Transcriptomic response of the hydrothermal mussel *Bathymodiolus azoricus* in experimental exposure to heavy metals is modulated by the Pgm genotype and symbiont content.

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28 **LIST OF ABBREVIATIONS:**

29 MG: Menez Gwen

30 LS: Lucky Strike

31 RB: Rainbow

32 Pgm: Phosphoglucomutase

33 PCA: principal component analysis

34

35 **Abstract**

36 Hydrothermal vent mussels belonging to the genus *Bathymodiolus* dominate communities at
37 hydrothermal sites of the Mid-Atlantic Ridge. The mussel *Bathymodiolus azoricus* harbors
38 thiotrophic and methanotrophic symbiotic bacteria in its gills and evolves in naturally highly
39 metal contaminated environments. In the context of investigations on metal tolerance/effect in
40 *B. azoricus*, we focused our work on the short-term adaptive response (15 days) of mussels to

different metals exposure at a molecular level using metal concentrations chosen to mimic natural situations at three vents sites. The expression of a set of 38 genes involved in different steps of the metal uptake, detoxication and various metabolisms was analysed by qPCR. Mussels were also genotyped at 10 enzyme loci to explore the relationships among natural genetic variation and gene expression. Relation between symbiont content (both sulfur-oxidizing and methanogen bacteria) and gene expression was also analysed. Our study demonstrated the influence of metal cocktail composition and time exposure on the transcriptome regulation with a specific pattern of regulation observed for the three metal cocktail tested. We also evidenced the significant influence of some specific Pgm genotype on the global gene expression in our experimental populations and a general trend of a higher gene expression in individuals carrying a high symbiont content.

Key words: hydrothermal vent, mussel, *Bathymodiolus azoricus*, heavy metals stress, genotype, symbiosis, transcriptomic.

1. Introduction

Hydrothermal vents are located along oceanic ridges of the deep-sea bed and have highly variable environmental characteristics both spatial and temporal composition including temperature, pH and gradients of oxygen, exceptional high concentration of metals, radionuclides, methane and/or sulphides that all exhibit strong heterogeneity (Von Damm et al., 1998; McMullin et al., 2000; Desbruyères et al., 2000). Among species evolving specialization for hydrothermal vent environments, the mussel belonging to the genus *Bathymodiolus* (Von Cosel et al., 1999) dominates communities at hydrothermal sites of the Mid-Atlantic Ridge and could represent both a good ecotoxicological marker of the vent

environment and a model for species naturally evolving under high concentrations of pollutants. In *Bathymodiolus azoricus*, accumulation of metals at high concentrations mainly takes place in the gills which represent the main interface between the organism and its environment and this species has been already used to evaluate the mercury concentrations in three hydrothermal vent fields localized along the Mid-Atlantic Ridge (Martins et al., 2001). Accumulation of metals has also been reported in the digestive gland and at a lower rate in the mantle (Rousse et al., 1998). *B. azoricus* also harbors a dual symbiosis with sulfur-oxidizing bacteria (SOX) and methanogen (MOX) bacteria both located in the gills (Fiala-Médioni et al., 2002; Duperron et al., 2006) but the involvement of symbionts in metal uptake and detoxification in the mussel physiology remains not clear (Cosson-Mannevy et al., 1988).

Metals are divided into two main categories including both physiologically useful trace metals such as iron, copper, magnesium and manganese which participate to the cellular homeostasis mainly as co-factors of numerous enzymes (Suzuki et al., 2002) and which can be toxic at high concentration and heavy metals such as cadmium, lead and mercury which exhibit an immediate toxic effect once they penetrate cells (Rousse et al., 1998). Metal-containing proteins are the main targets of toxic heavy metals and the subsequent cell toxicity has been assumed to result from the disturbance of proper metal-protein interactions that are essential for maintaining functional protein structure (Kang, 1997). Toxic effects of heavy metals include nonspecific intracellular complexation with thiol groups being particularly vulnerable (Rensing et al., 1997). Metals are also well known inducers of oxidative stress that stimulates reactive oxygen species (ROS) production by interfering with cellular metal-related processes and generating free radicals via ions with changeable valences such as in Fenton reactions in which hydrogen peroxide interact with iron to generate hydroxyl radicals (Cheeseman and Slater, 1993). ROS are comprised of several classes of molecules including superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxy radicals ($ROO^{\bullet}$), alkoxy radicals

(RO•) and have been shown to alter a large spectrum of cellular targets (Lushchak, 2011 for review). As other bivalve species, *B. azoricus* possesses a set of antioxidant molecules able to detoxify ROS, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and diverse glutathione peroxidases (GPx; EC 1.11.1.9) which activity vary according to the tissue, CAT and SOD activities being higher in gills than in mantle (Bebianno et al., 2005). Other biomarkers of the metal exposure such as DNA damages, lipid peroxidation (Cavaletto et al., 2002), metallothionein concentration and the SOD, CAT, GPx activities have been already monitored in *B. azoricus* (Géret et al., 1998; Bebianno et al., 2005; Pruski and Dixon, 2003; Company et al., 2010; Vidal-Liñán, 2010; Hardivillier et al., 2006) but not at the scale of gene expression. Pollution in general may provoke rapid genetic changes in exposed populations at different time scale (Medina et al., 2007). Numerous studies demonstrated the role of heavy metals in selection for or against specific allozymic variants in various species (Moraga and Tanguy 2000; Ma et al., 2000; Bickham et al., 2000; Belfiore and Anderson, 2001 for review). Quite often, local effluents of heavy metals create patches of altered habitats likely to promote diversifying selection in populations of benthic invertebrates (Lavie and Nevo, 1982, Nevo et al., 1984; Nevo 2001) in a few generations following theoretical predictions of Hedrick (1986) about the action of selection in heterogeneous environments. However, in some cases, genetic diversity could also be impacted by selecting against rare alleles (Lavie and Nevo, 1988; Benton and Guttman, 1990). In *Idotea baltica*, cadmium alone was shown to decrease heterozygosity at the PGM locus while a mix of zinc and cadmium increased it (De Nicolas et al., 1992). Exposure to metals may also act at different level such as the counter-selection of genotypes associated to sensitive phenotypes (Gardestrom et al., 2008, Van Straalen, 2003) in the fluid as mentioned in the text associated with the mutagenic effect of metals (Rogstad et al., 2003) or the selection for rare alleles in immune genes (Tennessen, 2005; Tennessen and Blouin, 2008). These studies suggest that some genotypes are able to perform

