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Vibrio diabolicus Immunomodulatory Effects on *Bathymodiolus azoricus* During Long-term Acclimatization at Atmospheric Pressure

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Abstract

Mussels belonging to the *Bathymodiolus* genus are the most abundant species inhabiting hydrothermal vent sites from the Mid-Atlantic Ridge. The presence of endosymbiont bacteria in vent mussel *Bathymodiolus azoricus* gills is regarded as an evolutionary feature that confers deep-sea vent mussels the ability to adapt to chemosynthesis-based environment while potentially driving host-immune gene expression. In the present study, the functional immunological capabilities of *B. azoricus* gill tissues were addressed during an acclimatization experiment in aquaria environment at atmospheric pressure, where vent mussels were exposed to *Vibrio diabolicus* stimulations, for recurrent periods of 6h, alternated with longer sea water incubation intervals, over a 3 weeks' time course.

The effect of *V. diabolicus* exposures were analyzed, at distinct time points, where mRNA transcript levels from both host-immune and endosymbiont genes potentially revealed gene expression interdependence between host and endosymbionts. qPCR results targeting selected host-immune and endosymbiont genes presented significant gene expression differences between sea water control and *V. diabolicus* exposed mussels. The effect of time of acclimatization and endosymbiont prevalence, on host-immune gene expression, suggested that distinct time-dependent immune gene responses in *B. azoricus* are tied to endosymbiont bacteria. The results reflect a direct effect of *V. diabolicus* on endosymbiont gene expression profiles as demonstrated by up-regulation of endosymbiont genes such as *ALDH*, CA, *CBB*, *MeDH*, *MMO* and *SOXB* particularly at 2 and 3 weeks' acclimatization. *V. diabolicus* stimulations caused up and down regulation of gene expression seen at 72 h and 1 week and 48 h, 2 and 3 weeks' acclimatization, respectively. These gene expression profile studies supported *B. azoricus* ability to mobilize its immune system and to react against *Vibrio* challenges. A putative protective role of endosymbionts was considered in light of the progressive decline of immune gene transcriptional activity beyond 2 weeks of acclimatization coincident with predictable endosymbiont loss in land-based aquaria systems at atmospheric pressure. A yet uncharacterized protective role of endosymbionts is here evoked, for the first time, and may extend to counteracting apoptosis induction resulting from *Vibrio* infections as suggested by *BCL2* and *p43* down-regulation gene expression seen for all the acclimatization time-points.

Keywords: Deep-sea hydrothermal vent; Atmospheric pressure acclimatization; Molluscan immune system; *Vibrio diabolicus*; Immune and endosymbiont gene expression; Marine invertebrates

Abbreviations: MAR: Mid-Atlantic Ridge; *B. azoricus*: *Bathymodiolus azoricus*; RNA: Ribonucleic Acid; LSU: Large Subunit; SSU: Small Subunit; Rrna: Ribosomal RNA; mRNA: Messenger RNA; tRNA: Transfer RNA; RNA-Seq: RNA Sequencing; cDNA: Complementary DNA; OUT: Operational Taxonomic Unit; 0 h: 0 Hours; 12 h: 12 Hours; 24 h: 24 Hours; 36 h: 36 Hours; 48 h: 48 Hours; 72 h: 72 Hours; 1w: 1 Week; 2w: 2 Weeks; 3w: 3 Weeks; 4w: 4 Weeks, 5w: 5 Weeks; KEGG: Kyoto Encyclopedia of Genes and Genomes; PCR: Polymerase Chain Reaction; *MMO*: Methane Monooxygenase; ATP: Adenosine Triphosphate; NADH: Reduced Form of Nicotinamide Adenine Dinucleotide; Fe: Iron; HSP: Heat Shock Proteins

Introduction

Deep-sea hydrothermal vents constitute unique ecosystems supporting a variety of endemic invertebrates' species adapted to extreme physico-chemical environments. The deep-sea mussel *Bathymodiolus azoricus* 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 [1,2]. Owing to the development of specialized epithelial cells harboring chemoautotrophic bacteria including methanotrophic and thiotrophic symbiotic bacteria, *B. azoricus* endosymbionts primary production ensures thus part of the host's nutrition [3,4]. In spite of the toxic chemical nature of deep sea hydrothermal vent surroundings, the problem of microbial threat and need for immunity exist in animals dwelling around the vents, amid fluids rich in sulfur compound, methane and high concentrations of trace metals [5]. The innate immune system is devised to provide an array of protective mechanisms that are readily available upon recognition of invading microbes, representing thus a fundamental defense system in all invertebrates [6]. From their earliest stages

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of development, animals use sophisticated mechanisms to manage their microbial environment when exposed to threatening levels of infective microbes [7]. Immune receptors interacting with highly conserved set of molecular structural motifs present on the surface of microorganisms and absent from host cells, will engage defense mechanisms triggered by the discrimination between self from non-self [8,9]. The innate immune system has evolved a large repertoire of germline-encoded receptors that detect molecular patterns associated with infections. Such pattern recognition receptors (PRRs) detect three broadly defined categories of patterns best described as "microbial non-self", "induced-self" and "missing-self" [8]. PRRs, in turn, have evolved to recognize conserved and c*ommo*n microorganism-associated molecular patterns (MAMPs) displayed on the pathogen cell surface [8-10]. As the guardian of the animal's internal homeostasis, the immune system coordinates cellular and humoral responses to alterations in the molecular landscape, creating a robust equilibrium between the healthy host and its normal microbiota [7]. While the immune system permanently interacts with the environment, it must adapt physiologically in accordance with a changing environment. It would seem unlikely that the animals´ immune system, programed to protect from microorganisms, is concomitantly supporting a large and often diverse microbiota that contributes to various host functions, including immune function and nutrition, however these functions turn out to be vital for the sustenance and health of the animal host [11-13]. The function of the immune system is thus predicted to be shaped by distinct selection forces aimed to protect against pathogens while maintaining beneficial microorganisms inside their physiological systems [14,15]. When the first line of defense barriers, is breached, permitting pathogen intrusion, recognition of MAMPs will ensue, followed by the activation of hemocytes or macrophage-like cells and humoral components in the hemolymph with antimicrobial, cytotoxic properties or with opsonin-like properties, facilitating phagocytosis [5,9]. The hemolymph assumes thus an important role in the immune system, circulating around the body of bivalves reaching the gills and mantle where potential pathogens interact with innate immune factors [5]. As filter-feeding animals, bivalves are constantly being exposed to pathogenic bacteria and environmental pollutants and for this reason they have been used in eco-toxicological studies to evaluate the quality of aquatic environments [16]. Responses to bacterial infection have, thus far, been largely characterized in bivalves, at the levels of both functional responses and gene expression in the immune cells, the hemocytes [17]. Acclimatization studies previously reported by our group 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 [18,19]. Furthermore, these studies have allowed analyses using immune challenged mussels comparatively to acclimatized control mussels, maintained under aquarium conditions. In view of current studies with vent mussels in our land-based aquarium system, the presence of endosymbiont bacteria, has been under investigation as a driving factor under which host-immune genes may transcriptionally be modulated [9,16,18,20]. The present study aims at further extending our knowledge about the impact of aquarium acclimatization on *B. azoricus* immune responses and its capacity to react to *Vibrio diabolicus* exposures during incubations using same *Vibrio* loads, for recurrent short periods of time, followed by clean sea water incubations, allowing animals to depurate and subsequently being re-exposed to *V. diabolicus,* over a 3 weeks' time-series acclimatization experiment. This particular experimental setting holds potential for demonstrating an acclimatization time and endosymbiont effects, on host immune gene expression, corroborating our previous findings suggesting that different time-dependent immune gene responses in *B. azoricus* are tied to endosymbiont bacteria inside vent mussel gills [18]. *Vibrio diabolicus* is a Gram-negative bacillus, facultative anaerobic, heterotrophic, mesophilic isolated from the polychaeta annelid *Alvinella pompejana* collected from a deep-sea hydrothermal field in the East Pacific Rise [21] and was used in this study as a preferred environmental pathogen from hydrothermal vents. The *Vibrio* recurrent-exposure experiment was opted over continuous *Vibrio* incubations, to allow comparison studies between both host and bacterial transcriptional activities whose gene expression levels and reliance may potentially reveal specific relationships between the timing of *Vibrio* exposures and the manifestation of acute phase-like responses, and a putative modulating role of *V. diabolicus* on host immune system- endosymbionts interactions. Following *V. diabolicus* stimulations, up and down regulation of gene expression was seen at 72 h and 1 week and 48 h, 2 and 3 weeks acclimatization, respectively. These gene expression profile studies supported *B. azoricus* ability to mobilize its immune system and to react against *Vibrio* challenges while endosymbiont gene expression also suggested that symbiotic prevalence and metabolism were actively higher during the first week of acclimatization. Endosymbiont gene expression responses to *V. diabolicus* recurrent infection is evocative of a yet uncharacterized protection role to the host, herein reported for the first time. *Vibrio* challenges were carried out while comparing both host and bacterial transcriptional activities whose expression levels are presumably revealing specific synergetic responses bringing thus, supporting evidence showing a putative modulating role of *V. diabolicus* on host immune system-endosymbionts interactions and on their gene expression reliance. This particular experimental setting enabled us to demonstrate an acclimatization time and endosymbiont effects on host immune gene expression. The present study corroborates our previous findings suggesting that different acute phase-like responses in *B. azoricus* and in the presence of *Vibrio* bacteria may be attributed to the prevalence of endosymbiont bacteria inside vent mussel gills [16].

Material and Methods

Ethics statement

No animal research was involved (vertebrate animals, embryos or tissues). Collection of wild specimens of *Bathymodiolus azoricus* was in compliance with the regulatory directive issued by the Regional Government of the Azores, through the Secretariat for Sea, Science and Technology, the Regional Science and Technology Directorate, Regional legislative decree n.º 9/2012/A, 20 of March, 2012. *Bathymodiolus azoricus* is not listed as an endangered or protected species.

Specimen collection

B. azoricus mussels were collected during the BIOBAZ cruise (July 2013) from the Mid-Atlantic Ridge (MAR) hydrothermal vent field Menez Gwen (850 depth, 37º50,8 -37º51,6N; 31º30-31º31,8W), with the French R/V Pourquoi pas? using the Remote Operated Vehicle, Victor 6000.

