ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Deep Sea Mussel *Bathymodiolus azoricus* Exposure to *Vibrio diabolicus* Induces the Expression of Apoptotic Genes

Eva Martins[1,2]*, Ricardo Serrão Santos[1,2,3] and Raul Bettencourt[1,2,3]

[1]*MARE- Marine and Environmental Sciences Centre; Rua Professor Dr. Frederico Machado, Horta, Portugal*

[2]*IMAR Institute of Marine Research-Azores, Horta, Portugal*

[3]*OKEANOS Marine Research Center/Department of Oceanography and Fisheries, Faculty of Science and Technology, University of the Azores, Horta, Portugal*

***Corresponding Author:** Martins, E, MARE-Azores, University of the Azores, IMAR-Center, University of the Azores, Department of Oceanography and Fisheries, Horta, Portugal.
E-mail: eva.biotec@gmail.com

Received: June 23, 2017; **Published:** August 03, 2017

Abstract

Apoptosis is a biological process known as programmed cell death that is found broadly in an array of organisms from invertebrates to vertebrates. This process has a key role in the maintenance of the immune system balance; in the normal cellular development and in the responses to environmental stress. The extreme conditions to which deep sea vent organisms have come to adapt represent an outstanding model to study immune responses and how they may be affected by the apoptosis pathway.

At such chemosynthetic habitats, surrounding microorganisms, reduced toxic chemical compounds from venting activity and high hydrostatic pressure constitute ecological and physiological challenges that require the symbiont bearing vent mussel *Bathymodiolus azoricus* to engage critical signalling pathways leading to immune and apoptosis survival mechanisms. Here we demonstrate how vent mussels when challenged with the hydrothermal vent-related *Vibrio diabolicus* bacterium and submitted to similar hydrostatic pressure found at Menez Gwen vent site, are able to regulate their immune and apoptosis-related genes. Fifteen differentially expressed immune and apoptosis genes were analysed by means of quantitative-Polymerase Chain Reaction (qPCR) in *B. azoricus* gill tissues from 3h, 12h, 24h and 48h post- *Vibrio* challenges and from seawater control mussels. Our results demonstrated that Caspase 8 was the highest up-regulated gene in 3h and 24h post-*Vibrio* challenge gill samples whereas, 12h and 24h post- *Vibrio* challenge gill samples showed remarkably different profiles for LITAF and TRAF6 genes whose expression was down-regulated. However, TRAF6 showed a contrasting up-regulation in *Vibrio* challenged mussels comparing to all the remaining genes at 48h post- *Vibrio* challenge. Control and *V. diabolicus* challenged mussels showed a clear significant expression difference for all genes tested. These results suggested that bacterial inoculation and IPOCAMP stimuli induced initial stress reactions leading to up-regulation of Caspase 8, MyD88, LITAF and GpxI genes. After 48h post-*Vibrio* challenge, gene expression was down-regulated, as shown for apoptotic genes including Caspases 8 and 9, BAX, and members of the Bcl-2 family. These novel results suggest that the down-regulation of apoptotic genes is involved in keeping active the immune gene transcriptional activity in deep-sea vent mussel *B. azoricus* possibly intertwining apoptotic signalling pathways.

Keywords: *Bathymodiolus azoricus; Deep Sea Vent Mussel; Innate Immunity; Apoptosis; Apoptotic Genes; Vibrio diabolicus; Differential Gene Expression; Hydrostatic Pressure; Acclimatization in Aquaria*

Introduction

Apoptosis or programmed cell death is an essential biological process with a pivotal role in the maintenance of immune system homeostasis [1-3]. Apoptosis regulates the elimination of unwanted or unneeded cells in normal body cells, as well as, during viral and bacterial infections [4-6]. This defense process also occurs when cells are damaged by the presence of diseases or harmful bacterial agents [7].

The health balance may thus be evaluated by the balance between cell proliferation, differentiation and death mechanisms, involving the precise regulation of apoptosis mechanisms. This regulation of complex apoptotic molecular mechanisms, may activate, or prevent the activation of caspases [8,9]. Caspases are cysteine aspartyl proteases with regulation, initiation and execution functions in apoptosis processes [9,10]. The initiation of apoptosis may release caspases 8 and 9, Fas ligand and Tumor Necrosis Factor (TNF) molecules as a consequence of drug stimulus and other signals. On prompting apoptosis, caspases 8 and 9 will consequently activate apoptosis executioner molecules such as caspases 3 and 7 [11,12].

Metazoans can eliminate unneeded cells via the extrinsic and intrinsic pathways. Extrinsic or death receptor pathway is started on receiving signals from the environment leading to the activation of cell death surface receptor Fas coupled to the Fas ligand (FasL) resulting in apoptotic cell death [13]. Cell death mediated via Fas/FasL interaction is important for homeostasis of cells in the immune system.

The intrinsic pathways also known as the mitochondrial pathway, is initiated when the outer mitochondrial cell membrane loses its integrity, releasing the cytochrome c and other apoptotic regulatory proteins of the Bcl-2 family [7,13-15]. Bcl-2 family includes evolutionarily conserved apoptosis regulators [16]. This family is composed of pro-apoptotic (BAX) and anti-apoptotic (Bcl-2 and Bcl-xL) members [17,18].

Crucial biochemical components of the programmed cell death-apoptosis pathways are remarkably conserved from invertebrates to vertebrates [19,20]. In mussels, apoptosis has been reported to occur in gills and hemocytes in response to toxicants and pathogens agents [19-24]. Also, an increase in apoptosis levels was found in *Crassostrea gigas* hemocytes relating to oxidative cell damage, in the presence of *Vibrio ssp*. bacteria [25,26]. *Bathymodiolus azoricus* mussel is a deep-sea vent species living in chemosynthetic habitats characterized by remarkably high heavy metals, methane and hydrogen sulfide concentrations which otherwise are highly cytotoxic to non-vent marine species. Acidic pH, darkness, extreme physico-chemical conditions and increased hydrostatic pressure are common environmental factors subsisting at hydrothermal vents, in addition to surrounding microbiota [27,28]. Additionally, this mussel species harbors methanotrophic and thiotrophic endosymbiotic bacteria in its gills providing nutritional support to its host, well adapted to this inhospitable environment [29,30]. To which extent the presence and prevalence of endosymbionts may support other physiological adaptations of *B. azoricus*, to hydrothermal vent environments, is not fully understood. Both the immune system and apoptosis mechanisms may be interlinked to maintain homeostasis in deep-sea vent animals. To further our understanding on signalling crosstalk between immune responses and apoptosis processes, in the deep-sea vent mussel *B. azoricus,* we set out to investigate differential gene expression responses in animals challenged with *Vibrio diabolicus* bacteria and submitted to equivalent hydrostatic pressure levels present at the Menez Gwen hydrothermal vent field using the hyperbaric IPOCAMP chamber [31].

