Chapter 8

An Insightful Model to Study Innate Immunity and Stress Response in Deep-Sea Vent Animals: Profiling the Mussel *Bathymodiolus azoricus*

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Abstract

Deep-sea environments are, in some cases, largely unexplored ecosystems, where life thrives driven by the geochemical features of each location. Among these environments, chemosynthesis-based ecosystems, in the Mid Atlantic Ridge, have an exclusive combination of high depth, high sulfur, and high methane concentrations. This is believed to modulate the biological composition of vent communities and influence the overall vent animal transcriptional activity of genes involved in adaptation processes to extreme environments. This opens, thus, the possibility of finding gene expression signatures specific to a given hydrothermal vent field. Regardless of the extreme physicochemical conditions that characterize deep-sea hydrothermal vents, the animals dwelling around the vent sites exhibit high productivity and thus must cope with toxic nature of vent surrounding, seemingly deleterious to the animals, while developing surprisingly successful strategies to withstand adverse environmental conditions, including environmental microbes and mechanical stress whether ensuing from animal predation or venting activity. The deep-sea vent mussel *Bathymodiolus azoricus* has adapted well to deep-sea extreme environments and represents the dominating faunal community from hydrothermal vent sites in the Mid-Atlantic Ridge, owing its successful adaptation and high biomasses to specialized exploitation of methane and sulfide sources from venting activity. Its extraordinary capabilities of adapting and thriving in chemosynthesis-based environments, largely devoid of photosynthetic primary production and characterized by rapid geochemical regime changes are due to symbiotic associations with chemosynthetic bacteria within its large gills. In an attempt to understand physiological reactions in animals normally set to endure extreme deep-sea environments, our laboratory has undertaken, for the

last few years, a series of investigations, aimed at characterizing molecular indicators of adaptation processes of which components of the host defense system has received most attention. This study reviews recent advances on the characterization of molecules and genes participating in immune reactions, using *in vivo* and *ex vivo* models, to elucidate cellular and humoral defense mechanisms in vent mussels and the strategies they have adopted to survive under extreme environments.

Keywords: innate immunity, deep-sea hydrothermal vents, chemosynthetic ecosystems, long-term acclimatization, host-symbionts interactions, endosymbionts, differential gene expression, transcriptomics, mollusc bivalve, hydrothermal vent mussel, *Bathymodiolus azoricus*

1. Introduction

Deep-sea hydrothermal vents were discovered 40 years ago in the Galapagos Rift [1] revealing for the first time, to the amazement of the scientific community, unusual life forms that have developed unique biochemical adaptations to high temperatures and toxic chemical nature of vent surrounding, otherwise harmful to life as we know it on the surface of the planet [2–5]. The animals dwelling around the vent sites exhibit high productivity and thus must cope with the seemingly deleterious physical and chemical conditions, while developing surprisingly successful strategies to withstand adverse environmental conditions, including environmental microbes and mechanical stress whether due to animal predation or from deep-sea volcanic eruptions [6–8].

At such depths and in the absence of light, life is thriving in chemosynthesis-based ecosystems where most abundant marine invertebrates have developed mutualistic relationships with chemosynthetic bacteria. These symbiotic interactions are believed to play a crucial role in the survival of hydrothermal vent animals, driving their transcriptional activities, and their successful adaptation strategies to subsist under extreme environmental conditions. They essentially rely on the establishment of endosymbiosis relationships between vent animals and sulfur-oxidizing (SOX) or methane-oxidizing (MOX) bacteria [9–13].

Deep-sea vent mussels of the *Bathymodiolus* genus are dominant members at hydrothermal vents and cold seep habitats. These mussels have the peculiarity of sheltering both endosymbiotic sulfide-oxidizing and methane-oxidizing bacteria in their gills [9–13], supporting thus their endurance within this type of environment. *Bathymodiolus azoricus* is also the dominant species in deep-sea hydrothermal vents in the Azores region and is well adapted to extreme conditions particularly to toxic concentrations of heavy metals, acidic pH, and absence of light [14–17].

In an attempt to understand physiological reactions of animals normally set to endure extreme conditions, in deep-sea environments, our laboratory has undertaken, for the last 6 years, a series of investigations aimed at characterizing molecular indicators of adaptation processes of which components of the immune and stress-related systems have received most of our attention [18, 19]. Central to our studies is the long-term maintenance of vent mussels to atmospheric pressure proven to be a useful model to study unique molecular relationships under which the regulation of gene transcription may be affected by aquaria conditions and

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by the gradual disappearance of endosymbiont bacteria from gill epithelia [20]. Nonetheless, vent mussels subsist for months at atmospheric pressure in aquaria supplemented with plain sea water in or with artificial diet. This has allowed us to focus on developing experiments to investigate new physiological responses of animals sustaining experimental challenges involving immunological and stress-related reactions and to provide new approaches to assess the effect of natural microorganisms and metal toxicity at vent environments [21, 22]. As a research model, the choice of the vent mussel *B. azoricus* is of great significance given its unique symbiosis with SOX and MOX bacteria. It has provided us with the means to understanding the molecular mechanisms underlying immune reactions in animals normally set to endure extreme deep-sea environments and the role of their symbiotic bacteria in controlling immune gene transcriptional activity.

In line with this, we have investigated main constituents of the vent mussel immune system and demonstrated how immune and stress genes could be modulated upon different experimental challenges in the absence of the characteristic high hydrostatic pressure found at deep-sea vent sites without methane and/or sulfide supplementation [21–23]. The proximity to the nearby hydrothermal vent fields, in the Azores region, has given us a geographical advantage for earning first insight into immediate physiological responses comprising both cellular and humoral responses of live mussels, freshly collected from the hydrothermal vents, which upon arrival, are acclimatized to our aquarium system, LabHorta [23–25]. The maintenance of live mussels in our laboratory is thus a key factor in gaining knowledge into the physiology of vent animals including the study of evolutionary conserved immune, inflammatory, and stress-related factors commonly found in other marine bivalves [19, 26].

2. Innate immunity in *Bathymodiolus azoricus*

The interaction between microorganisms and host defense mechanisms is a decisive factor for the survival of marine bivalves. They rely on cell-mediated and humoral reactions to overcome the pathogens that naturally occur in the marine environment [27]. Growing interest in deep-sea vent biology has turned the vent mussel *B. azoricus* into a model organism centered on research activities based on the premise that vent mussels clearly have need for an immune system to overcome microbial challenges in their natural surroundings. For this reason, our research strategies have been focused on the molecular characterization of molecules participating in immune reactions, using *in vivo* and *ex vitro* models, to elucidate cellular and humoral defense mechanisms in vent mussels and its survival strategies under extreme environments. As for other bivalves, the innate immune system of *B. azoricus* is based on cellular constituents and soluble hemolymph (blood) factors, which play a prominent role in protecting the animals against invading microorganisms. The circulating hemocytes or blood cells are mostly found in the hemolymph and extrapallial fluid. They are responsible for cell-mediated defense reactions such as phagocytosis and the activation of a variety of cytotoxic reactions including the release of lysozomal enzymes and antimicrobial peptides [28–30]. Moreover, the generation of highly reactive oxygen intermediates (ROIs) and nitric oxide also plays an important defense role against pathogens [30–33]. Besides their decisive role in protecting the host from microbial assaults, bivalve hemocytes have also been implicated in

