito et al., 2006), may also constitute an invaluable experimental system to study the expression of genes that typically respond to mechanical stress and contribute to a better understanding of the mechanism by which vent mussels are able to cope with elevated pressures not only inside the IPOCAMP chamber but also in its natural environment.

In view of the long-term experiments in our aquarium system LabHorta (Bettencourt et al., 2008; Colaço et al., 2010) for the assessment of aquarium conditions and its suitability to address *B. azoricus* physiological adaptations to atmospheric pressure, we set out to investigate the expression of the immune-related transcription factor Rel/nuclear factor-κB (NF-κB) and the antibacterial peptide Mytilin genes, following exposure to the marine bacteria *Vibrio parahaemolyticus* and the expression of metal-binding and stress-related protein Metallothionein (MT), ensuing from *de novo* pressurization in the IPOCAMP chamber.

Protein members of the transcription factor NF-κB family share a highly conserved 300 amino-acid domain known as the Rel Homology Domain (RHD). The RHD is essential for DNA binding and its function is important for the transcription of immune and inflammatory genes (Baltimore & Beg, 1995). The antibacterial gene Mytilin and their isoforms were initially identified in the shallow water mussels *Mytilus edulis* and *Mytilus galloprovincialis* and are essentially active against gram-positive bacteria including some pathogens for marine invertebrates (Mitta et al., 2000). Metallothioneins (MTs) are small cytoplasmic cysteine-rich proteins considered to be involved in the regulation of essential metals such as copper and zinc and in the detoxification of toxic metals, for instance, cadmium and mercury (Desclaux-Marchand et al., 2007). A protective role against free radicals and oxidative stress has also been attributed to MTs (Simes et al., 2003). It would thus appear reasonable to look at the MT gene expression in ecotoxicological and environmental marine studies using gill tissues from filter-feeding bivalves, particularly mussels, assuming that their gills constitute a key interface in the uptake of metals or in the interaction with stress-related factors (Hardivillier et al., 2004).

Even after prolonged aquarium maintenance at atmospheric pressure, mussels are still able to induce immune and stress-related genes as demonstrated by their transcripts (mRNA) analyses. Moreover, the pressure levels to which vent mussels were exposed, during IPOCAMP exposures, were not detrimental to the animal's physiology in regard to gene expression. In light of the results we concluded that long term acclimatization of vent mussels offers the opportunity to study genes involved in physiological adaptations under different laboratorial conditions after the animals have been maintained for months out from their natural environment, or subjected to *de novo* "deep-sea" hydrostatic pressure levels. Thus, the maintenance of live vent mussels in our laboratory is a key factor for gaining insights into the physiology of vent animals including the study of evolutionary conserved immune and metal-responsive factors commonly found in other marine bivalves and may contribute to a better

understanding of the effect of both, prolonged aquarium acclimatization and hydrostatic re-pressurization, on gene expression in vent mussels.

Material and Methods

Animal collection, maintenance and hyperbaric inductions.

The present study was carried out with mussels collected from the hydrothermal vent field Menez Gwen (850 m depth, 37º50.8-37º51.6N, 31º30-31º31.8W), in the Mid-Atlantic Ridge (MAR), with the French R/V *Pourquoi pas?* using the ROV Victor 6000 (MoMARETO cruise, August 6th – September 6th 2006). In the LabHorta aquarium, mussels were kept at 7- 8ºC in plastic containers filled with 20 litres of seawater and aerated to give an oxygen saturation of 10-50%. The stocking density was 1 animal.litre^{-1} of seawater, which was changed every other day to keep a pH of 7-8. Each container was supplied every other day, between seawater replenishments, with 5 ml of a food mixture consisting of freeze dried ocean plankton enriched with vitamins (Ocean Plankton, Hikari BIO-PURE® FD). This feeding regime was seemingly suitable for keeping mussels under aquarium conditions for several months, although growth assessment was not performed during the present studies (Bettencourt et al., 2008). For the hyperbaric stimulations the stainless steel pressure vessel IPOCAM 5 (AutoclaveFrance, France) was used as described previously by Shillito et al. (2006). The flow-through system consisted of a constant 4L/hour flow of chilled seawater from which temperature, oxygen saturation and pH parameters were monitored regularly, ranging from 8-10ºC; 40-60% and pH 7.3-7.9, respectively. A group of 6 animals was set inside the chamber and subjected to 20 bars daily increments until 80 bars were reached at the fourth day and after which a direct 170 bars stimulation was attempted on the 5th day for the remaining experimental period. On the seventh day of stimulation, the IPOCAMP vessel was depressurized and mussels brought to atmospheric pressure for immediate preservation at -80ºC for subsequent total RNA extractions or in 10% buffered formalin for further histological processing.