Acclimatization of *Bathymodiolus azoricus* challenge with *Vibrio diabolicus*

Mussels were acclimatized in the LabHorta laboratory system using aquaria filled with 20L of fresh cooled sea water (7°C to 8°C), directly pumped from a local coastal area, continuously treated with UV light and filtered through an external power canister filter (Eheim 600),

circulating through the aquaria system and without adding metals, gas or food sources. The air-oxygen supply provided oxygen saturation around 10% to 50% under atmospheric pressure. Experiments were performed in the dark except during water monitoring and sampling. From the 20 L initial batch of acclimatized mussels, 12 animals were retrieved for a time series experiment consisting of 0 h (T0), 48 h (48 h), 72 h (72 h), 1 week (1 w) and 3 weeks (3 w) acclimatization time points. For each time point mussels were transferred to 2 L sea water filtered beakers, with aeration, further divided into two experimental groups of 6 animals containing beakers, during 6 hours, corresponding respectively to, sea water control mussels, and sea water *Vibrio diabolicus* suspensions defined as a time-limited exposure or a 6 hours instant infectious exposure. 90 mL of overnight *Vibrio diabolicus* (HE800 strain, suspension obtained from Dr Valerie Cueff IFREMER, France) cultures grown in LB marine broth (Merck) were used as suspensions with an OD_{600}=1nm. 6 challenged and unchallenged mussels were sampled at 0 h (T0), 48 h (48 h), 72 h (72 h), 1 week (1 w) and 3 weeks (3 w) of acclimatization, after which time points, the gills were separated and frozen at 80°C for subsequent RNA extraction and gene expression analyses. The 0 h time-point sampling was considered as the beginning of the acclimatization experiment, when animals were brought onboard and immediately preserved.

Total RNA extraction and cDNA synthesis

Total RNA extractions were performed on 6 animal's gill tissues from non-*Vibrio* exposed animals (sea water control) and from 6 animal's gill tissues from *Vibrio* exposed animals, for each time pointed tested, using the TriReagent® (Ambion) and further purified with the RiboPure® Kit (Ambion) following the manufacturer's specifications and re-suspended in nuclease-free, DEPC-treated water.

Total RNA quality preparations and concentrations were assessed by the A260/280 and A260/230 spectrophotometric ratios using the NanoVue spectrophotometer (GE, Healthcare Life Sciences) and by visual inspection using denaturing agarose gels. RNA extractions were pooled to generate one RNA extract for each challenge and unchallenged (sea water control, sw) mussels for different time-points tested. The total RNA from gill samples was reverse-transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. 5 µg of total RNA per sample were used in all cDNA synthesis. The cDNA concentration was measured using the NanoVue spectrophotometer (A260/280 and A260/230 spectrophotometric ratios). The cDNA samples were prepared from a pool of 6 RNA gill purifications, for each time-point tested of challenged and unchallenged (control) mussels.

Gene expression analyses by Real-time PCR (qPCR)

Relative changes in gene expression were calculated according the $2^{-(\Delta\Delta Ct)}$ method normalized with 28S rRNA gene (housekeeping gene). Each value was calculated in reference to the mean of ΔCt of all conditions (relative expression = 1).

Gene expression analyses were conducted by means of 3 replicates qPCR using the 0 hours, 48 hours, 72 hours, 1 week, 2 weeks and 3 weeks cDNA samples from both challenged and unchallenged mussel.

The usefulness of 28S rRNA as a housekeeping gene and internal standard has been recently reported by our group in differential gene expression studies using *B. azoricus* as a model study [18,22]. In the present work 28S rRNA gene was selected as the most stable normalizing gene whose transcription is the least affected by experimental factors.

Based on sequences retrieved from the DeepSeaVent database (http://transcriptomics.biocant.pt/deepSeaVent/) primers were designed and previously tested, in RT-PCR experiments, to confirm the physical counterpart of *B. azoricus* genes putatively associated with immunity reactions and loss of symbionts. Primers were subsequently used to study gene expression profiles in gill samples. The primer pair efficiencies were analyzed in consecutive cDNA dilutions through the regression line of the Cycle thresholds (Ct) versus the relative concentration of cDNA using Microsoft Office Excel software [23]. The immune response triggered after *V. diabolicus* infection was evaluated through quantitative PCR (qPCR) gene expression analysis. Specific primers were used to perform a qPCR for immune genes with cDNAs synthesized from RNAs. The forty six genes selected for qPCR were chosen based on their relevance in the immune system, namely Actin (*ACT*), Aggrecan (*ACAN*), Allograft inflammatory factor (*AIF*), B-cell CLL/lymphoma 2 (*BCL2*), Calmodulin (*CALM*), Carcinolectin (*CL*), Caspase (*CASP*), Catalase (*CAT*), c-type lectin (*CLEC*), Cyclooxygenase (*COX*), Defensin (*DEF*), Epidermal growth factor (*EGF*), Fas Ligand (*FasL*), Ferritin (*FER*), Galectin (*GAL*), Glutathione peroxidase (*GPX*), Glycogen phosphorylase (GTPase), Heat Shock Protein 70 (*HSP70*), Immune Lectin Receptor (*ILR*), Interleukin-1 receptor-associated kinase (*IRAK*), Inhibitor of kappa B (*IκB*), JUN-Like (*JUN*), LPS binding/bactericidal-permeability-increasing protein (LPS-BPI), LPS induced TNF-alpha Factor (*LITAF*), Lysozyme (*LYZ*), Metalloproteinase Matrix (*MMP*), Metallothionein (*MT*), Mitogen activated protein kinase 7 (*MAPK-7*), Myeloid differentiation primary response (88) (*MyD88*), Nuclear factor kappa B (*NF-κB*), *p43*, *p53*, Plasminogen (*PLG*), Peptidoglycan recognition protein (*PGRP*), Rhamnose binding lectin (*RBL*), Scavenger Receptor Cysteine-Rich (SRCR), Serine Protease Inhibitor (*SERPIN*), Sialic acid-binding lectin (*SABL*), Signal Transducers and Activators of Transcription - Src-homology domain 2 (*STAT-SH2*), Transcription activator-like (*TAL*), Tissue inhibitor metalloproteinase (*TIMP*), TNF Receptor Associated Factor 6 (*TRAF6*), Toll like receptor-2 (*TLR2*), Tumor Necrosis factor receptor 1 (*TNFR1*), Tyrosine kinase-R (*TRK*) and Vascular endothelial growth factor (*VEGF*) represented in Table 1. 28S ribosomal RNA gene was chosen as the housekeeping gene. Target immune genes were selected according to previously defined categories of functional genes *i. e.* Recognition, Signaling and Transcription and Effector [10,18] (Table 1).

The 16S ribosomal gene is highly conserved between different species of bacteria [24]. In this study were used Sulfide oxidizer symbiont 16S and Methanotrophic Symbiont 16S as housekeeping genes [24] (Table 2) for bacterial gene expression study which is constitutively expressed and not affected by the bacteria challenge. The six bacterial genes selected for qPCR were chosen based on their relevance in the symbiotic interactions, namely Aldehyde dehydrogenase (*ALDH*), Rubisco Activation (*CBB*), Methanol dehydrogenase (*MDH*), Sulphate thiol ester (*SOXB*), Methane monooxygenase (*MMO*) and Carbonic anhydrase (*CA*). Quantitative PCR was performed to assess and quantify the expression of genes selected on the basis of results obtained from cDNA library screenings and whole transcriptome sequencing of *B. azoricus* for detecting systematically putative genes involved in innate immunity [10]. The same amount of cDNA concentration was used in Real-Time PCR reactions as determined by previous analysis of 28S rRNA as loading control and performed on the CFX96™ Real Time PCR System (Bio-Rad). Amplifications were carried out using 1 µl of specific primers (10 µM) (forward and reverse) and mixed to 10 µl of SYBR green (Fermentas) and nuclease-free water in a final volume of 20 µl per reaction. PCR cycling conditions were 95°C for 15 min, 94°C for 15 s and 60°C for 1 m followed by 40 cycles of 65°C for 5 min and 95°C for 5 min. Three technical replicates were performed for