other important physiological functions, including nutrient transport, digestion, wound healing and shell regeneration and/or mineralization, and excretion [34]. In addition, the hemolymph serum contains humoral defense factors such as lectins and cytokine-like molecules that are directly and indirectly involved in the killing of pathogens and in mediating cell-cell interactions, respectively. Lectins are important mediators of cellular reactions and exhibit opsonin properties, which facilitate the phagocytosis [35–39]. The hemolymph also contains antibacterial factors and lysozomal components that ensure, along with hemocyte phagocytic and cytotoxic processes, the clearance of pathogenic bacteria [38, 39]. Using a combination of light microscopy and staining procedures, three major hemocyte types are discernible in the extrapallial fluid and hemolymph of *B. azoricus*. The most abundant type was identified as granulocyte readily recognizable by their cytoplasmic granules [19]. They appear fairly homogeneous in size and showing a characteristic crescent, or half-moon shape morphology upon adherence to glass slides and before migratory movements. Granulocytes spread well onto the glass surface averaging 30–40 μm in length. In contrast, hyalinocytes presented smoother cytoplasm, i.e., a nongranular appearance due to a lower amount of cytoplasmic granules noticeable under phase contrast and differential interference contrast visualizations [19]. A third less common hemocyte type was also observed. They correspond to hemoblast-like cells and presented a spherical shape appearance with higher nucleus to cytoplasm ratio when compared to granulocytes and hyalinocytes [19]. *In vitro* phagocytic assays carried out with *B. azoricus* hemocytes revealed that 70% of the hemocytes containing more than two zymosan particles were granulocytes and to a lesser extent the percentage of phagocytic cells corresponding to hyalinocytes was 23%. In contrast, the percentage of hemoblasts containing ingested zymosan particles was 5–7%, the lowest revealed in our studies [19].

Along with hemocytes studies, we began to tackle signaling pathways putatively involved in the mediation of cellular responses in the presence of *Vibrio* spp. It was demonstrated that compounds of microbial origin could trigger detectable phosphorylation events in *B. azoricus* hemocyte extracts and likely involving the activation of different classes of mitogen-activated protein kinases (MAPKs). When challenged with a marine bacterium, *Vibrio parahaemolyticus* or a nonmarine bacterium, *Bacillus subtilis*, to stimulate hemocytes, cellular proteins were differently phosphorylated as demonstrated in Western blotting experiments using the MAPK/ERK, p38, and JNK rabbit polyclonal antibodies. Moreover, the differences seen in phosphorylation patterns could be attributed to inherent properties of the bacterial strain used, differences in the mechanisms of binding to hemocytes, or differential activation of cell membrane receptors and signaling pathways, resulting in different patterns of protein phosphorylation. Western blotting analyses suggest that *B. azoricus* hemocytes display receptors with binding affinities toward microbial molecules or to live bacteria [19].

3. *Ex vivo* experiments with gill tissues: new insights into tissue immune specificity toward microorganisms

As mytilid species, the deep-sea vent mussel *B. azoricus* exhibits large lamellated gills (ctenidia) arranged as numerous filament structures stacked together through ciliary junctions. Each filament is organized in two coalescent epithelial cell sheets overlaying a central lumen where

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hemocytes can be found. The thinness of gill filaments allows for the visualization of live hemocytes through the epithelium, and to monitor hemocyte motility directly under light microscopy [19]. Bivalve mollusk gills assume thus a strategic importance at the interface between the external milieu and the internal body cavities of the animal where contact with microorganisms is inevitable during feeding processes inasmuch as host defense responses may incur from interactions with infective pathogens during normal filtration [36–39]. For this reason, a number of typical cellular and humoral immune reactions are likely to take place in gill tissues and observable as hemocyte proliferation and phagocytosis, the activation of immune signaling pathways, and the activation of genes involved in immune, antioxidant and antibacterial responses against invading bacterial pathogens or the presence of metal toxicants [36–39].

To further test the gill's ability to mount immune reactions, a series of *ex vivo* experiments have been performed using gill tissues freshly dissected from vent mussels and subjected to short-term incubations in tissue culture well-plates and under different experimental settings. Different stimuli were carried out to demonstrate the expression of genes in gill tissues exposed to a mixture of endosymbionts previously obtained from gill extracts, and *V. parahaemolyticus*, in comparison with sterile sea water incubations (**Figure 1**). Differential gene expression results indicated that exposure to methanotrophic and thiotrophic endosymbiont preparations led to general upregulation of genes involved in immune recognition reactions, without the addition of hemolymph, while gill incubations with endosymbiont extract and to which hemolymph serum (hemocyte free) was added led to an opposite effect, resulting in a lower expression of immune recognition genes. These results contrasted with incubations performed with *V. parahaemolyticus* or with control sterile sea water. In this case, higher levels of gene expression were achieved when hemolymph was added to gill tissues incubated with *V. parahaemolyticus* or control sterile sea water (**Figure 1**). *Ex vivo* experiments as described bring evidence supporting a yet uncharacterized effect of hemolymph and its humoral constituents over endosymbionts, likely controlling immune gene expression of its host *B. azoricus*. This prompted the question of whether the host gill tissue would be able or not to recognize endosymbiont as self-particles and to which extent the host immune system does not disturb the acquisition of endosymbionts by horizontal transfer during the host larval stages. Moreover, the permanence and survival of endosymbionts within gill tissue would require a fitted control over the host immune system, acting on the transcriptional regulation of immune genes and at the level of pattern recognition receptors (PRRs) expressed by cells of the host innate immune system to detect microbial-associated molecular patterns (MAMPs) present on the surface of microorganisms [40, 41]. This endosymbiont effect over the host immune system would likely require the presence of hemolymph and its humoral constituents, as demonstrated by the gill *ex vivo* experiments (**Figures 1** and **2**). Since the gill tissue does grow over the mussel life's time we also probed different gill sections to determine levels of immune gene expression along the anterior-posterior axis, notably the "budding zone" on the posterior end, considered as the youngest section of the gill and through which endosymbionts are believed to make their entry. It was found that vent mussel gene expressions were markedly lower than other gill tissue sections, suggesting a thigh mechanism of transcriptional regulation of host genes in the presence of endosymbiont bacteria in the gill budding zone.

