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HOST-SYMBIONT INTERACTIONS IN THE DEEP-SEA VENT MUSSEL *BATHYMODIOLUS AZORICUS* - A MOLECULAR APPROACH

By

Inês Barros

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Inês Barros

Under the supervision of:

Doutor Ricardo Serrão Santos and Doutor Raul Bettencourt

Centro do IMAR da Universidade dos Açores

Departamento de Oceanografia e Pescas

Universidade dos Açores

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Abstract

The mid-oceanic ridges running around the Earth are the theatre of intense submarine volcanic activity creating oases such as deep sea hydrothermal vents for a specialized fauna where different species are distributed worldwide. Deep-sea mussels of the genus *Bathymodiolus azoricus* are dominant communities at hydrothermal vent sites between 800 to 2400 m depth in the Azores Triple Junction of the Mid-Atlantic Ridge. They have developed survival strategies including dual endosymbiosis with both methanotrophic (MOX) and sulfide-oxidizing (SOX) bacteria housed inside their specialized gill cells while exhibiting also unusual immune system capabilities, reflecting thus, their ability to adapt remarkably to environment changes. Their extraordinary physiological plasticity has been evidenced throughout this thesis work during different experimental acclimatization to aquarium environments. *B. azoricus* has been revealed as a suitable model to investigate the metabolism of the host at a molecular level, such as the description of genes involved in the innate immune system and symbiosis establishment in relation with bacteria.

The objectives of this work are to further characterize the adaptation of *B. azoricus* to long term acclimatization in aquaria conditions and its effects on host–symbiotic associations, endosymbiotic prevalence and host immune responses, in view of understanding the functional immunological capabilities of *B. azoricus* gill tissues. In order to study a comprehensive biological response profile, both immune and bacteria gene expressions were quantified by real-time PCR and by Fluorescence *In Situ* Hybridization approaches, which provided a direct way to determine the relative location and quantification of endosymbionts. The RNA-seq methodology was considered in order to reveal the specific microbial and functional variabilities in the *B. azoricus* holobiome structure.

The results herein presented, bring evidence supporting that vent mussels developed specific survival mechanisms, under different experimental conditions, which involved a repertoire of differentially expressed immune genes to endure different environmental parameters. The study of differential immune gene expressions brought evidence suggesting a physiological “alert point” translated into higher levels of transcriptional activity when vent mussels were acclimatized for more than one week in aquarium conditions at atmospheric pressure.

During the thesis work bacterial challenges were analyzed using *V. diabolicus* which presented a putative modulating role on *B. azoricus* host immune system-endosymbionts interactions within gill tissues. This was reflected by the successful bacterial recognition that prompted immune genes to increase their levels of transcriptional activity, predominantly genes involved in the Toll and apoptosis-related signaling pathways. Endosymbionts predominance was observed, during the first week of acclimatization, eliciting the increase their transcriptional activity, suggesting of a possible protection role to the host against bacterial challenges and following gradual loss over the time course.

To better understand these questions, a metatranscriptomic study was developed to analyze *B. azoricus* gill-microbe associations during an acclimatization experiment over a period of 5 weeks. This approach holds potential for the discovery of new host-symbiont associations, evidencing new functional transcripts and a clearer picture of methane metabolism during the loss of endosymbionts. To the best of our knowledge, the *B. azoricus* endosymbiont-host metatranscriptomic analysis provided, for the first time, insight into a gill-specific microbial diversity and host-endosymbiont gene expression patterns. Moreover, this work identified vent-related bacterial sequences that affiliated with Gammaproteobacteria, including fauna symbionts *Oceanospirillales, Methylococcales* and *Thiotrichales*.

Mussels from Menez Gwen and Lucky Strike hydrothermal vent fields were compared to address the hypothesis that physico-chemical characteristics and/or symbiont densities have an influence on *B. azoricus* transcriptional statuses. Genes encoding transcription factors, signaling pathways, effector and recognition molecules were investigated however, no clear immune gene expression signature was able to be depicted from this study given the variability of expression observed within and between the different functional immune genes from both Menez Gwen and Lucky Strike mussel gill samples. In sharp contrast, bacterial taxonomical structure clearly indicated a greater overall bacterial transcript distinction in Lucky Strike gill tissues when compared to Menez Gwen samples. The increased levels of bacterial transcripts in Lucky Strike gill samples could indicate a higher load of bacteria in gill tissues or/and an increased transcriptional activity from a relatively constant amount of bacteria associated to the gills.

My thesis work highlighted tight associations, unseen thus far, suggesting that host immune and bacterial gene expression patterns reflect distinct physiological

responses over the course of acclimatization under aquarium conditions. Taking together, *B. azoricus* is a suitable model to study how the prevalence of symbiotic bacteria is driving the expression of host immune genes, physiological plasticity, molecular interactions involving host-mediated immune recognition events and adaptation mechanisms to divergent environmental conditions.

Resumo

A dorsal média oceânica é caracterizada por apresentar intensa atividade vulcânica resultando na criação de ambientes invulgares, tais como as fontes hidrotermais, favoráveis ao estabelecimento de uma fauna especializada distribuída mundialmente. Os mexilhões de profundidade do género *Bathymodiolus azoricus* são as comunidades dominantes das fontes hidrotermais, encontradas entre os 800 e os 2400 metros de profundidade, e localizadas na junção tripla dos Açores da Dorsal Média do Atlântico. Estes desenvolveram estratégias de sobrevivência, tais como a dupla relação endosimbiótica com bactérias metanotróficas (MOX) e sulfuroxidantes (SOX) localizadas dentro de células especializadas - as brânquias, bem como um sistema imunológico adaptativo, manifestado pela sua capacidade em adaptar-se a extremas mudanças ambientais. *B. azoricus* apresentou uma extraordinária plasticidade fisiológica, nos trabalhos experimentais desenvolvidos nesta tese, sujeito a diferentes condições experimentais, quando aclimatizado em aquário. *B. azoricus* tem revelado ser um excelente modelo de estudo para compreender o metabolismo do hospedeiro a nível molecular, nomeadamente na descrição dos genes envolvidos no sistema imune inato e na sua relação simbiótica com bactérias.