Communication through canonical cell signalling pathways is being addressed in this study were different modes of cross-talk between the innate immune system and cell-programmed death were analysed by means of quantitative PCR targeting a selection of key signalling genes. Results herein bring evidence suggesting that the vent mussel innate immune and apoptosis reactions are tied together as demonstrated by gene co-expression studies targeting genes involved in the Toll and apoptosis signalling pathways.

Materials and Methods

Collection of animals

B. azoricus mussels were collected from the hydrothermal vent field Menez Gwen (MG4 site 850m depth, 37º50.70N 31º31.20W) onboard the French R/V *"Pourquoi Pas?"* using the Remotely Operated Vehicle (ROV) Victor 6000 (BioBAZ mission, 2-20 August 2013). A set of 54 mussels was collected to perform this study. Mussel size (mean length ± SD) was = 8.53 ± 1.25 cm.

V. diabolicus challenges and hyperbaric stimulus

Upon collection, *B. azoricus* mussels from Menez Gwen (MG) were maintained for one week in a 1L seawater plastic container. Subsequently, mussels were challenged with 7 mL *V. diabolicus* HE800 strain suspension. *V. diabolicus* suspension was prepared from overnight

cultures grown at room temperature in Marine Broth (Difco™ Marine Broth 2216) (OD_{600} = 1.2). 6 mussels were dissected prior to exposures to *Vibrio* and were considered as the experimental time point 0 (T0) preceding the plain seawater control and *Vibrio* incubations. Mussels were subsequently sampled at 3, 12, 24 and 48h for both pressure stimuli (Control). Additionally, 6 mussels were dissected at 3h, 12h, 24h and 48h post- *V. diabolicus* challenges.

In the LabHorta aquarium, the mussels were maintained in 20L filtered seawater vessels with aeration at 8ºC. After recovery, mussels were acclimatized to aquarium conditions for 7 days prior to the pressurization experiment which was performed at 80 bars to match equivalent Menez Gwen vent field pressure levels found at 800-meter depth. The pressurization was performed in the hyperbaric chamber IPOCAMP 6 *("Incubateur Pressurisé pour l'Observation et la Culture d'Animaux Marins Profonds"),* a 19L stainless steel pressure chamber, as previously described [31]. The general design of the pressure circuit was inspired by flow-through pressure systems utilized by Childress [32]. The experiment was conducted with a group of control mussel and an experimental group of mussels challenged with *V. diabolicus* and sampled at 3h, 12h, 24h and 48h time points during ongoing hydrostatic pressure stimulation and following normal operations with IPOCAMP pressure vessel [33].

Total RNA extraction

Total RNA was extracted from gill tissue with TriReagent® (Ambion) and further purified with GeneJet™ RNA Kit (Fermentas), following the manufacturer´s specifications and re-suspended in nuclease-free, DEPC-treated water. Total RNA quality and concentrations were assessed by the $A_{260/280}$ and $A_{260/230}$ spectrophotometric ratios using the NanoVue spectrophotometer (General Electric, Healthcare Life Sciences). cDNA was synthesized with Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR according to the manufacturer´s instructions, using 2 µg/mL total RNA per sample. The cDNA concentration was measured using the NanoVue spectrophotometer as above. The 3h, 12h, 24h and 48h cDNA samples were prepared from a mixture of 2 RNA gill purifications from the control (unchallenged) mussels and *V. diabolicus* challenge mussels.

Gene expression

Gene expression analyses were conducted using quantitative PCR (qPCR) following the MIQE guidelines [34]. The samples consisted of a mixture of gills from two different mussels' corresponding to four biological replicates. Samples from control mussels (unchallenged) and 3h, 12h, 24h and 48h post- *V. diabolicus* challenges mussels were analysed after repressurization at IPOCAMP chamber. The selection of genes was selected from *Bathymodiolus azoricus* cDNA sequences available from the DeepSeaVent database [35]; Fas ligand (FasL), Cysteinyl aspartic acid-protease 8 (Casp 8), Myeloid differentiation primary response gene 88 (MyD88), Tumor Necrosis Factor (TNF) Receptor Associated Factor 6 (TRAF6), Tumor Necrosis Factor Receptor 1 (TNFRI), Caspase precursor 3 (Casp pre 3), Caspase 9 (Casp 9), Bcl- 2 (B-cell lymphoma 2) Associated X protein (BAX), Bcl-2 Like protein I (Bcl-2LI), Jun-like, Lipopolysaccharide (LPS)-induced Tumor necrosis factor-alpha TNF-α factor (LITAF), Nuclear-Factor kappa B (NF-κB), Catalase, Glutathione peroxidase I (Gpx1) and Heat Shock Protein 70 (HSP70). Specific primers were designed based on conserved domain structure (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) found in each sequence. The primer pair (Table 1) efficiency was analysed in consecutive dilutions of cDNA through the regression line of Cycle thresholds (Ct) versus the relative concentration of cDNA [36]. qPCR assays were performed with the CFX96™ Real-Time (Bio-Rad) using the same amount of cDNA concentration together with 5 µl of Power SYBR®green PCR Master Mix (Applied Biosystems), 1 µl (10 µM) forward primer, 1 µl (10 µM) reverse primer and nuclease-free water in a final volume of 10 µl per reaction. Standard cycle conditions used in this experiment were 95 ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Gene expression was normalized using the 28S ribosomal gene as housekeeping gene. Data analyses were based on the $\Delta\Delta C_t$ method with normalization of the raw data to the housekeeping gene expression values. The forward and reverse sequences used in qPCR are shown in Table 1. Three technical replicates and four biological replicates (N = 4) were obtained from qPCR experiments and data were expressed as Mean and Standard Deviation. Also, for each gene was run one reverse transcription negative control (RT).