better than others in response to stressful conditions. In a previous study conducted on *B. azoricus*, Boutet et al. (2009) evidenced a global down-regulation of gene expression in response to increasing experimental temperatures but, also a clear counter-selection of some specific PGM genotypes during differential mortality experiments and, more surprisingly a direct relation of these frequencies and the gene expression of other genes and especially, stress oxidative genes. This clearly indicates that the short-term genetic response of the vent mussels to environmental pollutants is a complex process involving numerous genes in epistasis including regulatory ones, in which some of the genes may be under direct selection or linked to adaptive ones (i.e. genetic markers). In order to complete our knowledge on the putative importance of environmental stressors such as metals in shaping the adaptive polymorphism of *B. azoricus*, we investigated the expression of a panel of genes in response to metal exposure and explored the potential relationship between gene expression and both the symbiont content of the mussel (as an environmental proxy) and the frequency of allozymes currently known to vary in a heterogeneous habitat.

2. Materials and methods

2.1. Sample collection and experimental design:

2.1.1 Field samples:

Field *B. azoricus* samples were collected during the MoMARETO (Monitoring the Mid Atlantic Ridge: Ecology, Technology Observation) cruise (Sarrazin et al., 2006) at three vent sites: Menez Gwen (MG; 37°50'N, 31°30'W, 840 m depth) along the Mid-Atlantic Ridge (MAR), Lucky Strike (LS; 37°17' N, 32°17' W; 1700 m depth) and Rainbow (RB; 36°14' N, 33°54' W; 2350 m depth) with the N/O Pourquoi Pas? Research vessel and the Remote Operated Vehicle (ROV) Victor 6000 (see SUPPLEMENTAL MATERIAL Fig.S1). The mussels were collected and placed in hermetic boxes then brought onboard where the gills, mantle, digestive

gland and adductor muscle from 30 individuals of MG and LS populations and 24 samples of RB population were immediately dissected and frozen in liquid nitrogen.

2.1.2: Experimental population:

Mussels used for metal-exposure experiments were collected at MG because of their documented capacity to recover from depressurization and to support long-term laboratory maintenance at atmospheric pressure (Kadar et al., 2005). A total of 180 individuals were collected at the same time in hermetic boxes, brought on board and immediately placed in PVC tanks containing 40 L of normoxic sea-water for 24 h at 7°C before experimentation. All mussels exhibited an average shell length comprised from 3 to 5 cm. For the metal exposure experiment, three groups of 60 mussels were randomly distributed into tanks and placed in a cold room at a constant temperature of 7°C during all the experiment. The temperature chosen corresponded to the mean temperature registered at the bottom on the mussel bed. In each tank, a specific cocktail of metals called "MG condition", "LS condition" and "RB condition" was added. Composition and relative ratio of each metal in the three cocktails were chosen to reflect the metal concentrations of the three different vent sites MG, LS and RB (Douville et al., 2002, Charlou et al., 2002) (see SUPPLEMENTAL MATERIAL Table S1). Tanks water was renewed every day with freshly chilled sea water (7°C) and appropriate metal cocktail was added in corresponding tank in order to maintain heavy metal concentrations as stable as possible. Because mussels do not live directly in the fluid but close from the fluid, we postulate that they are exposed to a diluted fluid and then we choose to apply a dilution by ten to the metal concentration in our cocktails compared to the field conditions (Table 1). Oxygen concentrations and salinity were measured once a day and ranged from 6.3 to 6.7 mg L⁻¹ of oxygen and 37.2 to 38.2 ‰ in acclimatisation tanks. After 3 (T3), 7 (T7), 11 (T11) and 15 (T15) days of exposure, 10 mussels were sampled from each tank, measured and both gills and adductor muscle were dissected and swiftly frozen in liquid nitrogen until further analysis.

During the experiment, mussels were not subjected to methane or sulphide. Animals were manually checked for survival prior to dissection. No mortality has been observed and mussels kept their valves opened during the course of the experiment suggesting a relative normal behaviour.

2.2. Gene selection and mRNA quantification

The mRNA expression of 38 genes chosen to cover various metabolic pathways was analysed by real-time PCR. Gene sequences were collected from available *B. azoricus* libraries (Tanguy et al., 2008) and non-published sequence datasets. Gene name, primer sequences and metabolic pathways are provided (see SUPPLEMENTAL MATERIAL Table S2).

Total RNA was extracted from mussel gills by using Tri-Reagent (Sigma) according to the manufacturer's instructions. Both quantity and quality of RNA were assessed via UV absorbance (OD260/280/230) using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). Five µg of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega), an anchor-oligo(dT) primer (5'-CGCTCTAGAACTAGTGGATCT₍₁₇₎-3') and random hexamers (Promega). A volume of 2 µl of each diluted reverse transcription product (1:200) was subjected to real-time PCR in a final volume of 10 µl containing 70 nM of each specific primer and 1X ABsolute™ QPCR SYBR® Green Mix (ABgene). The amplification was carried out as follows: initial enzyme activation at 94°C for 15 min, then 40 cycles of 94°C for 15 sec and 60°C for 1 min. A dissociation curve was generated and PCR efficiency was estimated for each primer pair. All primer pairs tested generated a single peak in the dissociation curve and a PCR efficiency of 95 to 100%. Relative expression of each gene (fold-change to control) was calculated according to comparative Ct method using the formula: $RQ=2^{-\Delta\Delta C_t}$ (with $\Delta C_{t_{geneX}}=C_{t_{GeneX}}-C_{t_{RiboL15}}$; $\Delta\Delta C_t=\Delta C_{t_{geneX}}-\text{mean}\Delta C_{t_{geneX}}$). A fragment of ribosomal protein L15 gene (RpL15) from the host was used as

the internal PCR control. We validate the RpL15 after having observed very few (less than 5%) variation in its expression in all samples (both experimental and field individuals) for gill tissue.

