Immune responses in *Bathymodiolus azoricus* upon *Vibrio* challenges: approaches to characterize mussel survival strategies and physiological adaptations in deep-sea extreme environments

Respostas imunitárias em *Bathymodiolus azoricus* desafiados com *Vibrio*: abordagens para caracterizar as estratégias de sobrevivência do mexilhão e suas adaptações fisiológicas em ambientes extremos do mar profundo

A dissertation presented by Eva Lúcia Martins Graça to the University of the Azores in candidacy for the degree of Doctor of Philosophy in Marine Sciences, specialty in Marine Biology

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Acronyms & Abbreviations

AMP Antimicrobial peptide
BPI Bactericidal Permeability Increasing protein
CTL C-type lectin
EGF Epidermal Growth Factor
ESI-MS/MS Electrospray ionization mass spectrometry
GpxI Glutathione peroxidase I
HSP70 Heat Shock Protein 70
HPLC High Pressure Liquid Chromatography
ILR2 Immune Lectin Receptor 2
IRAK Interleukin-1 Receptor-Associated kinase
IRG Similar to immunity-related GTPase
LBP-BPI Lipopolysaccharide Binding Protein (LBP) a Bactericidal/Permeability-Increasing Protein (BPI)
LITAF Lipopolysaccharide (LPS)-induced Tumor necrosis factor-alpha TNF-α factor
LPS Lipopolysaccharide
LRR Leucine-Rich Repeats
LS Lucky strike
MAMPs Microbial Associated Patterns
MAR Mid-Atlantic Ridge
MG Menez Gwen
MT IB Metallothionein IB
MyD88 Myeloid Differentiation primary response gene-88
NF-κB Nuclear-Factor kappa B
PAMP Pathogen Associated Molecular Pattern
PCR Polymerase Chain Reaction
PGN Peptidoglycan
PGRP Peptidoglycan Recognition Protein
PRR Pathogen Recognition Receptor
qPCR quantitative-Polymerase Chain Reaction
RBL Rhamnose-Binding Lectin
ROS Reactive Oxygen Species
RTK Receptor Tyrosine kinase
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis method
SRCR Scavenger Receptor Cysteine-Rich
TLR Toll-like receptor
TNF Tumor Necrosis Factor
TRAF6 Tumor necrosis factor (TNF) receptor associated factor 6

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Abstract

The deep-sea environment is excellent source for investigating and improving our knowledge in the field of marine biology of organisms living in extreme habitats and their relationships with chemosynthetic-based ecosystems. Along with the study of chemosynthetic-based ecosystems the necessity to understand immune system function in deep-sea hydrothermal animals such as in the mussel *Bathymodiolus azoricus*, has emerged gradually. Indeed, these mussels live and thrive in extreme environments as the deep-sea hydrothermal vents. *B. azoricus* species has become an interesting model to characterize the survival strategies and physiological adaptations in these environments. At such depths and under hydrothermal physico-chemical conditions, their immune system is constantly challenged by foreign vent compounds and microorganisms (e.g. bacteria). However, these immune responses in *B. azoricus* upon *Vibrio* challenges are not thoroughly understood. *Vibrio* bacteria are frequently pathogens in the aquatic environment that cause a high mortality in bivalves. This thesis was conceived and achieved with the aim of better understanding the immune response in *B. azoricus* mussels after several experimental challenges with different *Vibrio* strains.

The core of this thesis consisted of elucidating signaling gene pathways and proteins involved in mussel defenses during bacterial stimuli. In this way, the thesis methods were undertaken using several molecular biology techniques, highlighting the qPCR. This technique was used: in gills from *B. azoricus* mussels infected with suspension of different marine bacterial pathogens (Chapter I); in comparative studies of gene expression and protein analyses between deep-sea hydrothermal vent mussel *B. azoricus* and the shallow-water mussel *Mytilus galloprovincialis* (Chapter II) and in an immune response tissue-specificity study in different tissues, and between Menez Gwen and Lucky Strike *B. azoricus* populations, after exposure to *Vibrio diabolicus* bacterium (Chapter III).