Gene	Accession no	Primer sequence (5´-3´)		Reference
28S	AJ786406.1	Sense	TTCTTTTCACTTTCCCTCACG	[37]
		Antisense	CTTGGAGTCGGGTTGTTGA	
FasL	HM756128.1	Sense	GATGCCAAGGAAAGAGATGC	Present work
		Antisense	ACATATTTTGCCGCCATTGT	
Casp 8	CAJ18374.1	Sense	CCCAAGCCAATGTTCATCTT	Present work
		Antisense	ACGAACCACCGGCTGTATTA	
MyD88	HM756130.1	Sense	GAGTTGTGAACTGCCTGCAA	[38]
		Antisense	TCGTCTGCTGACTGTTTTCG	
TRAF6	HM756134.1	Sense	TTGCACATTCGACCTTCAAA	Present work
		Antisense	AAGACGAGAGTTTGGCGAGA	
TNFRI	HM756131.1	Sense	GTCAGCTAGCGGAGACATCC	Present work
		Antisense	CAAGCTGCATCAGCACAAGT	
Casp pre 3	ACN11423.1	Sense	GAGCGCCTACCATTTTCAGA	Present work
		Antisense	CGACTGGTTATCTGCCCAAT	
Casp 9	XP_003455368.1	Sense	GGAATCTTGATCCGGGTGTA	Present work
		Antisense	CAGTTTTGTCCGGCTTTGTT	
BAX	AGK88247.1	Sense	GGTTGTCAAGGCATTGAATAAAA	Present work
		Antisense	TGATTCTGTGGTGTTCCAAAA	
Bcl-2LI	EKC30554.1	Sense	TGGTAGTAGTAAAATGGCACCAA	Present work
		Antisense	AGGCTTCAAACTACGTCCAGA	
Jun-like	HM756138.1	Sense	CATCGGCAACAACAACACTC	[38]
		Antisense	TGTGACGGGATTGACTTTGA	
LITAF	HM756126.1	Sense	ATGAGAGATACCCCCGTGAA	[38]
		Antisense	CACAAAACAACACCCAGCAT	
NF-κB	HM756140.1	Sense	GGCTGTGTTTGGTTGGACAT	[38]
		Antisense	AGTGGCGTATCACCGTTACA	
Catalase	HM756152.1	Sense	CTGTCCATATTCTGGGTCAGC	Present work
		Antisense	CGGGGGAAAAAGGTCTTAAA	
GpxI	HM756144.1	Sense	TTAACGGCGTCGCTTGG	[38]
		Antisense	TGGCTTCTCTCTGAGGAACAACTG	
HSP70	HM756159.1	Sense	TTGAAGAAAATGTGTGGTGACTTG	[38]
		Antisense	CCCTACCAGAACGACCTCAT	

Table 1: *B. azoricus Primer Sequences of the Housekeeping Gene (28S) and Target Genes (Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp 3, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70) used in qPCR analyses.*

Statistical Analyses

The statistical analyses were performed using PRIMER 6.1.12 and PERMANOVA 1.02 software [39]. The gene expression data were expressed as mean ± SD from control and *V. diabolicus* challenge mussels. Multivariate analysis was conducted based on a Euclidean

distance matrix. Differential gene expression induced by *V. diabolicus* challenge at 3h, 12h, 24h and 48h was evaluated using Permanova (Permutational MANOVA test with 999 permutations per sample). Post-hoc pair-wise tests were done with Primer and Permanova. The tests were considered statistically significant at $p < 0.05$. The Venn diagram was designed using Venny 2.1.0 BioinfoGP [40].

Results

B. azoricus mussels were challenged with *V. diabolicus* bacteria together with equivalent hydrostatic pressure levels found at the Menez Gwen vent site, during 3h, 12h, 24h and 48h for which times, gill tissues were dissected and gene expressions analysed. Statistical analyses showed 100% significant differences between control mussels and *V. diabolicus* challenge mussels (PERMANOVA tests, $p < 0.05$) for all time experimental time-points (Figure 1-4). In this study fifteen gene expression levels were analysed, namely Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI I and HSP70.

At 3h post- *V. diabolicus* challenge, Casp 8, MyD88, TRAF6, BAX, Jun-like, LITAF, Catalase and GpxI expressions were significantly up-regulated relatively to control conditions. Otherwise, Fas ligand, TNFRI, Casp pre-3, Casp 9, Bcl-2LI, NF-κB and HSP70 were down-regulated compared to control mussels. The highest expression was shown for the Casp 8 gene (Figure 1).

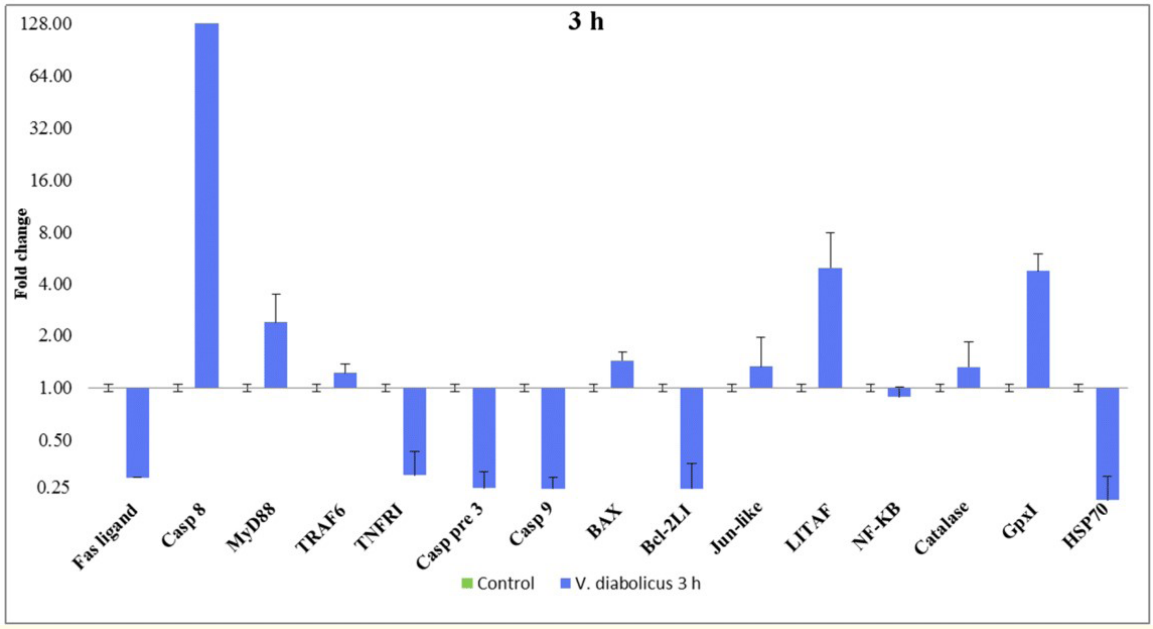

Figure 1: *Quantitative gene expression in B. azoricus after 3h pressure stimuli (Control and V. diabolicus post-challenge mussels). The genes selected were Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70. Data are expressed as means and Standard Deviation with three technical replicates and four biological replicates. Bars represent the expression level (fold change) of each target gene and normalized to the housekeeping gene 28S. Gene with fold change above 1 was up-regulated, whereas genes with fold change below 1 were down-regulated.*

At 12h post-challenged mussels, all immune and apoptosis genes tested were up-regulated relatively to control mussels (Figure 2). Otherwise, LITAF expression was down-regulated compared to control mussels. Emphasizing, Bcl-2LI and HSP70 expressions were higher in *V. diabolicus* challenged mussels at 12h (Figure 2).