2.3. *Symbiont ratio determination*

Genomic DNA of both mussel and bacteria was extracted together from gill tissue and muscle (used as a negative control in real-time PCR amplification) using a CTAB/PVP extraction procedure and the relative quantity of symbionts was estimated by real-time PCR amplification using 16S specific primers according to the protocol described in Boutet *et al.* 2011. All experiments were carried out using a Chroma4 thermal cycler (Bio-Rad Laboratories Inc, Hercules, CA) and 1X ABsolute™ QPCR SYBR® Green mix (ABgene, Epsom, UK), 70 nM of each primer, and diluted DNA (2.5 ng) in a final volume of 10 µl. A 120 bp-fragment of cytosolic malate dehydrogenase gene (MDH) from the host was used as an internal PCR control (gene primers are listed in SUPPLEMENTAL MATERIAL Table S2). The relative quantity of each symbiont type was estimated by using the comparative Ct method using the formula $RQ = 2^{-\Delta C_t}$ ($\Delta C_t = C_{t16S} - C_{tMDH}$).

2.4. *Individual genotyping*

Genotyping of ten allozyme systems was performed for all experimental and field samples. Proteins were extracted from adductor muscle of each individual in extraction buffer (0.01M Tris-HCl pH 6.8, 2mM EDTA, 0.5mM NADP, 0.05% β-mercapto-ethanol, 0.1mM PMSF) using the procedure described in Piccino *et al.*, 2004. The experimented mussels were genotyped for ten enzyme systems (Pgm-1 and Pgm-2, Mdh-1 and Mdh-2, Hk, Mpi, Aco, Odh, Gpi and Lap) following the protocols provided in Harris and Hopkinson (1976) and in Pasteur *et al.* (1987) (see SUPPLEMENTAL MATERIAL Table S3). Proteins were extracted from the adductor muscle for each individual in the extraction buffer using the procedure described in

Piccino et al. (2004). Electrophoreses were conducted for 4 to 6 hours at 80 mA using 12% starch gel. Loci were numbered according to the decreasing anodal electromorph mobility in multilocus systems. Alleles were assigned according to their relative distance to the most frequent allele (100) in the sample.

2.5. *Data analysis*

A canonical redundancy analysis (RDA) was used to estimate the fraction of variance in gene expression attributable to the explanatory variables. RDA was performed using the R-language 'rdaTest' function (<http://www.bio.umontreal.ca/legendre/indexEn.html>) on the data obtained from the metal exposure experiment using the mRNA expression of 38 genes as response variables, with the explanatory variables of "condition" (field population and the three metal cocktail treatments), exposure duration, genotype classes at nine loci (non-polymorphic loci were not retained for the analysis) and the symbiont content for both MOX and SOX. The explanatory variables were re-coded as a set of numerical variables; polynomial transformations (simple, power 1; square, power 2; cube, power 3) were applied to the exposure times, and genotype classes were coded as three binary variables corresponding to the genotypes 100/100, 100/X and X/X (X corresponding to alleles different from 100). In order to give the same weight to all response variables in the analysis, levels of gene expression were standardized to a mean of zero and a variance of one (z-score transformation) with the R-language 'decostand' function of the 'vegan' package (Vegan: Community Ecology Package v. 1.8.3; <http://cran.r-project.org/>). When necessary, the rare missing gene expression data (no amplification on the gene in the considered individual) was replaced by the mean expression across all individuals, to keep unaltered the position of the centroid of the response variables (Legendre and Legendre, 1998). A forward selection of the explanatory variables was performed using the 'packfor' R-language package (Packfor: forward selection with permutation. R package v.0.0-7; <http://biomserv.univ-lyon1.fr/dray/Software.htmlpackfor>), retaining the explanatory variables

with $p < 0.1$ after Monte Carlo permutation tests ($nperm = 9999$). In order to identify genes whose expression changed significantly according to their mussels' "condition", gene expression ratios were calculated according to the formula $RQ = 2^{-\Delta\Delta C_t}$ ($\Delta\Delta C_t = (C_{t_{gene\ X\ individual\ i}} - C_{t_{RpL15}}) / \text{mean } C_{t_{gene\ X\ all\ individuals}}$). Statistical analysis of mRNA expression data was conducted using the SAM procedure in the MeV 4.2 software (<http://www.tm4.org/mev.html>) (Saeed et al., 2003). Differentially expressed genes were identified using a fold-change of 2 and a False-discovery rate (FDR) of zero to avoid genes with non-acceptable error probabilities. FDR was estimated by analyzing permutations of the measurements and expresses the percentage of genes identified as significant by chance for a given value of a threshold parameter delta.

2.6. Relationship between genotypes and gene expression level

The differences between mean mRNA expression of the three genotype classes (100/100, 100/X and X/X, with X corresponding to any other allelic form than 100) for the loci showing a significant p-value in the RDA test were tested by using a non parametric Wilcoxon-Mann-Whitney test with the multiple test correction of Holm (1979) (R-language 'stats' package). The analysis was conducted on both experimental and field population samples.

2.7. Relationship between symbiont content and gene expression level in experimental individuals

The differences between mean mRNA expression of each SOX symbiont class (very low SOX content (0-15 U), low SOX content (16-30 U), moderate SOX content (31-60 U), high SOX content (> 61 U)) were tested by using a non parametric Wilcoxon-Mann-Whitney test with multiple test correction of Holm (1979) (R-language 'stats' package).

3. Results

259 *3.1. Relative effects of experimental and field populations, genetic components and symbiont*
260 *content on the variance of gene expression:*

261 The global expression pattern of all genes in the three metal cocktail experiments is presented
262 in SUPPLEMENTAL MATERIAL Figure S2. The first analysis identified significant variables
263 linked to the metal exposure experiment, and for the 38 genes studied, assessed the part of the
264 observed variance in mRNA expression among individuals associated with each of the
265 explanatory variables (metal cocktails, time of exposure, symbiont content, and enzyme loci).
266 The multivariate forward selection procedure showed that four of the explanatory variables
267 remained significant in explaining the variance of mRNA expression across individuals: "metal
268 cocktail" ($R^2 = 0.118$, $F = 13.38$, $p = 0.001$), "exposure time" in polynomial form (simple: $R^2 =$
269 0.071 , $F = 8.67$, $p = 0.001$; square: $R^2 = 0.060$, $F = 7.89$, $p = 0.001$; cube: $R^2 = 0.039$, $F = 4.97$, $p =$
270 0.012), Pgm-1 X/X ($R^2 = 0.027$, $F = 3.69$, $p = 0.05$) and SOX content ($R^2 = 0.035$, $F = 4.62$, $p =$
271 0.020).