Os objetivos desta tese incidiram na caracterização da adaptação do sistema imune do mexilhão *B. azoricus*, quando aclimatizado à pressão atmosférica durante um longo período de tempo, e os seus efeitos nas associações simbióticas bem como no estudo da prevalência das bactérias endosimbiontes, de forma a avaliar as capacidades imunológicas funcionais dos tecidos branquiais durante a adaptação fisiológica às alterações ambientais.

Para uma completa abordagem do perfil das respostas biológicas do *B. azoricus*, os níveis de expressão dos genes imunes e bacterianos foram quantificados por PCR em tempo real e por microscopia de fluorescência (*Fluorescence In Situ Hybridization*) que possibilitou localizar e quantificar os endosimbiontes presentes no tecido brânquial. Com o objetivo de estudar as variabilidades microbianas e funcionais na estrutura do holobioma do *B. azoricus*, o RNA foi sequenciado.

Os resultados aqui apresentados sugerem que os mexilhões das fontes hidrotermais desenvolveram mecanismos específicos de sobrevivência que envolvem a expresão diferencial de genes do sistema imune, evidenciado por um ponto fisiológico

de alerta, traduzido pelo aumento da atividade transcricional quando aclimatizado à pressão atmosférica mais do que uma semana.

Durante o trabalho desenvolvido nesta tese, os estímulos bacterianos nas brânquias do *B. azoricus* foram avaliados, usando a bactéria *V. diabolicus*, que apresentou um possível papel modulador no sistema imune do hospedeiro e nas interações com os endosimbiontes presentes nas brânquias. Esta capacidade foi comprovada pelo reconhecimento do hospedeiro aquando da infeção bacteriana, aumentando assim os níveis de atividade transcricional dos genes imunológicos, nomeadamente genes envolvidos nas vias de sinalização do Toll e da apoptose (morte celular). O aumento da atividade transcricional confirmou a presença das bactérias endosimbiontes durante a primeira semana de aclimatização, indicando uma possível proteção do hospedeiro contra infeções bacterianas e subsequente perda gradual ao longo do tempo.

Para um melhor entendimento das associações entre o hospedeiro e os endosimbiontes, durante a aclimatização experimental de 5 semanas, o metatranscritoma das brânquias do mexilhão *B. azoricus* foi sequenciado e analisado. Esta abordagem apresentou-se como uma informação potencial para novas descobertas nas associações hospedeiro-simbiontes, realçando novos transcritos funcionais e uma imagem mais definida do metabolismo do metano durante a perda dos simbiontes. A análise metatranscricional do hospedeiro e endosimbiontes do *B. azoricus* evidenciou, pela primeira vez, os padrões da diversidade microbiana bem como as relações entre o hospedeiros e os endosimbiontes. Adicionalmente, foram idenfificadas sequências bacterianas associadas ao género Gammaproteobacteria, nomeadamente à fauna simbiótica *Oceanospirillales, Methylococcales* e *Thiotrichales.*

Os tecidos brânquiais de *B. azoricus* provenientes de duas fontes hidrotermais diferentes, Menez Gwen e Lucky Strike, foram comparados para perceber se as características fisico-químicas e/ou a carga simbiótica teriam uma influência no estado transcricional do *B. azoricus*. Para tal, os genes que codificam para fatores transcricionais, vias de sinalização, moléculas efetoras e de reconhecimento, foram analisados. No entanto, a expressão dos genes imunes testados não mostraram ter uma assinatura específica para cada fonte hidrotermal dada a variabilidade de expressão dos genes imunológicos, tanto para amostras de Menez Gwen como de Lucky Strike.

Contrariamente, a estrutura taxonómica bacteriana indicou claramente uma maior distinção entre as brânquias de Lucky Strike e Menez Gwen pois o aumento dos

níveis de transcritos de bacterias provenientes da fonte hidrotermal Lucky Strike são indicativos de uma maior carga bacteriana e/ou de um aumento da atividade de transcrição dos genes bacterianos associados aos endosimbiontes. Os estudos desenvolvidos nesta tese dão especial destaque às associações entre o *B. azoricus* e respetivos endosimbiontes, apresentando padrões para a expressão dos genes imunes do hospedeiro e das bactérias que refletem respostas fisiológicas distintas, ao longo da aclimatização em condições de aquário.

Desta forma, *B. azoricus* é um modelo adequado para entender de que forma a prevalência de bactérias simbióticas induzem a expressão de genes imunes do hospedeiro, bem como as adaptações fisiológicas e interações moleculares, que envolvem eventos de reconhecimento do sistema imune, mediadas por mecanismos de adaptação face às mudanças das condições ambientais.

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ACAN	Aggrecan
ACT	Actin
AIF	Allograft inflammatory factor
ALDH	Aldehyde dehydrogenase
AMP	Antimicrobial Peptide
AP1	Activator Protein 1
ATP	Adenosine Triphosphate
B. azoricus	*Bathymodiolus azoricus*
BCL2	B-cell CLL/lymphoma 2
CA	Carbonic anhydrase
CALM	Calmodulin
CAR	Carcinolectin
CASP	Caspase 3
CAT	Catalase I
Cbb	RuBisCO activation Cbb
cDNA	complementary DNA
CH_4	Methane
CL	Carcinolectin
CL	Cyclooxygenase
CLEC	c-type lectin
CO_2	Carbon Dioxide
COX	Cyclooxygenase
Ct	Cycle thresholds
Cyt	Cytolysin
DEF	Defensin
DIC	Differential Interference Contrast
EGF	Epidermal growth factor
FasL	Fas Ligand
Fe	Iron
FER	Ferritin
FISH	Fluorescence In Situ Hybridization
GAL	Galectin
GPX	Glutathione peroxidase
GTPase	Guanosine triphosphates hydrolase
H_2S	Hydrogen sulfide
HCNO	formaldehyde
HKG	Housekeeping gene
HSP	Heat Shock Proteins
HSP70	Heat Shock Protein, molecular weights range from 66 – 78 kDa
IκB	Inhibitor of kappa B