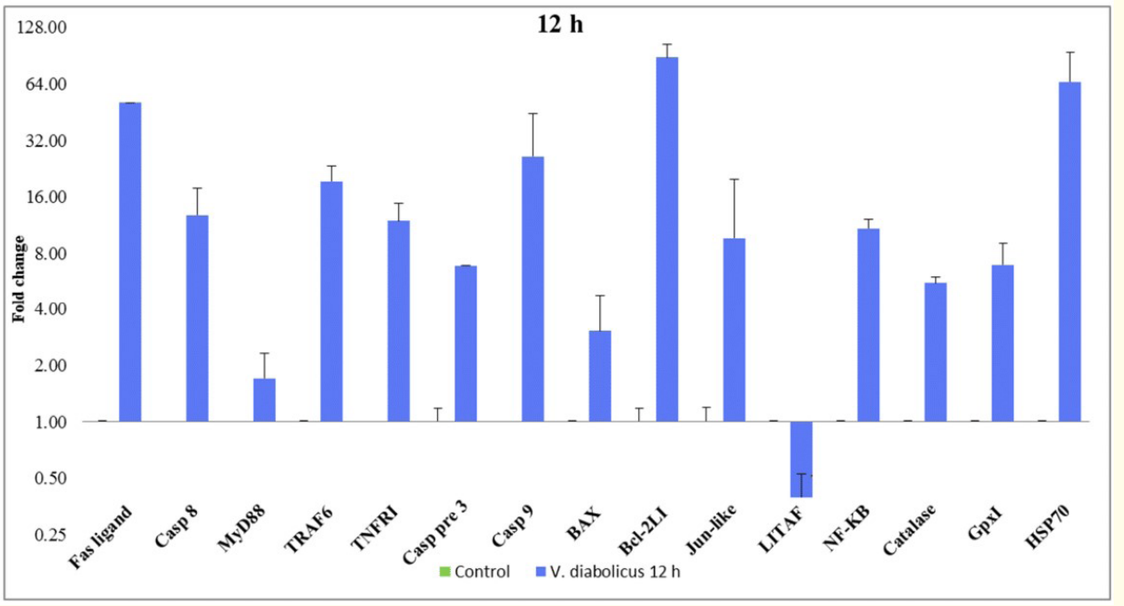

Figure 2: *Quantitative gene expression in B. azoricus after 12h pressure stimuli (Control and V. diabolicus post-challenge mussels). The genes selected were Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70. Data are expressed as means and Standard Deviation with three technical replicates and four biological replicates. Bars represent the expression level (fold change) of each target gene and normalized to the housekeeping gene 28S. Gene with fold change above 1 was up-regulated, whereas genes with fold change below 1 were down-regulated.*

Up-regulation of Fas ligand, Casp 8, MyD88, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70 genes were found at 24h post-*V-diabolicus* challenged mussels. These genes were significantly expressed compared to contrl mussels at 24h (Figure 3). TRAF6 expression was significantly down-regulated compared to control mussels at 24h time point challenge (Figure 3).

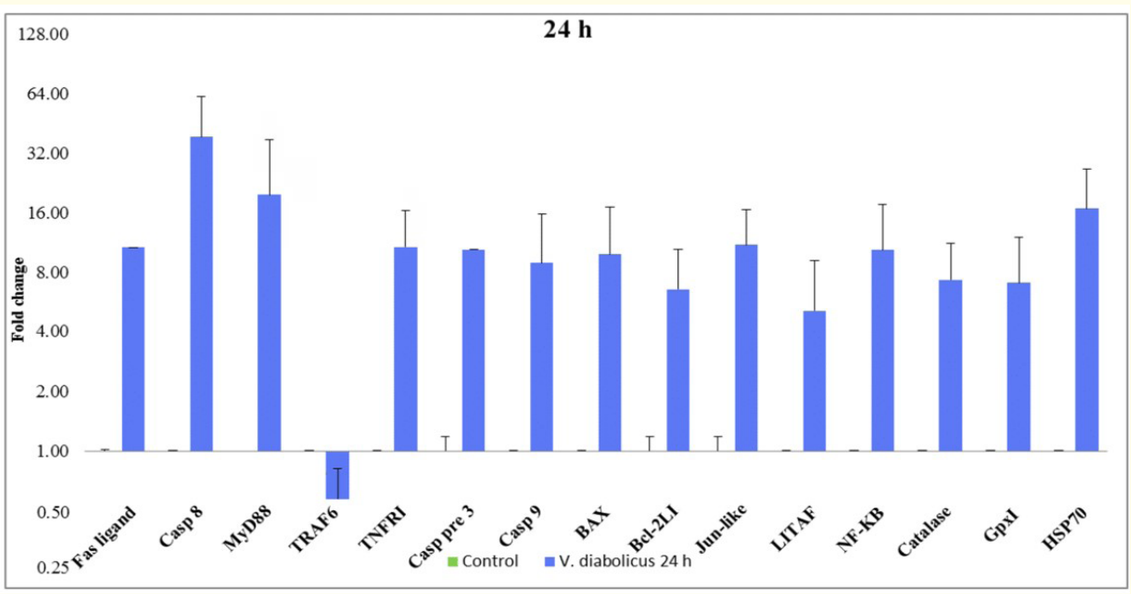

Figure 3: *Quantitative gene expression in B. azoricus after 24h pressure stimuli (Control and V. diabolicus post-challenged mussels). The genes selected were Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70. Data are expressed as means and Standard Deviation with three technical replicates and four biological replicates. Bars represent the expression level (fold change) of each target gene and normalized to the housekeeping gene 28S. Gene with fold change above 1 was up-regulated, whereas genes with fold change below 1 were down-regulated.*

At 48h post-stimulus, TRAF6 gene was up-regulated relatively to control mussels (Figure 4). Indeed, Fas ligand, Casp 8, MyD88, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI I and HSP70 gene were significantly down-regulated compared to control mussels at 48h (Figure 4).