Chapter I present a study on differential immune system responses in deep-sea mussels submitted to different bacterial strains, consisting of *Vibrio splendidus, Vibrio alginolyticus,* or *Vibrio anguillarum* and a pool of these bacteria as well as *Flavobacterium* bacterium. This study investigated the discriminatory capabilities of the *B. azoricus* immune responses. Results demonstrated a general gene expression pattern, decreasing from 12 h to 24 h post-infections. Among the animals tested, *Flavobacterium* is the bacterium inducing the highest gene expression level in 12 h post-infection animals, whereas the 24 h infected animals with *V. splendidus* showed a greater gene expression levels. SDS-PAGE analysis also pointed at protein profiles differences between 12 h and 24 h, particularly evident for proteins of 18-20 KDa

molecular mass. Multivariate analyses demonstrated that immune genes, as well as, experimental infections, clustered in discrete groups in accordance with gene expression pattern induced by the marine bacteria tested.

Chapter II presents a comparative immune responses study in two bivalve species from distinct marine habitats. The two species are the deep-sea hydrothermal vent mussel *B. azoricus* and the shallow-water mussel *M. galloprovincialis*. Both species were exposed to *V. splendidus, V. alginolyticus, V. anguillarum* and to a mixture of these mentioned *Vibrio* strains. The aim of this study was to evaluate the features of signaling pathways in different bivalve species. Gene expression results revealed that these bivalve species exhibited significant expression differences between 12 h and 24 h post-challenge times, as well as between bacteria tested. *V. splendidus* bacterium induced the strongest gene expression level in the two bivalve species, whereas the NF-kB and Aggrecan genes were the most differentially expressed in *B. azoricus* and *M. galloprovincialis*. HPLC- ESI-MS/MS analyses resulted in different peptide sequences in gill tissues from *B. azoricus* and *M. galloprovincialis*, suggesting that naive animals present differences, at protein synthesis level, in their natural environment.

Chapter III was focused on the immune responses tissue-specificity using gill, digestive gland and mantle tissues from MG and LS *B. azoricus* populations challenged with *V. diabolicus* bacterium. Results demonstrated a significantly tissue-specific gene expression in both *B. azoricus* populations. However, MG mussels showed gill and digestive gland gene expression levels remarkably higher than LS mussels. In mantle tissue, most genes were down-regulated in MG mussels and up-regulated in LS animals.

Resumo

O ambiente marinho profundo representa uma excelente fonte de investigação para desenvolver e melhorar os conhecimentos sobre os animais que habitam em ambientes extremos e sobre as relações quimiossintéticas estabelecidas nesse ecossistema marinho.

Dessa premissa surge a necessidade de entender o funcionamento do sistema imunitário em animais do fundo marinho, nomeadamente do mexilhão do mar profundo *Bathymodiolus azoricus*. O facto de este mexilhão habitar e desenvolver-se em ambientes extremos, como as fontes hidrotermais marinhas de profundidade, torna-o num interessante modelo para caracterizar as suas estratégias de sobrevivência e adaptações fisiológicas nesses ambientes. Nestas fontes hidrotermais, o seu sistema imunitário está constantemente a ser desafiado por compostos estranhos e microrganismos, como as bactérias do meio ambiente circundante. Contudo, as respostas imunitárias no *B. azoricus* após contacto com *Vibrio* não estão completamente compreendidas. As bactérias do género *Vibrio* são frequentemente patogénicos marinhos que causam grande mortalidade em bivalves. Assim, a presente tese pretende compreender as respostas imunitárias dos mexilhões após a exposição a diferentes estirpes de *Vibrio,* de modo a elucidar as principais vias de sinalização dos genes e proteínas intervenientes na defesa dos mexilhões durante um determinado estímulo bacteriano.