ILR	Immune Lectin Receptor
ILR	IκB
IMD	Immune Deficiency pathway
IRAK	Interleukin-1 receptor-associated kinase
JUN	JUN-Like
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBP-BPI	LPS binding/bactericidal-permeability-increasing protein
LITAF	LPS induced TNF-alpha Factor
LPS	Lipopolysaccarides
LSU	large subunit
LYZ	Lysozyme
MAMPs	Microbe-Associated Molecular Patterns
MAPK	Mitogen-Activated Protein Kinase
MAPK-7	Mitogen activated protein kinase 7
MAR	Mid-Atlantic Ridge
MeDH / MDH	Methanol dehydrogenase
MIQE	Minimum Information for Publication of Quantitative
MMO	Methane Monooxygenase
MMO	Methane monooxygenase
MMP	Matrix Metalloproteinase
MOX	Methane-Oxidizing
MOX 16S	Housekeeping gene Methanotrophic Symbiont 16S
mRNA	messenger RNA
MT	Metallothionein
MyD88	Myeloid differentiation primary response gene 88
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor-κappaB
O_2	Oxygen
O^{2-}	Oxide ion
OTU	Operational Taxonomic Unit
p43	cytoplasmic protein, molecular weight 43 kDa
p53	cytoplasmic protein, molecular weight 53 kDa
PAMPs	Pathogen-Associated Molecular Patterns
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PGNs	Peptidoglucans
PGRP	Peptidoglycan recognition protein
PLG	Plasminogen
pMMO	particular Methane-oxidizing
PRPs	Pattern Recognition Proteins
PRRs	Pattern-Recognition Proteins
qPCR	quantitative PCR
RBL	Rhamnose binding lectin
RNA	Ribonucleic acid

RNA-seq	RNA sequencing
ROIs	Reactive Oxygen Intermediates
rRNA	ribosomal RNA
RuBisCo	Ribulose-1,5-Bisfosfato Carboxilase oxigenase
S^0	elemental sulfur
SABL	Sialic Acid Binding Lectin
SERPIN	Serine Proteinase Inhibitor
sMMO	soluble Methane-oxidizing
SO_3^{2-}	sulfite
SO_4^{2-}	sulfate
SOX	Sulfur-Oxidizing
SOX 16S	Housekeeping gene Sulfide oxidizer symbiont 16S
SOXB	Sulphate thiol ester SOXB
SPSS	Statistical Package for the Social Sciences
SRCR	Scavenger receptor cysteine-rich domain
SSU	small subunit
STAT-SH2	Signal Transducers and Activators of Transcription - Src-Homology Domain 2
TAL	Transcription activator-like
TIMP	Tissue inhibitor metalloproteinase
TLR2	Toll like receptor-2
TLRs	Toll-like receptors
TNF factor III	Tumor necrosis factor factor 3
TNFR	Tumor necrosis factor receptor
TRAF 6	TNF Receptor Associated Factor 6
TRK	Tyrosine kinase-R
tRNA	transfer RNA
UV	ultraviolet
V. diabolicus	*Vibrio diabolicus*
VEGF	Vascular endothelial growth factor
VEGF receptor	Vascular endothelial growth factor receptor

CHAPTER I

GENERAL INTRODUCTION

General characteristics of deep-sea hydrothermal vents

The discovery of deep-sea hydrothermal vents and associated animal communities in 1977 was one of the most exciting oceanographic discoveries of the 20th century and has challenged our way of thinking about biological systems [1, 2]. Hydrothermal vents, also known as deep-water seeps, deep-sea springs, and deep-sea vents, are the result of a volcanic eruption due to shifting of the plates that form the Earth's crust. The shifting causes cracks to form when the earth's plates are pulled apart along the Mid-Ocean Ridges [3].

Deep-sea hydrothermal vents are characterized by different physical and chemical factors, including, high pressure, high temperature gradients and high hydrostatic pressure, complete absence of light, low pH, elevated concentrations of methane, sulfur and heavy metals [4]. Hydrothermal vents are one of the most spectacular features on the seafloor. They form in places where there is volcanic activity, such as along the Mid-Ocean Ridge and occur in waters ranging from 30 to 3600 meters depth. Superheated water rushing from the sea bottom can reach temperatures as high as 400°C [5]. Hydrothermal-vent ecosystems are localized areas of the seabed where heated and chemically modified seawater exits the seafloor as diffuse or focused flow and where microbial chemoautotrophs are at the base of the food web [6]. Most vent ecosystems tend to be linearly distributed on hard substrata (basalt) associated with new ocean crust along seafloor spreading centers, though there are sites where active vents on spreading centers are sediment-hosted [6] and associated with seamount volcanic systems. Environmental conditions at hydrothermal vents are extreme and variable, so abiotic factors are generally thought to be most important in structuring populations and communities.

Azores Triple Junction

Azores Triple Junction area is a geologic junction where the boundaries of three tectonic plates intersect: the North American Plate, the Eurasian Plate and the African Plate. The hydrothermal vent communities are currently distributed in three major vent fields located on three segments of the south eastern limb of the Azores Triple Junction: Rainbow, Lucky Strike and Menez Gwen (Figure I-1).