Gene	Label	DeepSeaVent Accession #	Function	Forward primer (5´-3´)	Reverse primer (5´-3´)
Actin	ACT	mussel_c325	Cell motility; Structure; Integrity	ATCGTGTAGATGTGTGATGCCAG	GGAGATGATGCCCCAAGAGCCG
Aggrecan (c-type lectin domain)	ACAN	mussel_c11731	Extracellular matrix component	GCACCCGAACAGAGTTGG	CCATCGCCAGTCACCATTA
Allograft inflammatory factor	AIF	mussel_c2558	Wound repair; Cellular response	TTTTCACTTGGGGAAAGGCGC	CCAAAAGCTTTGCCACCCTGG
B-cell CLL/lymphoma 2	BCL2	mussel_c90	Apoptotic signaling pathway	CTGGACGTAGTTTGAAGCCTAA	TCAACACCTGAAATCGGTCA
Calmodulin	CALM	mussel_c407	Ca^{2+} transport, binding	GGGGAGAAAAATGAAAGACACTGC	CGGCGCGCATTGAAAATGACTGG
Carcinolectin	CL	mussel_c2366	MAMPs recognition (lectin family)	AACGGTCATGCCTTTACCAC	TGAATACCACCAGCCTCCTT
Caspase 3	CASP	mussel_rep_ c66691	Apoptotic signaling pathway	GAGCGCCTACCATTTTCAGA	CGACTGGTTATCTGCCCAAT
Catalase I	CAT	mussel_c17081	Antioxidant response	CATGTTAGCAGGCACTCCAGA	TACGGCCGGGGGAAAAAGGT
c-type lectin	CLEC	mussel_rep_ c66293	MAMPs recognition (lectin family)	ATTCACCCGAACAGAGTTGG	CATATAGCCATCGCCAGTCA
Cyclooxygenase (prostaglandin-endoperoxide synthase)	COX	mussel_c61123	Inflammatory response	CCAGTGGGGAGGACATGGGG	CCTGGCAGAGCGCCAAAGAA
Big defensin	DEF	mussel_c8746	Antimicrobial peptide	AACGCAGAGTGGGCCATACG	TCACTGGTGCGAACCGTTTGT
Epidermal growth factor EGF-like-domain, multiple 6	EGF	mussel_c9134	Cellular proliferation; Differentiation; Survival	GTGGTTGCCTAGCTGGTTTGC	TCGGAGTTGCTCCATGTCGTC
Fas Ligand	FasL	mussel_c24457	Apoptotic signaling pathway	GATGCCAAGGAAAGAGATGC	ACATATTTTGCCGCCATTGT
Ferritin	FER	mussel_c2719	Stress; Immune response	TTCGATAGGGATGACGTAGC	TTTCATCAGCTTTTCAGCATGT
Galectin	GAL	mussel_c14771	MAMPs recognition (lectin family)	GCTGGGCTGGATACTGATAATG	ACAAGGTCGCTGTAAATGGAAG
Glutathione peroxidase	GPX	mussel_rep_ c23951	Antioxidant response	TTAACGGCGTCGCTTGG	TGGCTTCTCTCTGAGGAACAACTG
Guanosine triphosphates hydrolase (similar to immunity-related GTPase family)	GTPase	mussel_c1036	Signal transduction; Cellular differentiation	GGCACATCCATCAACTTTCA	CCAGGGTTCCAAAATCAAAT
Heat Shock Protein 70	HSP70	mussel_rep_ c66502	Stress response (chaperon protein)	TTGAAGAAATGTGTGGTGACTTG	CCCTACCAGAACGACCTCAT
Immune related-Lectin Receptor	ILR	mussel_rep_ c70917	Pattern Recognition Receptor (PRR)	TGGACACTGCTACCATTATGG	CGATTGGTCATAGCTCCAACG
Interleukin-1 receptor-associated kinase	IRAK	mussel_c2397	Signal transduction mediator (immune response)	GAGTGGCCATTACGGCCGGG	GCTTGCATCGATCTGGCGGGT
Inhibitor of kappa B	**IkB**	mussel_c244	Transcription factor modulator	TGAGGCAGCACTGAACGGAC	ACGCAGAGTGTGCCAACGC
JUN-Like	JUN	mussel_c38	Transcription factor	TGTGACGGGATTGACTTTGA	CATCGGCAACAACAACACTC
LPS binding/ bactericidal-permeability-increasing protein	LBP-BPI	mussel_c27903	MAMPs recognition	CACATTTAACACCTTGACATACCAG	AACAAAACATTGACTTTTCCTTCAG
LPS induced TNF-alpha Factor	LITAF	mussel_c1273	Mediator of the LPS-induced inflammatory response	ATGAGAGATACCCCCGTGAA	CACAAAACAACACCCAGCAT
Lysozyme	LYZ	mussel_c15166	Antimicrobial peptide (AMP)	GCTGTATCTGTCAGGTTGAATCGC	TGGTCCTCCGTTATGGATCCTGGC
Matrix Metalloproteinase	MMP	mussel_c33576	Cellular functions; Apoptosis; Host defense	CTTGCCCATGCCTTCTTCCCA	GGTGGTCTTGTTGGTGGTGGT
Metallothionein IB	MT-IB	mussel_c72489	Metal-binding; Stress-immune response	TTTCCACAACCACTTCCACA	TGCCCAAAGAACAAAGGATG
Mitogen activated protein kinase 7	MAPK-7	mussel_rep_ c28417	Immune response signaling	TTACGGCTTGCTTTAGGAAAC	ACTCGATGGTACAGAGCCCCA
Myeloid differentiation primary response gene 88	MyD88	mussel_c1511	Immune response signaling	CTGCCACACCCAACACAGCA	TGCAGACTGAGGTTCTCGCAG
Nuclear factor kappa B	NF-kB	mussel_c69932	Transcription factor; Immune and inflammatory responses, Apoptosis	GGCTGTGTTTGGTTGGACAT	AGTGGCGTATCACCGTTACA
p43	p43	mussel_c25175	Wound repair; Cellular proliferation; Apoptotic signaling pathway	AGTGGAACACCAGATGCCATG	TCACATGCATTGTCTGTCGGC
p53	p53	Present work	Cellular proliferation regulator; Apoptotic signaling pathway	CCAGCTCCAACACATTTATGTCG	CTTCTACTGCCCGTCTACCTAGC
Plasminogen	PLG	mussel c12003	Inflammatory response mediator	CCATTGTAGTCGCTGCCTGTG	GGGAACGTCCACACCGCATAA
Peptidoglycan recognition protein	PGRP	mussel_rep_ c24000	MAMPs recognition	TGTTGGTGAAGATGGCAAAA	CCGTGAGTGGTGTGTGTGT

Rhamnose binding lectin	RBL	mussel_c4578	MAMPs recognition (lectin family)	ACAATGGGTTGATTTGTTTGC	CCGGGGGCCTGAAAGTTGGT
Scavenger receptor cysteine-rich domain	SRCR	mussel_rep_ c53412	MAMPs recognition	ACACCCCAACCATCTGATTT	ATCATTTGGAAACGGGACAG
Serine Proteinase Inhibitor	SERPIN	mussel_c9943	Immune and inflammatory responses; Proteolytic cascades	TTAGGGTTGTGCGTGAAGTG	TGGGCAAGTCAGTTTGATGA
Sialic Acid Binding Lectin	SABL	mussel_rep_ c71109	MAMPs recognition (lectin family)	GGTTGTGGSAGTTGTAAAGCGT	GGGCGGTCCAAGCAAGTTCAA
Signal Transducers and Activators of Transcription - Src-Homology Domain 2	STAT-SH2	mussel_c3642	Inflammatory, Immune response transcription factor	AGCTGAAACAGGGCGTGGTC	GACAAATCCAGCCACATGCCC
Transcription activator-like	TAL	mussel_c37245	Hemocyte proliferation	GTTGACGCCATCGCTCTCGG	GCCATTACGGCCGGGGTTTAG
Tissue inhibitor metalloproteinase	TIMP	mussel_rep_ c23916	Wound repair; Extracellular matrix regulator	CAGGATCAGACGCTGCTTGTG	TCATTATTCCACCGGGGCTCC
TNF Receptor Associated Factor 6	TRAF 6	mussel_c2678	NF-κB signaling mediator; inflammatory; Apoptotic signaling	TTGCACATTCGACCTTCAAA	AAGACGAGAGTTTGGCGAGA
Toll like receptor-2	TLR2	mussel_c2881	PRR; Innate immune signaling pathway	TCTCCAGTCAGTGGTGCAAG	CAGGAGGACTCGGATGACAC
TNF Receptor Associate Factor 1	TNFR1	mussel_c684	Inflammatory signaling	ACTGTCCAAGCTGCATCAGCA	CATTACGGCCGGGGACTCCA
Tyrosine kinase-R	TRK	mussel_c31000	Proliferation; differentiation; Phagocytosis mediator	GATTGTGGCTCTTAAGTAGTCC	CTGTCCAGTTGTCTCATATCACTC
Vascular endothelial growth factor	VEGF	mussel_c29359	Growth factor; Cellular proliferation	AGCTGCATGGAGACTTGAACC	AGGTGGGGTGGTACTTGCTCC
Housekeeping gene – 28S	HKG-28S			AAGCGAGAAAAGAAACTAAC	TTTACCTCTAAGCGGTTTCAC

Table 1: Details of *B. azoricus* genes and primers used in the quantitative real-time PCR expression analysis.

Gene	Label	DeepSeaVent Acession #	Forward primer (5´-3´)	Reverse primer (5´-3´)
Aldehyde dehydrogenase	ALDH	mussel_c49271	CGTGGTGGGTCTGTTCGATG	CATTACGGCCGGGGCACCAG
Rubisco Activation Cbb	Cbb	mussel_c53956	TTCTAGCGCGGCGTGCGATT	AGGCGCGCCAAGATACCACA
Methanol dehydrogenase	MeDH	mussel_c40905	ACGGCCGGGGCTTAACAGGT	CCAGCCCCAAGTTGTACCGCC
Sulphate thiol ester SOXB	SOXB	mussel_c24142	TGGGCGGGCATCGTATTGCAG	CCGACCACACGCGATGCCAT
Methane monooxygenase	MMO	mussel_c589	GAGTGGATTAACAGATATTTGAACTTC	CATACCACCAACAACAGCTGT
Carbonic anhydrase	CA	mussel_c26839	ATTGGCGAGCTGGCGGCATT	GAGTGGCCATTACGGG
Housekeeping gene Methanotrophic Symbiont 16S	MOX 16S	[23]	GTGCCAGCMGCCGCGGTAA	GCTCCGCCACTAAGCCTATAAATAGAC
Housekeeping gene Sulfide oxidizer symbiont 16S	SOX 16S	[23]	GAGTAACGCGTAGGAATCTGC	CGAAGGTCCTCCACTTTACTCCATAGA

Table 2: Details of *B. azoricus* bacterial genes and primers used in the quantitative real-time PCR relative expression analysis.

each gene tested in real time PCR reactions. Melt curves profiles were analysed for each gene tested. The 28S rRNA and 16S rRNA were used to normalize the expression of immune genes and to assess the expression of bacterial genes and the relative abundance of endosymbiotic bacteria within gill tissues, respectively.

The primer pair efficiencies were analyzed in consecutive cDNA dilutions through the regression line of the Cycle thresholds (Ct) versus the relative concentration of cDNA.

Data analyses are based on the ΔΔCt method with normalization of the raw data to the housekeeping gene expression values. Three technical replicates were carried out per experimental condition. Fold change units were calculated by dividing the normalized expression values in *Vibrio* challenges by the normalized expression values from unchallenged animals. Standardization of expression ratios took into consideration a fold change unit equal to 1 in control samples. Fold changes above 1 were considered as up-regulation of genes (higher mRNA abundance than in the control). Conversely, fold changes below 1 were considered as down-regulation of genes.