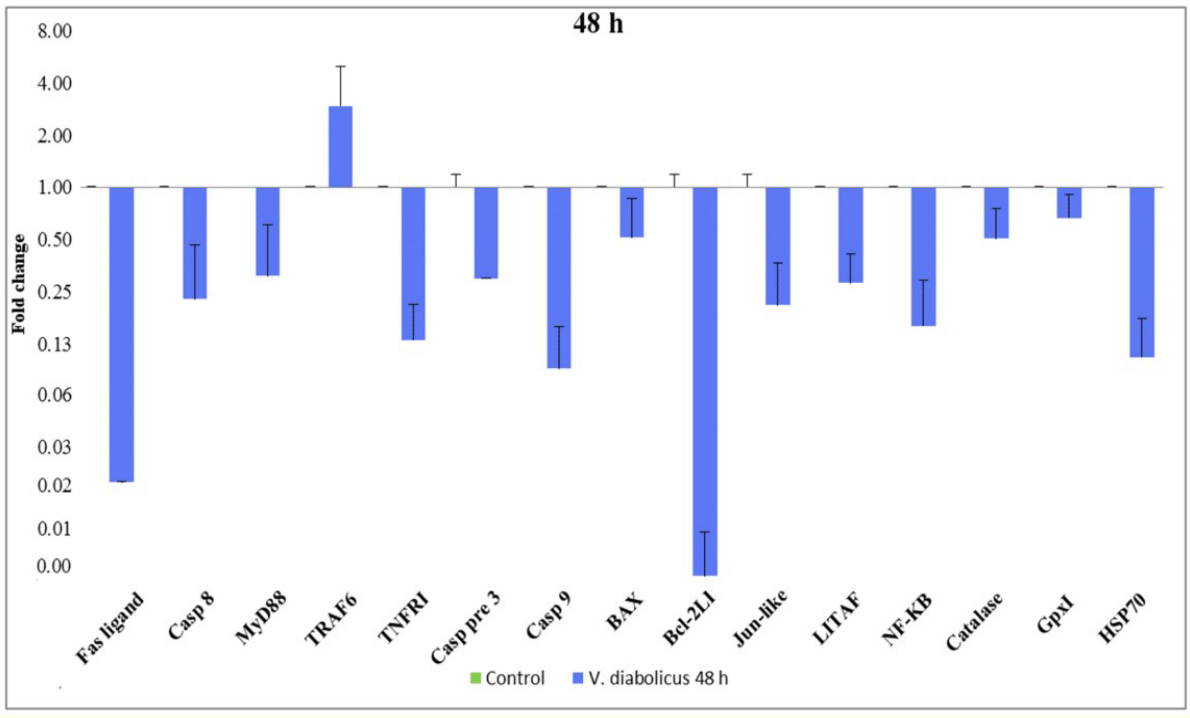

Figure 4: *Quantitative gene expression in B. azoricus after 48 h pressure stimuli (Control and V. diabolicus post-challenged mussels). The genes selected were Fas ligand, Casp 8, MyD88, TRAF6, TNFRI, Casp pre-3, Casp 9, BAX, Bcl-2LI, Jun-like, LITAF, NF-κB, Catalase, GpxI and HSP70. Data are expressed as means and Standard Deviation with three technical replicates and four biological replicates. Bars represent the expression level (fold change) of each target gene and normalized to the housekeeping gene 28S. Gene with fold change above 1 was up-regulated, whereas genes with fold change below 1 were down-regulated.*

According to the Venn diagram (Figure 5) the percentage of down-regulated and up-regulated genes were 46.7 % and 53.4 % respectively at 3h, and 6.7 % and 93.4 % respectively at 12h. The percentages of down-regulated and up-regulated genes, at 24h were 6.7% and 93.3% respectively and 93.3% and 6.7% at 48h respectively.

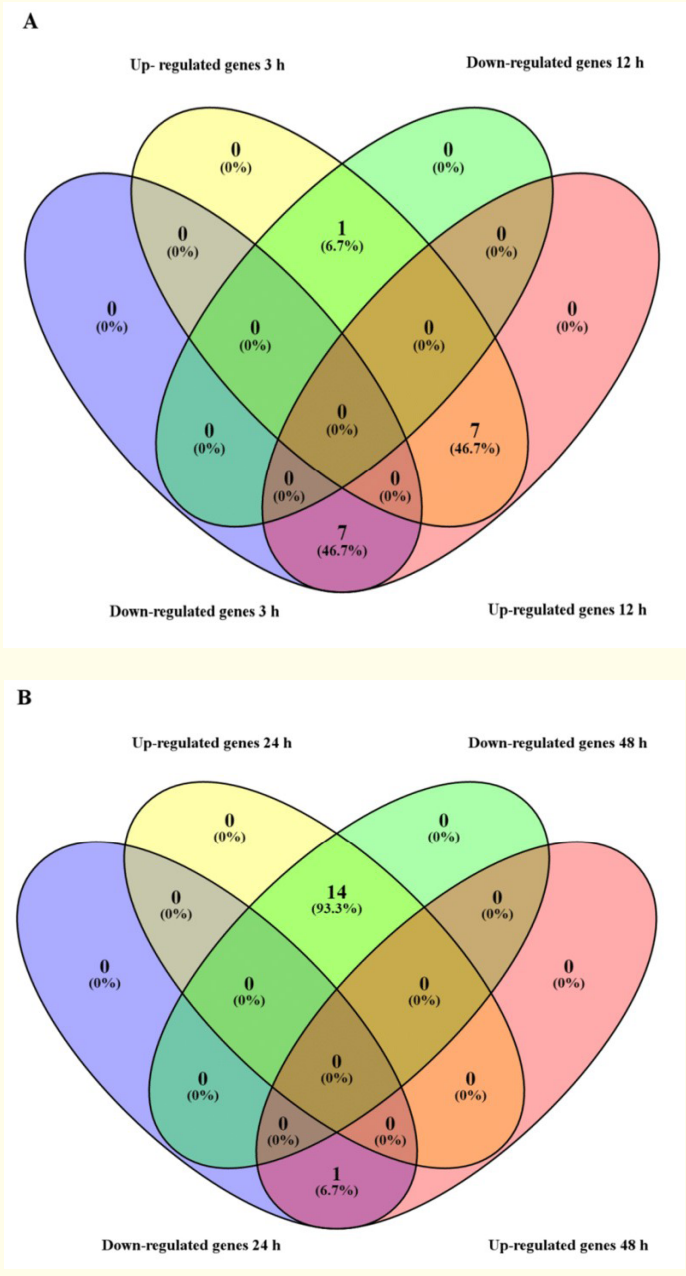

Figure 5: *Venn diagram showing the percentage of down-regulated and up-regulated genes in B. azoricus mussels. A: Comparison of the gene percentage between 3h and 12h. B: Comparison of the gene percentage between 24h and 48h.*

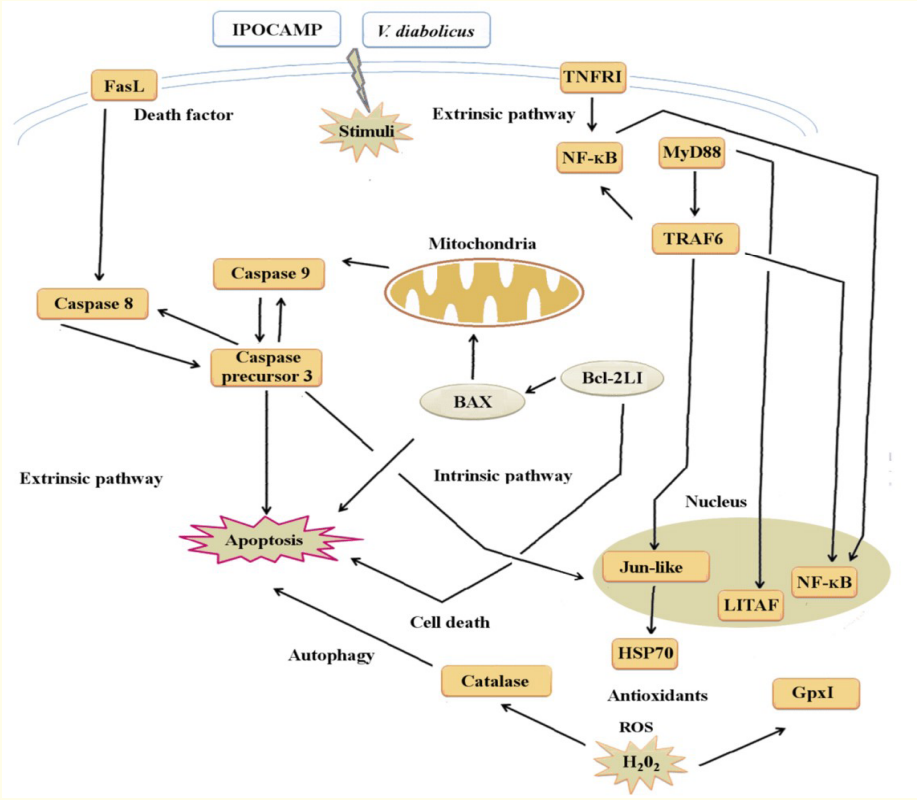

Figure 6: *Apoptotic and Immune Genes Signaling Pathways. Interaction between Apoptotic and Immune Genes after IPOCAMP and V. diabolicus Stimuli in B. azoricus Mussels.*