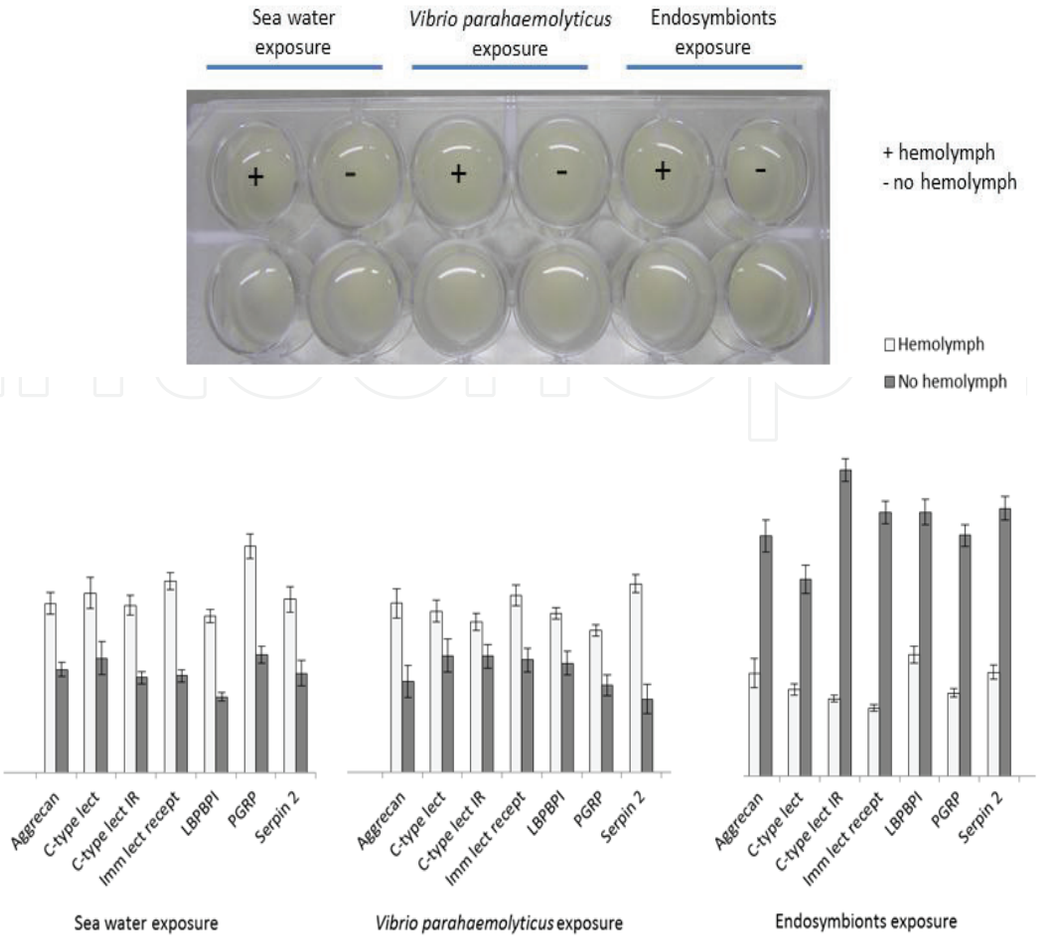

Figure 1. *Ex vivo* experiments performed with dissected gills. Gill fragments were incubated with *V. parahaemolyticus* and with an enriched preparation of endosymbionts freshly obtained from gill homogenates and gradient centrifugation. The effect of hemolymph humoral factors was tested by incubating gill fragments with and without hemolymph in the presence of *V. parahaemolyticus* and endosymbiont mixture. Results were compared to control incubations with plain sterile sea water. Gene expression was performed by qPCR targeting the immune recognition genes Aggrecan, C-type lectin, C-type lectin immune receptor, immune lectin receptor, lipopolysaccharide-binding protein-bactericidal/permeability-increasing protein, peptidoglycan recognition protein, and serine protease inhibitor-2 [50]. Incubations performed with endosymbiont preparations distinctively induced immune recognition genes in the absence of hemocyte-free hemolymph.

As filter feeders living most of their lives attached to a substrate, bivalves are exposed to constant biologically available pollutants over an extended period of time [42–44]. They have been studied as biological models to assess the impact of pollution in the environment and used as a biomonitoring "tool" due to their capacity of bioaccumulating high concentrations of trace metals, mostly in soft tissues such as gills and digestive gland [45–47]. The large surface of the gills and their involvement in gas exchange and feeding processes bring bivalves to constant and intimate contact with their environment where pathogens may also find their route of entry and encounter the bivalve first-line immune defense reactions.

While marine bivalves living in sandy, rocky intertidal, and shallow subtidal environments may rely on well-established humoral and cellular immune reactions to counteract pathogenic microorganisms, a new level of molecular intricacy may be seen between endosymbiont-bearing

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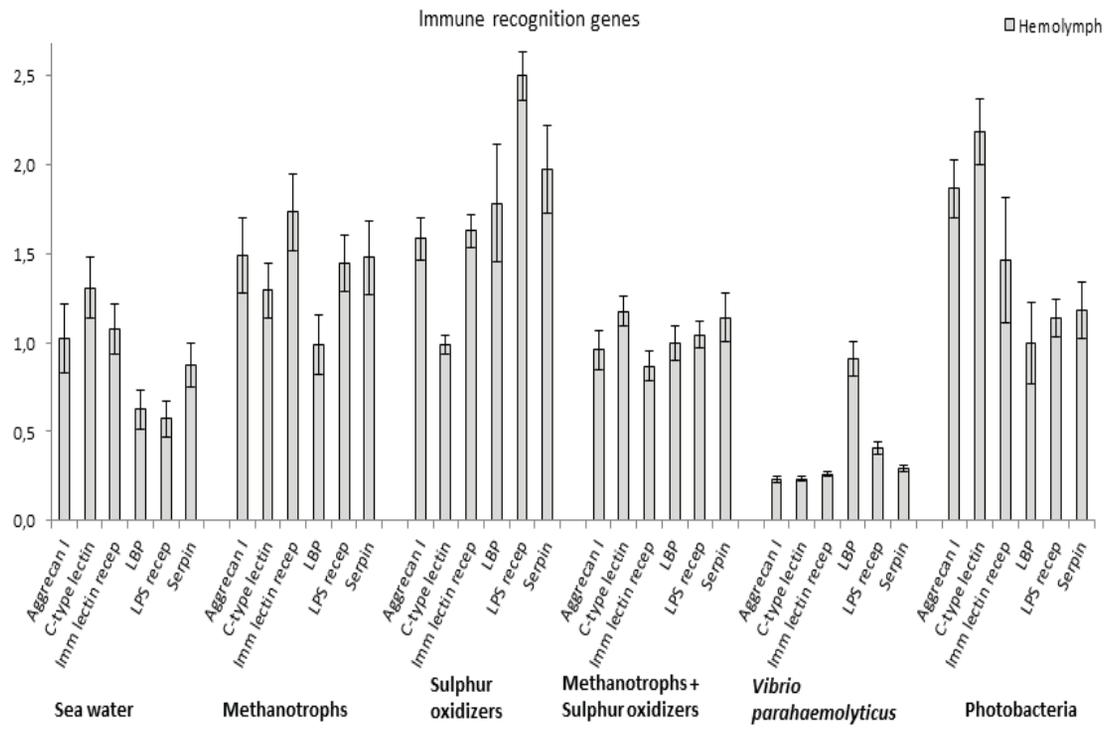

Figure 2. *Ex vivo* experiments performed with dissected gills. As in **Figure 1**, gill fragments were incubated with distinct bacterial stimulants: an enriched methanotrophic bacterial preparation; an enriched thiotrophic bacterial preparation, a mixture of methanotrophic and thiotrophic bacterial preparations, *Vibrio parahaemolyticus* and *Photobacteria* bacterium. Results were compared to control incubations with plain sterile sea water. Gene expression was performed by qPCR targeting same immune recognition genes as in **Figure 1**. Separate methanotrophs and thiotrophs preparations induced higher levels of immune gene expressions when compared to a mixture of the two endosymbiont bacteria preparations. Incubations using *Vibrio parahaemolyticus* resulted in drastic downregulation of immune recognition genes.

bivalves living in anaerobic and sulfide-rich environments and the pathogenic microorganisms they encounter. These natural molecular interactions would account for the role of endosymbionts in modulating the host immunity by controlling the transcriptional activity of immune genes. Bivalve associations with chemoautotrophic endosymbionts are now well known and widely distributed across a range of different chemosynthetic environments, including deep-sea hydrothermal vents (*Bathymodiolus* spp., *Calyptogena* sp.); gas seeps, mud volcanoes, and petroleum seeps (*Bathymodiolus* spp., *Calyptogena* sp.); whale and wood falls (*Idas* spp., *Adipicola* spp., *Vesicomya* sp., *Axinodon* sp.); and shallow water anoxic sediments mediated by sulfate reduction (*Solemya* spp., Codaki spp., *Anodontia* spp., *Lucina* spp.) [7].