Neste sentido, os métodos apresentados nesta tese têm como base a utilização de diferentes técnicas da biologia molecular, com destaque para o PCR quantitativo em tempo real baseado no princípio da reacção em cadeia da polimerase. Esta técnica foi realizada: em brânquia de mexilhões *B. azoricus* submetidos ao contacto com diferentes suspensões bacterianas de patogénicos marinhos (Capítulos I); no estudo comparativo da expressão de genes e proteínas entre o mexilhão de profundidade *B. azoricus* e o mexilhão costeiro *Mytilus galloprovincialis* (Capitulo II) e no estudo sobre a especificidade da resposta imunitária em diferentes tecidos, entre populações de *B. azoricus* provenientes das fontes hidrotermais Menez Gwen e Lucky Strike, após contacto com a bactéria *Vibrio diabolicus* (Capitulo III).

No capítulo I desta tese, foi realizado um estudo diferencial das respostas imunitárias em mexilhões de profundidade submetidos em contacto com diferentes estirpes bacterianas, nomeadamente *Vibrio splendidus, Vibrio alginolyticus,* ou *Vibrio anguillarum* e um pool das estirpes de *Vibrio* mencionadas anteriormente, assim como *Flavobacterium.* Este estudo teve como finalidade investigar a capacidade selectiva de resposta do mexilhão *B. azoricus*. Os resultados da expressão dos genes foram significativos nos mexilhões infectados com diferentes bactérias marinhas após 12 h e 24 h de exposição. Também os resultados demonstraram um padrão na expressão dos genes com decréscimo do nível da expressão das 12 h para 24 h. Entre os animais testados com diferentes bactérias, os animais infectados com *Flavobacterium* apresentaram níveis da expressão dos genes superiores às 12 h, enquanto os animais infectados

com *V. splendidus* apresentaram genes com uma maior expressão às 24 h. Ao nível proteico utilizando a técnica SDS-PAGE, diferenças entre 12 h e 24 h foram visualizados principalmente nas proteínas com peso molecular entre 18-20 KDa. Análises multivariadas demonstraram agrupamento hierárquico dos animais infectados e a sua expressão de genes estando de acordo com o padrão da expressão dos genes induzidos pelas bactérias testadas.

No capítulo II, foi realizado um estudo comparativo das respostas imunes em duas espécies de bivalves provenientes de distintos ecossistemas, o mexilhão das hidrotermais *B. azoricus* e o mexilhão costeiro *M. galloprovincialis*. Ambas as espécies de mexilhões foram submetidas em contacto com *V. splendidus, V. alginolyticus, V. anguillarum* ou pool das bactérias anteriores. Este estudo teve como finalidade avaliar se as vias de sinalização da expressão dos genes é uma característica comum em diferentes espécies de bivalves. A expressão dos genes revelou que estas espécies de bivalves exibem diferenças significativas entre as 12 h e 24 h após desafios bacterianos assim como das bactérias testadas. Os resultados mostraram que *V. splendidus* bactéria induz elevada expressão dos genes em ambas as espécies de bivalves, enquanto os genes NF-kB e Aggrecan apresentam níveis de expressão diferentes em *B. azoricus* e *M. galloprovincialis*. Os resultados da HPLC- ESI-MS/MS identificaram diferentes sequências peptídicas em brânquias nativas de *B. azoricus* e *M. galloprovincialis,* sugerindo que animais apresentam diferenças ao nível da síntese proteica no seu meio ambiente.

No capítulo III realizou-se um estudo sobre a especificidade da resposta imunitária com uma abordagem ao nível dos tecidos de brânquia, glândula digestiva e manto, em populações de *B. azoricus* provenientes das fontes hidrotermais Menez Gwen (MG) e Lucky Strike (LS), após estímulo com uma estirpe bacteriana de *V. diabolicus*. Os resultados demonstraram especificidade significativa ao nível dos tecidos em ambas as populações de *B. azoricus*. Contudo, mexilhões de MG mostraram níveis de expressão dos genes superiores em brânquia e glândula digestiva do que dos mexilhões de LS. A maioria dos genes no manto apresentou baixa expressão nos mexilhões de MG e elevada expressão nos animais de LS.