Discussion

Our previous studies reported differences in immune gene expression in *Bathymodiolus azoricus* after *Vibrio* exposure [37,38,41,42]. These gene expression differences were linked to multifactorial factors, such as the strain of bacterium used to perform the bacterial challenges, the type of vent mussel's body tissue (gill, digestive gland and mantle) and the time of exposure to *Vibrio* spp. bacteria. Gene expression studies enable the identification of transcripts and their transcriptional activity involved in specific molecular pathways. Expression level of mRNA can significantly vary under different experimental conditions, such as exposure to different microorganisms. In the present study, the *B. azoricus* innate immune system was challenged through a combination of hydrostatic pressure, time and *V. diabolicus* stimuli. In spite of the significant gene expression differences observed, our results pointed to a similar gene expression pattern within each experimental time point (3h, 12h, 2h and 48h). Most of gene expression differences were shown in mussels' exposure to *V. diabolicus* at 3h. Up-regulation of GpxI and down-regulation of HSP70 suggested a good physiological health condition in mussels after initial stimuli (repressurization, time and bacterial challenges). A higher level of mRNA transcripts was generally found in mussels at 12h and 24h (Figure 2 and 3) after *V. diabolicus* stimulus. According to Ziegier and Groscurth (2004), several hours are required, since the initiation of the cell-death cellular program, to undergo the signalling apoptotic pathway which will depend on the cell type, the stimulus and the activation of different signalling molecules across the apoptotic pathway [43]. In deep-sea mussels, the timing control and their gene expression levels may have a vital role in their encoded functions possibly leading to higher protein expression levels. Pattern of antioxidant enzyme (Catalase and GPXI) is identical for all time points (3h, 12h, 24h and 48h) suggesting a similar cell role that promotes

a "cross-protection" effect during the duration of the experiment. This possible role was reported by Jiang., *et al.* 2006 in GPX expression [44]. *B. azoricus* HSP70 levels were up-regulated at 12h and 24h (Figure 2 and 3) due to cell stress reaction likely resulting from IPOCAMP and *Vibrio* stimuli. As reported in the literature, Heat Shock proteins assist other proteins involved in the protection of cell membranes, counteracting the effects of newly induced stress reactions for up to 1h after decompression [44]. The expression of the proapoptotic BAX and antiapoptotic Casp 8 genes were up-regulated at 3h, 12h and 24h and their expressions were down-regulated at 48h.

These results showed the activation of the apoptosis pathway at an early stage of our experiments. On the other hand, proapoptotic Casp 3 and antiapoptotic Casp 9 genes showed higher levels of mRNA transcripts at 12h and 24h, and lower expression at 48h, respectively. These results suggest an interaction between proapoptotic and antiapoptotic signaling genes matching a co-expression pattern of up-regulation and down regulation of BAX, Casp 8, Casp 3 and Casp 9 (Figure 6). The proapoptotic Bcl-2 family members have been proposed to play a central role in regulating apoptosis [45]. The Fas receptor is also a receptor protein of the Tumor Necrosis Factor Receptor (TNFR) family that induces apoptosis upon binding the Fas ligand. Fas cell antigen was originally identified by a monoclonal antibody recognizing a cell surface antigen ("F" as in antibody fragment) that induced apoptosis in human cell lines ("as" as in apoptosis stimulating) [46,47]. The Fas ligand (FasL) is a member of the TNF cytokine family. Studies with spontaneous mutant mice, gene-targeted mice and cells from human patients have shown that FAS and FASL play critical roles in the immune system, in particular in the killing of pathogen infected target cells and the death of no longer needed, potentially deleterious as well as autoreactive lymphocytes. On binding to its ligand, members of the TNFR trigger apoptosis through activation of the intracellular adaptor death domain containing-protein FADD (Fas-Associated protein with Death Domain, also called MORT1) mediating the recruitment and activation of the aspartate-specific cysteine protease, Caspase 8 [48]. This activation involving members of the TNFR receptor gene superfamily is also called extrinsic signalling pathway that initiates apoptosis through transmembrane receptor-mediated interactions involving death receptors [3,49]. Members of the TNFR family share similar cysteine-rich extracellular domains and have a cytoplasmic "death domain" of about 80 amino acids. The "death domain" plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways [3,50]. The intrinsic signalling pathways initiates apoptosis through a diverse array of non-receptor-mediated stimuli that produce intracellular signals acting directly on targets within the cell. The main intrinsic pathway is characterized by mitochondrial dysfunction, promoting the release of cytochrome C, the activation of Caspase 9 and subsequently of Caspase 3. These mitochondrial-initiated events produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis [3]. Tumor necrosis factor receptor-associated factors (TRAFs) were initially discovered as adaptor proteins that couple the tumor necrosis factor receptor family to signalling pathways. Several members of the TRAF family have been identified playing important roles in a variety of signalling pathways [51,52].

Different members of TRAF family mediate different signal. TRAF6, a member of the TRAF family, was first identified as a transducer of CD40 and interleukin-1 receptor (IL-1R) signals [53]. TRAF6 is a unique TRAF family member and possesses a unique receptor-binding specificity, which is important for its crucial role as the signalling mediator for not only the TNF receptor superfamily but also the IL-1R/Toll-like receptor superfamily [54]. The downstream signals activated by TRAF6 mainly include NF-κB and AP-1, while NF-κB and AP-1 play an important role in the transcription and expression of numerous genes (including inflammation, apoptosis) in organisms [54]. It also plays an important role in tumorigenesis, invasion and metastasis. Unlike other TRAFs, the spectrum of biological functions of TRAF6 reflects its pivotal role in mediating signalling pathway from the IL-1R/TLR superfamily and hence its involvement in immune defense against pathogens and positive regulation of the NF-κB pathway [55]. TRAF6 protein also interacts with various protein kinases including IRAK1/IRAK which provides a link between distinct signaling pathways, playing thus a key role in the regulation of innate immune responses by mediating signals from both TNF receptors (TNFRs) and interleukin-1 receptors (IL-1Rs)/Toll-like receptors (TLRs). The present study represents, for the first time, the description of inflammatory-like responses in *B. azoricus* deep-sea vent mussels, caused by a combination of *V. diabolicus* and IPOCAMP stimuli and involving the activation of immune and apoptosis signalling pathways. This