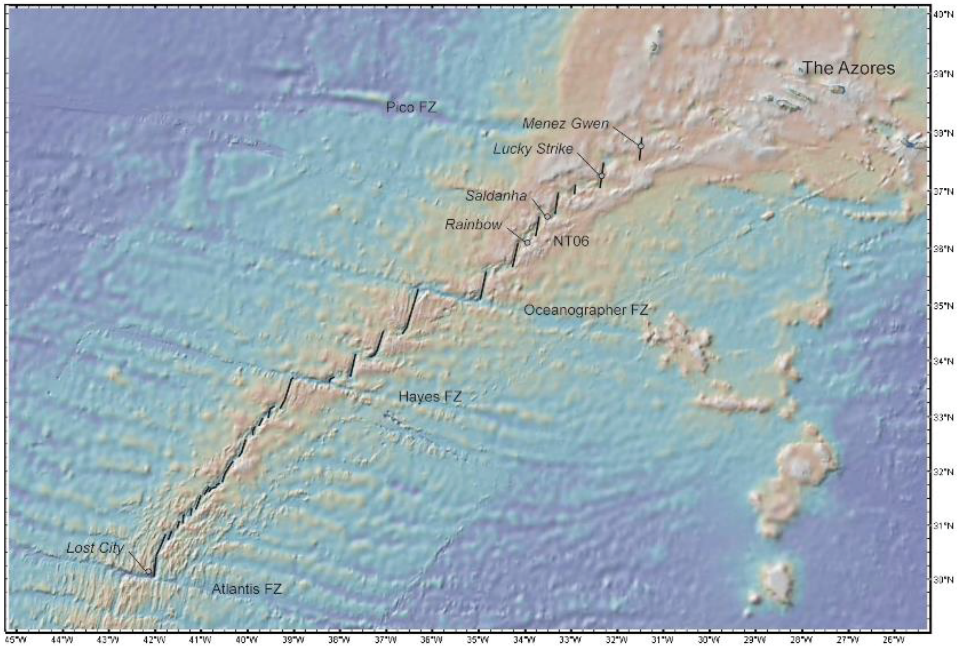

Figure I-1 Bathymetric map of the Mid-Atlantic Ridge axis south of Azores Triple Junction, showing the location of the hydrothermal vent fields adopted in ref [7].

For the present work the south-western arm of the triple junction is of particular interest because it is where Lucky Strike and Menez Gwen hydrothermal fields are located.

Study areas

The Lucky Strike and Menez Gwen hydrothermal fields are both located in the Mid-Atlantic Ridge (MAR), southwest of Azores islands, in two different ridge sections. These systems were discovered in the nineties, Lucky Strike in 1993 [8] and Menez Gwen in 1994 [9]. Lucky Strike and Menez Gwen are two active hydrothermal sites intensively explored since their discovery. The hydrothermal vents (active or inactive) at Lucky Strike hydrothermal field are distributed around the lava lake in the depression formed between the three volcanic tops. The site is at latitudes ranging from 37º17'15''N to 37º17'45''N, and longitudes from 32º16'15''W to 32º17'15''W – an extension of nearly 1 km^2.

The Menez Gwen site was discovered after Lucky Strike and is located at latitudes ranging from 37º50'12''N to 37º50'36''N, and longitudes from 31º31'00''W to 31º31'36''W. For the discovery of Menez Gwen it was very important the use of a new strategy of exploration where both CH_4 and H_2S concentrations were measured in the seawater samples collected near the bottom. High concentrations of these

compounds were found in the fluids collected during the dives before the discovery of the site. This vent field is located near the top of a young volcano emerging from the rift valley and extending over an area of nearly 200 m^2. Mounds and chimneys are of modest size and the maximum recorded fluid temperature was 281°C, although diffuse venting reaching 25°C was observed through-out the area [10].

Invertebrates at hydrothermal vents

Marine invertebrates constitute the largest group of macroscopic species in the sea [11]. Marine bivalves are an important component of the ecosystem and biodiversity [12], which have abundant species distributed worldwide from the intertidal zones to hydrothermal vents and cold seeps [13–15]. Deep-sea hydrothermal vents constitute unique ecosystems supporting a variety of endemic invertebrates species adapted to extreme physico-chemical environments. Bathymodiolin mussels (family Mytilidae) are one of the most ecologically successful metazoans in the deep-sea; they are ubiquitous within these habitats, colonizing hydrothermal vents and cold seeps to wood falls, whale carcass, and oil impregnated muds from oil-drilling platform [16, 17]. The occurrence of bathymodiolin mussels in this broad range of environments may be attributed to their nutritional flexibility; they are capable of obtaining nutrition from filter feeding [18] in addition to receiving nutrition from chemosynthetic symbionts [19].

Bathymodiolus azoricus – The study model

The deep-sea mussel *Bathymodiolus azoricus* (Bivalvia: Mytilidae) is generally found in dense populations at the Mid-Atlantic Ridge (MAR) hydrothermal vent fields due to successful adaptation strategies implicating a flexible feeding regime supported by dual symbiosis enabling vent mussels to colonize sulfide and methane rich environments [20, 21]. They owe their success and high biomass, at the Menez Gwen hydrothermal vent site, to their extraordinary capabilities to adapt and thrive in chemosynthesis-based environments [22, 23].

Chemosynthesis at deep-sea hydrothermal vents - The primary production

For a long time it was thought that the input of energy for the heterotrophic production of biomass in the absence of light was limited to the sedimentation of particulate organic matter from the photosynthetically productive surface waters to the bottom of the ocean. The decomposition and mineralization of this organic carbon occurs largely in the upper 200-300 m layer of the world oceans averaging at about 95% of their total primary productivity. With the discovery of metabolic activities in the permanently dark deep-sea, based on geothermal rather than solar energy, a new dimension was added to this general notion [24]. In geothermal systems, water rock interactions at high temperature generate hydrothermal fluids enriched in reduced inorganic chemical species that provide a source of energy, or "geofuels" for microbial oxidations. Microorganisms transform chemical energy into biochemical energy (ATP), which is then used to fix carbon dioxide [5,7]. This process, called chemosynthesis or chemolithoautotrophy, emphasizes that not only the carbon source but also the energy source is inorganic, providing a basis for primary production of organic carbon in the deep-sea hydrothermal vents [19]. These ecosystems often referred to as "chemosynthetic communities", have proved to be unsurpassed when compared to any other biological system on Earth in terms of biomass production rate [25].

Deep Sea hydrothermal vents ecosystem and host-symbiont interactions

There are numerous environments in the biosphere where the biogeochemistry prompt the colonization and emergence of chemosynthetic metabolisms. These sites are unified by the simultaneous availability of reduced compounds and molecular oxygen. Deep-sea hydrothermal vents were the first habitats in which chemosynthesis-driven primary production was shown to fuel large animal communities [26]. At almost all hydrothermal vents explored to date, dense assemblages of host animals are found clustered around vent orifices in order to provide their symbionts access to chemicals in venting fluid [27, 28] .