Statistical analyses

All analyses were performed (n=3) and are presented as means ± standard deviation (SD). Student t-tests were performed in order to test differences for post challenge ratios relatively to control (i.e., sea water vs. *V. diabolicus*) through time course (48 h, 72 h, 1 week, 2 weeks and 3 weeks). The analyses were performed for host and bacterial gene expression. Also, the same procedures were realized per gene. Prior to the analyses, in all cases the variables conformed to the assumptions of parametric statistics of normality and homogeneity of variances was investigated by normal probability plots and Levene's test respectively [25,26]. For all statistical tests, the significance level was set at p-value ≤ 0.05. All calculations were performed with IBM SPSS Statistics 22. A principal component analysis (PCA) was performed to evaluate distribution patterns based on the immune genes (host and bacterial) and the time-point sampling. PCA is used to reduce the dimensionality of a data set, while retaining as much of the original information (variability) as possible. The most important principal components (PC1 and PC2) are calculated by linear combination of original

variables and they adequately represent the original data. The positions of original variables in the principal component plot relevantly represent their interrelations. Thus, if the variables are in the opposite position, then the given variables are weakly associated. However, if the variables are very closely located, their interrelation is strong and positive. Hence, graphical representation of the objects investigated in the plot is very useful in detecting their possible association. Moreover, in the principal component biplot, simultaneously representing the objects and the variables, it is possible to detect those variables which are associated with the group formed from closely located objects and in this way the mutual relationships among the objects and variables can be discovered. Canoco for Windows 4.5 [27] software was used to perform graphs.

Fluorescence *in situ*-hybridization (FISH)

The presence of *V. diabolicus* bacteria in *B. azoricus* gill tissue was determined according to Duperron et al. [1] with slight modifications [28]. Gill tissues were fixed in 5% buffered formalin and processed for paraffin embedding according to standard protocol. Transverse sections (7 µm thick) were subjected to deparaffinization and re-hydration through a decreasing ethanol series (100%, 95% and 70%) and rinsed with distilled water. The sections were incubated with Proteinase K (RT for 2 minutes) followed by 3X PBS wash (RT for 5 minutes). Prior to hybridization and to increase the probe signal the tissue sections were incubated with Triethanolamine (0.1 M-ph8) for 5 min at RT. Tissues were pre-treated for 15 min with hybridization solution at 48°C (0.9 M NaCl, 0.02 M Tris/HCl ph8.0, 0.01% SDS, 30% formamide) and then subjected to hybridization solution containing *V. diabolicus* hemolysin gene probe for 5 hours at 48°C followed by a wash solution (0.1 M NaCl, 0.02 M Tris/HCl, pH8.0, 0.001% SDS, 5 mM EDTA) for 15 min, individualized by a circle of PAP pen. The *V. diabolicus* hemolysin Cy3 Red fluorescent probe (Integrated DNA Technologies®) were used to target respectively *V. diabolicus* bacteria in FISH experiments. Control experiments were carried out with oligos corresponding to non-complementary sequences. Gill filaments were visualized under fluorescent light and differential interference contrast (DIC) microscopy using a Leica DM6000 digital microscope (Leica Microsystems CMS GmbH, Germany) with the 63X objective. The Rhodamine Red fluorochromes were visualized with appropriate red (Abs560/Em580) channels according to their spectrum of absorbance (Abs) and emission (Em).

Results

Experiments with *Vibrio diabolicus*

The *Vibrio* challenge was carried out with *V. diabolicus* [21] and results were analyzed taking into consideration how the presence of *V. diabolicus* affects gene expression levels. The qPCR-based expression patterns for these genes showed different profiles (Figure 1) as demonstrated by relevant expression differences measured over the course of *Vibrio* exposure and evidencing a time-dependent gene expression pattern in *B. azoricus* subjected to aquaria acclimatization and subsequent gradual loss of endosymbiont bacteria.

Host gene expression

Standardization of gene expression results and description of the number of regulated genes in fold change groups are summarized in Figure 1. As represented, the highest levels of up regulated genes were observed at 48 h in *B. azoricus* acclimatized to sea water (44 up-regulated genes of 46 genes studied). *Vibrio* temporary infection, for the same time-point, resulted in a lesser immune gene expression level yet above expression levels observed for the remaining time course acclimatization experiment (34 up-regulated genes of 46 genes studied). *V. diabolicus* challenge also induced the transcriptional activity of immune genes at 72 h and 1 week compared with sea water conditions. The level of up- and down- regulated immune genes observed at 0 h, 48 h, 72 h, 1w, 2w and 3w in sea water and post-*V.*

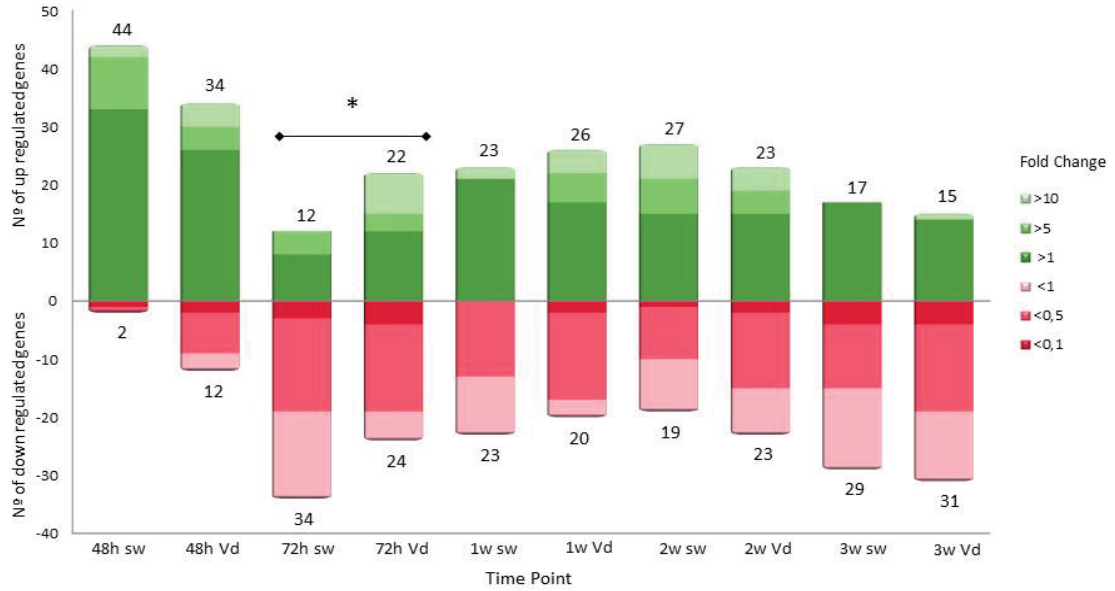

Figure 1: Immune genes expression through acclimatization time. Different time-points are represented: 48 hours in sea water (48 h sw); 48 hours post-*Vibrio* challenge (48 h Vd); 72 hours in sea water (72 h sw); 72 hours post-*Vibrio* challenge (72 h Vd); 1 week in sea water (1w sw); 1 week post-*Vibrio* challenge (1w Vd); 2 weeks in sea water (2w sw); 2 weeks post-*Vibrio* challenge (2w Vd); 3 weeks in sea water (3w sw); 3 weeks post-*Vibrio* challenge (3w Vd). The gene expression is subdivided according to intensity (fold change) and up or down-regulation orientation. The cardinal numbers represent the nº of up and down regulated genes. Symbol (*) represents statistical significant differences (Student t-tests, p-value<0.05).

diabolicus challenge time-points was analyzed by qPCR. Statistically significant differences were detected when compared seawater and *V. diabolicus* challenge (Student t-test, $t_{(274)}$=-3.248, p-value=0.001 at 72 h (Figure 1). The immune genes were grouped by functional categories, classified as recognition, signaling, transcription and effector. Thus, all functional groups were analyzed and all time-point tested by comparing the control condition (sea water) with post-*V. diabolicus* challenge as represented in Figure 2. Student t-test was used for all genes selected for qPCR, therefore were considered the genes (Figure 2) that presented statistically significant differences. For each functional group, statistically significant differences for the majority of genes were detected (Student t-tests, p-value<0.05; Figure 2). In order to summarize the statistically significant results presented in Figure 2, a color code expression map was used, as shown in Figure 3 to highlight that statistical differences between seawater and *Vibrio* challenge conditions were most significant at 48 h and 2 weeks' time-points, and characterized by a down-regulation gene expression trend. In contrast, up-regulated gene expression levels were significantly statistically different at 72 h (Figure 3). The expression of 11 recognition immune genes was analyzed (Figure 2A). Statistical differences between seawater condition and *Vibrio* challenge were shown for the expression of 8 genes at 2 weeks, 8 genes at 48 h, 6 genes at 72 h, 5 genes at 1 week and 3 genes at 3 weeks (Figures 2 and 3) As above, the expression of 11 signaling immune genes was analyzed (Figure 2B). Statistical differences between seawater condition and *Vibrio* challenge were shown for the expression of 10 genes at 2 weeks, 9 genes at 48 h, 6 genes at 72 h and 1 week and 6 genes at 3 weeks (Figures 2 and 3). As presented in color code expression map (Figure 3), 48 h time-point has shown a down regulation gene expression with statistical differences between seawater and *Vibrio* challenge condition. The time-point of 72 h revealed the highest transcription levels for the recognition immune genes tested. The expression of 8 transcription immune genes was analyzed (Figure 2C). Statistical differences between seawater condition and *Vibrio* challenge were shown for the expression of 5 genes at 48 h, 4 genes at 2 weeks and 3 genes at 72 h, 1 week and 3 weeks (Figures 2 and 3). As shown in color code expression map (Figure 3) 48 h was the strongest time-point represented by a down regulation gene expression with statistical differences between seawater and *Vibrio* challenge conditions and 1 week revealed the highest transcription levels for the transcription immune genes tested. *IκB* gene showed down regulation gene expression at 48 h and up regulation gene expression at 1 and 2 weeks (Figure 2). For JUN gene the time-points that exhibited statistical differences were at 72 h, 2 and 3 weeks and were influenced by the up regulation gene expression (Figure 2). The expression of 8 effector genes was analyzed (Figure 2D). Statistical differences between seawater condition and *Vibrio* challenge were shown for the expression