was evidenced by the activation of extrinsic and intrinsic apoptotic pathways as demonstrated by differential gene expression results for Caspase-8 and Caspase 9/Caspase-3 respectively (Figure 6). The expression levels of TRAF6 however, showed distinct results compared to other genes tested at 24h and 48h post-stimuli. All genes were down-regulated except for the TRAF6 gene at 48h. TRAF6 plays a key role in the regulation of innate immune response by mediating signals from both TNF and interleukin-1 receptors /Toll-like receptors. The distinct gene expression results of TRAF6 bring evidence supporting a yet uncharacterized TRAF6 role, in deep sea vent mussels, antagonizing cell death during TNF and Toll-like signalling pathways when mussels are met with bacterial inoculation together with pressurization events. In this way TRAF6 may assume a protective role in deep sea vent cells, dependent on the continuous integration of cell survival and cell death signals from the extracellular environment [56]. qPCR results pointed at a reduction of mRNA transcription of apoptosis genes. The expression results after 4h pressure and *Vibrio* challenge, suggests a suppressive effect at all the apoptotic genes studied implicating the innate immune system of *B. azoricus* mussels in survival strategies that would require a tight control over the apoptosis signalling pathway. This would probably involve its down regulation, to promote cell survival, where only the TRAF6 transcription gene is up-regulated at 48h (Figure 4). Moreover, our results demonstrated that the levels of genes changed according to the time of bacteria exposure and time of hyperbaric stimuli. Our results also further extend the understanding of mechanism involved in aquatic toxicology caused by the progressive increase of ammonia in the animal's body and the animal's ability to respond to the stress and environmental conditions such as high amount of toxic substances or pathogenic microorganisms.

Conclusions

B. azoricus capacity to survive and to maintain its physiological adaptiveness after retrieval from deep-sea environment prompted us to investigate the molecular mechanisms underlying innate immune and apoptosis responses when animals were shortly acclimatized to aquarium environment at atmospheric pressure and subsequently subjected to *Vibrio diabolicus* challenges while repressurized in the IPOCAMP chamber. We investigated the expression of selected immune and apoptosis genes to better understand the vent mussel physiological reactions after 3h, 12h, 24h and 48h *V. diabolicus* challenges and to repressurization to levels compared to that of deep sea hydrostatic pressure. Results showed that gene expression is tied to the animal's response in presence of microbiological and environmental stimulus. The experimental setting was focused on the activation of apoptotic and immune gene pathways which seemed to follow a physiological pattern that is confirmed by the correlated gene expression profiles observed, highlighting the role of HSP70 and GPX genes in protecting cell animals as high levels of mRNA were visualized at 12h and 24h. Furthermore, the gene expression studies herein presented bring evidence supporting the activation of more than one signalling pathway in an animal set to endure extreme environmental conditions and thus making *B. azoricus* mussels an insightful model of research to further investigate the molecular mechanisms underlying immune and apoptosis signalling crosstalk in deep sea mussels.

Acknowledgments

The authors gratefully acknowledge the captain and crew of the R/V *"Pourquoi Pas?"* during the BIOBAZ 2013 mission (IFREMER); all members of LabHorta facility for technical assistance with laboratory work conducted in the aquaria system (University of the Azores, Faial Island) and Valerie Cueff (IFREMER, France) who provided *Vibrio diabolicus* bacterium strain. Acknowledgements are due to IMAR-Marine Institute-Research and Development Unit #531 in the Azores (Thematic Area E) and the LARSyS-Associated Laboratory # 9 funded by the Portuguese Fundação para a Ciência e Tecnologia (FCT) through FCOMP-01-0124-FEDER-007376 and the pluri-annual and programmatic funding programs-OE-FEDER-POCI2001-FSE and through the PEst project-OE/EEI/LA0009/2011-2014, and by DRCTC-Regional Government of the Azores through a pluri-annual funding scheme. This study also received the support from the Fundação para a Ciência e Tecnologia, through the strategic project UID/MAR/04292/2013 granted to MARE; and through FCT doctoral grant awarded to E. Martins- SFRH/ BD/ 73152/ 2010. The authors are thankful to Sergio Gomes (IMAR, Azores), Edmundo Pamplona (DRDA, Terceira/ Azores) for logistical support with liquid nitrogen used in BioBaz mission and Renato Bettencourt (DOP/Uaç, Azores) for IPOCAMP technical support.

Bibliography

1. Opferman JT and Korsmeyer SJ. "Apoptosis in the development and maintenance of the immune system". *Nature Immunology* 4.5 (2003): 410-415.

2. Malemud CJ., *et al.* "Immune Response and Apoptosis-Introduction". *Journal of Clinical and Cellular Immunology* S3 (2012): e001.

3. Elmore S. "Apoptosis: a review of programmed cell death". *Toxicologic Pathology* 35.4 (2007): 495-516.

4. Ellis RE., *et al.* "Mechanisms and functions of cell death". *Annual Review of Cell Biology* 7 (1991): 663-698.

5. Vaux DL and Korsmeyer SJ. "Cell death in development". *Cell* 96.2 (1999): 245-254.

6. Cohen JJ., *et al.* "Apoptosis and programmed cell death in immunity". *Annual Review of Immunology* 10 (1992): 267-293.

7. Norbury CJ and Hickson ID. "Cellular responses to DNA damage". *Annual Review of Pharmacology and Toxicology* 41 (2001): 367-401.

8. Cairrão F and Domingos PM. "Apoptosis: Molecular Mechanisms". In: ELS. John Wiley and Sons Ltd: Chichester. (2010): 1-8.

9. Thornberry NA and Lazebnik Y. "Caspases: enemies within". *Science* 281.5381 (1998): 1312-1316.

10. Cryns V and Yuan J. "Proteases to die for". *Genes and Development* 12.11 (1998): 1551-1570.

11. Nicholson DW and Thornberry NA. "Caspases: killer protease". *Trends in Biochemical Sciences* 22.8 (1997): 299-306.

12. Schulze-Osthoff K., *et al.* "Apoptosis signaling by death receptors". *European Journal of Biochemistry* 254.3 (1998): 439-459.

13. Feig C and Peter ME. "How apoptosis got the immune system in shape". *European Journal of Immunology* 37 (2007): S61-70

14. Strasser A., *et al.* "Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis". *The EMBO Journal* 14.24 (1995): 6136-6147.

15. Green DR. "Apoptotic pathways: paper wraps stone blunts scissors". *Cell* 102.1 (2000): 1-4.

16. Adams JM and Cory S. "The Bcl-2 protein family: arbiters of cell survival". *Science* 281.5381 (1998): 1322-1326.

17. Kluck RM., *et al.* "The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis". *Science* 275.5303 (1997): 1132-1136.