272 In a second analysis, only the three experimental populations (MG condition, LS condition and
273 RB condition) were analyzed. The multivariate forward selection procedure showed that four
274 of the explanatory variables remained significant in explaining the variance of mRNA
275 expression across individuals: "metal cocktail" ($R^2 = 0.109$, $F = 10.92$, $p = 0.001$), "exposure
276 time" in polynomial form (simple: $R^2 = 0.071$, $F = 8.67$, $p = 0.001$; square: $R^2 = 0.060$, $F = 7.89$, $p =$
277 0.001 ; cube: $R^2 = 0.039$, $F = 4.97$, $p = 0.012$), Pgm-2 X/X ($R^2 = 0.027$, $F = 3.69$, $p = 0.034$) and
278 SOX content ($R^2 = 0.035$, $F = 4.62$, $p = 0.020$). Both variables "experimental condition" and "time
279 exposure" explained 10.92% ($p = 0.001$) and 6.34% ($p = 0.001$) of the variance respectively. The
280 SOX content and the Pgm-1 X/X genotype explained 2.84% ($p = 0.02$) and 2.07% ($p = 0.054$)
281 of the variance respectively. In a third analysis, only the three **field** populations were used and
282 the variable "condition" was excluded. In MG population, the multivariate forward selection
283 procedure showed these four variables remained significant in explaining the variance of

mRNA expression across individuals: Pgm-2 X/X ($R^2=0.20$, $F=5.25$, $p=0.11$), Pgm-1 (100/X) ($R^2=0.095$, $F=3.02$, $p=0.028$), Gpi X/X ($R^2=0.100$, $F=2.87$, $p=0.046$) and SOX content ($R^2=0.067$, $F=2.25$, $p=0.083$). In the two others populations, LS and RB, no variables remained significant.

3.2 Treatment effect: global comparison of experimental conditions versus *field* population

Using SAM analysis, 18 genes were identified as being significantly up-regulated in MG condition, 20 in LS condition and 11 in RB condition compared to the MG population used as the reference and two genes (AF and NuRed1) showed a significant down-regulation only in RB condition. Ten genes (HSP20, Defensin, MT4, MT3, Timp, HSF1, SeGPX, PGM, GluRed and NiFu) are shared between the three conditions (Fig. 1). This group of 10 genes could represent the global response to heavy metal exposure and/or laboratory conditions (e.g., pressure, oxygen, absence of methane and sulfurs).

3.3 Time exposure effect

Genes identified as significantly up- or down-regulated in all comparisons using the SAM procedure are presented in SUPPLEMENTAL MATERIAL Table S4. After 3 days of exposure, individuals from LS condition exhibited a significant up-regulation for 16 genes compared to MG and 16 genes compared to RB, with 9 genes common to both sets. Individuals from MG condition also presented 18 up-regulated genes compared to RB condition. Four genes (NuRed1, MnSOD, AF, and DualOx) showed a clear expression pattern with $LS > MG > RB$. After 7 days of exposure, very few genes were significantly regulated between the three conditions. In LS condition, 5 genes were up-regulated and only GST sigma gene was down-regulated compared to MG. Compared to RB, only the ferritin AF gene was up-regulated in LS while 4 genes (Timp, HSF1, MT3 and GF2) were down-regulated. Four genes (HSF1, MT3,

MT4 and ThioRed3) were down-regulated and 2 genes (GSTsi and AF) were up-regulated in MG versus RB. After 11 days of exposure, the main result was a strong up-regulation observed for 17 genes in LS compared to MG condition and 13 genes compared to RB condition (9 in common). After 15 days of exposure, RB condition is characterized by a global down-regulation which is significant for 12 genes compared to MG and 8 genes compared to LS. Between LS and MG conditions, only 3 genes (GSTsi, AF and GF1) are down-regulated in LS condition.

3.4 Metal cocktail effect

All the genes identified as significantly regulated between sampling times in the three metal cocktails tested are presented in the SUPPLEMENTAL MATERIAL Tables S5 to S7. In MG condition, the main observation is the identification of a set of 15 genes up-regulated at T3, T7, T11 and T15 compared to T0. The comparison between T3, T7 and T11 showed a limited number of genes being regulated, with 3 down-regulated genes (PC, ThioRed2 and GPX4) at T7, T11 and T15 compared to T3 and one up-regulated gene (MT4) at T11 and T15 compared to T7. These results suggest a limited physiological effect of the MG cocktail on individuals during this exposure duration. At T15, a peak of expression is noticed with 16 genes being significant up-regulated compared to T11, 6 of which are related to iron response and 4 genes related to ROS detoxification. In LS condition, we identified a set of 17-19 genes up-regulated at T3, T7, T11, T15 compared to T0, most of them being common with those from MG cocktail. At T3, a strong event of global up-regulation is observed for many genes (12 to 19) compared to T7, T11 and T15, and a second peak of expression is observed at T11 compared to T7 with 17 genes being up-regulated. In RB condition, few genes present a significant differential between T3 and T0 (only the MT3 gene was up-regulated and AF was down-regulated. A first peak of up-regulation (18 genes) was observed at T7 compared to T3 and 20 genes are also up-regulated

between T11 and T3, 14 genes being in common. This down-regulation at T3 highly contrasts with the expression pattern observed in the two other conditions. No significant differences was observed between T7 and T11 except for AF which was down-regulated and at T15, were a significant low regulation (1 to 2 genes up-regulated and 1 to 4 genes down-regulated) compared to T0 or T3 was seen but a strong down-regulation compared to T7 (17 genes) and T11 (6 genes).

3.5 Correlations between gene expression and genotype in experimental population

Out of the 10 enzymatic loci, 7 of them exhibited polymorphism (Pgm-2, Pgm-1, Gpi, Mdh-h, Mdh-b, Odh and Lap) and only Pgm-2 locus was contributing to the overall variance of gene expression associated with metal exposure. For each of the 38 genes, the mean level of mRNA expression was estimated in association with each genotypic class of Pgm-2 for the whole set of mussels exposed to the three metal cocktails. Almost all the 38 genes clearly displayed the same trend: individuals that possessed the genotype 100/100 had a higher mRNA expression than the other two classes (100/X or X/X) and this relation is significant for 13 of them (Fig. 2).