Vibrio infections

Mussels were experimentally exposed to *Vibrio para-haemolyticus* using 18 ml of overnight cultures (OD_{600} = 1) mixed in vessels containing 2 litres of sea water in which 10 animals were kept for 48 h in aerated conditions. Gill tissues were dissected, preserved at -80ºC for subsequent RNA extractions or in 10% buffered formalin for further histological processing and observations using the Hematoxylin and Eosin (H&E) staining, *in situ* hybridization experiments (Bettencourt et al., 2009) and detection of intracellular Ca^{2+} variations, using Fura-2 AM (Molecular Probes®, Invitrogen). Paraffin-embedded histological sections were processed for deparrafinization and rehydration according to standard procedures. After the last ethanol wash followed by a short phosphate saline buffer rinse, tissue sections were labeled with Fura-2 in final concentration of 1 µM in sea water hemocyte preparations, for 30 min at 4 ºC, after which the preparations were covered with a glass cover slip for immediate observations under UV light. Cell labeling conditions were according to the manufacturer's indications (1 µM or 1:1000 dilution of original purchased solution). For total hemocyte counts following *Vibrio* exposure a minimum of 6 microscopic slides per individual were examined and compared to slides from non exposed mussels.

Fluorescence in situ-hybridization (FISH)

The presence of the REL homology domain and the Metallothionein gene in *B. azoricus* gills was determined by FISH according to Bettencourt et al. (2008). Tissues sections were incubated with the hybridization solution containing specific single-stranded oligo-probes, consisting of 5'CCTAAAACGTTAACGTAGCTTTGTTTGCAC3' labeled with Alexa Fluor 555 (Molecular Probes®, Invitrogen) to target the MT mRNA or the RHD specific probe, 5'ATACTCACAGCCCCTTCTAGACGACCCT-CATAAGGACCGC3' labeled with Alexa Fluor 488, to target Rel Homology Domain (RHD) containing transcripts. Control experiments were carried out with oligos corresponding to non-complementary sequences to the mRNA. Gill filaments were visualized under fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany).

RNA extraction, Semi-quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA from *B. azoricus* gill tissues was extracted with the RiboPureTM kit (Ambion®) according to manufacturer's instructions and re-suspended in nuclease-free, DEPC-treated water. The quality of total RNA preparations was assessed in formaldehyde agarose gel electrophoresis using the standard procedure and using the NanoVue spectrophotometer (General Electric, Healthcare Life Sciences) for concentration and purity estimations. The first strand cDNA synthesis or reverse transcriptase reaction (RT) was performed with the M-MLV reverse transcriptase enzyme (Promega®) according to manufacturer's instructions and using 2 µg total RNA in 25 µl total volume reactions. Subsequently, 2 µl of RT

reactions were mixed to a 100 µl Polymerase Chain Reaction (PCR) mix containing appropriate PCR buffer, deoxyribonucleotide triphosphates (dNTPs) (10 mM), GoTaq® DNA Polymerase (Promega) and the following sense and antisense sequence-specific oligonucleotides primers 5'GGATTTTGCAATTGCATCGA3' and 5'GCAGGAGCACCCGGATTCGC3'; and 5'GTTATTCTGGCTATCGCTCTTG3' and 5'GTATAATGTCAAACAGAACGGGTC3', to generate PCR amplicons from the MT, and the mytilin genes respectively. Thermocycling conditions were performed according to Bettencourt et al. (2007). PCR products were examined on 1.5% agarose gel electrophoresis using standard Tris-boric acid-EDTA buffer and ethidium bromide for DNA visualization.