Our recent results from *ex vivo* gill tissue experiment proven to be a valuable system for the study of tissue-specific immune responses where the thin epithelial cell layers of gill filaments would make it possible to signal pathogen-sensing directly through gill epithelia and affecting adjacent methanotrophic or thiotrophic endosymbionts which in turn would functionally prime host immune cells, the hemocytes, into altering their transcriptional activity (**Figure 3**). The endosymbiont immunomodulatory effect on the host immune system, as discussed in detail further below, is still under current investigations by our research team as the complexity of host-endosymbiont interactions in the deep-sea vent mussel *B. azoricus* remains to be

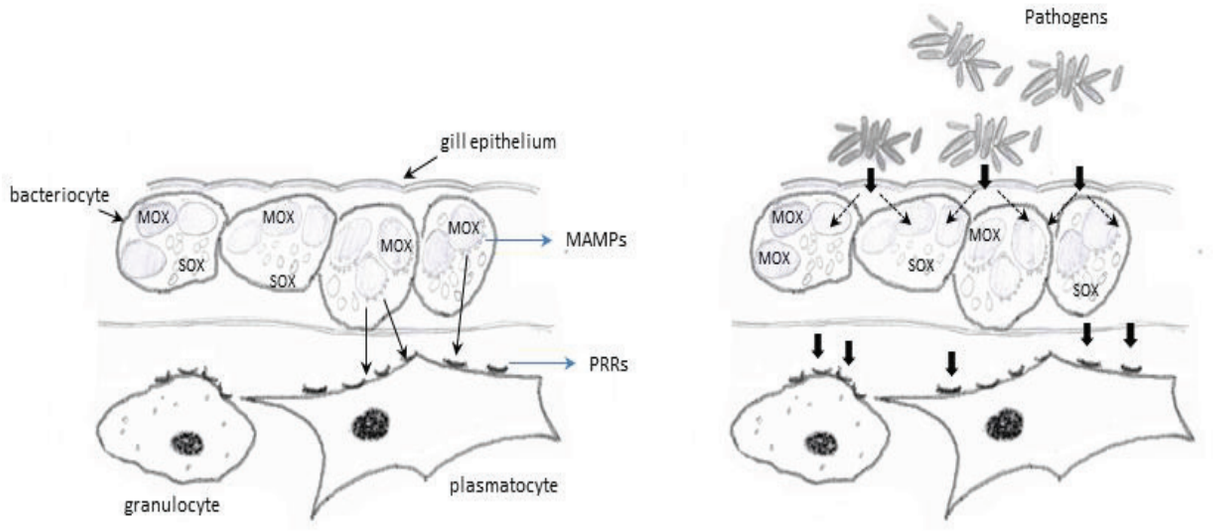

Figure 3. Hypothetical model representing the host-endosymbiont-mediated immune responses against pathogens. In a normal immunological state, hemocytes PRRs are being sensitized by host-endosymbiont interactions allowing the vent mussel immune system to remain active and tolerant to the presence of MOX and SOX bacteria. Upon interacting with extracellular pathogens, host-symbiont interactions are altered and incur in higher endosymbiont genes transcriptional activity [74] and subsequently affecting host hemocytes by triggering its immune repertoire via PRRs activation.

fully understood. This model is consistent with the hypothesis that innate immune receptors are required to promote long-term colonization by microbiota. This emerging perspective challenges current paradigms in immunology and suggests that PRRs may have evolved, in part, to mediate the bidirectional cross-talk between microbial symbionts and their hosts [48, 49].

4. Posttranscriptomics studies in *Bathymodiolus azoricus*

In 2010 a high-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *B. azoricus* was reported by our group [50]. It represented the first tissue transcriptional analysis of a deep-sea hydrothermal vent animal, using next generation sequencing technology, enabling the creation of a searchable catalog of genes that provided a direct method of identifying and retrieving vast numbers of novel coding sequences which could then be applied in gene expression profiling experiments, using quantitative polymerase chain reaction (qPCR), from a nonconventional model organism [50]. It provided the most comprehensive sequence resource for identifying novel genes currently available for a deep-sea vent organism, in particular, genes putatively involved in immune and inflammatory reactions in vent mussels. This first transcriptional analysis of gill tissues from the deep-sea hydrothermal vent *B. azoricus* was organized as a searchable catalog of

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genes providing a direct method of identifying and retrieving vast numbers of novel coding sequences, which can be applied in gene expression profiling experiments. The assembled and annotated sequences were organized in a dedicated database, accessible through the website http://transcriptomics.biocant.pt/deepSeaVent [50].

With an unprecedented high number of gene sequences available from our transcriptomic data, we were able to tackle signaling pathways and compare gene expression profiles in a series of experiments aiming at better understanding innate immunity in animals physiologically programmed to endure deep-sea vent conditions. Responses to bacterial infections with different strains of *Vibrio* wound experiments, long-term acclimatization in aquarium conditions and pressurization experiments with the hyperbaric chamber IPOCAMP [51] became the main focus of our research, setting thus the grounds for more in-depth analyzes revealing distinct gene expression profiles behind unique molecular relationships under which the regulation of gene transcription may be affected by biotic factors including microorganisms, the presence of endosymbiont bacteria and shell damage incurring in opportunistic infections or by abiotic factor as the hydrostatic pressure. The majority of the genes comprising four functional categories as described by Bettencourt et al. [50] and relating to immune recognition, signaling transduction, transcription, and effector molecules mechanisms were analyzed by qPCR.

The long-term aquarium maintenance of vent mussels to atmospheric pressure has long been central to our studies and proven to be a useful model to study unique molecular relationships under which the regulation of gene transcription may be affected by the gradual disappearance of endosymbiont bacteria from gill epithelia [20, 52]. Nonetheless, vent mussels from Menez Gwen hydrothermal vent site subsist for months at atmospheric pressure in aquarium conditions, in plain sea water or supplemented with methane and sulfide. This has allowed us to focus on developing experiments to investigate new physiological responses of vent mussels sustaining experimental challenges involving bacterial pathogens of the *Vibrio* genus, even in the absence of the characteristic high hydrostatic pressure found at deep-sea vent sites and without methane and sulfide supplementation [21, 22].

Earlier results from experimental exposures to *Vibrio splendidus*, *Vibrio alginolyticus*, *Vibro anguillarum*, and *Flavobacterium* sp. pointed at the immune discriminatory capacity of *B. azoricus* to distinguish different *Vibrio* strains, and at significant differences of immune gene expression levels between 12 and 24 h exposure times. These studies concluded that the immune gene transcriptional activity was modulated at two levels, i.e., over the course of time and according to the bacterial strain tested, suggesting thus, a selective response toward *Vibrio* spp. when vent mussels were experimentally challenged during 24 h [53, 54]. Additional experiments were carried out with *Vibrio diabolicus* aiming at the analysis of gene expression differences between distinct vent mussel populations from the hydrothermal vent sites Menez Gwen (MG, 800 m depth) and Lucky Strike (LS, 1700 m depth) both located on the Mid-Atlantic region, near the Azores islands. These comparative studies revealed unique immune transcriptional specificities at the gill, digestive gland, and mantle tissues level providing further evidence supporting different usage of transcription factors at the promoter region of immune genes possibly linked to the hydrothermal vent environment Furthermore, Menez Gwen (MG) and Lucky Strike (LS) *B. azoricus* showed significant

gene expression differences during *V. diabolicus* challenges over time demonstrating that immune genes are differentially expressed within the same mussel populations regardless of their hydrothermal vent origin suggesting thus site-related tissue-specific gene expression patterns [55]. Moreover, these results also suggested different tissue tolerance to decompression and adaptation to atmospheric pressure not seen so far. Mantle tissues from LS mussels seemed unaffected by deep-sea retrieval showing significantly higher levels of immune gene expressions as compared to MG mantle tissues. Thus, the decompression effect on the animal's internal organs may be evaluated by ways of its ability to respond, at the immune transcriptional level, to *V. diabolicus* challenges. For that reason, mantle tissues from LS animals appear to be decompression-resistant and immune competent toward bacterial challenges. On the other hand, the digestive gland revealed the most increased gene expression levels in MG animals showing how the tissue microenvironment is relevant to *in situ* immune responses. Gill immune transcriptional activity in both MG and LS mussels was not as significantly different as for the other tissues tested which may be attributed to the presence of endosymbiont bacteria in gill epithelia acting as a driving factor likely to affect host-gene expression and the overall physiological statuses of MG and LS vent mussels while interacting with *V. diabolicus*. Even though gill tissues have been the main focus of most of our previous investigations in the deep-sea vent mussel *B. azoricus*, the digestive gland and mantle tissues hold the potential for highlighting specific immune responses in tissues other than gills and how they can modulate the outcome of the animal's overall immune responses [55].