*3.6 Correlations between gene expression and genotype in **field** populations*

For the MG population, out of the 7 polymorphic enzyme loci analyzed, **only Pgm-2, Pgm-1 and Pgi loci** were contributing to the overall variance of gene expression observed. For each of the 38 genes, the mean level of mRNA expression was estimated in association with each genotypic class of the three loci. Individuals having Gpi(X/X) genotype had a significantly higher mRNA expression for 9 of the genes, but no global pattern of expression was observed between genotypes. For the Pgm-1 locus, individuals having Pgm-1(X/X) genotype had higher mRNA expression compared to other genotypes, with a significant relation for 22 of them (Fig.

3) Individuals having Pgm-2(X/X) genotype had a globally lower expression but the relation was not significant. The same analysis conducted on LS and RB populations did not show any significant correlation between mRNA expression and genotypes at the different loci.

3.7 Vent site effect on mRNA expression

The SAM analysis conducted on the three field populations revealed a common set of 11 genes as being significantly up-regulated in MG compare to LS and RB; one was specifically up-regulated in MG compared to LS, 4 genes were specifically up-regulated in MG compared to RB, and 3 genes were significantly up-regulated in LS compare to RB (see SUPPLEMENTAL MATERIAL Table S8). The global expression pattern of all genes is presented in SUPPLEMENTAL MATERIAL Figure S3.

3.8 Correlations between gene expression and symbiont content in experimental population and field population

The methanotrophic and thiotrophic symbiont content of experimental individuals showed a significant decrease according to time of metal exposure compared to T0 (Fig. 4A and B). A similar pattern of depuration was observed in the three conditions but with a surprising higher symbiont content in all individuals from the LS experimental condition compared to MG and RB. Using the multivariate forward selection procedure, only the thiotrophic content was correlated with gene expression. For each of the 38 genes, we calculated the mean level of mRNA expression for each of the four thiotrophic content classes for the whole set of mussels exposed to the three metal cocktails. We observed a clear pattern in which individuals hosting the highest thiotrophic symbiont content had higher mRNA expression. This correlation was highly significant for 17 of the 39 genes (Fig. 5). A similar analysis was performed on the three field populations. In the MG population, in individuals harboring the highest thiotrophic content, we observed a tendency of higher mRNA expression for 17 genes (including the five

metabolic genes PGM, MDH, SDH, MDH and G3PDH), but with no significant correlation. In the LS and RB populations, no significant relation or tendency was found.

4. Discussion

4.1 Global response of *B. azoricus* to metal exposure

In this study, we focused on the synergistic effect of a cocktail of metals present naturally in the hydrothermal environments rather than on response to a specific metal. *B.azoricus* mussels were exposed to a constant concentration of three cocktails that mimic the metallic signature of three contrasted vent sites MG, LS and RB. Using a set of genes involved in different physiological processes that may be directly or indirectly affected by metals, we identified three different patterns of gene regulation related to stress intensity and time of exposure. We hypothesized that the MG cocktail condition was very close to the field condition and so could be considered as a control population.

We first identified a set of 10 genes showing a global up-regulation in all experiments compared to the population collected in field condition. Two main factors, heavy metals and laboratory conditions can explain this result. In hydrothermal vent environments, metal-sulphide complexes buffer the concentration of bioavailable metals before physiological detoxification reactions occur (Edgecomb et al., 2004). Due to the absence of any sulfur in our experiment, we suggest that metal concentrations available in our experiment may be higher than those naturally encountered by mussels. Indeed, in our experiment, mussels are permanently exposed to metals when in field condition, variations in the fluid composition and fluid circulation may occur at small temporal scale. More, mussels being able to move thanks to their foot, we cannot reject the possibility that mussels are able to not be in contact with the fluid for short periods and so escape a constant exposure to metals. Experiments were also conducted at atmospheric

408 pressure and with seawater containing an oxygen level close to 20% versus an average
409 concentration of 10% in field conditions. These differences in the laboratory environment could
410 contribute to partly modify the physiological conditions of the mussel mainly by generating a
411 global oxidative stress. However, the differences in gene regulation patterns observed according
412 to time and metal concentration indicate that the changes in oxygen concentration, pressure
413 effect and absence of sulphur do not explain all the gene expression changes. In the MG
414 condition, we observed a global up-regulation after 15 days that could be explained by the fact
415 that the mussels were continuously exposed to the metallic cocktail contrary to their field
416 conditions where concentrations can fluctuate quickly. This strong heterogeneity in metal
417 exposure is confirmed by previous analyses of metal composition in mussel shell that show
418 large variations in concentrations from shell to shell, and even between mussels collected from
419 the same location (Cravo et al., 2007; 2008). In LS condition, we observed one major event of
420 up-regulation after 3 days of exposure and a second moderate event of up-regulation after 11
421 days of exposure (which could also have happened between day 7 and 11). At these metal
422 concentrations (mainly higher copper and iron content), mussels first show an activation of the
423 transcription of almost all genes which seems sufficient to cope with the metals until day 11
424 when a second event of up-regulation occurred, probably linked to additional needs for
425 detoxifying molecules. In RB condition, we observed a global down-regulation of the genes
426 compared to MG and LS conditions that could correspond to a global depression of
427 transcription, indicating a shutdown of the metabolism as a response to a very toxic event.
428 Similar phenomena of hypometabolism have been observed in many species as a protective
429 response to an abiotic event such as hypoxia (Hochachka et al., 1996; Larade and Storey, 2007)
430 but also during hibernation, torpor, aestivation, anaerobiosis, diapause and anhydrobiosis
431 (Bocharova et al., 1992; Van Breukelen et al., 2002; Storey and Storey, 2004). Such metabolic
432 depression leads to decreased use of ATP in ATP-consuming processes such as transcription

and translation (Larade and Storey, 2007). Heavy metals, particularly cadmium, have also been shown to strongly interfere with energy metabolism in aquatic organisms by inhibiting mitochondrial function and efficiency and by binding to metabolic enzymes, causing reduction in their activity and so act as modulators of the stress response (Casalino et al., 2000, Cannino et al., 2009).