For comparative studies, MT gene expression analysis, was carried out with freshly collected mussels, 3 months acclimatized mussels and mussels subjected to the IPOCAMP stimulations. Loading controls were assessed by visualization of the abundance of the ribosomal rRNA gene 28S and careful measurement of equal amounts of total RNA used in RT and cDNA in subsequent PCR reactions. During cycling reactions, 10 µl of PCR products were collected from each 100 µl PCR reactions, at 15, 20, 25 and 30 cycles time point for following analyses by agarose electrophoresis and image densitometry (image processing software ImageJ, http://rsb.info.nih.gov/ij/). For Mytilin gene expression analysis, total RNA from gill tissues dissected from *Vibrio* exposed animals was utilized in semi-quantitative RT-PCR as described above. Additionally, to further characterize the transcriptional responsiveness of gill and hemocyte tissues to hydrostatic pressure, the following primers were tested in RT-PCR to assess relevant transcripts previously detected in our recently created *B. azoricus* gill cDNA library: lysozyme (5'GGTTGAATCGCACTGCCTACCCA3' and 5'GGTCCTCCGTTATGGATCCTGGC3'); fibrinogen (5'GTTGGCGGATATGTGG GTAGCGC3' and 5'GGAGTAATGATAACCCTTCCACTG3'); receptor kinase (5'GATTGTGGCTCTTAAGTAGTCC3' and 5'CTGTCCAGTTGTCTCATATCACTC 3'); HSP70 (5'TGGGTGGTGAAGATTTTGAC3' and 5'CCCTCGAACAGAGAGTCAAT3'); sarcoplasmic calcium-binding protein (5'CAACCATGATGGCGTTATAACGCG3' and 5'AGTGATGTCATAAAAGCATGCGCG3'); lectin aggrecan (5'GCAGTAAACACCCTCAGGAACGC3' and 5'CTATGACCCCATTCAAGGACTTGCC3'); ferritin (5'GTCGGCGATCTTATGAGGTCAAGG3' and 5'GGCCTACTATTTTGACCGTGACG3'); calmodulin (5'GCTGATATAGATGGTGATGGAC3' and 5'CCTGCTCTCACTTTAGACTC3') and actin (5'ATCGTGTAGATGTGTGATGCCAG3' and 5'GGAGATGATGCCCCAAGAGCCG3'). To confirm the nucleic acid identity of the amplicons, PCR products were gel-extracted with the Wizard SV GEL and PCR Clean-Up System (Promega) and submitted to nucleotide sequencing at the BMR-Genomics sequencing facility (http://www.bmr-genomics.it/).

Results and Discussion

Vibrio infections

The effect of *Vibrio* exposure was manifested in gill tissues as an increase in hemocyte counts was clearly assessed microscopically (Fig. 1A). This could be an indication that *B. azoricus* hemocytes are susceptible to *V. parahaemolyticus* and could perform specific responses against the marine bacteria while proliferating at the site where bacteria are in contact with gill tissues, given the strategic significance that gill tissues take on as filtering interfaces between the external milieu and the animal's body cavity. Thus, it is presumed that the presence of immune genes should be prevalent where host tissue organisms meet foreign microorganisms. In line with this supposition we further demonstrated the activities of hemocytes, at the time of infection, by comparing the levels of *mytilin* and *Rel*-like expressions in gill tissues (Fig 1B), by means of semi quantitative RT-PCR and *in-situ* hybridization experiments, respectively. While the first Rel/NF-κB molluscan homologue was characterized in the Pacific oyster, *Crassostrea gigas* (Montagnani et al., 2004), evidence for additional evolutionary conserved constituents of regulatory pathways such as the Toll signaling pathway (Naitza & Ligoxygakis, 2004; Royet et al., 2005) leading to the transcription of immune genes remain elusive in marine bivalves. Our results suggest that the higher expression of Rel domain containing messengers within gill hemocytes (Fig. 2), following *Vibrio* exposure, constitute thus far some of the best evidence for the presence of transcription factor members of the immune-related Rel/NF-κB family in *B. azoricus* and their involvement in host defense responses against marine bacteria. Furthermore, the antibacterial Mytilin gene expression results, clearly induced by the exposure to *Vibrio* bacteria, constitute yet another evidence of the strategic significance of antibacterial genes in gill tissues representing thus a natural defense barrier to environmental microorganisms (Fig. 1).

The Ca^{2+} indicator Fura-2 AM was also utilized to examine the intracellular calcium variation during stimulation with *Vibrio* exposures. The hemocytes within gill tissues dissected from animals exposed to *Vibrio*, revealed higher intracellular Ca^{2+} levels (Fig 2, punctate structures) than in the gills from control animals maintained in sea water, as determined by imaging analysis of relative fluorescence intensity within the hemocyte cells (Fig 2.). Although *V. parahaemolyticus* is not pathogenic to marine bivalves, it was used in our experiments to demonstrate that