Vent ecosystems are typically dominated by benthic invertebrate taxa (e.g., vestimentiferan tubeworms, bathymodiolin mussels, vesicomyid clams, provannid

snails, rimicarid shrimp, yeti crabs) that host symbiotic, chemoautotrophic microorganisms [2]. Chemosymbiotic bivalves were found in a range of environments where sulfur and methane compounds, originate from decaying organic matter these environments, provides the biogeochemistry necessary for chemosynthetic metabolism. Differences in symbiotic communities are often observed among vent fields within the same region that have differing chemistry or geology [17, 29, 30], regarding to the concentrations of particular reduced compounds and sources of nutrients available to the symbioses (Table I-1).

Table I-1 Typical pore water concentrations of reduced compounds in different habitats [31].

Habitat	Sulfur	Methane
Hydrothermal vents	3–40 mmol/kg	0.1–3.4 mmol/kg
Cold seeps	0.57–19.43 mmol/kg	0.06–0.8 mmol/kg
Seagrass beds	5–35 μmol/kg	2–20 μmol/kg

Mutualistic associations between bacteria and eukaryotes occur ubiquitously in nature, forming the basis for key ecological and evolutionary innovations. These so-called 'holobiont' (host-symbiont) taxa often exhibit unusual morphological, physiological, and biochemical adaptations to characteristics of vent environments, including loss of the digestive system in vestimentiferan tubeworms, novel photoreceptors in swarming shrimp on black smoker chimneys, sulfide-binding proteins in vesicomyid clams and [32]. Holobiont taxa are also often foundation species, creating complex 3-dimensional habitat (e.g., worm aggregations, bivalve beds, snail aggregations) that serves as substratum for microbial growth and as refuge for juvenile invertebrates and habitat for associated organisms, including primary consumers (e.g., limpet grazers on microbial biofilms) and secondary and tertiary consumers (e.g., scavenging and predatory crustaceans and fishes) [6].

The term "symbiosis" was created to describe associations in which different species live closely together, in relationships ranging from mutualisms to parasitism. Symbiosis has played a major role in shaping the evolution and diversity of eukaryotic organisms. Some of the most prominent examples of these symbioses are chemosynthetic bacteria and marine invertebrates living in the absence of sunlight at deep-sea hydrothermal vents and in sediments rich in reduced sulfur compounds. Here, chemosynthetic bacteria living in close association with their hosts. The host provides

access to reduced compounds (e.g., hydrogen sulfide) and oxygen that the bacterium uses to drive the formation of fixed carbon from single carbon molecules (either CO_2 or CH_4). These symbionts require a source of electron donors (e.g., sulfide in vent fluid), a source of electron acceptors (e.g., O_2 in seawater), and a source of inorganic carbon (e.g., CO_2 or CH_4 in vent fluids, CO_2 in seawater) [29, 30].

Bivalves typically harbor their symbionts in large and conspicuous gills, often accounting for more than one third of the animal's total soft tissue weight [35]. However, chemosynthetic symbioses within Bivalvia are excellent model systems for studying the evolution of bacteria–eukaryote interactions, as they display a range of intimacies with some symbionts being housed intracellularly within specialized gill cells called bacteriocytes [17].

Bathymodiolin mussels and dual symbiosis

Bathymodiolins appear to be more versatile than vesicomyids and vestimentiferans, because the mussels are mixotrophic, retaining a functional digestive tract while hosting nutritional endosymbionts [18]. Some species, like *Bathymodiolus thermophilus* from east Pacific vents, harbor only thiotrophic bacteria, while others, like *Bathymodiolus childressii* from the Gulf of Mexico, have only methanotrophic symbionts [36]. A dual symbiosis, in which a single host harbors both thiotrophic and methanotrophic bacteria, has been described for four species, two from cold seeps in the Gulf of Mexico (*Bathymodiolus brooksii* and *Bathymodiolus heckerae*) [37, 38] and two from vents along the Mid-Atlantic Ridge (*Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis*) [15, 35, 39].

Endosymbiosis by autotrophic sulfur-oxidizing bacteria (thiotrophs) or methane-oxidizing bacteria (methanotrophs) occur in more than 200 marine invertebrate species that represent 5 or more phyla, depending on phylum classifications [23, 26]. It has been a common consensus that most host animals harbor a single thiotrophic or methanotrophic species.

In nature, sulfide exposed to oxygen is inorganically oxidized however, specialized bacteria can also mediate this oxidation which leads to intermediate oxidation state compounds (S^0, SO_3^{2-} and SO_4^{2-}), The amount of energy that results from this oxidation process is very important for the enzymatic CO_2-fixation cycle and resulting synthesis of carbon compounds.

Thiotrophic symbionts carry out chemolithoautotrophic organic production via the Calvin-Benson cycle, using ATP and NADPH generated from sulfur oxidation [40], in which energy for CO_2 fixation by the enzyme RuBisCO derives from sulfide oxidation [41]. Carbonic anhydrase is known to be involved in the transfer of CO_2 from the environment to the cell in many animal symbioses. This enzyme catalyzes the reversible hydration of CO_2 and was found to be regulated at the transcriptome level according to the state of symbiosis, in both plants and animals, but also in *B. azoricus* in response to temperature variations [13]. It had been proposed that hydrogen sulfide-oxidizing and oxygen-reducing chemoautotrophs potentially sustain the primary production in these unique ecosystems [42]. However, anoxic hydrothermal fluids contain several reduced compounds such as H_2, CH_4, and reduced metal ions in addition to H_2S [43]. Recent studies have demonstrated that these chemicals are all used as energy sources for chemoautotrophs, indicating the great diversity of chemoautotrophic energy metabolic processes in the ecosystems [44–46].

In contrast, methanotrophic symbionts assimilate carbon derived not from CO_2 but from methane, and oxidize part of methane to gain energy for metabolism [38]. Free living methane-oxidizing (MOX) bacteria start to oxidize methane to carbon dioxide through sequential reactions catalyzed by Methane Monooxygenase (MMO). MMO enzyme present two forms, the particular membrane bound form (pMMO) and a soluble cytoplasmic form (sMMO). MOX bacteria then use methanol dehydrogenase (MeDH) to oxide methanol to formaldehyde (HCNO), which can be assimilated to form intermediates of the central metabolic pathways.[40].