18. Vander Heiden MG., *et al.* "Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria". *Cell* 91.5 (1997): 627-637.

19. Kiss T. "Apoptosis and its functional significance in molluscs". *Apoptosis* 15.3 (2010): 313-321.

20. Sokolova IM. "Apoptosis in molluscan immune defense". *Invertebrate Survival Journal* 6 (2009): 49-58.

21. Micić M., *et al.* "Induction of apoptosis in the blue mussel Mytilus galloprovincialis by tri-n-butyltin chloride". *Aquatic Toxicology* 55 (2001): 61-73.

22. Micić M., *et al.* "DNA damage and apoptosis in the mussel Mytilus galloprovincialis". *Marine Environmental Research* 53.3 (2002): 243-262.

23. Châtel A., *et al.* "Induction of apoptosis in mussel Mytilus galloprovincialis gills by model cytotoxic agents". *Ecotoxicology* 20.8 (2011): 2030-2041.

24. Gao LY and Kwaik YA. "The modulation of host cell apoptosis by intracellular bacterial pathogens". *Trends in Microbiology* 8.7 (2000): 306-313.

25. McFall-Ngai MJ. "Consequences of evolving with bacterial symbionts: insights from the squid-vibrio associations". *Annual Review of Ecology, Evolution, and Systematics* 30 (1999): 235-256.

26. Takahashi KG and Mori K. "NADPH oxidase-like activity in hemocytes of the Pacific oyster Crassostrea gigas". *Fish Pathology* 35.1 (2000): 15-19.

27. Bettencourt R., *et al.* "Deep sea immunity: Unveiling immune constituents from the hydrothermal vent mussel Bathymodiolus azoricus". *Marine Environmental Research* 64.2 (2007): 108-127.

28. Colaco A., *et al.* "LabHorta: a controlled aquarium system for monitoring physiological characteristics of the hydrothermal vent mussel Bathymodiolus azoricus". *ICES Journal of Marine Science* 68.2 (2010): 349-356.

29. Salerno J., *et al.* "Characterisation of Symbiont Populations in Life-History Stages of Mussels from Chemosynthetic Environments". *Biological Bulletin Publications - Marine Biological Laboratory* 208.2 (2005): 145-155.

30. Bettencourt R., *et al.* "Innate immunity in the deep sea hydrothermal vent mussel Bathymodiolus azoricus". *Comparative Biochemistry and Physiology* 152.2 (2008): 278-289.

31. Shillito B., *et al.* "Temperature resistance of Hesiolyra bergi, a polychaetous annelid living on deep-sea vent smoker walls". *The Marine Ecology Progress Series* 216 (2001): 141-149.

32. Quetin LB and Childress JJ. "Observations on the swimming activity of two bathypelagic mysid species maintained at high hydrostatic pressures". *Deep Sea Research Part I: Oceanographic Research Papers* 27.5 (1980): 383-391.

33. Shillito B., *et al.* "The IPOCAMP pressure incubator for deep-sea fauna pressure aquarium". 22.1 (2014): 97-102.

34. Bustin SA., *et al.* "MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments". *BMC Molecular Biology* 11 (2010): 74.

35. Bettencourt R., *et al.* "High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel Bathymodiolus azoricus". *BMC Genomics* 11 (2010): 559-576.

36. Livak K and Schmittgen TD. "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method". *Methods* 25.4 (2001): 402-408.

37. Martins E., *et al.* "Vibrio diabolicus challenge in Bathymodiolus azoricus populations from Menez Gwen and Lucky Strike hydrothermal vent sites". *Fish and Shellfish Immunology* 47.2 (2015): 962-77.

38. Martins E., *et al.* "Comparative study of immune responses in the Deep-sea hydrothermal vent mussel Bathymodiolus azoricus and the Shallow-water mussel Mytilus galloprovincialis challenged with Vibrio bacteria". *Fish and Shellfish Immunology* 40.2 (2014): 485-499.

39. Primer-E Ltd., editor. Primer 6.1.12 and Permanova +1.0.2. Plymouth, United Kingdom. 2009.

40. Oliveros JC "Venny. An interactive tool for comparing lists with Venn's diagrams". 2007-2015.

41. Martins E., *et al.* "Finding immune gene expression differences induced by marine bacterial pathogens in the Deep-sea hydrothermal vent mussel Bathymodiolus azoricus". *Biogeosciences* 10.11 (2013): 7279-7291.

42. Bettencourt R., *et al.* "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 and Shellfish Immunology* 39.2 (2014): 343-353.

43. Ziegler U and Groscurth P. "Morphological features of cell death". *News in physiological sciences* 19 (2004): 124-128.

44. Jiang Z., *et al.* "Effects of High Hydrostatic Pressure on Expression Profiles of *in Vitro*-Produced, Vitrified Bovine Blastocysts". *Reproduction, Fertility, and Development* 2016.

45. Lindsten T., *et al.* "The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues". *Molecular Cell* 6.6 (2000): 1389-1399.

46. Trauth BC., *et al.* "Monoclonal antibody-mediated tumor regression by induction of apoptosis". *Science* 245.4915 (1989): 301-305.

47. Yonehara S., *et al.* "A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor". *The Journal of Experimental Medicine* 169.5 (1989): 1747-1756.

48. Waring P and Müllbacher A. "Cell death induced by the Fas/Fas ligand pathway and its 582 role in pathology". *Immunology and Cell Biology* 77.4 (1999): 312-317.

49. Locksley RM., *et al.* "The TNF and TNF receptor superfamilies: integrating mammalian biology". *Cell* 104.4 (2001): 487-501.

50. Ashkenazi A and Dixit VM. "Death receptors: signaling and modulation". *Science* 281.5381 (1998): 1305-1308.

51. Wixted JH., *et al.* "Identification of functionally distinct TRAF proinflammatory and phosphatidylinositol 3-kinase/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (PI3K/MEK) transforming activities emanating from RET/PTC fusion oncoprotein". *The Journal of Biological Chemistry* 287.6 (2012): 3691-3703.

52. Inoue J., *et al.* "Tumor necrosis factor receptor-associated factor (TRAF) family: adapter proteins that mediate cytokine signaling". *Experimental Cell Research* 254.1 (2000): 14-24.

53. Naito A., *et al.* "Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice". *Genes Cells* 4.6 (1999): 353-362.

54. Meng Q., *et al.* "TRAF6 regulates proliferation, apoptosis, and invasion of osteosarcoma cell". *Molecular and Cellular Biochemistry* 371.1 (2012): 177-186.

55. He L., *et al.* "TRAF6 Regulates Cell Fate Decisions by Inducing Caspase 8-dependent Apoptosis and the Activation of NF-kB". *The Journal of Biological Chemistry* 281.16 (2006): 11235-11249.

56. K Yoon., *et al.* "TRAF6 deficiency promotes TNF-induced cell death through inactivation of GSK3b". *Cell Death and Differentiation* 15 (2008): 730-738.

Volume 10 Issue 3 August 2017