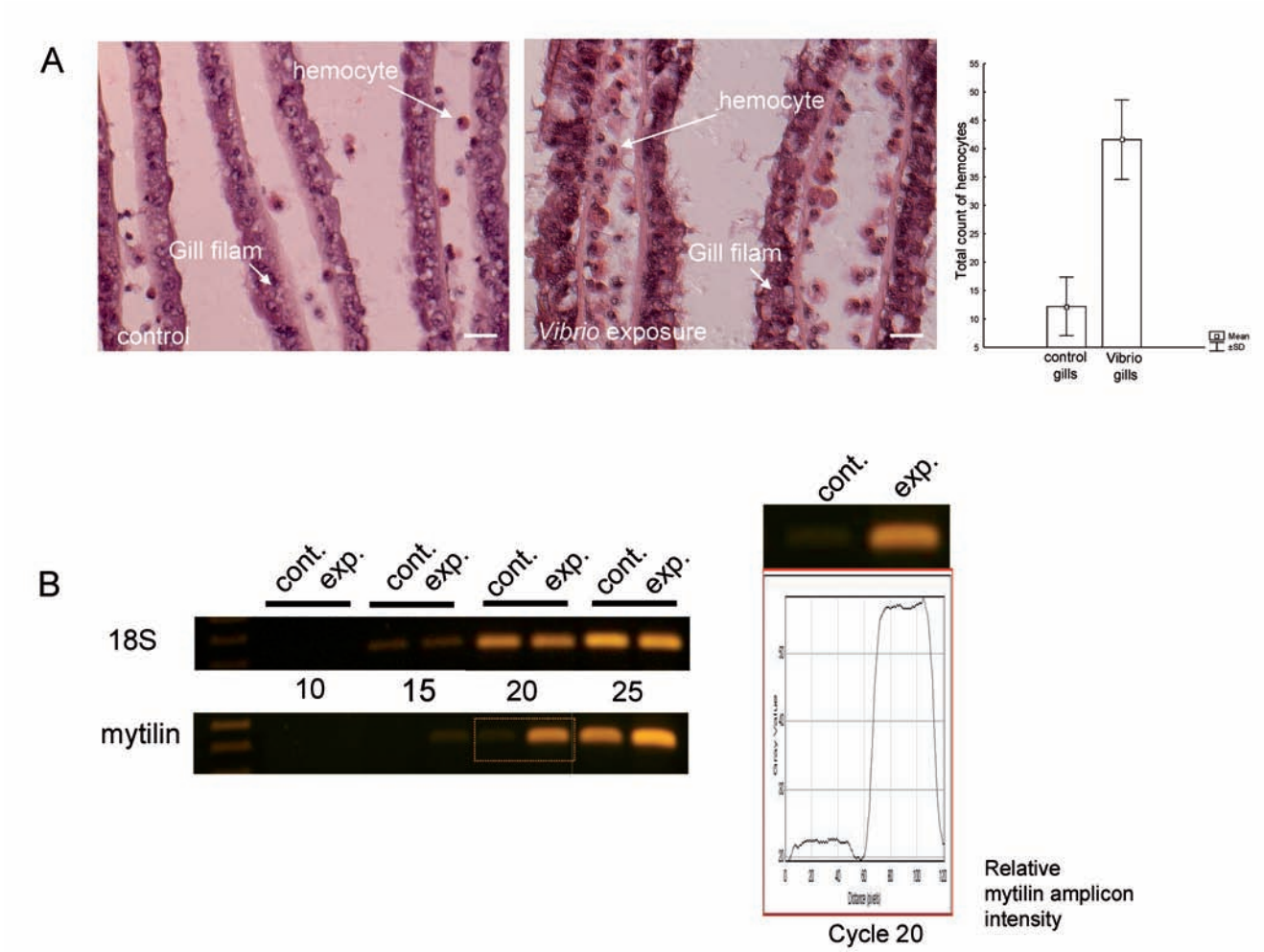

Figure 1. *Bathymodiolus azoricus.* The effect of *Vibrio* on gill hemocytes and mytilin gene expression. **A.** The exposure of *B. azoricus* to *Vibrio parahaemolyticus* resulted in a clear increase of free-circulating hemocytes within gill filaments. Total hemocyte counts in gill tissues are shown for control and *V. parahaemolyticus* exposed animals as the means ± s.d. (n = 6). **B.** A comparative expression analysis of the antibacterial gene mytilin revealed that increased levels of mytilin mRNA were obtained by RT-PCR in animals exposed to *Vibrio* as shown by the relative intensity of mytilin amplicons at cycle 20. The 18S gene was used as a suitable endogenous control for determining gene expression in gill preparations.