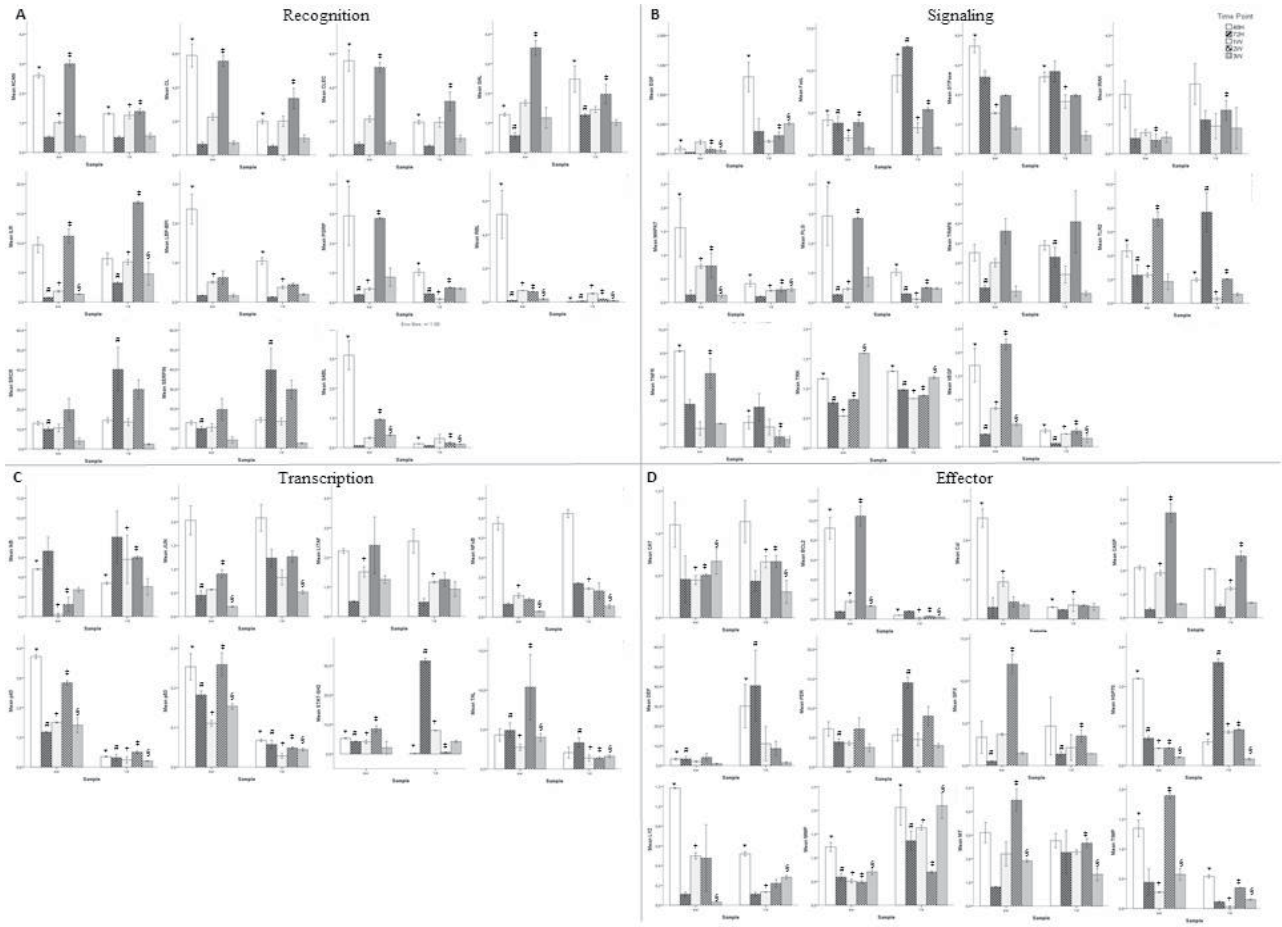

Figure 2: Immune gene expression analysis following 48 h, 72 h, 1 week, 2 weeks and 3 weeks in sea water and *V. diabolicus* challenge. Time-points were coded as in Figure 1 and host genes labeled as in Table 1. Functional groups were organized as: (A) Recognition genes; (B) Signaling genes; (C) Transcription genes; (D) Effectors genes. Significant differences between seawater control condition (sw) and *V. diabolicus* challenge (p<0.05, ANOVA, Dunnett's test) at *48 hours; # 72 hours; † 1week; ‡ 2 weeks and § 3 weeks, are shown. Error Bars : +/- 1 SD.

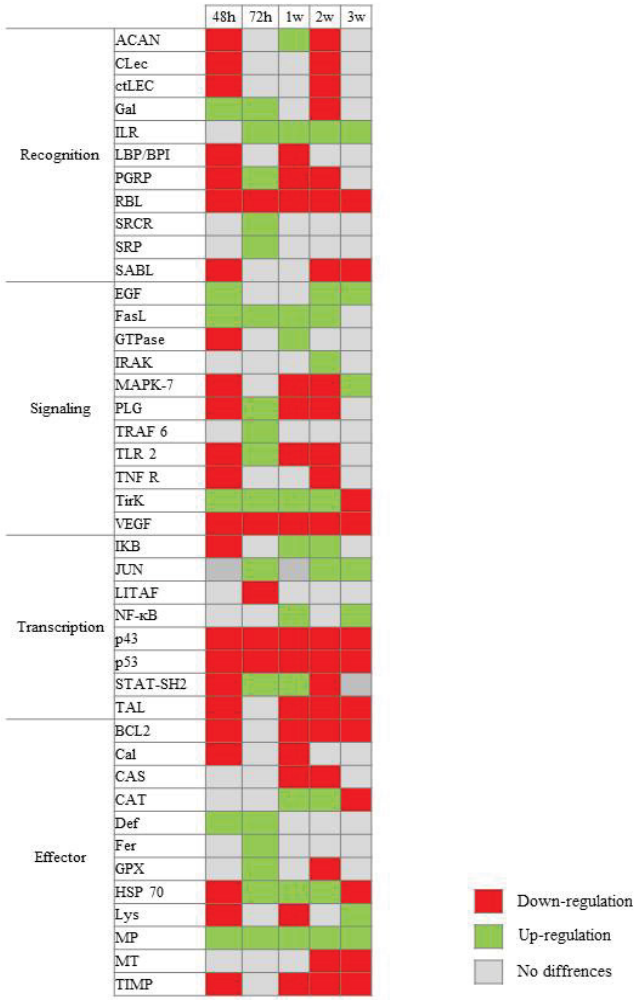

Figure 3: Statistical differences between seawater and *Vibrio* challenge condition color code expression map at 48 h, 72 h, 1 week, 2 weeks and 3 weeks. Time-points were coded as in Figure 1 and host genes labeled as in Table 1. Red squares represent down-regulation gene expressions; green squares represent up-regulation gene expressions and grey squares represent unaltered gene expressions.

of 6 genes at 1 week and 5 genes at 48 h, 2 weeks and 3 weeks (Figures 2 and 3). As shown in color code expression map (Figure 3) 1 week was the time-point that presented a down regulation gene expression with statistical differences between seawater and *Vibrio* challenge conditions. The time-point of 72 h is characterized by the up regulation gene levels for the effectors immune genes tested. Data obtained from qPCR were subject to the PCA (Figure 4). As a dimension-reducing technique, the PCA results led us to two principal components that together accounted for 57.3% of the overall variability of the data (Figure 4). The PCA biplot illustrates the grouping of time-points relative to a 2- or more fold change expression, corroborating how data originated from acclimatization conditions may be grouped on the basis of quantitative gene expressions similarities. Variables that presented higher distances from the center of the diagram were the most relevant to explain the total variability of the data and patterns revealed by the results. Six main groups were considered. The unchallenged condition (sea water) at 48 h is strongly represented in group 2 followed by group 1, namely for *p43*, *CALM, SABL, VEGF, RBL, BCL2, PLG, PGRP* immune genes. Group 3 represented by *CLEC, CL, CAS* and *TNFR* showed higher gene expression levels at 48 h and 2 weeks in sea water. *NF-κB, TLR2, GAL, TRAF6 ILR* and *HSP70* genes included in group 4 presented a higher expression levels at 48 h post-*Vibrio* challenge. The fifth group including *SERPIN, SRCR, FasL, FER* and *DEF* genes was higher expressed at 48 h, 72 h and 2 weeks post-*Vibrio* challenge. The sixth group is represented by *IκB* and *STAT-SH2* genes showing higher levels of transcription, at 72 h, 1 and 2 weeks post- *Vibrio* challenge. At 3 weeks of acclimatization, the challenge condition (*V. diabolicus*) is strongly associated with 72 h in sea water. The genes grouped into group 2 and 3 shown the same pattern at 48 h and 2 weeks in seawater condition. In contrast, were observed homogeneity of gene expressions into group 5 for the same time-points in *Vibrio* challenge. We identified two PCA "biological components" in the data set diagram (Figure 4). The first "biological component" that we regarded as "apoptosis regulation" is characterized by the positive correlation between *p43*, *CAS* and *BCL2* genes and the variables 48 h and 2 weeks, in sea water conditions time-points, this being consistent with its role during the time dependent response. A second "biological component", regarded as "immuno-inflammatory and cellular stress pathways" is characterized by the positive correlation between immune genes, belonging to the groups 1 (*p43*, *CALM, SABL, VEGF, RBL*), 2 (*BCL2*, *PLG*, *PGRP*) and 3 (*CLEC, CL*, *CAS, TNFR*), and the variables 48 h and 2 weeks in sea water. Groups 5 and 6, represented by *DEF, FER*, *FasL, SRCR* and *SERPIN* genes which highest transcriptional activities were evidenced at 72 h post-*Vibrio* momentary challenge.