General introduction

Since the discovery of the first hydrothermal vent in 1977, during exploration along the Galapagos Rift, vent communities have been investigated due to their remarkable and yet not fully understood capacity to live in one of the most extreme environments of the world. Some invertebrate communities that live in harsh environments are colonizers from the surrounding deep-sea, whereas others originated from shallow-water species (Van Dover et al. 2002, Miyazaki et al. 2010). For this reason, deep-sea environments are considered a rich biodiversity spot.

Hydrothermal vents are mainly found in active volcanic sites located in mid-ocean and back-arc spreading centers and seamounts (Lonsdale 1977, Childress and Fisher 1992). Deep-sea vents are characterized by extreme physicochemical conditions such as, absence of light, acidic pH, high temperature variation, elevated hydrostatic pressure and toxic chemistry fluids (Childress and Fisher 1992, Van Dover et al. 2002). Another characteristic of hydrothermal vents is the presence of high concentrations of metals (Cd, Cu, Fe, Hg, Mn and Zn) originating from toxic fluids and sulfide-rich water emanations (Sarradin et al. 1998 and Cosson et al. 2008). Metals like Fe, Zn, Cu and Mn are essential for deep-sea bivalve species. On the other hand, Cd, Ag, Ba and Sr metals are non-essential and toxic in deep-sea species (Rousse et al. 1998). High levels of Cu, Hg and Zn metals have been reported in mussels, crabs, shrimps and polychaetes (Colaço et al. 2006). The metazoan species from hydrothermal vents include approximately 600 species, such as barnacles, bivalve mollusks, limpets, shrimp, tubeworms and gastropods members (Grassle 1985, Dover et al. 2002, Desbruyères et al. 2006). To live in such deep-sea environments some vent communities from *Mytilidae* family involves symbiotic bacteria interactions from which most animal energy is derived as result of organic matter produced by chemosynthetic bacteria or from the refractory organic matter present in the water column (Fiala-Medioni and Felbeck, 1990). Chemosynthesis is the biological conversion of 1-carbon molecules (carbon dioxide and methane) into carbohydrates using the oxidation of inorganic compounds such as sulfur dioxide and hydrogen gas for energy (Howe 2008).

The phylum Mollusca is one of the most diverse groups of animals with at least 100,000 living species. These species are spread in terrestrial and aquatic habits as well, in extreme environments such as hot thermal vents and cold seeps (Coyne 2011). The class Bivalve is characterized by the presence of pair of shells connected by muscles and ligaments including's animals as clams, mussels, oysters, scallops. These animals have gills with ciliated filaments which are responsible for suspension feeding on plankton, algae, bacteria, and micro detritus (McMahon and Bogan 2011).

Bathymodiolus azoricus

The Mytilid *Bathymodiolus azoricus* (Cosel & Comtet 1998) is an endemic mussel from the deep-sea hydrothermal vent communities at North Atlantic vent fields, located on the Mid Atlantic Ridge (MAR). *B. azoricus* is the dominant species in Menez Gwen (37°51′N, 31°31′W, 850 m), Lucky Strike vent sites (37°17′N, 32°16′W, 1700 m) and outnumbered by the shrimp species *Rimicaris exoculata* at the Rainbow (36°13′N, 33°54′W, 2300 m) vent site. These mussels are subjected to different hydrostatic pressure, temperature and hydrothermal fluid composition with variations of pH, dissolved oxygen, methane and sulfide concentrations (Charlou et al. 2000). These three vent sites have been extensively characterized and the differences in fluid composition are reported in several studies (Charlou et al. 2000, Desbruyères et al. 2001). The survival of these mussels in deep-sea environments depends on their efficient metal regulation including their accumulation, excretion or detoxification metal capacities (Chassard-Bouchaud et al. 1986). The metal bioaccumulation process is specific- vent dependent and relying on chemical composition fluid found at each hydrothermal vent site. Intense studies of metal bioaccumulation have been reported in *Bathymodiolus azoricus* (Company et al. 2006, Cosson et al. 2008 and Martins et al. 2011).

