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Seasonal variations of gene expression biomarkers in *Mytilus galloprovincialis* cultured populations: Temperature, oxidative stress and reproductive cycle as major modulators

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HIGHLIGHTS

- Gene expression biomarkers were evaluated in cultured populations of mussels.
- Study carried out in a relatively unpolluted area during a complete annual cycle.
- All biomarkers showed seasonal variations.
- Temperature and oxidative stress postulated as major abiotic modulators.
- Reproductive status is believed to be the major biotic factor.

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ABSTRACT

The blue mussel *Mytilus galloprovincialis* has been used as monitoring organism in many biomonitoring programs because of its broad distribution in South European sea waters and its physiological characteristics. Different pollution-stress biomarkers, including gene expression biomarkers, have been developed to determine its physiological response to the presence of different pollutants. However, the existing information about basal expression profiles is very limited, as very few biomarker-based studies were designed to reflect the natural seasonal variations. In the present study, we analyzed the natural expression patterns of several genes commonly used in biomonitoring, namely ferritin, metallothionein, cytochrome P450, glutathione S-transferase, heat shock protein and the kinase responsive to stress KRS, during an annual life cycle. Analysis of mantle-gonad samples of cultured populations of *M. galloprovincialis* from the Delta del Ebro (North East Spain) showed natural seasonal variability of these biomarkers, pointing to temperature and oxidative stress as major abiotic modulators. In turn, the reproductive cycle, a process that can be tracked by VCLM7 expression, and known to be influenced by temperature, seems to be the major biotic factor involved in seasonality. Our results illustrate the influence of environmental factors in the physiology of mussels through their annual cycle, a crucial information for the correct interpretation of responses under stress conditions.

1. Introduction

Mussels of the genus *Mytilus* are among the commonest marine mollusks and constitute an important element in the ecology of coastal waters. Three taxa or forms of the genus *Mytilus* inhabit along the European coast, two of them being predominant: *Mytilus edulis*, which occupies temperate to cold areas along European Atlantic coasts, and *Mytilus galloprovincialis*, a warm-water form that occurs in the Mediterranean and extends northward to the coast of France and the United Kingdom. Apart from their ecological importance and economic value, mussel species have gained an important role as bioindicators because they are sessile and filter feeders, which results in the accumulation of

Abbreviations: actin, β-actin; AU, arbitrary units; bp, base pair; CYP, cytochrome P450 family members; DO, dissolved oxygen; Fer, ferritin; KRS, kinase responsive to stress; GSTpi1, glutathione S-transferase pi1; HKG, housekeeping gene; HSE, heat shock element; HSP70, heat shock protein 70; Imm, immature; L13A, ribosomal protein L13A; L19, ribosomal protein L19a; MT, metallothionein family members; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PC1,2,3, principal component 1,2,3; S3, ribosomal protein S3; Temp, temperature; TG, tested gene; VCLM7, vitelline coat lysin M7.

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contaminants in their tissues. They also provide the opportunity of allowing comparison between cultivated and wild populations, making them especially interesting for biomonitoring (Marigómez et al., 2013; Serafim et al., 2011). As consequence, mussels have been commonly used as monitoring organisms in several environmental studies, and a battery of pollution-stress biomarkers have been developed, including biochemical, cytological, genetic and gene expression-based assays (Tanguy et al., 2008; Porte et al., 2006; Saavedra and Bachere, 2006; Venier et al., 2006, 2003).

Among the gene biomarkers used in biomonitoring are those relating to oxidative stress and metal and organic contamination. However, although some of them have been successfully applied in several environmental studies (Serafim et al., 2011; Bebianno et al., 2007), there are still some limitations that may compromise their validity when using mussels as sentinel organisms (Forbes et al., 2006). One of the major drawbacks refers to the current limited knowledge of the invertebrate physiology, which makes difficult to distinguish between natural physiological responses from those considered as stressful. In addition, the fact that mussels colonize intertidal environments influenced by daily and annual cycles may increase natural variability and hinder interpretation of results (Gracey et al., 2008). So far, most of the environmental studies using gene biomarkers have been focused on determining causation between exposure to pollutants and changes in gene expression, and only very few have considered natural seasonal variations as gene expression modulators (Schmidt et al., 2013; Banni et al., 2011). This lack of information is particularly relevant since a number of genes have been demonstrated to respond to several biotic and abiotic factors (Schmidt et al., 2013; Banni et al., 2011; Luedeking and Koehler, 2004). In this respect, the study of populations located in unpolluted areas is postulated as a mandatory task to fully understand the natural gene expression profiles and, in turn, to interpret correctly responses under stress conditions.