The distribution of symbiont types among various mussel hosts has been summarized elsewhere [34, 47, 48]. All work done to date suggests that the bathymodiolin symbionts are acquired from the environment [47] and other studies suggested that symbionts are reacquired from the surrounding seawater after induced loss [49].

Invertebrate Immune system

The immune system, within all animals, is based on two fundamental systems: recognition, to distinguish between self and non-self, and effector systems. Through evolution, species have developed sophisticated solutions to manage invading threats like infectious microbes, i.e. pathogens, and other non-self-molecules. Nowadays the

comparative study of invertebrate and vertebrate immunity represents an important part of basic science and a promising field of research. The character of the immune system of the species reflects its surrounding environment. The immune reactions in different animals are dependent on their way of living and how they have evolved together with their threats. Thus, their susceptibility to environmental stressors may differ.

The invertebrates are efficient against intruding microbes in spite of, in a number of cases, relying on immune systems that lack many of the components familiar from mammalian immunology. Understanding invertebrate immunity has been dominated by the idea that a relatively small number of germ-line derived pattern-recognition proteins (PRRs) bind to a few molecules, in particular the major constituents of cell walls or other surface structures of potential pathogens, and this initial recognition event in turn sets in motion a limited number of relatively fixed early responses such as: phagocytosis, encapsulation, coagulation, melanisation and the production of oxygen radicals and other short-lived toxic compounds, followed by more long-term effects such as the antimicrobial peptide (AMP) synthesis.

Immune recognition proteins are essential constituents of innate immunity, which recognize structural motifs commonly referred to as microbe-associated molecular patterns (MAMPs) [50, 51] represented by a diversity of sugars, proteins, lipid bearing molecules and nucleic acid motives, that initiate a cascade of extracellular and intracellular events leading to the activation of immune genes. Thus, the cell surface composition is of primary importance during cellular responses to environmental stimuli and, in this context, glycoconjugates are important for specific recognition between microorganisms and host cells, mediating the interaction of carbohydrate-binding proteins or lectin-like molecules [52]. Lectins are membrane-associated and soluble proteins with specific carbohydrate recognition domains which can promote opsonization, phagocytosis and the activation of the complement system through mutualistic interactions between host and microbiota [53, 54].

Invertebrate AMP defensins have been found in the hemolymph (plasma and hemocytes) and in certain epithelial cells of arthropods (e.g. insects) and mollusks [55] The immune system is mastered to distinguish beneficial microbes from pathogens and to coordinate appropriate immune responses [56]. As symbiotic microbes presumably share similar MAMP's with pathogens, how they immunologically elude host immune recognition, remains an open question and a challenge to lifelong microbiota prevalence inside vent mussel gill epithelia. Emerging evidence, however, point at evidence

showing certain microbes directly engage the immune system, in some cases, into active shaping of beneficial host immune responses [56]. Symbiosis is often achieved through microbial molecules that are sensed by PRRs. As the first eukaryotes evolved in a world inhabited by bacteria, PRRs appear to have facilitated a wide range of microbial interactions [56] including chemolithoautotrophic bacteria living in extreme environments.

The receptors (PRRs) are able to identify non-self by pathogen-associated molecular patterns (PAMPs). These molecules, for example lipopolysaccarides (LPS), peptidoglycans and β-1-3-glucans, stimulate the immune system unspecific ally since they are present on the surface of large groups of bacteria and other microorganisms [57, 58]. Especially peptidoglycans (PGNs) are excellent targets for recognition by the eukaryotic immune system, because PGN is an essential cell wall component of virtually all bacteria and it is not present in eukaryotic cells [59]. PGN is especially abundant in Gram-positive bacteria, in which it accounts for almost half the cell wall mass. In Gram-negative bacteria, a relatively thin PGN layer surrounds the cytoplasmic membrane under the LPS-containing outer membrane that is also a unique molecule to be recognized [60] .This general response to compounds such as peptidoglycans, lipopolysaccharides, β-1-3-glucans, which are present in many microorganisms, certainly constitutes the support of invertebrate immunity, but from recent research a more complex picture is starting to emerge. Separate bacterial strains or species, in the same host, may trigger an immune response that differs considerably, both quantitatively and in terms of which immune effectors are used [61, 62].

The innate immunity uses a set of sensors to recognize foreign patterns as mentioned earlier, which are found either intracellular, on cell surfaces or excreted in the hemolymph of the host for an instant reaction [58]. In general invertebrates have an open or semi-open circulatory system and aquatic invertebrates live in continuous contact with potential pathogens [63]. This makes them dependent on minute reaction of defense mechanisms. In the semi open circulatory systems of e.g. bivalves, the blood is called hemolymph and the blood cells hemocytes.

Invertebrates and molluscan immune responses are notorious for their ability to defend themselves against bacteria, fungi, and parasites [40, 64]. Their first lines of defense against infectious agents are physical and chemical barriers, such as the shell and exoskeleton, and deterrent chemical compounds. Once these barriers are breached, humoral and cellular reactions are set to function through hemolymph constituents and

hemocytes respectively [65]. Also, in bivalves, cellular and humoral components are required for defense responses allowing them to overcome pathogens that are naturally present in marine environments [66]. The main cellular immune response against pathogens in molluscan is phagocytosis [67].

Moreover, the generation of highly reactive oxygen intermediates (ROIs) and nitric oxide also plays an important defense role against pathogens. 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 [68]. Also, the hemolymph serum contains humoral defense factors such as lectins that are directly and indirectly involved in the killing of pathogens. They are important mediators of cellular reactions and exhibit opsonin properties, which facilitate the phagocytosis. The hemolymph also contains antibacterial factors and lysosomal components that ensure, along with hemocyte phagocytic and cytotoxic processes, the clearance of pathogenic bacteria [66]

Many invertebrates have the capacity to synthetize immune proteins with an enormous range of sequence variability. Together this seems to suggest that invertebrate immune reactions to pathogens may be as varied and complex as their vertebrate counterparts. The existence of the hypervariable proteins has led to speculation that they could constitute part of a system that would allow immune memory, or at least immune specificity, in invertebrates. Although there are some intriguing data suggesting the possibility of an immune memory or immune priming in invertebrates [69].