In addition to *ex vivo* experiments and *Vibrio* exposures to live vent mussels, we were able to carry out long-term acclimatization experiments with vent mussels kept in aquaria and at atmospheric pressure. These experiments were devised to assess the effect of such prolonged aquarium conditions on immune and stress-related reactions as mussels were gradually releasing their endosymbiont bacteria from gill bacteriocytes. These studies provided a basis for understanding the interactions between host-immune and endosymbiont gene expressions during postcapture long-term acclimatization in plain sea water and represented an ideal model for investigating *B. azoricus* immune genes transcriptional activity and symbiont bacteria prevalence, in view of changes in the availability of chemical-based energy sources during acclimatization at atmospheric pressure. It also pointed out the relevance of gene expression studies while addressing the swift changes affecting metabolic adaptations and food intake fluctuations, whether induced by or as a result of the gradual loss of endosymbionts and subsequent presence of symbiont bacteria in the aquarium environment, altering thus the physiological homeostasis of *B. azoricus* [56]. These studies demonstrated that the transcriptional activity profiles for immune and bacterial endosymbiont genes followed a time-dependent mRNA transcriptional pattern evidenced at 24 h, 1 week, and 3 weeks acclimatization. Furthermore, after 1 week acclimatization, vent mussels were under the influence of what appears to be a concomitant host-immune and endosymbiont gene expression, possibly indicating a physiological transition point which induces higher levels of transcriptional activity [56]. Under such circumstances, survival of vent mussels will require immune gene repertoire switching involving the differential expression (DE) of recognition, signaling, transcription, and effector genes tied to environmental parameters and to the symbiotic relationships in *B. azoricus*. Metabolic adaptations and food intake changes, whether induced as a result of

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the gradual loss of endosymbionts and subsequent release in the aquarium environment, are likely to affect gene transcription activities and prevalence of symbionts in gill tissues [56–58].

The geographic proximity to the nearby hydrothermal vent fields, in the Azores region, gave our laboratory a positional advantage for earning first insight into immediate physiological responses comprising both cellular and humoral responses of freshly collected mussels from different hydrothermal vents, which upon arrival, are acclimatized to our aquarium system, LabHorta [23].The maintenance of live mussels from the shallower vent field, Menez Gwen, became thus a key factor in gaining knowledge into the physiology of vent animals including the study of evolutionary conserved immune, inflammatory and stress-related factors commonly found in other marine bivalves [18–22].

Taking advantage of the LabHorta facility, comparisons studies were made possible, with live vent mussels subjected to *V. parahaemolyticus* infection, wound injury, hyperbaric pressurization, and 3 months acclimatization (**Figure 4**). These experiments allowed for the characterization of the differential activation of signaling pathways and the relative quantification of immune genes expressed during each type of stimulation. Differential gene expression results indicated that the four experimental conditions tested were distinctively inducing the immune genes of vent mussels to different levels of transcriptional activity of which the immune and signal transduction genes showed the highest expressions (**Figure 5**).

Of the four challenging conditions *V. parahaemolyticus* infections resulted in the highest number of genes with higher level of expression during this comparison study based on qPCR and selected genes targeting immune recognition, signal transduction, transcription, and synthesis of effector molecules processes (**Figure 5**). Also, cross-talk between signaling pathways may occur in *B. azoricus* individuals subjected to *Vibrio* infections, wound responses, and hyperbaric stimulations, i.e., same immune or pro-inflammatory signaling molecules may serve different signaling pathways whether they are conspicuously more expressed or not during such experiments. Cleary, the activation of signaling pathways involved in *Vibrio* infections was distinct from that of wound and hyperbaric reactions and thus conferring the animal model presented here with the physiological versatility to cope with deep-sea hydrothermal vent environments. These experiments were important to elucidate the molecular mechanisms under which, physiological responses to bacterial infections, would responses, hyperbaric stimulations and long-term maintenance in aquaria conditions, may be involved in *B. azoricus* adaptation processes whether in deep-sea vent environments or at atmospheric pressure. However, in-depth analysis of different signaling genes and pathways involved in such experimental challenges remained fragmentary and elusive.

One the most common goals of RNA Sequencing (RNA-Seq) profiling is to identify genes or molecular pathways that are differentially expressed (DE) between two or more biological conditions [59–63]. Changes in expression can then be associated with differences in physiological reactions, providing clues for further investigation into potential mechanisms of action [64, 65]. In order to gain additional insight into the different signaling genes involved in *Vibrio* infection, wound response, long-term acclimatization, and hyperbaric repressurization, we sequenced the full transcriptome of gill tissues from each of these experimental challenges to which deep-sea vent mussels were subjected and compared their differential

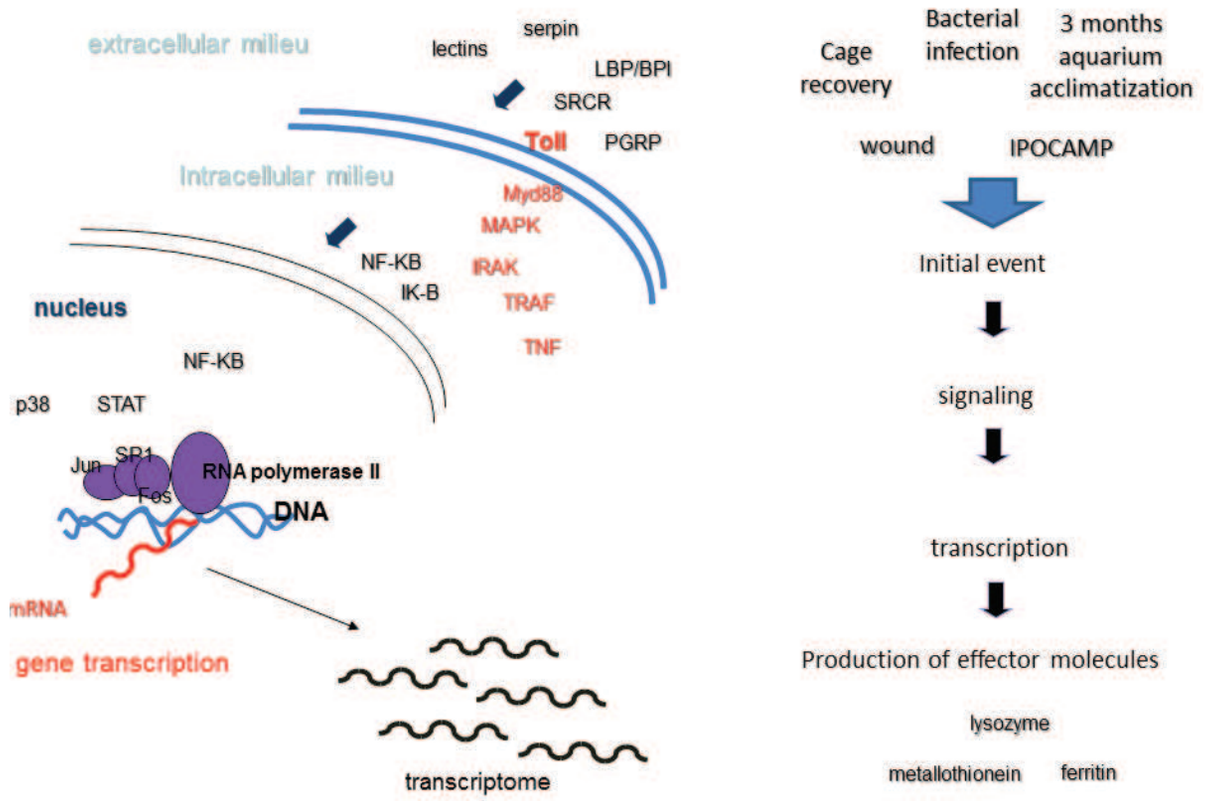

Figure 4. Schematic representation of immune signaling activation. After initial events characterized by immune recognition and stress-related reactions, signal transduction pathways are induced into transmitting a series of protein phosphorylation events, through the intracellular milieu, which ultimately result in the translocation of transcription factors into the nucleus that initiates the transcription of genes encoding immune effector molecules, here represented as lysozyme, metallothionein, and ferritin.