Figure 1. *Bathymodiolus azoricus.* L'effet de *Vibrio* sur les hématocytes de branchies et l'expression du gène mytilin. **A.** L'exposition de *B. azoricus* à *Vibrio parahaemolyticus* induit une nette augmentation des hématocytes libres dans les filaments branchiaux. Les nombres totaux d'hématocytes dans le tissu des branchies sont donnés pour les individus témoins et ceux exposés à *V. parahaemolyticus* (moyenne ± écart-type, n = 6). **B.** L'analyse comparative de l'expression du gène antibactérien mytilin montre une augmentation d'ARNm mytilin, obtenue par RT-PCR chez des animaux exposés à *Vibrio*, révélé par l'intensité relative observée au cycle 20. Le gène 18S a été utilisé comme contrôle endogène pour déterminer l'expression du gène dans les préparations de branchies.

bacterial products may interact with hemocytes in the way that an increase of the intracellular Ca^{2+} concentration, could acts as a second messenger of signal transduction, an early key feature in the immune cell activation (Grafton & Thwaite, 2001; Lewis, 2001). The rise in intracellular Ca^{2+} distinctively visualized as fluorescent blue labeled structures was clearly consequent from live *V. parahaemolyticus* interactions with hemocytes. For this reason, the variation in intracellular Ca^{2+} levels may be regarded as another immune-related parameter contributing to intracellular signaling processes involved in mussel hemocytes immune activation (Baus et al., 1996).

IPOCAMP stimulations

To confirm the specificity and transcriptional responsiveness of gill and hemocyte tissues to hydrostatic pressure we