Table 1 *B. azoricus* morphology and biology features (Desbruyères et al. 2006).

Morphology	Biology
-Size : Shell length up to 121 mm -Shell variable, more or less elongate-modioliform, -Animal with large gills. -Mantle lobes on anterior half of ventral side separate. -Valvular siphonal membrane short, narrow and rather strong.	In dense clusters byssally attached to hard substrate around the hydrothermal vent, mostly on the walls and flanges of active edifices, at temperatures from 6° to up to 30°C. Endemic to vents.

The capacity to associate with chemosynthetic bacteria is one adaptive characteristic in bivalves (Miyazaki et al. 2010). In the case of *B. azoricus*, these mussels harbor thiotrophic and methanotrophic bacteria in their gills. During experimental conditions, the number of endosymbiont bacteria is decreasing until complete absence from gill tissue in approximately 2-3 weeks (Bettencourt et al. 2008). These endosymbiotic bacteria use substances from vent environments to produce organic compounds and energy to the mussels (Boutet et al. 2011).

B. azoricus is a unique model to study physiological adaptations because these mussels survive decompression from deep sea retrieval (Bettencourt et al. 2008). For this motive, these deep mussel species are an interesting model to study their innate immune system.

Bivalve innate immune system

Bivalves are soft-bodied ectotherms, enclosed by calcified shell valves into which they can retract their tissues in presence of foreign and pathogenic agents (Abele and Philipp 2012). The bivalve shells offer a protective barrier in deep-sea species from environmental impacts, as the wave's movement or tectonic movements due to volcanism activity. Also the extrapallial cavity and hemolymph constitute barriers of protection from the external milieu (Ridgway et al. 2010).

The function of the immune system is to protect the organisms against pathogens that invade their body to avoid damage in their tissues (Chi and Flavell 2008). Most investigations of the molluscan immune system at molecular level have been construed from information of invertebrate organisms, as the fruit fly *Drosophila melanogaster* and as well as, the innate conserved features found between invertebrate and vertebrate immune systems (Silverman and Maniatis 2001).

Bivalve mollusks possess an innate immune system which includes a cellular and humoral components that act together to safeguard the animal against infections. Primarily, bivalve have chemical and physical barriers such as the cuticle, shells and mucus layer that act as first obstacle to foreign substances and pathogenic agents as bacteria, fungi and parasites (Canesi et al. 2002).

The survival of marine bivalve species depends on their defense mechanisms, in the case of deep-sea mussels; they mobilize cell-mediated and humoral reactions to overcome the pathogens that naturally occur in aquatic environment (Bettencourt et al. 2009). The extrapallial cavity in bivalve species is predominantly constituted by hemolymph or plasma and circulating hemocyte cells. These humoral and cellular constituents act as part of innate immune system with the purpose of protecting the host from pathogenic agents. Hemolymph is constituted by several humoral factors such as lectins that are involved in the killing of pathogens by mean of agglutination processes, as well as, by lysosomal enzymes, and several antimicrobial peptides. These substances are important humoral defense factors in bivalve species (Pipe 1990, Bettencourt et al. 2009 and Sokolova 2009).

Antimicrobial peptides (AMPs) are universal immune effectors which are conserved along evolution. AMPs such as defensins, mytilins, myticins and mytimycin, have been reported in mussel species with a defense role (Mitta et al 2000a, Mitta et al. 2000b, Bettencourt et al. 2007 and Boutet et al. 2009).