gene expression levels with that of gene expression in animals immediately retrieved from the vent sites with the help of acoustically trigged cages that were recovered at the sea surface. Transcript sequences for the five cDNA libraries were obtained from the Illumina RNA-sequencing platform and *de novo* assembly of RNA-Seq transcripts performed with Trinity [66, 67] followed by differential expression (DE) analyses using the edgeR package [68–70]. DE results were presented as Heatmaps clusters (transcriptional cluster report for edgeR DE analysis). The advantage of Heatmaps is that it can display the expression pattern of the genes across all the RNA samples. Visualization of the results is aided by clustering together genes that have correlated expression patterns [68].

Here we present examples of expression plots for some of the most DE genes across the five different experimental conditions referred to as "cage," animals freshly collected with acoustically triggered cages, from the bottom of the deep-sea vent floor; "3 months," same animals as in "cage" acclimatized for 3 months in aquaria environment at 1 atm; "Vibrio," same animals as in 3 months exposed to *V. parahaemolyticus*; "Wound," same animals as in 3 months with shell injury caused by mechanical abrasion to expose the mantle; "IPOCAMP," same animals as in 3 months subjected to 80 bar hydrostatic pressure for 72 h. The top-scoring BLAST hit for each of the gene exemplified is shown on top of the respective expression plot (**Figure 6**).

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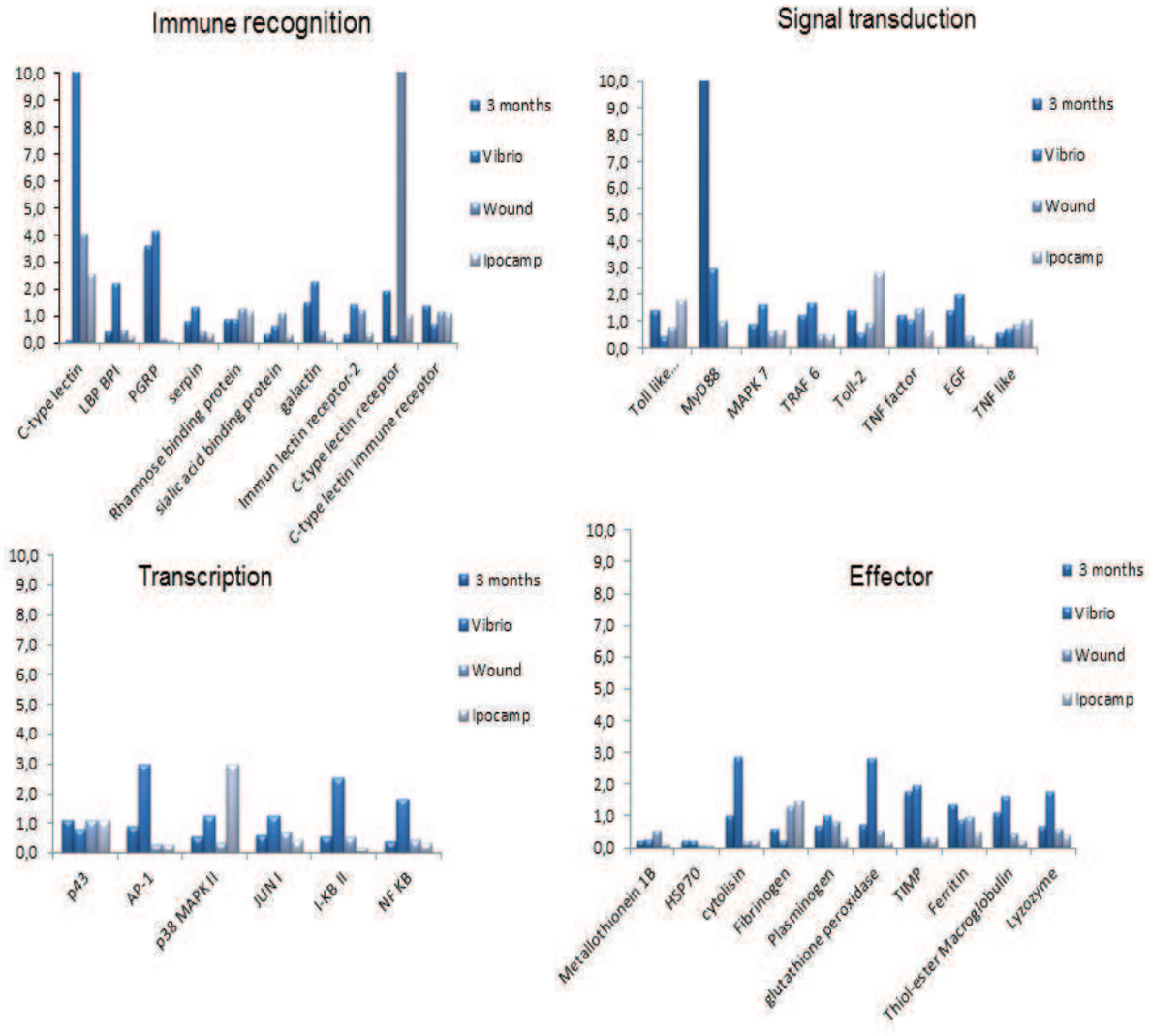

Figure 5. Comparative gene expression profiles from vent mussels subjected to 3 months acclimatization in aquaria at atmospheric pressure; *Vibrio parahaemolyticus* exposures; wound injury, and repressurization in the IPOCAMP chamber. Results are presented as relative expression folds calculated by qPCR and targeting immune genes from recognition, signaling transduction, transcription and effector functional gene categories as defined in Bettencourt et al. [50].