4.2 Does *B. azoricus* possess a specific metal detoxification pathway?

Very few detoxification processes of heavy metals have been described within molluscan species. The major process of metal depuration involves the participation of the metal binding protein called metallothionein (MT) that has been studied at both the molecular and protein level in many mollusc species such as the oysters *Crassostrea gigas* (Tanguy et al., 2001; Tanguy and Moraga, 2001; Boutet et al., 2002) and *Crassostrea virginica* (Unger et al., 1991), the mussels *Perna viridis* (Khoo & Patel, 1999), *Mytilus edulis* (Barsyte et al., 1999) and *B. azoricus* (Hardivillier et al., 2006). MTs are also inducible by many biotic and abiotic factors such as parasite infection (Desclaux-Marchand et al., 2007), hypoxia (Romero-Isart and Vasak, 2002; David et al., 2005), radioactive discharge (Farcy et al., 2007) and temperature changes (Farcy et al., 2009). Other mechanisms involving the presence of low molecular weight ligands have also been demonstrated in the oyster *Ostrea edulis* (Frazier & George 1983) and the production of mucus as a chelator of metals in *B. azoricus* has been suggested (Kadar et al., 2005). In previous work done on *B. azoricus* in response to copper (Company et al., 2008) or cadmium (Company et al., 2010), MTs did not appear to be the main metal detoxification mechanism and similar results have been observed in *B. thermophilus* after short exposure to different metals (Hardivillier et al., 2006). MT levels in *Bathymodiolus* sp. remained relatively high and that the amount of metal bound to this protein was relatively low which suggested a limited involvement of MTs in metal detoxification (Hardivillier et al., 2004). In our study, the

two isoforms studied (named MT3 and MT4) are characterized by many amino acid differences in their sequence compare to the MT10 and MT20 isoforms described by Hardvillier et al. (2006). Both MT3 and MT4 displayed a significant up-regulation in our experiments compared to the field population but no strong pattern of regulation according to time exposure in the three conditions. Our results suggest that these two MT isoforms could play a role in response to heavy metal depuration and/or ROS detoxification since MT also acts as an effective cell protector against oxidative stress (Viarengo et al.,1999; Cavaletto et al., 2002). Complementary studies are needed to better address the question about the exact role of the MT family in metal regulation in *B. azoricus*.

In biochemical pathways described for other species, phytochelatins play a role in metal binding. Phytochelatins (PCs) are enzymatically synthesized peptides known to be high-affinity chelators of metals and are involved in the detoxification of Cd²⁺ and arsenate in plants (Hall, 2002; Clemens et al., 1999). They were first described in *Schizosaccharomyces pombe* (Kondo et al., 1984), then in plants and algae (Pal and Rai, 2010) and recently in some annelid species such as *Eisenia fetida* (Brulle et al., 2008), *Caenorhabditis elegans* (Clemens et al.,2001; Vatamaniuk et al., 2001), but never in molluscs. Genome analyses also indicate that humans and other mammals do not have genes for PCs. In *E. fetida*, Brulle et al. (2008) hypothesized that PCs could have a immediate and essential role at low cadmium concentrations while MTs could be involved at higher concentrations. *B. azoricus* is the first mollusc species in which a gene encoding phytochelatin synthase has been identified and studied at a transcriptional level. Our results did not reveal any significant regulation of phytochelatin synthase in the three conditions tested and only a limited significant up-regulation was observed in LS conditions at T7 and T11 and in RB conditions at T7, suggesting a potential but limited involvement of PCs in metal detoxification, at least in gill tissue.

4.3 Involvement of antioxidant pathway in *B. azoricus* response to heavy metal

Regulated production of free radicals in organisms and maintenance of “redox homeostasis” are essential for the physiological health of organisms (Ames et al., 1993). Production of ROS by heavy metals results in the depletion of cell antioxidant defense systems, destabilization of cell membranes and DNA damage; this has been reported in many species (Franco et al., 1995; Lushchak, 2011). The antioxidant system in aquatic animals is comprised of both low molecular mass molecules such as reduced glutathione, ascorbic acid, carotenoids, retinol and tocopherol (vitamin E), and high molecular mass molecules such as superoxide dismutases, catalases, Se-dependent glutathione peroxidases, DT diaphorase, glutathione reductase and glucose-6-phosphate dehydrogenase (Livingstone, 2001). In our study, we focused on the expression of several of these enzyme families that possess different isoforms. Our results revealed different expression patterns for the different isoforms according to the conditions tested and the sampling time, demonstrating a complex regulation. We identified several members of the GPX, SeGPx and GR families, in particular, as being strongly up-regulated in all experimental individuals compared to the natural population. Similar observations in *M. edulis* showed induction of Se-GPx by selenium (Trevisan et al., 2011) and in *M. galloprovincialis* in response to copper (Regoli and Principato, 1995). SOD and GPx activities also increased in *B. azoricus* exposed to cadmium (Company et al., 2004). Also, Bebianno et al. (2005) found a distinct pattern for antioxidant enzyme systems in populations of MG, LS and RB with different regulation in GPx and catalase suggesting that resistance of mussels to metal toxicity at these vent fields decreases from the less heavy metal-impacted site (MG) to the most impacted (RB). Despite the absence of a clear pattern in the mRNA expression of antioxidant enzymes in our experiments, they were regulated, confirming the presence of ROS in our experimental conditions. Our results also agree with other studies showing contrasted regulation according

507 to species, pollutant concentration and season (Vidal-Liñán, 2008; Verlecar et al., 2008;
508 Bebianno et al., 2005).

509 Glutathione S-transferases (GSTs) are comprised of dimeric enzymatic proteins mainly
510 involved in the detoxification of endogenous and xenobiotic compounds and in oxidative stress
511 resistance (Hayes and Pulford, 1995). In our experiment, we also observed an up-regulation of
512 the three GST isoforms studied in response to metal exposure with various pattern of regulation
513 according to the condition and the sampling time, with a surprising strong inhibition of a GST
514 sigma in MG condition at all sampling times. In molluscs, GST enzyme activity has been
515 shown to be either inducible or unchanged by exposure to various xenobiotics (Petushok et al.,
516 2002; Boutet et al., 2004, Vidal et al., 2010). Canesi et al (1999) showed that copper was able
517 to induce a decrease in glutathione content that could be mainly related to a stimulation of GST
518 activity, particularly in mussel gills. In the intertidal copepod *Tigriopus japonicus*, a study
519 conducted on both the expression and kinetic properties of GST sigma in response to pro-
520 oxidant chemicals suggested a putative role against oxidative stress (Lee et al., 2007). Analysis
521 of the co-expression of various GSTs in *Chironomus riparius* in response to cadmium and silver
522 showed a pattern of up- or down-regulations to varying levels according to the concentration
523 and duration of exposure (Nair and Choi, 2010), an high up-regulation being observed for a
524 particular isoform from the GST sigma family. When considering the regulation of all
525 antioxidant enzymes analyzed in this study, we did not observe specific or unusual pattern of
526 regulation in *B. azoricus* and our results are in concordance with what has been observed in
527 other species in comparable experiments using heavy metals (single or complex mixtures),
528 suggesting that hydrothermal vent mussels react as their coastal homologs do.