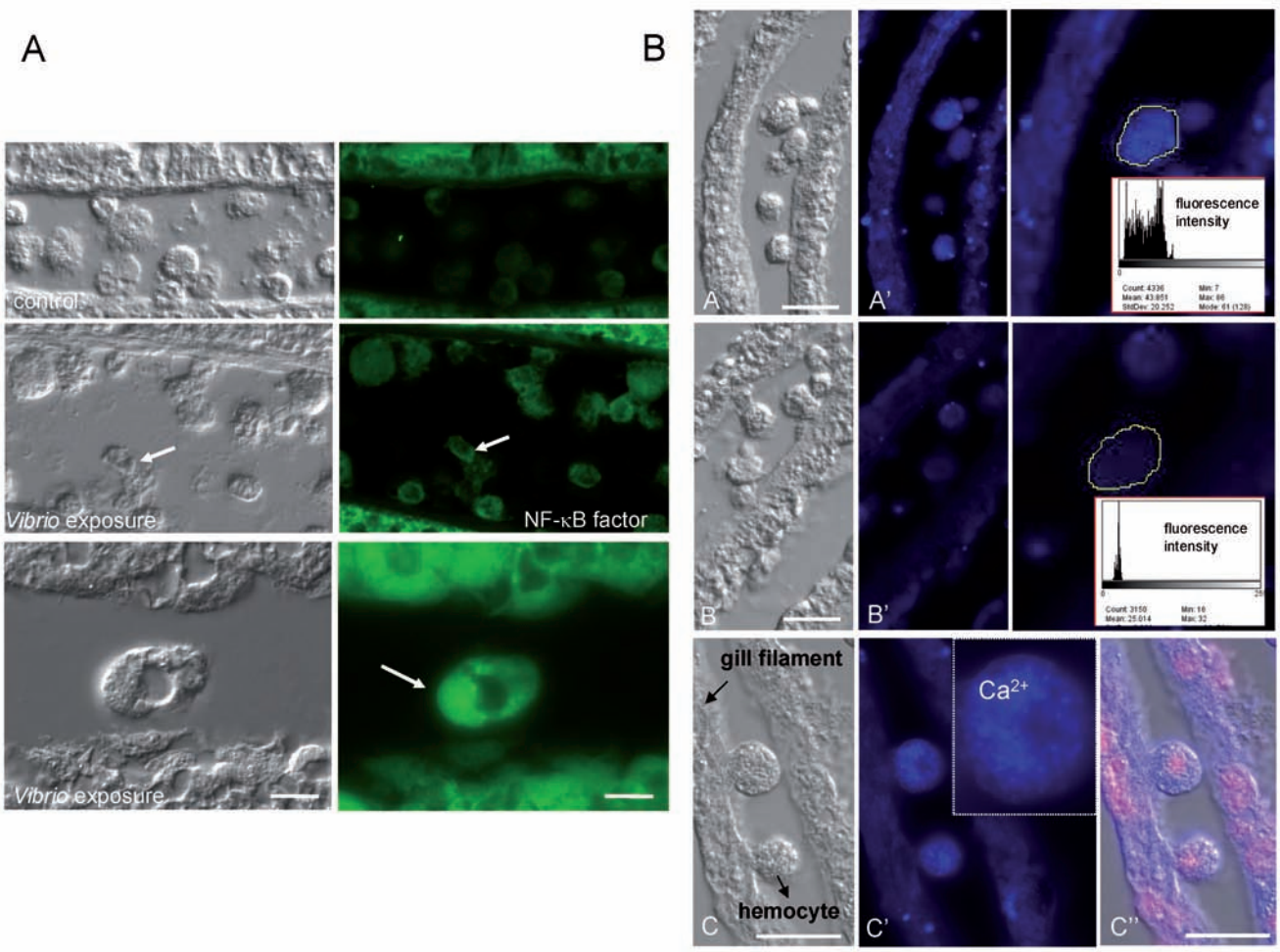

Figure 2. *Bathymodiolus azoricus.* Rel Homology Domain fluorescence *in-situ* hybridization and intracellular Ca^{2+} variation. **A.** As a result of *Vibrio* exposure, the RHD fluorescence probe intensity was markedly higher inside gill hemocytes than hemocytes from animals only incubated in plain sea water. **B.** *Vibrio* exposed animals exhibited higher levels of intracellular Ca^{2+} (A'') than control animals (B'') as indicated by imaging analysis and relative fluorescence intensity inside the hemocyte cells (A'' and B'', yellow line around cell). Punctate fluorescent structures are visualized inside hemocyte as shown in higher magnification (C', inset). A, B and C, DIC optics. A'-A''; B'-B''; C'-C'', fluorescence visualization with Leica 6000 DM. Scale bar: 20 µm.

Figure 2. *Bathymodiolus azoricus.* RHD FISH et variation intracellulaire en Ca^{2+}. **A.** L'exposition à *Vibrio* augmente l'intensité du marquage par la sonde fluorescente RHD dans les hématocytes de la branchie. **B.** L'exposition à *Vibrio* augmente le niveau de Ca^{2+} intracellulaire (A'' versus B''), mis en évidence par l'analyse d'image et l'intensité relative de la fluorescence dans les hématocytes (A'' et B'', cellules entourées d'une ligne jaune). Des structures ponctuées fluorescentes sont visibles ans les hématocytes à fort grossissement. Echelle : 20 µm.

also examined the levels of expression for the Metallothionein gene and other genes that were identified from our recently constructed *B. azoricus* gill cDNA library and from transcriptome studies (Bettencourt et al., 2010, in press). A comparative analysis was then performed, between gills and hemocyte preparations, from freshly collected animals and animals subjected to the effect of re-pressurization from 1 to 170 bars in the IPOCAMP chamber. No other additional effect of the stainless-steel chamber was taken into consideration during the pressure experiments as the system consisted of a constant flow-through of chilled seawater with temperature, oxygen saturation and pH parameters ranging from 8 to 10ºC, 40-60% and pH 7.3-7.9 respectively. Increased levels of MT mRNA were present in gill tissues from animals maintained in aquaria for 3 months and then subsequently subjected to hyperbaric stimulations (Fig. 3B) since a higher MT amplicon intensity was visualized after 30 cycles, in IPOCAMP challenged mussels (Fig 3A), in comparison to freshly collected mussels and 3 months acclimatized to