Bacterial gene expression

The highest level of up regulated genes was observed at 2 weeks (Figure 5). However *V. diabolicus* challenge induced the transcriptional activity of bacterial genes at 48 h, 72 h and 1 week compared with sea water conditions. The level of up- and down- regulated immune genes observed at 0 h, 48 h, 72 h, 1w, 2w and 3w in sea water and post-*V. diabolicus* challenge time-points was analyzed by qPCR. Figure 5 summarizes the standardization results of the gene expression and describes the number of up and down-regulated genes. No statistically significant differences were detected (Student t-test, p-value > 0.05) for all time-point tested in this study. Moreover, all genes were analyzed and all time-point tested by comparing the control condition (sea water) with post-*V. diabolicus* challenge (Figure 6). Thus, statistically significant differences for some genes were detected (Student t-tests, p-value<0.05; Figure 6). The bacterial genes showed statistically

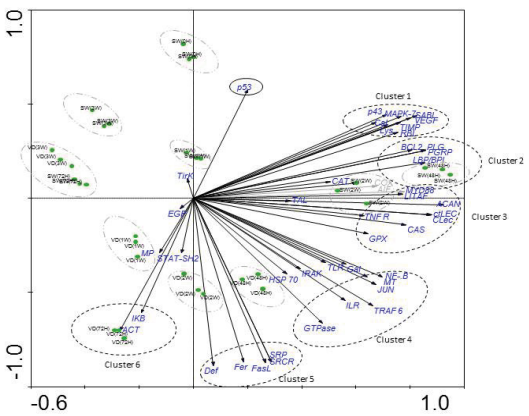

Figure 4: PCA biplot displaying the position of host genes as well as of time-points in principal component plane PC1 vs. PC2, based on 2- or more fold change expression level criterion. Time-points were coded as in Figure 1 and host genes labeled as in Table 1.

significant differences for *ALDH* gene at 1, 2 and 3 weeks acclimatization. *CBB* gene presented statistically significant differences for all time-points tested. *MDH* gene pointed differences at 48 h, 2 and 3 weeks and SOX B gene presented statistically significant differences at 48 h and 72 h. Statistically significant differences were notice at 72 h and 3 weeks for *MMO* and *CA* genes (Figure 6), as a result of the up regulation gene expression. Data obtained from qPCR were subject to a PCA (Figure 7). As a dimension-reducing technique, the PCA results led us to two principal components that together accounted for 82.1% of the overall variability of the data (Figure 7). The PCA diagram (Figure 7) illustrate the grouping of time-points related to the expression of six immune genes, supporting how data originated from these experiments could be assembled on the basis of similarities of quantitative gene expressions. Three main groups were considered: Group 1 is represented by *CA*, *ALDH* and *MDH* genes which presented the highest expression levels at 3 weeks in both unchallenged and challenged conditions. The

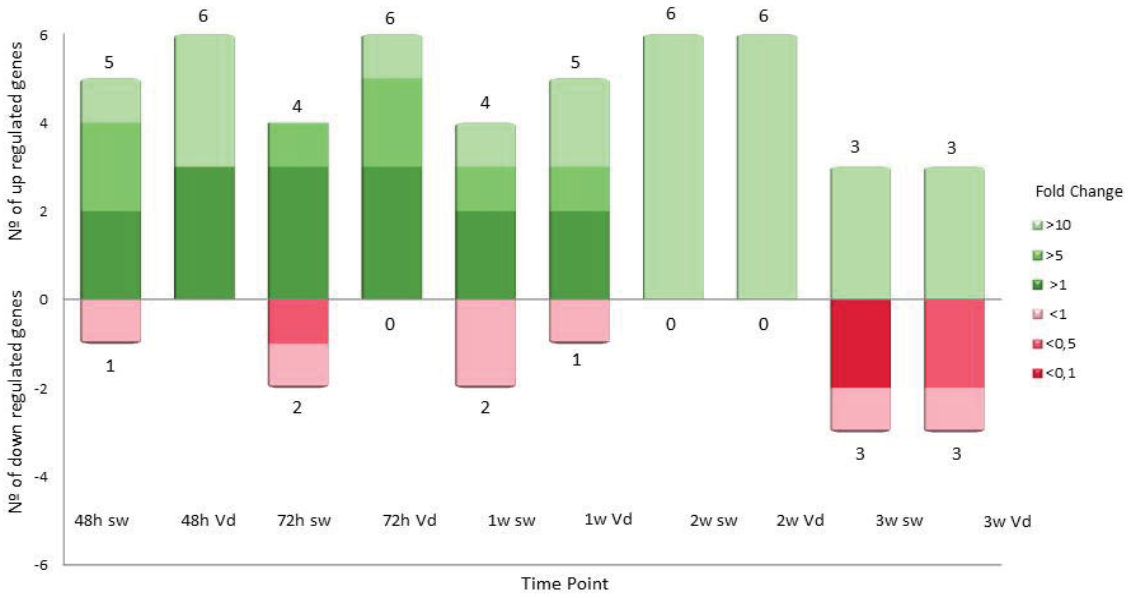

Figure 5: Bacterial genes expression through acclimatization time. Time-points were coded as in Figure 1 and host genes labeled as in Table 2. The gene expression is subdivided according to intensity (fold change) and up or down-regulation orientation. The cardinal numbers represent the nº of up and down regulated genes.

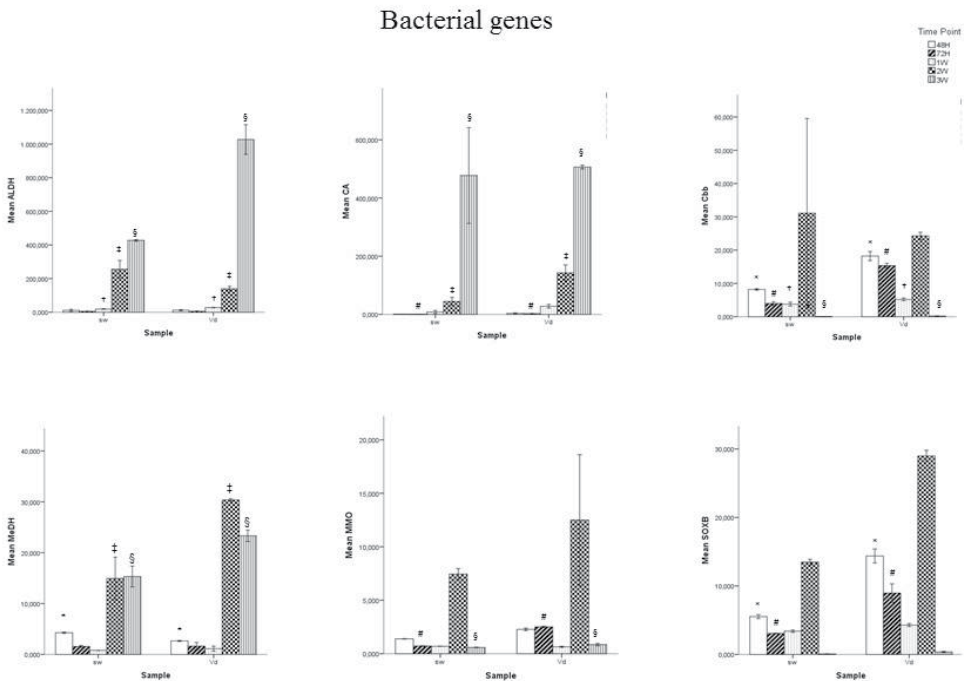

Figure 6: Bacterial gene expression analysis following 48 h, 72 h, 1 week, 2 weeks and 3 weeks in sea water and *V. diabolicus* challenge. Time-points were coded as in Figure 1 and host genes labeled as in Table 1. Significant differences between seawater control condition (sw) and *V. diabolicus* challenge ($p<0.05$, ANOVA, Dunnett's test) at *48 hours; # 72 hours; † 1week; ‡ 2 weeks and § 3 weeks, are shown. Error Bars : +/- 1 SD.

second group including *MMO* gene was expressed at 2 weeks in both unchallenged and challenged conditions. The third group represented by SOX B and *CBB* presented a strong correlation with the variables 48 h in both experimental conditions and 72 hours post *Vibrio* challenge. The sampling time-point 72 h, 1 week in sea water conditions and 1 week post-*Vibrio* challenge showed a negative correlation with the bacterial genes included in group 2 - *MMO*. We identify three principal "biological components" in the multivariable data set (Figure 7): The first, designated as "time component", is characterized by strong positive correlation between 0h, 72 h and1 week in sea water condition and less prominent correlation at 1 week after *V. diabolicus* shot challenge. This group of variables was weakly associated with 2 weeks after *V. diabolicus* challenge and 2 weeks sea water. The 48 h time-point in both conditions tested and 72 h post-*Vibrio* challenge were also weakly associated with 3 weeks in sea water and post-*Vibrio* challenge. The second "biological component", here termed as "CO_2 regulation", is characterized by the positive relationship between genes included in group 1 at 3 weeks in both conditions tested. The third "biological component", termed as "MOX and SOX pathway", is characterized by the positive correlation between bacterial genes belonging to the group 2 and 3 as a requirement to repair the damaged induced by the infection challenge. *CBB* and *CBB* genes (group 3) presented a positive correlation at 48 hours and 2 weeks of acclimatization in both experimental conditions and at 72 h post-*V. diabolicus* momentary challenge. *MMO* (group 2) were strongly expressed at 2 weeks in both experimental conditions. All genes revealed the highest transcriptional activity at 2 weeks post-*Vibrio* shot challenge.

Fish

Gill tissues sections were subjected to FISH experiments to assess for *V. diabolicus* bacteria distribution and prevalence over acclimatization time-course under aquarium environment. *V. diabolicus* bacteria were explicitly detected with the *V. diabolicus* hemolysin Cy3 probe showing the characteristic bacterial morphotypes present within the modified epithelial cells, the bacteriocytes. At 3 weeks of acclimatization the animals presented the most abundant and high prevalence of bacteria distribution patterns (Figure 8).

Discussion

Of several physiological reactions involving deep-sea vent organisms, *B. azoricus* has been the role model organism to investigate innate immune responses under controlled laboratory conditions. During the course of acclimatization, under aquarium conditions and at atmospheric pressure, the number of endosymbiont bacteria is frequently reduced until apparent total disappearance from gill tissues of mussels maintained for at least 2-3 weeks in plain sea water-supplied aquaria [18,20]. Whether or not long-term aquarium acclimatization may affect host immune genes directly influenced by endosymbionts loss, has only been addressed recently. In line with our previous post-capture investigations, *B. azoricus* long-term acclimatization challenges with *V. diabolicus* also holds potential for revealing new immunological responses providing alternative approaches to assess endosymbionts and host immune system interactions under experimental conditions [26,27]. Moreover, exposure to the deep-sea vent related *Vibrio diabolicus* bacterium constitutes an ideal model to study signaling cascades set to initiate immune responses and transcriptional regulation of immune-related genes ensuing from recognition of pathogenic microorganisms. To ascertain the full extent of the inhibitory or stimulating effect of bacterial infections on *B. azoricus* immune gene transcriptional activity, under aquarium acclimatization, a 6 hours' time-defined "pulse-like" or instant exposure with *V. diabolicus* was recurrently analyzed throughout 3 weeks acclimatization. While *Vibrio* MAMPs likely prompted the activation of signaling cascades leading the production of effector molecules, the time series *Vibrio* instant exposure suggested otherwise, a progressive weakening of host immune transcriptional activity tied to the gradual disappearance of endosymbiont bacteria from gill tissues [17,18]. *Vibrio* challenges were carried out while comparing both host and bacterial transcriptional activities whose expression levels are presumably revealing specific synergetic responses bringing thus, supporting evidence showing a putative modulating role of *V. diabolicus* on host immune system-endosymbionts interactions and on their gene expression reliance.