529 In response to oxidative stress, ferritins are known to play a key role in sequestering iron which
530 participates as free ferrous iron (Fe^{2+}) in the Fenton reaction catalyzing hydroxyl radical
531 formation and then decreasing ROS formation (Storey, 1996). In this study, we analyzed the

expression of four distinct ferritin isoforms and observed a different pattern of regulation for each of them. The GF1 isoform showed a strong up-regulation in the three experimental conditions compared to the natural population, especially the MG condition, confirming its involvement in metal response. In contrast, the isoform GF2 showed a clear down-regulation in the three conditions compared to the natural population. The Yolk ferritin isoform showed a higher expression in MG, a lower expression in LS, and a very low expression in RB condition, highlighting an inhibitory effect of heavy metals, particularly iron, on this specific isoform. Finally, the AF isoform also exhibited a very surprising strong up-regulation in MG conditions compared to the natural population and a gradient of down regulation in LS and RB conditions indicating, also, an inhibition of this isoform to increasing heavy metals concentration. Interestingly, we observed that three of the four isoforms were down-regulated in the presence of increasing iron concentration. Ferritin up-regulation has been described in response to situations of anoxia/ischemia such as in snail (Larade and Storey, 2004), and frog (Storey, 2004). In the mollusc *Tegillarca granosa*, a ferritin subunit similar to GF1 was found to be up-regulated after 24h of exposure to iron then down-regulated after 48h, suggesting a complex pattern of expression for ferritin (Jin et al., 2011). Typical yolk protein precursors are developmentally regulated proteins and show a tissue- and sex-specific expression (Wahli, 1988). Yolk ferritins are also able to store iron together with some phosphate (Bottke et al., 1982) and the presence of the ferroxidase center in the yolk ferritin suggests a possible ability of this protein to accelerate iron oxidation. Interpretation of the particular regulation of the AF isoform in our study was difficult because of the lack of literature available on this particular ferritin isoform. Complementary studies on the ferritin family in the hydrothermal mussels are needed to better understand their regulation, particularly in response to iron.

4.4 Adaptability of *B. azoricus* to heavy metal: plasticity versus adaptive selection?

557 In order to survive in heterogeneous habitats, organisms can adopt different evolutionary
558 strategies (Scheiner and Lyman, 1989) such as the fixation of a generalist genotype able to cope
559 over a broad range of environmental parameters or to adapt to a specific environmental
560 parameter by heritable variation in a particular trait. Phenotypic plasticity classically occurs
561 when the phenotype expressed by a given genotype is modified by changes in environmental
562 parameters and allows adjustments at both the biochemical and molecular levels, such as the
563 induction of specific genes which limit negative effects induced by contaminants (Pigliucci,
564 2001, Bijlsma and Loeschke, 2005; Morgan et al., 2007). In the MAR, *B. azoricus* species
565 colonized different vent sites, including MG, LS and RB, that strongly differ by many
566 environmental parameters, including gas and heavy metals concentrations, temperature and
567 pressure (Douville et al., 2002, Charlou et al., 2002), suggesting a potential high plasticity in
568 this species. In this study, we explored the potential correlation between genotype and
569 phenotype (i.e., gene expression) to evidence a putative adaptive strategy/selection in this
570 species. In our metal exposure experiments, mussels exhibited different patterns of gene
571 expression according to both time exposure and metal concentration with a strong decrease of
572 gene expression in the presence of the highest metal concentrations. Interestingly, when
573 comparing gene expression in the three field populations, we also observed that mussels living
574 at the RB vent site exhibited a globally lower gene expression. Using the same approach,
575 differences in transcriptome regulation have previously been observed in this population for
576 another set of genes (Boutet et al., 2011). The down-regulation observed in mussels from RB
577 could reflect a globally lower metabolism as a response to the particularly stressful conditions.
578 Overall however our results suggest, despite the environmental heterogeneity of the different
579 vents, a low plasticity for this species. We suggest that adults and/or settled individuals are not
580 any more able to cope with abrupt changes in the metallic composition of the fluid, and thus
581 not very plastic (stressed) to environmental changes. Allozymic studies in numerous species

support selection on specific genotypes in response to anthropogenic xenobiotics, for example in crustaceans (Nevo et al., 1981, Nevo et al., 1983, Nevo et al., 1984), gastropods (Lavie and Nevo, 1986; DeNicola et al., 1992), and molluscs (Tanguy et al., 1999; Moraga and Tanguy, 2000, Ma et al., 2000). Among all allozymic markers investigated, the Pgm locus remains the most studied. Due to its key position in the glycolytic pathway branch and the importance of glycogen in the energy metabolism of bivalves, variations in Pgm activity contribute to the regulation of carbohydrate storage and exert significant physiological effects (Gabbot ,1983; de Zwaan, 1983). Correlations between Pgm polymorphism, glycogen content and Pgm activity have been shown in many species (Leigh and Brown, 1977; Fucci et al., 1979; Sugie et al., 1988). In our study, we found that under experimental conditions, individuals who possess Pgm-2 genotypes associated with allele 100 (as homozygote or heterozygote associated with any other allele) displayed a globally higher gene expression in response to metal exposure. In contrast, the same genotypes for Pgm-2 showed the opposite pattern in mussels from the natural MG population (even not significant). We also showed that in the MG population, individuals having Pgm-1(X/X) genotype presented a clear pattern of higher mRNA expression compared to other genotypes. Similar significant correlation has not been observed in the other two populations. These results are in accordance with those found by Boutet et al (2009) who evidenced that individuals carrying Pgm-1(X/X) genotypes were more able to cope with increasing temperature and were associated with a higher global transcriptome expression. Comparison of the different genotype classes distributed among the three populations did not show significant differences in gene expression though differences in specific allele frequencies has been observed (data not shown). These results suggest that the two Pgm loci are not affected the same way under experimental conditions compared to the field condition and that some genotypes seem to be able to perform better than others under different conditions.