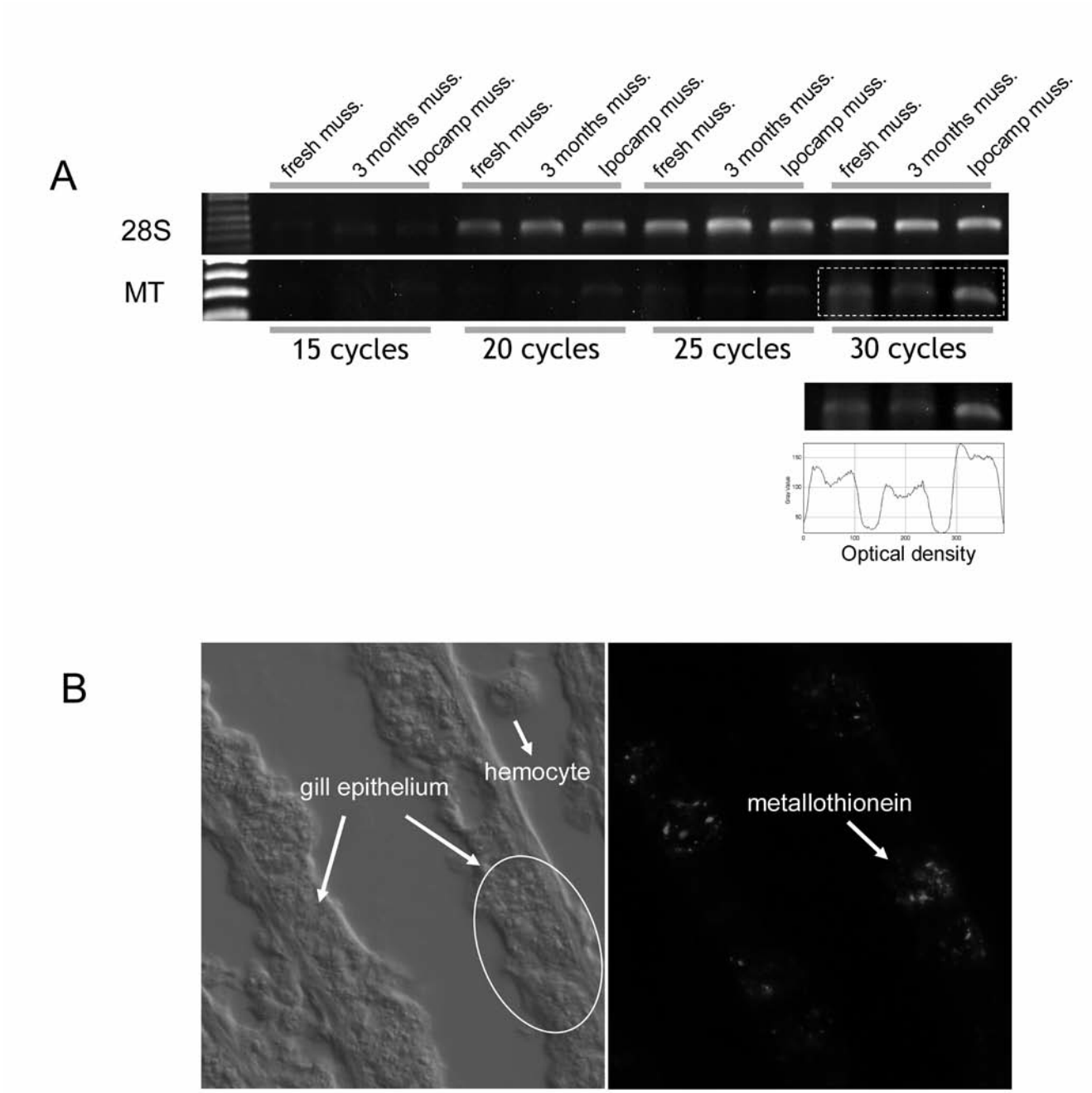

Figure 3. *Bathymodiolus azoricus.* MT Semi-quantitative RT-PCR and MT Fluorescence *in-situ* Hybridization. **A.** The relative MT gene expression level was evaluated in a comparative RT-PCR experiment using gill RNA from freshly collected animals (fresh mussels), animals acclimatized at atmospheric pressure (3 months mussels) and mussels acclimatized for 3 months to atmospheric pressure and subjected to de novo hyperbaric stimulation (Ipocamp mussels). The 28S gene was used as a suitable endogenous control for determining gene expression in gill and hemocyte preparations. **B.** FISH experiments showing the Metallothionein gene expression within gill epithelium. Fluorescence signals were not detected at the hemocyte level.

Figure 3. *Bathymodiolus azoricus.* RT-PCR semi-quantitative et FISH du gène métallothionéine (MT). **A.** Le niveau relatif d'expression du gène MT a été évalué par RT-PCR à partir d'ARN de branchie d'individus frais, d'individus acclimatés à la pression atmosphérique (3 mois) et d'individus acclimatés 3 mois à la pression atmosphérique puis soumis de nouveau à une stimulation hyperbarique (Ipocamp). Le gène 28S a été utilisé comme contrôle endogène pour la détermination de l'expression du gène dans la branchie et les préparations d'hématocytes. **B.** Mesures de FISH montrant l'expression du gène MT dans l'épithélium de la branchie. Les signaux de fluorescence ne sont pas détectés au niveau de l'hématocyte.