Using a set of genes involved in different immunological and inflammatory processes, under direct or indirect effect of *Vibrio* recurring infections, we identified three different temporal patterns of gene transcription in relation to the infection and acclimatization time-

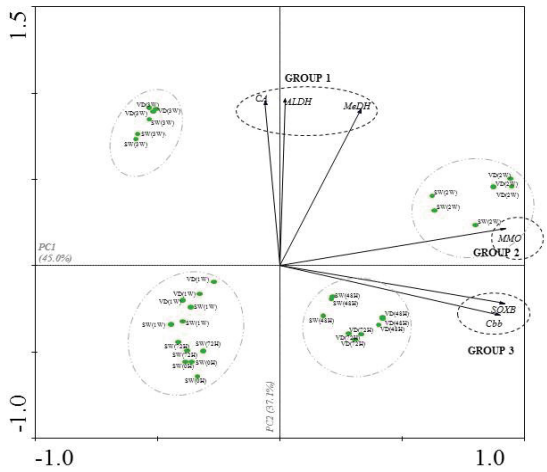

Figure 7: PCA biplot displaying the position of bacterial genes as well as of time-points in principal component plane PC1 vs. PC2. Time-points were coded as in Figure 1 and bacterial genes labeled as in Table 2.

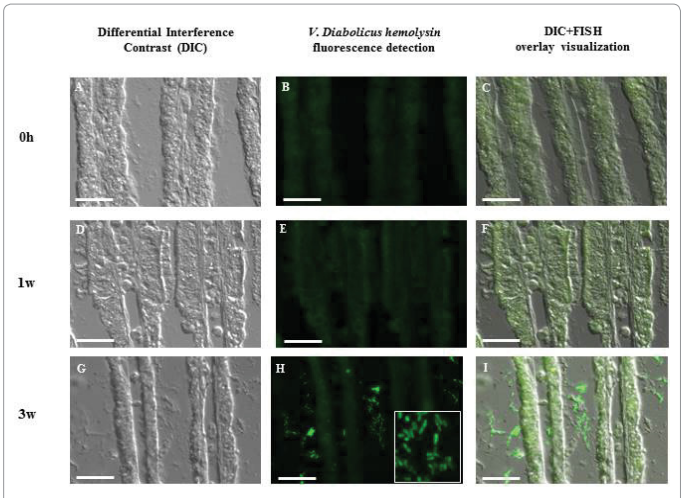

Figure 8: Fluorescence *in situ* hybridization. Hemolysin -based fluorescent probe was used to detect *V. diabolicus* bacteria in *B. azoricus* gill filaments transverse sections observed at 60x magnification. [A, D, G] Differential Interference Contrast, [B, E, H] *V. diabolicus* hemolysin fluorescence detection and [C, F, I] overlay visualization. Hemolysin detection appears as fluorescent green. Insets represent higher digital magnifications to enhance bacterial visualization. Scale bar indicates 20 µm.

points (Figure 1). Gene expression levels were assessed and fold changes means compared in Student t-test analyses across different sampling points (Figure 2). The first pattern was noticed in the first 48 h acclimatization, reflecting initial adaption processes to aquaria conditions inducing higher transcriptional levels in sea water conditions. This is represented by a general up-regulation gene expression for 44 of 46 genes tested (Figure 1). In contrast, a decline in immune gene transcriptional activity following *V. diabolicus* challenges was observed for the same acclimatization time-point. Statistically significant gene expression differences were found for *CL, CLEC, PGRP, RBL, SABL, PLG, TLR2, TNFR, VEGF, p43, STAT-SH2, TAL, BCL2, CAL* and *HSP70* genes (Figure 2). The second pattern was evidenced at 72 h and 1 week of acclimatization as demonstrated by a gene expression surge upon a *Vibrio* temporary infection (Figure 1). Statistically significant gene expression differences were observed at 72 h for *GAL, ILR, PGRP, SRCR, SERPIN, FasL, PLG, TRAF6, TLR2, DEF, FER, GPX, HSP70* and *STAT SH2* genes (Figure 2). At 1 week acclimatization the up-regulation gene expression with statistically significant differences was observed for ILR and *IκB* genes (Figure 2). The third pattern was revealed at 2 weeks and 3 weeks acclimatization as evidenced by a transcriptional activity decrease after *Vibrio* challenge (Figure 1). At 2 weeks acclimatization time-point in sea water statistically significant gene expression differences were detected for *CL, CLEC, GAL, PGRP, SABL, PLG, TLR2, TNFR, VEGF, p43, STAT-SH2, TAL, BCL2* and *GPX* genes. At 3 weeks' time-point ILR gene presented statistically significant differences. Further statistical analyses were carried out to provide adequate gene expression correlations between host immune and bacterial symbiont responses over acclimatization time. Changes associated to *Vibrio* exposure were analyzed in light of its significant influence (more than 2-Fold change) on host immune responses interactions, providing further knowledge on "instant" *Vibrio* challenge effects for each time-point in comparison to unchallenged animals while both groups of acclimatized vent mussels undergo endosymbiont loss over time. This particular experimental setting enabled us to demonstrate a dual acclimatization time and endosymbiont effect on host immune gene expression. The present study corroborates our previous findings suggesting that different acute phase-like responses between *B. azoricus* and *M. galloprovincialis* challenged *Vibrio* bacteria may be attributed to the prevalence of endosymbiont bacteria inside vent mussel gills [16]. As supported by principal components analysis (Figure 4), based on 2- or more fold change expression level criterion, the physiological changes induced by *Vibrio* exposure were noted by a strong positive correlation represented by the transcription category genes *STAT-SH2* and *IκB* (grouped into cluster 6), and the 72 h, 1 week and 2 weeks times points. Such correlation suggests a transcriptional capability persisting over the course of acclimatization likely to involve the Janus kinase/STAT pathway activation following microbial infection [29]. The *STAT* gene is involved in mediating intracellular functions often associated with innate immune reactions, proliferation and differentiation of epidermal cells [30]. The epidermal growth factor (EGF) is activated by the signal transducer *STAT-SH2*, whose mRNA transcripts was up-regulated upon *Vibrio* challenge in the present study. Upon microbe sensing all TLR signaling pathways culminate in the activation of the transcription factor nuclear factor-kappaB (NF-κB), which controls the expression of an array of inflammatory cytokine genes [31]. 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 [31]. As presented in gene expression results (Figure 2C) and PCA analysis (Figure 4) NF-κB and IκB, followed an inverse gene expression relation, confirming thus, a significant role in the NF-κB signaling pathway. Our novel results of *IκB* mRNA tied to NF-κB mRNA expression levels brings evidence supporting a possible mechanism of Toll recognition of *Vibrio diabolicus* bacterium. These genes included into cluster 6 (*IκB* and *STAT-SH2*) were weakly associated with genes belonging to group 1, 2 and 3 as well as with the 48 h and 2 weeks' time-points group (sea water conditions). Positive correlation was seen between the "immuno-inflammatory and cellular stress pathways component" groups 1 (*p43, CALM, SABL, VEGF, RBL*), 2 *(BCL2, PLG, PGRP*) and 3 (*CLEC, CL, CAS, TNFR).* This positive correlation is reflecting key physiological time-points during aquarium acclimatization through which gene signal expressions follow a phasic distribution evidenced at 48 h and 2 weeks. The former may be considered as an immune system adaptation time period to aquaria shortly after deep-sea retrieval, regardless of the mussel capacity to react in presence of *V. diabolicus* at 48 h (Figures 1 and 4). The latter, on the other hand, seems to relate to endosymbiont shedding time period usually occurring at 2 weeks [18]. As a consequence of *Vibrio* challenge, apoptosis-related reactions may incur in *B. azoricus* mussel. For this reason, *p43, BCL2, CAS, MMP, p53, TRAF6* and *FasL* were evaluated for their putative role in apoptosis molecular mechanisms in addition to an involvement in cellular differentiation, proliferation and wound repair. The high transcriptional gene expression observed for *p43* gene at 48 h, in sea water condition, follows the general trend seen for other immune genes for the same time-point, otherwise swiftly down-regulated by *Vibrio* recurrent exposures throughout the entire acclimatization experiment. An 'apoptosis regulation component' was considered in the PCA (Figure 4). This particular component is characterized by a positive correlation between *p43, CAS* and *BCL2* genes and 48 hours, 2 weeks sea water conditions time-points, suggesting a role of apoptotic-related genes during initial acclimatization and longer periods of time in which endosymbiont population may change dynamically. Our results also corroborate a down-regulation of immune genes following *Vibrio* exposure whereas endosymbiont genes are up-regulated supporting thus a bacterial interactivity between endosymbiont and *V. diabolicus* not described previously. The endosymbiont population is seemingly affected by *Vibrio* during at least 1 week acclimatization as demonstrated by bacterial gene expression analyses (Figures 5 and 6). Moreover, *BCL2, p43* and FasL gene expressions are in agreement with an apoptosis induction effect following *V. diabolicus* exposure (Figures 2a and 2b, Figure 4). 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 [32]. Regulation of apoptosis is conferred by families of pro- and anti-apoptotic molecules. The *BCL2* family proteins (anti-apoptotic molecules) are key regulators of molecular mechanisms of programmed cell death [33]. *BCL2* gene has been characterized in non-model invertebrates but recently new information regarding marine mollusks was described [34]. *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 [35]. The concomitant expression levels of *p43* and *BCL2* genes, in response to *Vibrio* infection is evocative of apoptotic events, resulting in down-regulation of transcriptional activity throughout acclimatization experiments The *PGRP* gene expression is also tied to the "apoptosis regulation component" (group 2). This is suggestive of an apoptotic signaling pathway possibly under the influence of microbial sensing involving *PGRP* known to act as a signal-transducing innate immune receptor in the IMD pathway [36]. Previous studies have shown *PGRP*

gene expression is strictly associated with endosymbionts release [37]. The strong expression of *PGRP* gene at 2 weeks' time-point in control sea water conditions may relate to the endosymbionts release time-point event. Other positive correlations were seen between groups 4 (NF-κB, *TLR2, TRAF6, ILR* and *HSP70*) and 5 (*SERPIN, SRCR, FasL, FER* and *DEF*) together with the 48 h, 72 h and 2 weeks *Vibrio* challenge conditions.