Hemocytes interact during cellular immune responses such as encapsulation, phagocytosis and enzymatic digestion (Canesi et al. 2002 and Labreuche et al. 2006). These hemocytes are subdivided into hyalinocytes (agranular cells) and granulocytes (containing cytoplasmic granules) that are larger than hyalinocytes (Carballal et al. 1997). Granulocytes are the most

abundant type of hemocyte and main phagocytic cells in *B. azoricus* mussels (Bettencourt et al. 2009). The hemocyte population appears to be capable of discriminating between stress factors, and even between two *Vibrio* species as demonstrated in the mussel *M. galloprovincialis*, using *V. splendidus* and *V. anguillarum* (Li et al. 2008). After bacterial challenge, an increase of AMPs molecules (e.g. mytilins) are synthesized in granulocyte cells and released into the plasma (Mitta et al. 1999 and Mitta et al. 2000a). This feature evidence the role of hemocyte in innate immune response in bivalve species including mussels. Thus, immunocompetent cells as hemocytes and molecular effectors as AMPs, cytokines and chemokines, provide an essential line of defense against pathogens in bivalves. These immunocompetent cells once activated by contact to Microbial Associated Patterns (MAMPs) or Pathogen Associated Molecular Patterns (PAMPs) present on the surface of pathogenic cells and Pathogen Recognition Receptors (PRRs) found on host cells, display defense reactions with an important role in the clearance of pathogenic agents from host animal body.

Pattern recognition receptors

PRRs participate in the innate immune system for host defense against the invasion of foreign and pathogenic agents with an essential role in animals without an adaptive immune system such as bivalve species. PRRs are evolutionarily conserved families of soluble, membrane-bound or cytosolic molecules whose function can be referred to a limited number of protein domains (Pålsson-McDermott and O´Neill. 2007). Some PRRs were identified in marine bivalves and highlighted by the Peptidoglycan Recognition Proteins (PGRPs), C-type lectin receptors (CLRs) and Galectins, SRCR, Toll-like receptors (TLRs) that recognize a wide variety of conserved MAMPs derived from bacteria, viruses, protozoa. All PRRs are able to initiate downstream signaling cascades which lead to modification of gene expression of a variety of immune and inflammatory genes (Pålsson-McDermott and O´Neill. 2007).

PGRP

Peptidoglycan recognition receptors (PGRPs) are innate immunity molecules and found in most invertebrate and vertebrate animals. These molecules recognize and bind the peptidoglycan component found in bacterial cell wall (Royet and Dziarski 2007). The first bivalve PGRP cDNA sequence was cloned from the bay scallop *Argopecten irradians* (Ni et al. 2002).

CLRs and Galectins

C-type lectins (CTLs) are a large class of animal lectins with one or more carbohydrate-binding sites. CTLs have a role in pathogen recognition, functioning as mediators in endocytosis, induction of phagocytosis and encapsulation processes and in anti-bacterial defense mechanisms (Goudine et al. 2007, Jing et al. 2011 and Yang et al. 2011). Generically, CTLs specifically bind a variety of MAMPs on the surfaces of pathogens and consequently, they recognize an

extensive number of pathogens (Kilpatrick 2002 and Devi et al. 2010). In bivalve species, as *Chlamys farreri* scallop has been reported an increase of C-type lectin levels after stimulation with MAMPs, evidencing their function in innate immune response (Yang et al. 2011). A relative high abundance of C-lectin transcripts were reported in deep-sea mussels (Bettencourt et al. 2010). C-type lectins and galectins are two of major lectin families in animal kingdom (Kilpatrick 2002). Galectins are a widespread class of soluble lectins that specifically bind β-galactoside sugars and, they participate in a variety of biological processes as in regulation of immune homeostasis, and recognition of microbial pathogen (Feng et al. 2015). 14 galectin genes were found in the oyster *Crassostrea gigas* genome suggesting the importance of this family of receptors in bivalve innate immunity (Zhang et al. 2012).

SRCR

Scavenger Receptor Cysteine-Rich (SRCR) is an abundant superfamily of scavenger receptors extremely versatile while in association with different co-receptors. These receptors have the ability to recognize and to remove modified lipoproteins, pathogen clearance, lipid transport, as well as, cell transport (Canton et al. 2013). SRCR is found in bivalve species such as the deep-sea mussel *B. azoricus* and Oyster *Crassostrea virginica* (Bettencourt et al. 2010, McDowell 2012).