We also noticed that the presence of heavy metals may not be the main selective stress in the MG population since Pgm-1 genotypes were not associated with higher global mRNA expression in our experimental conditions. Gillespie and Guttman (1993) suggested that while the selection of tolerant genotypes may appear beneficial at first, it may result in increased susceptibility to other selection pressures due to reduced diversity. The differences in enzymatic kinetic, metabolic efficiency and the sensibility to the metallic ions of the different allozymic alleles of Pgm could modify the fitness under stress conditions in response to increasing energetic demand (Nevo, 2001). This processes of activation and inhibition of Pgm by chelating agents and metallic ions has also been widely studied (Milstein, 1961a, b, c; Hashimoto et al., 1966). In the clam *Sphaerium novaezelandiae*, individuals carrying a specific Pgm genotype showed a better resilience in response to heavy metal challenge (Phillips and Hickey, 2010). We also showed in the natural population that individuals having Gpi(X/X) genotype showed a globally higher mRNA expression suggesting that selection could operate on some genes involved in energy metabolism in *B. azoricus*. Clearly, the biochemistry of Pgm and Pgi variants in the hydrothermal mussel need further investigation. The advantage of the work presented here is that we now have a good prediction that some allozymic variants should be metabolically more efficient under heavy metal exposure.

4.5 Does symbiont content influence transcriptomic response of *B. azoricus* to heavy metal stress?

We investigated the potential influence of the symbiont content on the mussel transcriptome regulation in both experimental and field populations. Previous studies using stable isotopes and histology demonstrated that nutrient transfer from symbionts to mussel tissues was the result of the digestion of symbionts rather than nutrient translocation (Fisher and Childress, 1992). Boutet et al. (2009) showed that part of the *B. azoricus* transcriptome expression was

modulated by both environmental parameters and symbiont content, but they did not looked for correlation between global gene expression and symbiont content. Symbionts represent an important nutrition source. We can assume that mussels harboring a higher symbiont content also possess a higher energy stock available to provide the ATP needed for their metabolism, such as for transcription and translation. In our experimental populations, we did not provide any source of sulfur or methane to the mussels and observed a decrease in symbiont content over time with a surprising more limited decrease in mussels exposed to the LS conditions. Analysis of the relation between thiotrophic symbiont content and gene expression in experimental samples showed a significant correlation between symbiont content and a higher mRNA expression. The same analysis performed according to methanotrophic symbiont content did not reveal any expression tendency. The same analysis was conducted on the three field populations and only the MG population showed a similar but not significant tendency of higher mRNA expression. Notably, the thiotrophic symbiont content was lower in the LS and RB populations. We also noticed that all metabolic gene comparisons show the same relationship to symbiont content. All these results support the idea of a potential link between symbiont content, available energy and metabolic activity. The present study also showed that in stress condition, the quantity of symbiont available to the mussel can be an important biological factor able to modulate and influence the transcriptomic response. Further studies are needed to confirm these results.

5. Conclusion

We assume that despite the fact that some genotypes seem better adapted to high heavy metals concentrations, they do not dominate in field populations because of the frequent spatial and temporal variations in the metals concentrations in and around the vent fluids and the ability of the mussel to escape stressful conditions by using their foot to move. Moreover, heavy metals

are not the only environmental parameters able to strongly influence hydrothermal mussel populations. We also demonstrated that the use of gene expression profiling based on a panel of genes involved in different physiological functions allow to provide a better view of the regulations that occur at a cellular level than the study of a single molecular marker, even if considered as specific to metal response. We also discovered a distinct pattern of response according to metal concentration and exposure time and highlighted the importance of symbiont content in the ability of *B. azoricus* to increase its transcription in response to stress. Clearly, complementary studies on functional differences (i.e., the effect of different metallic ions on enzyme activity) between genotypes at the Pgm-1 locus and the other metabolic loci using bivalent cations as cofactors are also needed.

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Legends of Figures and Tables

Figure 1: Venn diagram illustrating the relationships between the significantly regulated genes among the three metal conditions tested (Menez Gwen condition, Lucky Strike condition and Rainbow condition).

Figure 2: Gene mRNA expression (standardized mean ΔCt) as a function of Pgm-2 genotypes (homozygotes 100/100, heterozygotes 100/X and other homozygotes and heterozygotes X/X) of *B. azoricus* mussels exposed to heavy metals. Only the 13 genes showing a significant p-value (Wilcoxon-Mann-Whitney test) are presented. Standard deviation bars have not been added for more clarity.

Figure 3: Gene mRNA expression (standardized mean ΔCt) as a function of Pgm-1 genotypes (homozygotes 100/100, heterozygotes 100/X and other homozygotes and heterozygotes X/X) of *B. azoricus* mussels in field Menez Gwen (MG) population. Only the 22 genes showing a significant p-value (Wilcoxon-Mann-Whitney test) are presented. Standard deviation bars have not been added for more clarity.

Figure 4: Content in methanogen (MOX) and sulfur-oxidizing (SOX) bacteria in *B. azoricus* mussel exposed to metal in the three conditions at T0 (field population), T3, T7, T11 and T15.

Figure 5: Gene mRNA expression (standardized mean ΔCt) as a function of SOX symbiont content in experimental *B. azoricus* mussel population. Only the 17 genes showing a significant p-value (Wilcoxon-Mann-Whitney test) are presented. Standard deviation bars have not been added for more clarity.

1050 **TABLES**

1051 **Table 1** : Concentrations of each metal used in the three cocktails for the metal exposure
1052 experiments.

	MG	LS	RB
Cu ²⁺ (µM)	190	158	14220
Mn ²⁺ (µM)	6	27.3	21.8
Cd ²⁺ (µM)	8.9	35	124
Fe ²⁺ (µM)	2.5	36	254
Pb ²⁺ (nM)	0.38	0.57	1.42
Zn ²⁺ (µM)	0.3	3	15

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