The majority of the genes grouped into groups 4 and 5, related to bacterial recognition and apoptosis mechanism, revealing higher transcriptional activity at 72 h post-*Vibrio* challenge.

SRCR immune recognition gene has been shown to function along in with the Toll-like receptor signaling pathway, an essential component in innate immunity [38]. Other extracellular signaling events upstream of Toll receptor may involve the participation of immune recognition molecules as the serine proteases [39] and serine protease inhibitors upon which *Vibrio diabolicus* may exert its modulating effect. 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) [40] 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 [41]. Defensin is an antimicrobial peptides (AMPs) acting as an effector molecule in the innate immune system providing successful defense against invading pathogens. Invertebrate AMP defensins have been found in the hemolymph (plasma and hemocytes) and in certain epithelial cells of arthropods (e.g. insects) and mollusks [42]. *B. azoricus* Defensin, was the most significantly differentially expressed immune gene showing higher transcriptional levels during initial 2 weeks after temporary infection challenge, particularly at 72 h post-*Vibrio* infection, followed by a markedly down-regulation at 3 weeks acclimatization. This is agreement with FISH results coincidently showing the presence of *Vibrio* in gill tissues at the same time-point (Figure 8). The immune system is mastered to distinguish beneficial microbes from pathogens and to coordinate appropriate immune responses [43]. 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. However emerging evidence point at evidence showing certain microbes directly engage the immune system, in some cases, into active shaping of beneficial host immune responses [43]. 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 [43] including chemolithoautotrophic bacteria living in extreme environments.

To further characterize host-immunity and endosymbiont interactions in *B. azoricus* we analyzed bacterial symbiont-related gene expression profiles to evaluate the endosymbiont influence on host immune gene transcriptional activity following *Vibrio* challenges. Using a set of primers targeting bacterial genes we identified three different patterns of gene regulation relative to *Vibrio* instant exposures and acclimatization time-points (Figure 5). The first pattern was noticed at 1 week of acclimatization, reflecting higher transcriptional levels activity upon *V. diabolicus* challenges. The second pattern was observed at 2 weeks of acclimatization, prompting highest transcriptional levels in sea water and *V. diabolicus* challenge, for all bacterial genes tested. At 3 weeks of acclimatization, the third pattern showed an up-regulation for half of the genes tested and a down-regulation for the other half. Carbonic anhydrase (CA), Aldehyde dehydrogenase (*ALDH*) and RuBisCO (*CBB*) were characterized as the most induced genes at 3 weeks of acclimatization time. The significantly up-regulated CA, *ALDH* and *CBB* was most noticed in the presence of *V. diabolicus* constituting yet another evidence suggesting a direct bacterial interaction between *V. diabolicus* and endosymbionts. Moreover, the 3 weeks acclimatization time corresponds also to the best seen gill-*Vibrio* physical interaction as demonstrated by FISH (Figure 8).

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 [24]. The fixation of CO_2 occurs primarily via the RuBisCO, in the Calvin Benson cycle, using ATP and NADPH generated from sulfur oxidation [18]. SOX B gene expressed the higher transcriptional level at 2 weeks acclimatization, this suggests that an increase of transcriptional activity prompted by in loco sulfur oxidation could contribute for the variation of bacterial mRNA transcripts seen over the course of time. Methanotrophs can oxidize methane to carbon dioxide through sequential reactions catalyzed by a series of enzymes including *MMO* and *MeDH* [18]. The production of methanol is also possible from methane by methanotrophic bacteria which explains the gene expression of *MMO* and *MeDH* genes (Figure 6) over the course of acclimatization. *MMO* and *MeDH* showed the highest transcriptional activity at 2 weeks in both experimental conditions tested however *V. diabolicus* challenge increased the gene expression values. The PCA analysis (Figure 7) showed distinct groups that were strongly associated to 72 h and 1 week in sea water condition and post-*Vibrio* challenge. The same gene expression pattern (Figure 5) is reflected by the same number of down and up-regulated genes in sea water condition. The bacterial genes tested presented the higher expression levels at 2 weeks of acclimatization in both experimental conditions. A hypothetical representation is depicted in Figure 9 where we suggest, based on our results, that the endosymbiotic activity increased when the mussel were exposed to *V. diabolicus* challenge, likely producing signals sensed by the host prompting it to activate the immune system against infection. The gradual reduction of the number of endosymbiont bacteria and eventually their total disappearance from gill tissues during acclimatization at atmospheric pressure has been documented in several Fluorescence *in situ* Hybridization-based studies including our previously work [18]. In this work, FISH was used to investigate how *V. diabolicus* interact within *B. azoricus* gills. Results bring evidence suggesting that *V. diabolicus* association with gill tissues are likely under the influence of endosymbiont occurrence and affected by its gradual disappearance during acclimatization in aquaria at which time, host immune defenses may be compromised, unable to eliminate the pathogen threat. Unlike other authors showing a direct inhibitory effect of *Vibrio* infections over shallow water bivalves host defense reactions [44,45], *B. azoricus* does react against *V. diabolicus* by inducing up-regulation of immune genes within 72 h post –acclimatization and regardless of temporary infection events. This immunological framework is likely enhanced by the presence of endosymbiont bacteria still present in gill tissues during initial days of acclimatization. However, a general decline of immune gene

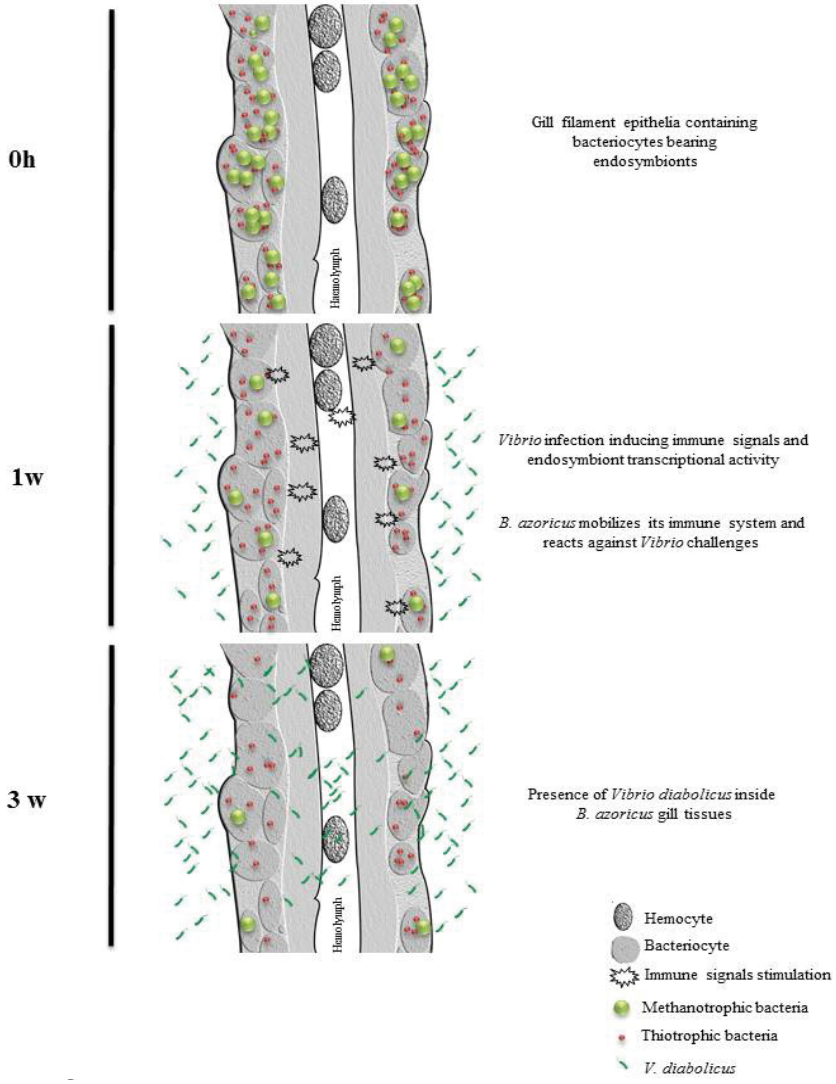

Figure 9: Hypothetical schematic representation of gill and *V. diabolicus* interaction in aquarium condition. (0h) host-endosymbiont interactions in sea water condition. (1w) *V. diabolicus* temporary challenge (for 6 hours) on 1 week acclimatized mussels. (3w) *V. diabolicus* temporary challenge (for 6 hours) on 3 weeks acclimatized mussels. *V. diabolicus* was seen in greater abundance on the surface and inside gill filaments (Fig. 8). Methanotrophic (larger green spheres) and Thiotrophic (smaller red spheres) endosymbiont bacteria are representatively depicted inside of bacteriocytes. Immune signals induced by *Vibrio* infection are illustrated as "flashing stars".

transcriptional activity was seen after 2 weeks of acclimatization, even though instant infections only lasted for 6 h for each acclimatization time-point. The loss of endosymbionts during the acclimatization process (Figure 8) was considered as detrimental to host immune defenses since an overall down-regulation of immune gene expression was observed at 2 and 3 weeks.

Conclusion

A recurrent time-defined challenge experiment using *V. diabolicus* as a *bone fide* immunostimulant agent throughout a time series experiment 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. Irrespectively of the putative environmental effect, on acclimatized vent mussels, the time series experiment allowed for comparative expression analyses for both host and endosymbiont genes, after *V. diabolicus* challenges, showing a time-dependent mRNA transcriptional pattern evidenced during the first week acclimatization with most immune genes expressed at 48 h followed by a reduced number of immune mRNA transcripts at 2 and 3 weeks of acclimatization which is likely tied to the loss of the endosymbionts in an aquarium setting. 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. Evidence presented, suggest successful *V. diabolicus* recognition prompting 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* 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.

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Competing Interests

The authors have declared that no competing interests exist.

Authors' Contributions

RB collected the species and performed the experiment design. IB prepared the RNA and DNA synthesis. IB and JD prepared the libraries and Illumina sequencing. GM and HF supervised the bioinformatics data analysis. IB, RB and HF drafted the manuscript. PG, MC provided the lab facilities at Harvard University. All authors contributed to revising the manuscript and approved the final version.

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