Notably, these mechanisms would require that the host be able to recognize its symbiont, differentiate the symbiont from other bacteria, and directly or indirectly influence the growth of the population. Despite possessing very similar PAMPS on their surfaces, different microbial strains are able to activate a variety of immune responses in invertebrates [64, 66, 70, 71]. The immune system has the ‘double-edged’ task of discriminating and eliminating pathogenic non-self while minimizing damage to self. Specific immune priming permits an induced response upon secondary exposure to the same threat [67]. While immunological memory was traditionally considered a hall-mark of the vertebrate adaptive immune system [72], there is growing evidence that invertebrate immune responses are also modulated upon repeated infections [73, 74].

Signaling pathways in invertebrate immune and stress response

A wide variety of signaling pathways regulate immune and stress response in invertebrates. The invertebrate immune response recognizes pathogenic motifs through Toll-like receptors and pattern recognition proteins (PRPs). The dogma that invertebrates do not possess an adaptive immune response, activated by multivariate recombination events, may actually be oversimplifying the invertebrate immune system. For example, oysters have an experimentally determined anticipatory response to infection, not a trait expected from a static immune response [75]. Also, shrimp injected with *Vibrio harveyi* were shown to have heightened levels of circulating PRPs, retained some recognition of bacterium and showed evidence of immune "priming" [76]. The fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* are extensively utilized model organisms for studies of such signaling pathways in invertebrates. Intriguingly, major signaling pathways in immune response in *Drosophila* and *C. elegans*, as represented by the Toll and IMD pathways. On the other hand, the mitogen-activated protein kinase (MAPK) pathways play not only in immune response but also in response to various abiotic stressors such as heat shock, ultraviolet (UV) irradiation, oxidative stress and osmotic shock [77].

Toll-like receptors (TLRs) are critical pattern recognition receptors (PRRs) that recognize MAMPs consisting of specific molecular "signatures" expressed by microbe cell membrane surfaces. Upon microbe sensing all TLR signaling pathways culminate in activation of the transcription factor nuclear factor-kappaB (NF-κB), which controls the expression of an array of inflammatory cytokine genes [78]. The expression and activation of transcription factor NF-κB are tightly regulated by the inhibitory protein IκB whose phosphorylation and subsequent degradation leads to NF-kB translocation to the nucleus [78]. TLR activation leads to the recruitment of several intracellular factors, including the adaptor protein MyD88, resulting in signal transduction events which ultimately lead to the degradation of Iκ-B allowing NF-κB translocation to the nucleus and subsequent activation of NF-κB-driven transcription of target immune genes [79, 80]. In-deed TLRs are membrane associated molecules which require conformational changes such as receptor heterodimerization upon ligand binding to promote signal transduction and subsequent MyD88 intracellular homodimerization [81]. Another possibility consists of the occurrence of a MyD88 independent TLR signaling pathway that could also be involved, for instance, in the induction of interferon or in the

mediation of NF-kB and MAPK activation and also contribute to inflammatory responses in deep-sea vent mussels [82, 83]. Generally, TLR do require limited transcription whereas intracellular adaptors are constantly being degraded and replenished hence their increased gene expression upon immune signal reactions. Additionally, the Toll signaling induces the production of pro-inflammatory cytokines such as interleukins, interferon, TNF, responsible for direct innate response and for triggering adaptive immune cells [84].

The TNF pathway presumably plays an important role in the first line of defense in marine bivalves along with the pathogen sensor Toll pathway, mediating inflammatory responses and the macrophage-like granulocytes reactions during cytokine-dependent host cellular defenses [85]. The role of TNF in invertebrates has been associated to pathogenic infections with *Vibrio* bacteria responsible for TNF inducible gene expression in Molluscs [86].

The involvement of Janus kinase/STAT pathway also is correlated with microbial infection [87]. The STAT gene is involved in mediating intracellular functions often associated with innate immune reactions, proliferation and differentiation of epidermal cells [88]. The epidermal growth factor (EGF) is activated by the signal transducer STAT-SH2.

SRCR immune recognition gene has been shown to function along in with the Toll-like receptor signaling pathway, an essential component in innate immunity [89]. Other extracellular signaling events upstream of Toll receptor may involve the participation of immune recognition molecules as the serine proteases [90] and serine protease inhibitors upon which *Vibrio diabolicus* may exert its modulating effect.

Whether or not vent mussels may actively control their bacterial symbiont population through apoptotic processes is still an open question. It is possible that different symbiont contents in gill tissues may induce different patterns of apoptosis [91]. Regulation of apoptosis is conferred by families of pro- and anti-apoptotic molecules. Fas ligand is a member of the TNF superfamily that plays an important role by inducing apoptosis, and homeostasis of immune responses and control microbial infection by inducing O^{2-}, H_2O_2 and other Reactive Oxygen Species (ROS) [92] that are generated during mitochondrial oxidative metabolism as well as in cellular response to bacterial invasion. Ferritin is an iron chelating protein which has been classified as a stress protein due to its similarity with proteins involved in detoxification processes triggered by various stresses and the iron is involved in respiratory burst activity, which

leads to the production of reactive oxygen species. Hence, ferritin can regulate iron concentration to destroy microbial agents and at the same time protect cells from oxidative stress [93].

The BCL2 family proteins (anti-apoptotic molecules) are key regulators of molecular mechanisms of programmed cell death [94]. BCL2 gene has been characterized in non-model invertebrates but recently new information regarding marine mollusks was described [95]. p43, a mitochondrial apoptotic gene, is considered as a marker of cellular stress in mussels and it is also secreted as a cytokine controlling angiogenesis, immune responses, tissue regeneration [96]. Also tied to the apoptotic signaling pathway, PGRP gene act as a signal-transducing innate immune receptor in the IMD pathway [97]. Previous studies have shown PGRP gene expression is strictly correlated with endosymbionts release [98].