Comparison of DE across the five experiment revealed interesting correlations as for "cage" and "3 months" mussels indicating that vent mussels endured well aquarium conditions for as long as 3 months, as demonstrated by similar levels of gene expression. *Vibrio* infections and IPOCAMP pressurization also showed clustering patterns of gene expression which would seem to indicate that once mussels are acclimatized to atmospheric pressure, repressurization stimulus is impacting vent mussels in similar ways as in *V. parahaemolyticus* challenges, suggesting thus the occurrence of stress-related reactions in both types of stimulations. The expression pattern seen for wound injury was particularly distinct as compared to the other four experimental conditions. Wound injury seemed to affect drastically the vent mussel transcriptional activity which some of its genes were severely downregulated probably due to the damaging effect caused by the mechanical abrasion and direct exposure of the

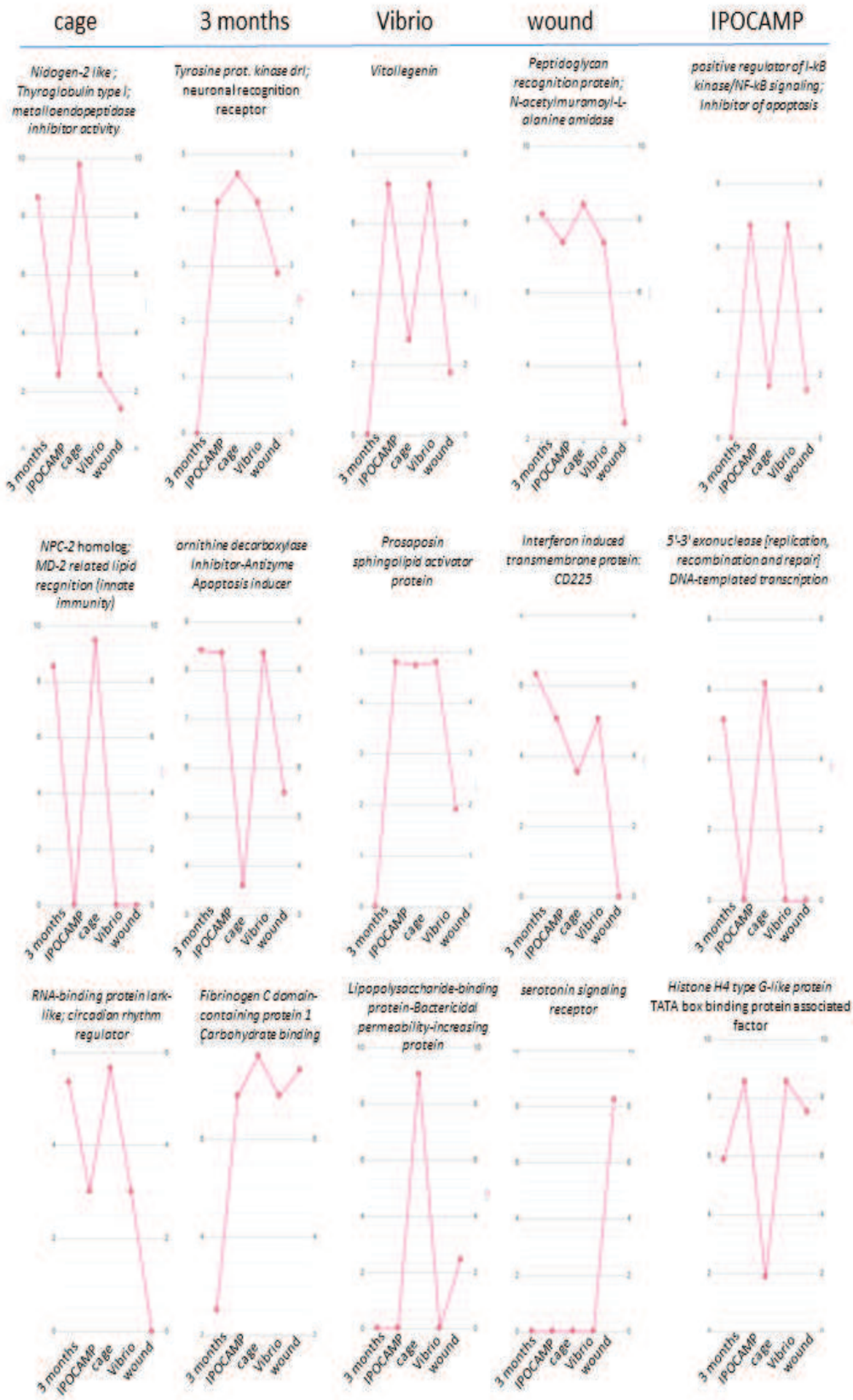

Figure 6. Expression plots across the five experimental conditions, "3 months"; "IPOCAMP"; "cage"; "*Vibrio,*" and "Wound," representing differential gene expression analyses using EdgeR. The top-scoring BLAST hit for each of the genes exemplified is shown on top of the respective expression plot.

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mantle to the aquarium environment. Taken together these experiments proven to be insightful in demonstrating the contrasting behavioral expression of given important physiological transcripts such as the peptidoglycan recognition and LBP-BPI proteins, both involved in innate immune responses, when vent mussels are met with distinct environment factors.

5. Host-endosymbiont interactions: implications for host immunity and defense mechanisms against bacterial pathogens

The transcriptome sequencing of gill tissues from the mussel *B. azoricus* revealed a set of genes of bacterial origin, providing a functional insight into the microbial vent community [71]. The transcripts supported a metabolically active microbiome and a variety of bacterial mechanisms and pathways, among which the fixation of carbon, the use of nitrate as a terminal acceptor of electrons and oxidation of sulfur and methane. The bacterial genes ensued from this sequencing work were deemed relevant to evaluate the influence of abiotic and biotic environmental conditions on *B. azoricus* transcriptional activity and also potentially useful to assess symbiont density differences in vent animals originated from distinct hydrothermal vent sites, respectively, to their environmental settings [72]. Keeping in line with the assumption that geographically distinct vent mussels will adopt different physiological statuses in relation to their environmental conditions, we also surmised that the relative abundance of methanotrophic and sulfide oxidizing endosymbiotic bacteria would differ between the Menez Gwen and Lucky Strike mussels as previously reported by other researchers [12, 58, 73]. We hypothesized that geographically distinct *B. azoricus* individuals may be experimentally traced back to their original hydrothermal vent sites based on their bacterial transcriptional activity and bacterial gill densities at the time animals were retrieved from the shallower Menez Gwen and deeper Lucky Strike vent sites. A taxonomical structure of the vent mussel gill's microbiome was also assessed to determine the bacterial community composition of gill tissue from MG and LS mussels to infer the symbiont densities differences between animals from both vent sites. Results from the ribosomal RNA amplicon sequencing of the V6 hypervariable regions, by massive parallel 454 pyrosequencing, indicated that the percentage of sequences obtained was from endosymbiont bacteria at nearly the same proportion between Menez Gwen and Lucky Strike samples. Moreover, comparative analyses based on BLAST searches in the RDP database, using the 16S rRNA OTU sequences, revealed that the thiotrophic endosymbiont represented 90% of all the sequences and methanotrophic endosymbiont almost 5% of the sequences from vent mussel samples originated from the distinct Menez Gwen and Lucky Strike hydrothermal vent fields [72].

While the majority of our experiments using live vent mussels were performed shortly after their retrieval from the Menez Gwen hydrothermal vent, long-term studies with vent mussels acclimatized to atmospheric pressure conditions have hardly been addressed until recently. As above-mentioned, long-term acclimatization experiments in aquarium systems have allowed us to study the expression of bacterial symbionts genes, particularly methanotrophic and thiotrophic bacteria, over time of acclimatization while their mussel host is faced with drastic physiological challenges, metabolic adaptations, and food intake changes in an effort to adapt to an aquarium environment at atmospheric pressure and without supplementation

of methane and sulfur [56]. The physiological adaptation to aquarium environment is likely to be aggravated by the expelling of endosymbionts into the aquarium environment, progressively emptying the gill tissue of its autotrophic bacteria, essential for the host vent mussel nutritional sustenance. Long-term aquarium acclimatization represents thus a model study to investigate the presence and maintenance of symbiotic associations between chemosynthetic bacteria and vent animals, which depend on controlled cell-cell communication between host and endosymbionts and the role of the host immune system [56, 74].