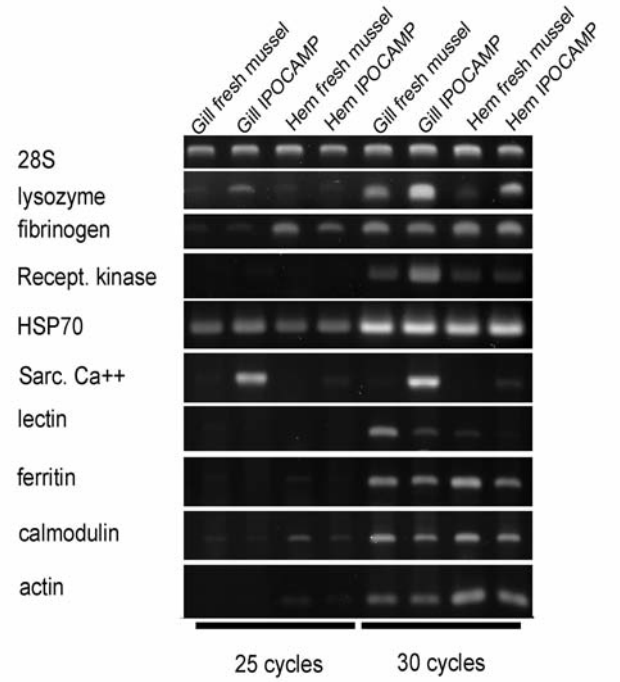

Figure 4. *Bathymodiolus azoricus.* Semi-quantitative RT-PCR. The *B. azoricus* lysozyme, fibrinogen, receptor kinase, HSP70, sarcoplasmic calcium-binding protein, lectin aggrecan-like, ferritin, calmodulin and actin genes were tested to confirm the specificity and transcriptional responsiveness of gill and hemocyte tissues to hydrostatic pressure in a comparative analysis between gills and hemocyte preparations from freshly collected animals and animals subjected to the IPOCAMP chamber. PCR results are presented at 25 and 30 cycles. The 28S gene was used as a suitable endogenous control for determining gene expression in gill and hemocyte preparations.

Figure 4. *Bathymodiolus azoricus.* RT-PCR semi-quantitative. Les gènes de lysozyme, fibrinogène, kinase récepteur, HSP70, protéine collectrice de calcium sarcoplasmique, lectine aggrecan-like, ferritine, calmoduline et 'actine ont été testés pour confirmer la spécificité et la sensibilité de transcription de la branchie et des hématocytes à la pression hydrostatique entre les individus frais et ceux soumis à l'incubation dans la chambre IPOCAMP. Les résultats de PCR sont montrés à 20 et 30 cycles. Le gène 28S a été utilisé comme contrôle endogène pour déterminer l'expression des gènes dans la branchie et les préparations d'hématocytes.

atmospheric pressure mussels (Fig. 3A). Indeed, the intensity level of MT PCR product from IPOCAMP mussels was comparable to the freshly collected mussels suggesting that the levels of MT gene expression are probably under the influence of hydrostatic pressure, in the natural environment of deep-sea vent mussels whereas animals acclimatized to sea-level conditions seemed to have reduced expression of MT (Fig. 3A) indicating that the MT gene transcriptional activity was influenced by aquarium conditions, for instance, the low metal concentration in the seawater supplied. A direct causal effect of hyperbaric pressure on vent mussels could be further observed in *in-situ* hybridization experiments using MT fluorescent probes. In this case, MT transcripts could be detected in gill filament epithelium and not in free-circulating gill hemocytes (Fig. 3B).

Furthermore, the relative abundance of amplicons obtained in semi-quantitative RT-PCR experiments indicated that not all tissue analyzed responded the same way. IPOCAMP stimulations was distinctly evident in the induction of lysozyme and sarcoplasmic Ca^{2+}-binding gene in gill tissues whereas other genes such as the lectin aggrecan, calmodulin and ferritin were negatively affected by hyperbaric stimulations and this inhibition of gene expression, comparatively to freshly collected animals, was more evident among hemocyte preparations (Fig. 4). Contrary to our expectations neither HSP70 gene expression showed differences between freshly collected animals and animals subjected to hyperbaric stimulations nor HSP70 expression differences were seen between gill and hemocytes tissues. Given its protective nature against stress it is possible that levels of the Heat Shock Protein 70 mRNA were already elevated at the time of deep-sea collection and persisting throughout the experiments, unaffected by the IPOCAM stimulation. However, it remains to be determined whether the *B. azoricus* HSP70 gene is up-regulated or not, following bacterial infections.

The RT-PCR experiments revealed that vent mussel tissues such as gills and hemocytes respond differently to hyperbaric stimulations suggesting that the mechanisms of adaptation in *B. azoricus* is conferred with some level of specificity involving the up-regulation and down-regulation of critical genes in important processes of acclimatization whether at atmospheric pressure or deep-sea pressure levels. Not only gene expression differs between the tissues analyzed, for some of the genes tested, gills and hemocytes express differently, irrespectively of the hydrostatic pressure and it also appears more susceptible among hemocytes for which the effect of IPOCAM stimulation was more pronounced.

Taken together our data provide direct evidence for a more prominent role of gill tissues during acclimatization processes to sea-level conditions or to deep-sea vent pressure levels upon which, stress and immune-related gene expression is likely being modulated, as demonstrated in our IPOCAMP stimulations. In addition to harbouring endosymbiont bacteria, gill tissues may contribute to the overall adaptations processes, keeping in line with the argument that deep-sea vent organisms have to cope with adverse environmental conditions. In addition to providing

markers for monitoring levels of heavy metals in the water, gill tissues can serve as environmentally responsive sensors to study expression profiles of genes typically involved in stress and immune responses in marine bivalves.

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