

## ***Interactive comment on “Finding immune gene expression differences induced by marine bacterial pathogens in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*” by E. Martins et al.***

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Received and published: 30 May 2013

We thank Referee #1 and #2 for taking the time to review our discussion paper. We understand the paper in its present form, reveals some confusing interpretations partially due to an apparent overlap between the “Results” and “Discussion” sections of the paper and we will take this into consideration in a revised version of the manuscript where results on differential gene expression will be discussed and will be put into the context of innate immunity in marine bivalves. The aim of our study was to address the problem of microbial threat and the need for immunity that must exist in the

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deep-sea mussels as for their congeners living in estuarine environments and them too, subjected to microbial threat. We have focused our gene expression analyses on “canonical” immune genes already evidenced in our and others previous studies more so when new sequencing techniques were made available such as next generation sequencing methods, high-throughput deep sequencing, applied to de novo transcriptome sequencing (454 and illumina). Our transcriptome sequencing, published in 2010, was then and is today still, a work of reference and one of pioneering nature, revealing tens of thousands cDNAs from the deep-sea vent mussels providing a source of data to discover and identify new genes of which several putative immune genes were revealed and their physical nature put in evidence by means of qPCR. Most of the papers that were subsequently published on marine bivalves, after our first transcriptome sequencing project (Bettencourt et al. BMC Genomics, 2010), dealt with the same high-throughput deep sequencing approach and were mostly focused on sequence analyses and functional annotations (Venier et al. BMC Genomics, 2011; Moreira et al. PlosOne, 2011) and really not addressing on experimental issues and physiological experiments. Even the published work by Gagnière et al. BMC Genomics, 2010 and Nyholm et al. PlosOne, 2012, mentioned by the referees, are dealing with high-throughput transcriptomics and real-time PCR (qPCR) results with very little physiological context, as far as the gene expression of immune genes following standard infection experiments is concerned. Thus, we regard our work as contributing for the understanding of immune gene expression in the deep-sea vent mussel *Bathymodiolus azoricus*, in ways never reported before with an additional approach that includes a multivariate statistical analyses to better illustrate how vent mussels respond to bacterial infections while up-regulating and down-regulating immune genes during bacterial infections and to provide evidence to whether or not their innate immune system is capable of discriminating different *Vibrio* strains. We were compelled to use representatives of the four groups of functional genes that were described in our important paper on *Bathymodiolus azoricus* gill transcriptome and thus saw an opportunity to test “recognition genes”, “signaling genes”, “transcription genes” and “effector genes” while developing

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experimental infections with different strains of *Vibrio*. This would not only demonstrate the inducibility and responsiveness of immune genes but also would bear the possibility of showing the animal's discriminatory capabilities to distinguish distinct *Vibrio* strains. The three *Vibrio* strains *V. alginolyticus*, *V. splendidus*, *V. anguillarum* were obtained as a gift from Prof Figueras, IIM, Vigo, himself involved in the study of innate immunity in the shallow water mussel *Mytilus galloprovincialis*. Experimental infections carried out by *Flavobacterium* were somewhat unexpected as this bacterium was used as a non-pathogenic bacterium. It was originally isolated from one of our seawater screens and identified as *Flavobacterium* on the basis of a PCR test using primers targeting the 16S ribosomal gene. *Flavobacterium*. The remaining *Vibrio* strains were obtained as pure bacterial isolates from Prof Figueras. The cDNA sequences based on which the gene expression studies were carried out were obtained from our previous transcriptome sequencing achievement in 2010. These sequences have been annotated and their homologies circumscribed to putative immune genes in both mammals and invertebrates. This is the case for the "recognition and signaling genes" PGRP and Toll-like receptor both identified in high-throughput sequencing studies in clams and the deep-sea vent tubeworm (Moreira et al., Plos1, 2012 and Nyholm et al., Plos1, 2012, respectively). qPCR experiments conducted with the vent worm *Ridgeia piscesae* indicated increased immune gene fold expression in animals maintained under in situ conditions denoting their putative role in protecting the host against microbial challenges. Similarly, the work we presented showed markedly higher expression in infections experiments carried out for 12h and 24h as compared to individuals not challenged with *Vibrio* and *Flavobacterium*. The pathogenicity of *Flavobacterium* was not immediately noticed until experiments were performed and differential gene expression addressed for several immune gene targets. As it turns out, the gene targets tested showed differences in expression over infection time and nature of the bacterium used and thus pointing at a dual modulation of gene expression and immune discriminatory capabilities of the host vent mussel. Similarly, Lorget et al., Plos1, 2011, revealed by means of quantitative PCR analysis pathogen-specific signatures in oyster gene regulation and

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identified a number of up-regulated genes related to cellular and immune function that characterize the oyster capability to survive pathogenic *Vibrio* infections. Among the targets, the C-type lectin 2, L-rhamnose-binding lectin and the metallothionein genes were shown to be up-regulated upon *Vibrio* infections much in concordance to our results. In another work published by Xue et al. BMC Evolutionary Biology, 2010, evidence for the involvement of a new lysozyme in the oyster *Crassostrea virginica* was reported which also agrees with our finding that lysozyme is involved in our deep-sea vent mussel model given the up-regulation of this gene in *Flavobacterium* infections.

In summary, we analyzed the effect of bacterial infections in our mussel species while focusing our experiments on infections conducted at atmospheric pressure and not simulating "natural environment" found at hydrothermal vents. In this way, our deep-sea vent mussel became a "shallow-water" animal model while keeping its deep-sea vent genomic "make-up" and transcriptional capabilities (responsiveness), in our land-based aquarium system, without the characteristic hydrostatic pressure levels found at the deep-sea vent environments. Our results concur with other's published work, in the field of innate immunity, combining high-throughput and transcriptome sequencing analyses with experimental data obtained by means of quantitative PCR and pointing at the up-regulation of canonical genes involved in innate immunity.

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Interactive comment on Biogeosciences Discuss., 10, 2675, 2013.

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