In the present study, we analyzed the natural expression patterns of several gene biomarkers during the course of an annual life cycle in the mantle-gonad of cultured populations of *M. galloprovincialis*; β-actin (actin) as structural protein often used as reference gene, vitelline coat lysin M7 (VCLM7) as male-specific maturation stage biomarker, ferritin (Fer) as indicator for anoxia, metallothionein (MT-10) for metal contamination, cytochrome P450 (CYP4Y1) and glutathione S-transferase (GSTpi1) for oxidative stress, and heat shock protein (HSP70) and kinase responsive to stress (KRS) as general stress biomakers. Because the study was carried out with samples from Ebro Delta, a well-known relatively unpolluted area (Sole et al., 2000, 1994), the observed seasonal variations are considered to be presumably associated to natural expression patterns.

2. Materials and methods

2.1. Physical and chemical data

Temperature data at Alfacs bay (Ebro Delta, NW Mediterranean Sea) at the time of mussel collection was obtained from an HOBO Water Temperature Pro v2 Data Logger (ONSET Computer Corporation), deployed at the mussel farm. Missing values (two samplings, in May and June 2006) were parameterized using the corresponding data from the Ebro River at the Tortosa station, just at the beginning of the Delta (data from the Confederación Hidrografica del Ebro, CHE, www.chebro.es). Changes on the salinity levels at the Delta bays were calculated from the historical record (1990–2004, Llebot et al., 2011).

2.2. Mussel sampling

A commercial culture of *M. galloprovincialis* from Alfacs bay (Ebro Delta) was monitored during an annual cycle (March 2005–July 2006) in order to study growth, mortality and reproduction. To analyze the expression of the genes, four to six individuals were sampled monthly (64 mussels in total) and their sex and maturation stage (mature and immature) checked under a dissection microscope. Total length and weight of the specimens were measured. A fragment of mantle tissue (about 100 mg), which includes the gonad when the mussel is mature, was dissected, frozen in liquid nitrogen and stored at −80 °C until processing.

2.3. RNA preparation and qRT-PCR analysis

Total RNA was extracted from individual frozen mantles using Trizol (Sigma-Aldrich, Buchs SG, Switzerland) as previously described (Garcia-Reyero et al., 2004). Total RNA concentration was estimated by spectrophotometric absorption at 260 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Delaware, DE). RNA integrity was checked in a Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA). One to five micrograms of RNA per sample was afterwards treated with DNase I (F. Hoffmann-La Roche Ltd., Basel, Switzerland), retrotranscribed to cDNA (Omniscript, Qiagen, Valencia, CA) and stored at −20 °C. Specific transcripts were quantified by Real Time PCR in a Abi Prism 7000 SDS (Applied Biosystems, Foster City, CA) using the SYBR Green chemistry (Power SYBR Green PCR Master Mix, Applied Biosystems). Primer sequences and Gene Bank references are detailed in Table 1. Preliminary results showed that the variability among individuals may act as confounding factor in some cases. Consequently, samples were considered individually rather than in pools based on month or maturation status.

2.4. Data analysis and statistics

Relative expression values were calculated according to Eq. (1) using threshold cycle (Ct) values from triplicate assays as previously described (Pfaffl, 2001):

$$\frac{\mathrm{mRNA_{TG}}}{\mathrm{mRNA_{HGK}}} = \frac{E_{HGK}^{(Ct_{HGK})}}{E_{TG}^{(Ct_{TG})}} \times 1000 \qquad (1)$$

in which TG and HKG indicate tested and reference genes, respectively. Evaluation of the suitability of different reference genes was tested by the BestKeeper programm (Pfaffl et al., 2004). PCR efficiency values for reference and tested genes, E_{HGK} and E_{TG}, were calculated as described (Pfaffl, 2001), both of them being close to 100%. The sequence of amplified PCR products (amplicons) was confirmed by DNA sequencing in Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). Amplified sequences were compared to the corresponding references in GenBank (Table 1) by the BLAST algorithm at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

All statistics, including Partial Correlations and Principal Component Analysis or PCA, were performed using the SPSS 19 (SPSS Inc. 2002) package. The rationale of using PCA was to group the data set into few variables that could explain most of the variance associated to the samples. Data from qRT-PCR were analyzed using ΔCp values (Cp reference − Cp target), as this parameter follows a normal distribution, assessed by Kolmogorov–Smirnov test. These values were used to calculate mRNA levels, expressed as mRNA copies of target gene per 1000 copies of the reference gene mRNA (‰ of reference gene, $1000 \times 2^{\Delta Cp}$). Statistical comparison of mean values was done using one way analysis of variance (ANOVA) plus Tukey's tests. Correlograms were drawn using the corrgram v.15 library in R.

3. Results

3.1. Determination of reference genes for qRT-PCR analysis in the developing mussel gonad

To determine the suitability of different candidates for reference genes in *Mytilus*, we analyzed Ct values for β-actin and the three ribosomal proteins S3, L13A and L19 in16 individuals (4 males, 4 females