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Involved in the cell cycle, apoptosis and in mitigating putative cell stress, HSP 70 gene has been widely accepted as a biomarker for the assessment of unhealthy environmental factors. In previous studies, a positive correlation between the levels of DNA strand breakage and HSP 70 expression, in response to decompression stress, was found by Pruski and Dixon [99]. These authors showed that HSP 70 revealed protective functions following environmental stresses at atmospheric pressure rather than high stress temperature variations.

Others immune transcription-factor genes including AP-1 and Jun has a pivotal role at the crossroad of the signaling network in invertebrates, including mussels [54] . Jun interacts with Fos to engage the transcription factor AP-1 heterodimer activity, regulated by a variety of extracellular stimuli, including growth factors, cytokines, cell–matrix interactions, and genotoxic stress, among others [100]. Once activated, the AP-1 signal transduction pathway regulates immune, inflammatory and stress responses.

The study of innate immunity in *B. azoricus* has been largely focused on the demonstration of the conservation of the immune system and its constituents [68, 101],

apparently homologous to that of insects and other bivalves, involving the participation of NF-κB transcription factors and antibacterial genes [14, 50, 102].

Based on the transcriptome and subsequent gene expression studies of *B.azoricus*, symbiont bacteria stimulate the expression of host-immune genes throughout acclimatization [14, 101] and transcriptional activity profiles revealed the possibility of using specific immune or stress-related genes in response of different environmental conditions and bacterial challenges [40].

Objectives and thesis outline

The thesis intends to contribute towards a better understand how deep-sea vent mussel *B. azoricus* can be used as a model organism to study the immune system during acclimatization in aquaria conditions and the symbiotic bacteria influence on expression of host immune genes. In an attempt to understand the deep-sea mussel *B. azoricus* adaptations to extreme environments and mechanisms through which it overcomes environmental microbial challenges, the present thesis aimed at investigate the innate defense reactions and the role of immune recognition molecules. Thus, advances in sequencing technologies provide the opportunity to study the entire genetic make-up of microbial communities in terms of their taxonomic and metabolic potential to analyze expressed genes under experimental conditions.

The thesis is composed of four research based chapters:

Chapter II The relevance of gene expression studies demonstrated that the swift changes affected the physiological homeostasis of *B. azoricus*. It has provided insights into the understanding of post-capture acclimatization and adaptation processes at atmospheric pressure. The results suggested that after 1 week acclimatization vent mussels are under the influence of what appears to be a concomitant host-immune and endosymbiont gene expression, possibly indicating a physiological alert point translated into higher levels of transcriptional activity. The objective of this chapter was recognized *B. azoricus* as a suitable model to study physiological plasticity and adaption processes to new environmental conditions at atmospheric pressure.

Chapter III A pulse challenge experiment using *V. diabolicus* as a *bone fide* immunostimulant agent was envisaged to demonstrate a general progressive incapacity of vent mussel *B. azoricus* to induce immune gene transcriptional activity over the course of acclimatization time. Expression analyses for both host and endosymbiont genes, after *V. diabolicus* challenges, showed a time-dependent mRNA transcriptional pattern evidenced during the first week acclimatization. The results herein presented support a putative modulating role of *V. diabolicus* on host immune system-endosymbionts interactions and on their gene expression reliance to an extent which, host-immune and endosymbiont genes are mutually dependent during the first weeks of acclimatization. Successful bacterial recognition prompted immune genes to increase their levels of transcriptional activity particularly for genes involved in the Toll-like receptor signaling and apoptosis-related pathways during first days of acclimatization in aquarium environments. *B. azoricus* was 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.

Chapter IV A metatranscriptomic study was developed to analyze *B. azoricus* gill-microbe associations during an acclimatization experiment in sea-water aquarium environment and at atmospheric pressure. rRNA sequencing analyses from 11 transcriptomic data sets, corresponding to distinct acclimatization time points, highlighted a variable distribution of taxonomical and functional assignments, consistent with changes in symbiont metabolic activity. The aim of this chapter was confirmed by Next-generation sequencing the results obtained in chapter II. The results confirmed the *B. azoricus* immunological response trend at 1 week of acclimatization concomitantly with the gradual loss of endosymbiont.

Chapter V To address the hypothesis that geographically distinct *B. azoricus* individuals may be experimentally traced back to their original hydrothermal vent site, the specific gene expression levels for both bacterial genes and host-immune related genes were compared between animals from the shallower Menez Gwen and the deeper Lucky Strike vent sites. A taxonomical structure of the vent mussel gill's microbiome was also evaluated to determine the bacterial community composition of Menez Gwen and Lucky Strike gill tissue samples. The same specimens of *B. azoricus* presented

different transcriptional activities most likely at the level of the gill's microbiome, which is presumably under direct influence of the hydrothermal vent environment from which mussels were originated.

The chapters of this thesis were based on the following manuscripts:

Chapter II

Barros, I., Divya, B., Martins, I., Vandeperre, F., Santos, R.S. & R. Bettencourt (2015). Post-capture immune gene expression studies in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* acclimatized to atmospheric pressure. Fish & Shellfish Immunology, 42, 159-170. **DOI: 10.1016/j.fsi.2014.10.018**

Chapter III

Barros, I., Mendes, S., Rosa, D., Santos, R.S. & R. Bettencourt. *Under review*. *Vibrio diabolicus* immunomodulatory effects on *Bathymodiolus azoricus* during long-term acclimatization at atmospheric pressure. PLOS ONE – Invertebrate physiology

Chapter IV

Barros, I., Froufe, H., Marnellos, G., Delaney, J., Clamp, M., Santos, R.S. & R. Bettencourt. *Under review.* A metatranscriptomics approach to address host-microbial interactions in the deep-sea hydrothermal vent *Bathymodiolus azoricus*. BMC Genomics – Research notes

Chapter V

Bettencourt, R., Rodrigues, M., Barros, I., Cerqueira, T., Freitas, C., Costa, V., Pinheiro, M., Egas, C., & R.S. Santos (2014). Site-related differences in gene expression and bacterial densities in the mussel *Bathymodiolus azoricus* from the Menez Gwen and Lucky Strike deep-sea hydrothermal vent sites. Fish & Shellfish Immunology, 2, 343-53. **DOI: 10.1016/j.fsi.2014.05.024.**