Presumably, the loss of endosymbiont induces a dramatic change in host gene expression profiles especially if endosymbiont genes exert some transcriptional control over host gene expression. For this reason, acclimatization studies have been instrumental to further our understanding of *B. azoricus* immune system. These studies have provided insights into physiological principles underlying mechanisms of adaptation to aquarium conditions at sea level pressure while taking advantage of the remarkable capacity of vent mussels to survive well decompression once brought to surface [21, 22, 56]. Furthermore, these studies have allowed analyses using immune challenged mussels comparatively to acclimatized control mussels, maintained under aquarium conditions. In view of our previous experiments performed with live gill tissues and postcapture immune gene expression studies in *B. azoricus* acclimatized to atmospheric pressure, the presence of endosymbiont bacteria is now being under investigation as a driving factor under which host-immune genes may transcriptionally be modulated and reciprocally endosymbiont genes may transcriptionally be modulated by the host [53–56]. Moreover, the impact of aquarium acclimatization on *B. azoricus* immune responses and its capacity to react to *V. diabolicus* challenges was recently evaluated during recurrent incubations with *V. diabolicus* during short periods of time, followed by clean sea water incubations allowing animals to depurate and subsequently be reexposed to the same load of *V. diabolicus* over a period of 3 weeks acclimatization experiment [74]. As previously described, we found a time-dependent immune gene response in *B. azoricus* tied to the endosymbiont presence inside the vent mussel gills. The vent mussel's immune defense capabilities were affected by the gradual loss of symbiont bacteria suggesting a symbiont-mediated defense mechanism under which the transcriptional regulation of host immune genes is directly affected by symbiont density and/or activity. The host-immune system-endosymbiont interactions were actively higher during the first week of acclimatization as a result of *Vibrio* exposures, demonstrating the ability of *B. azoricus* to increase the transcription of immune genes while endosymbiont gene expression also correlated with an increased symbiotic metabolism and prevalence. A synergistic response was proposed to counteract the presence and potential infection by *V. diabolicus* bacterium while modulating *B. azoricus* immune defenses-endosymbiont interactions to an extant, which host-immune and endosymbiont genes are mutually reliant during the first weeks of acclimatization [74]. The evidence presented suggests successful *V. diabolicus* recognition prompting immune genes to increase their levels of transcriptional activity particularly for genes involved in the Toll-like receptor signaling [75, 76] and apoptosis-related pathways [77] during first day of acclimatization in aquarium environments. In agreement with this, *B. azoricus* is presented as a suitable model to study molecular interactions involving host-mediated immune recognition events and adaptation mechanisms, to mitigate apoptosis harmful effects induced by *Vibrio* exposure against which, endosymbionts were prompted to increase their transcriptional activity, evocative of a possible protection role to the host [74]. This work brings to light other questions relating to

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how the host-immune system regulates the symbiont population within their gills and conversely symbionts avoid being recognized and eliminated by the host. These topics are being further investigated in our group and focused on finding and characterizing the molecular mechanisms underlying the establishment (recognition and acquisition) and functioning of symbiosis between deep-sea vent mussel *B. azoricus* and the methanotrophic and thiotrophic bacteria (gene expression, energy metabolism, regulation of symbiont population).

Interestingly, the study of intricate associations with chemosynthetic symbiont-bacteria living in the gills of deep-sea vent animals led us to the conception of a new pathogenesis model system based on an unconventional host-symbiont model system. This new marine invertebrate model system, as for the ecotoxicological model *Mytilus* spp. [78] relies, instead, on its unique host-immune-symbiont bacteria interactions believed to play a crucial role in counteracting infectious pathogens. The establishment of invertebrate host pathogen systems may serve as suitable and useful models to study pathogenicity. The molecular mechanisms through which pathogens are able to colonize and overtake host's immune system, particularly during the initial phase of infection when molecular recognition of MAMPs is occurring, as the pathogen defines its route of entry, are expected to reveal new molecular strategies that could help developing new therapies in aquaculture diseases. Using the deep-sea vent mussel *B. azoricus* as an alternate invertebrate model system to study pathogenesis brings a new perspective into the search for new drug targets that could directly interfere with pathogen recognition processes and/or with *in situ* inflammatory process where immune cells (i.e., hemocytes) and cytokine-like molecules are being mobilized. Indeed, in such host-endosymbiont model systems, the role of endosymbiont-derived molecules could have an important influence in mediating pathogenesis and in counteracting the deleterious effect of pathogens on the host immune system. From an experimental approach, several genera of bacterial fish pathogens may be used in *B. azoricus*, as infectious agents, e.g., *Vibrio*, *Flavobacterium*, *Pseudomonas*, *Aeromonas*, *Streptococcus*. Host and endosymbiont gene expression profiles may be studied during infection experiments carried with a given bacteria and genes that are markedly upregulated or downregulated further analyzed and their cDNA sequences determined by traditional sequencing methods.

Particular attention should be given to genes whose encoded proteins are participating in signal transduction pathways directly influencing the outcome of immune effector molecules, as antibacterial peptides; immune recognition lectins and antioxidant products such as superoxide dismutase, ferritin, metalloproteinases, metallothioneins, and heat shock proteins [26]. Synergistic effects resulting from interactions between host immune and endosymbiont activity, in counteracting infectious pathogens, may now be studied at the molecular level, for future therapies design, targeting key steps during pathogen infection processes, for instance, host recognition events; production of the anti-inflammatory factor TNF-alpha and cytokine-like growth factors; enhancement of antibacterial molecules synthesis.

6. Concluding remarks

In an attempt to understand physiological reactions of animals normally set to endure extreme conditions, in deep-sea environments, our laboratory has undertaken, for the last 6 years, a

series of investigations aimed at characterizing molecular indicators of adaptation processes of which components of the immune and antioxidative stress response systems have received most of our attention. As a research goal, long-term maintenance of vent mussels to atmospheric pressure was instrumental to further our understanding on molecular relationships under which the vent mussel-endosymbiont interactions are affected by aquaria conditions and by the gradual disappearance of endosymbiont bacteria from gill epithelia. Hence, the maintenance of live mussels in our aquarium laboratory system has been a key factor in gaining knowledge into the physiology of vent animals including the study of evolutionary conserved immune, inflammatory, and stress-related factors commonly found in other marine bivalves. *In vivo* and *ex vivo* experiments conducted with live mussels and their excised gill tissues as primary tissue cultures, allowed the specific host-endosymbiont interactions to be revealed, and further characterized in the deep-sea vent model *B. azoricus*, establishing distinct genetic signatures for the expression of endosymbiont genes and host-immune genes in relation to different environmental conditions. Increasing evidence now support the role of gills as a *bone fide* immune-responsive tissue in *B. azoricus*, consistent with a suitable study model for exploring molecular interactions involving host-endosymbiont-mediated immune recognition events and adaptation mechanisms to deep-sea hydrothermal vent environments. Such adaptation mechanisms are likely to be influenced by the microbial community composition surrounding the mussel beds at hydrothermal vents and therefore it is important to continue metatranscriptomic and metagenomic studies [79] from the gill-associated microbial diversity and surrounding hydrothermal vent sediments [80, 81] in view of the broader ecological organization and evolutionary importance of animal-bacterial microbiomes in chemosynthetic-based ecosystems in the deep sea [82, 83].

In recent years, researchers have turned to the human microbiome for its functional role in human health [84] and both composition and alterations in the microbiome have been found associated with diabetes, inflammatory bowel disease, obesity, asthma, rheumatoid arthritis, and susceptibility to infections [85]. Other microbiomes from nonmammalian and nonvertebrate species have also been characterized, for instance in insects where it was found to be highly dependent on the environment, species, and populations and affecting the fitness of species. These fitness effects may have important implications for the conservation and management of species and populations [82, 83]. Given the temporal instability of deep-sea hydrothermal vents and their constant fluctuations of physical and chemical environmental conditions, vent animal-microbiome associations have become critical for our understanding of invasion of nonnative species, responses to pathogens, and responses to chemicals and global climate change in the present and future [82] particularly when deep-sea mining activities are projected to have a major impact on deep-sea vent ecosystems [86].

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