TLRs

TLRs are among the best-characterized PRRs and have been found in a similar domain organization in vertebrates and invertebrates. TLRs are PRRs made up of Leucine Rich Repeats (LRRs) extracellular variable region and TIR [Toll/IL (interleukin)-1 receptor] intracellular domain. The LRR and TIR domains are conserved protein structures along evolution scale (Khan et al. 2004, Leulier and Lemaitre 2008). In the Animal's kingdom as well as, in Bivalves species are present multiples TLRs, in which distinct TLRs provide a specific properties for pathogen binding site (Leulier and Lemaitre 2008 and Toubiana et al. 2013). In *Mytilus galloprovincialis* mussels 23 Toll-like receptors were identified with crucial roles in host immune response (Toubiana et al. 2013).

TLRs are able to bind several MAMPs, generally the Lipopolysaccharides (LPS) or Peptidoglycan (PGN) found on the surface of pathogens bacteria. Subsequently, TLR recruits intermediary elements like as the Myeloid Differentiation primary response gene-88 (MyD88) adaptor, Interleukin-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) Wang et al. 2014 and Xie 2013). In general, MyD88 is a universal cytoplasmic adaptor in invertebrates, containing a single death domain (Death) associated to a single TIR domain, which allowing to connect through a TIR domain with TLR (Muzio et al. 1997). It was reported three MyD88 adaptors in *M. galloprovincialis* (Toubiana et al. 2013). Subsequently, TLR activates transcription factors such as Nuclear-Factor kappa B (NF-κB). This transcription

factor has an essential role in immunity, inflammation, cell proliferation, differentiation, and survival (Oeckinghaus and Ghoshi 2009).

The transcriptome sequencing analyses constitutes a valuable tool for researching of innate immune genes. In 2010, *B. azoricus* transcriptome sequencing and analysis provided an indispensable source for the development of our knowledge on genes involved in immune responses, survival strategies and physiological reactions in deep sea vent mussels. Table 2 shows some functions and genes putatively involved in immune responses and inflammatory reactions from the *B. azoricus* transcriptome database (Bettencourt et al. 2010).

Table 2 Functions of *B. azoricus* genes putatively involved in immune response and inflammatory reactions found in the deep-sea mussel transcriptome (Bettencourt et al. 2010).

Function	**Gene Ontology n°**	**Gene Ontology description**
Recognition		
PGRP	GO: 0008745	N-acetylmuramoyl-L-alanine amidase activity
Galectin 4-like protein	GO:0005529	Sugar binding
Rhamnose-binding lectin	GO:0005529	Sugar binding
SRCR	GO:0005044	Scavenger receptor activity
Serpin (serine protease inhibitor)	GO:0004867	Serine-type endopeptidase inhibitor activity
LBP/BPI	GO:0008289	Lipid binding
Signaling		
TLR	GO:0045087 GO:0007165	Innate immune response; signal transduction
MyD88	GO:0004888	Transmembrane receptor activity
TRAF	GO:0007165 GO:0042981	Signal transduction; regulation of apoptosis
IRAK	GO:0019221 GO:0051092	Cytokine-mediated signaling pathway; regulation of NF-κB
TNF receptor	GO:0007165 GO:0042981	Signal transduction; regulation of apoptosis
Transcription		
NF-κB	IPR015681 (InterPro)	Regulation of NF-κB activity
Effector and modulatormolecules		
Defensin (big defensin)	GO:0006952	Defense response
Cytolysin	GO:0009404	Toxin metabolic process
LITAF	GO:0006955 GO:0006952	Immune response; defense response
Metallothionein	GO:0046872	Metal ion binding
Ferritin	GO:0006879	Cellular iron ion homeostasis

Objectives of the experimental work

This study aims at contributing towards a better understanding of the innate immune system in the deep-mussel *Bathymodiolus azoricus* in response to *Vibrio* challenges. The deep-sea mussels' chemosynthetic habitats support the interaction with microbial communities assuming an important role in the survival strategies of vent mussels. Thus, the deep-sea vent biological systems represent the opportunity to study and provide new insights into the basic principles that govern the defense mechanisms in vent animals and how they cope with different environmental microorganisms. This thesis intends to study the question whether or not the *Vibrio* challenges induce transcriptional responses and to investigate which genes are expressed in response to these bacterial challenges, as well as, to identify protein sequences variations found in natural mussel populations of deep-sea mussels. The objectives were:

(i) To study the immune responses in *B. azoricus* upon experimental challenges with different *Vibrio* strains and their immune discriminatory capabilities.

(ii) To understand the immune responses in two bivalve species from distinct marine habitats: the progressive adaptation of *Bathymodiolinae* mussels to deep-sea environments and their immune reactions after *Vibrio* challenges comparing with their Mytilid ancestor.

(iii) To survey two deep-sea mussel populations from different vent depths and their tissue response specificities to *V. diabolicus* bacterium.

This thesis contains three fundamental chapters followed by a general discussion, final conclusions and suggestions for future research.

Thesis synopsis

The investigation focused on the innate immune system in deep-sea animals can help elucidate general mechanism used by animals to survive in harsh environment such as the deep-sea chemosynthetic-based ecosystems. This study brings insights into physiological adaptive mechanisms that bivalves may have adopted in view of biotic and abiotic environmental parameters changes over time, including the biodiversity and prevalence of marine pathogens. The first chapter of this thesis relates to five different responses in *B. azoricus* after exposure to distinct marine bacteria. *B. azoricus* mussels display different gene expressions and immune discriminatory capabilities as reported in this chapter. Results were compared to control animals maintained in plain seawater. This chapter sets the stage for the study of gene expression after *Vibrio* bacterial challenges in deep-sea mussels. Actin protein was found in control and challenged samples. Chapter I was designed to meet the first goal of the thesis, in which *B. azoricus* immune discriminatory capabilities were studied.

Bivalvia included several invertebrate species widespread in different geographic locations. An example of two bivalve species from distinct marine habitats is the deep-sea mussels *Bathymodiolus azoricus* and the shallow water mussels *Mytilus galloprovincialis.* This chapter provides the baseline to meet the second major goal of this dissertation. This chapter compares the gene expressions and protein sequences found in these mussels species challenged with *V. splendidus*, *V. alginolyticus*, *V. anguillarum* and to a mixture of *Vibrio* sp. The aim of this chapter was to evaluate the immune signaling pathway in *B. azoricus* and *M. galloprovincialis*. Results demonstrated significantly differentially expressed genes between these two species. Gene transcription mechanism differences were found in these two species, as well as, their unique immune reactions in presence of *Vibrio*, whereas *V. splendidus* bacterium induced the strongest gene expression (Chapter II).

Most immune studies in *B. azoricus* have targeted gill tissues due to the presence of endosymbiotic bacteria harbored in modified gill epithelial cells. However, in this dissertation, gill, digestive gland and mantle tissues were also studied with the aim of demonstrating gene expression differences in different organs of the animals' body during *V. diabolicus* challenges. This third chapter addresses Menez Gwen (MG) and Lucky Strike (LS) mussel populations and their tissue specificities in response to *V. diabolicus* bacterium. Results demonstrated a significantly tissue-specific gene expression in MG and LS mussels. MG mussels showed gill and digestive gland gene expression levels remarkably higher than LS mussels, whereas in mantle most genes were up-regulated in LS animals. Significantly differences were demonstrated in different tissues from MG and LS mussels **(**Chapter III).

The appendix 1 comprises the *B. azoricus* protein sequence from SDS-PAGE methodology. The appendices 2 and 3 include the *B. azoricus* and *M. galloprovincialis* protein sequences respectively, from HPLC- ESI-MS/MS analyses. The appendix 4 concerns the permanova results and the global significance of gene expression levels differences in gill, digestive gland and mantle tissues between MG and LS mussels.