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Interactive effects of nutrition, reproductive state and pollution on molecular stress responses of mussels, *Mytilus galloprovincialis* Lamarck, 1819

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ABSTRACT

Marine bivalves including mussels *Mytilus galloprovincialis* are commonly used as sentinels for pollution monitoring and ecosystem health assessment in the coastal zones. Use of biomarkers to assess the pollution effects assumes that the effects of pollutants on the biomarkers exceed the natural background variability; yet this assumption has rarely been tested. We exposed mussels at different reproductive stages and nutritive states to two concentrations of a polycyclic aromatic hydrocarbon (fluoranthene, 3 and 60 μg L^{-1}) for three weeks. Expression levels of the molecular biomarkers related to the detoxification and general stress response [cytochrome P450 oxidase (CYP450), glutathione S-transferases (GST-α; GST-S1; GST-S2), the multixenobiotic resistance protein P-glycoprotein (PgP), metallothioneins (MT10 and MT20), heat shock proteins (HSP22, HSP70-2; HSP70-3; HSP70-4), as well as mRNA expression of two reproduction-related genes, vitellogenin (Vitel) and vitelline coat lysin M7 (VCLM7)] were measured. The mussels' nutrition and reproductive state affected the baseline mRNA levels of molecular biomarkers and modulated the transcriptional responses of biomarker genes to the pollutant exposure. Thus, mussel physiological state could act as a confounding factor in the evaluation of the response of pollution through molecular biomarkers. The biomarker baseline levels must be determined across a range of physiological states to enable the use of biomarkers in monitoring programs.

1. Introduction

Mussels are commonly used as sentinel organisms in integrated monitoring programs due to their sedentary nature, wide-spread geographical distribution, capacity to accumulate contaminants and easy sampling (Kimbrough et al., 2008; Sericano et al., 2014). Biological responses (biomarkers) of mussels such as antioxidant enzymes, metallothioneins or physiological parameters are regarded as sensitive early warning signals to assess the environmental quality of coastal areas (Cajaraville et al., 2000; Lam, 2009) and have been incorporated by different international pollution monitoring programs (OSPAR Commission, 2012; ICES, 2013). However, the natural variability of environmental conditions between areas makes it difficult to interpret the data obtained in monitoring programs and establish the links between chemical pollution and biological effects.

Earlier research on large-scale monitoring programs showed a strong effect of the physiological condition of mussels on some biochemical and physiological biomarkers (Albentosa et al., 2012; Bellas et al., 2014; González-Fernández et al., 2015a). In these studies, mussel condition (CI), which is an indicative of the nutritive and reproductive states, significantly affected biomarker responses, showing an inverse relationship between antioxidant activities and mussel CI. Laboratory studies confirmed these field observations and showed that under controlled conditions mussel nutrition (González-Fernández et al., 2016a, 2015b) and reproduction (González-fernández et al., 2016b) can modulate responses of the biochemical and physiological biomarkers to pollutant exposure. This natural background of biomarker variability complicates the interpretation of biomarker responses in field studies. To

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overcome this challenge, multi-biomarker approaches targeting different levels of biological organization have been recommended (Hagger et al., 2008).

Transcriptomic biomarkers have emerged as a result of recent progress in bivalve genomics (Saavedra and Bachère, 2006) and provide novel mechanistic information on the effects of multiple stressors such as complex mixtures of pollutants, temperature and nutritive stress on the organisms, which cannot be obtained by classical biochemical and physiological approaches (Bourlat et al., 2013). Transcriptomic changes are among the earliest signals of stress and could forecast changes at higher levels of biological organization, which makes them useful for monitoring of environmental pollution (Lacroix et al., 2014). Marine mussels are commonly used in environmental monitoring programs such as "Mussel Watch", but to date, there are few studies that use transcriptomics biomarkers to assess pollutant effects in the field (Rola et al., 2012; Coimbra Rola et al., 2012; Núñez-Acuña et al., 2012; Lacroix et al., 2014). To some degree, this reflects a challenge in distinguishing the pollutant signatures from the background variation in mRNA levels caused by other stressors and physiological state of the organisms (Venier et al., 2006). Therefore, studies are urgently needed to assess the effects of mussels' physiological state on expression of the potential transcriptomic biomarkers under the controlled laboratory conditions in sentinel species such as mussels.

The aims of our present work were to determine the transcriptional responses of genes involved in pollutant detoxification and general stress response to environmentally relevant levels of polycyclic aromatic hydrocarbons (PAHs), identify the molecular biomarkers that can serve as potential early signals of PAH-induced stress and determine whether the molecular signature of PAH exposures can be reliably detected against the background of natural physiological variation of mussels. We used fluoranthene as a model PAH and analyzed the effects of fluoranthene as well as nutritional and reproductive states on the mRNA expression patterns of stress-related biomarkers in the gills of *M. galloprovincialis*. Fluoranthene (FLU) is one of the most toxic PAH for marine biota (Ben Othman et al., 2012) found in high concentrations in sediments, particulate matter, and water (Baumard et al., 1998; Van Hattum et al., 1998; Viñas et al., 2010). FLU is included in the lists of priority substances in the field of water policy of the European Commission (EC) and the United States Environmental Protection Agency (USEPA). We exposed mussels at different stages of the reproductive cycle and/or nutritionally conditioned by different feeding regimes, to two concentrations of FLU (3 and 60 μg L^{-1}) for three weeks and assessed mRNA expression of molecular biomarkers in gill tissues. We chose mRNA expression of genes involved in Phase I and II biotransformation of xenobiotics, cytochrome P450 oxidase (CYP450) and glutathione S-transferases (GST-α; GST-S1; GST-S2), a multixenobiotic resistance protein P-glycoprotein (PgP), metal-binding and redox-active proteins, metallothioneins (MT10 and MT20), as well as molecular chaperones, heat shock proteins (a small heat shock protein HSP22, and members of HSP-70 family, HSP70-2; HSP70-3; HSP70-4). Expression levels of vitellogenin (Vitel) and vitelline coat lysin M7 (VCLM7) mRNA were used as reproductive markers of female and male mussels, respectively. The findings of this study are important for understanding the molecular mechanisms of interaction between the mussels' physiology and FLU toxicity and have implications for the biomarker development for large-scale monitoring programs of PAHs that encompass field populations of mussels in different physiological (including reproductive and nutritional) states.

2. Materials and methods

2.1. Mussel collection, conditioning and exposure

2.1.1. Experiment A: effects of the mussels' reproductive cycle on biomarker expression

Wild mussels, *Mytilus galloprovincialis*, were collected in February and September of 2013 from an unpolluted site of Galicia (Santa María de Oia, NW Spain), and transported within 24 h under cool humid conditions to Spanish Institute of Oceanography (IEO, Murcia, Spain). At each season, mussels were acclimated to standardized laboratory conditions for 1 week (0.5 μm filtered seawater at 15 ± 1 °C, 36 salinity in an aerated closed system) and fed with *Isochrysis galbana*, clone T-ISO (0.17% of microalgal organic matter per mussel live mass and day). Mussels sampled in February (when mussels are expected to be in a reproductive stage, RS-1) showed (mean ± s.d) length: 44.93 ± 1.90 mm, total dry mass: 347.02 ± 68.61 mg, condition index (CI): 10.48 ± 1.76, gonadosomatic index (GSI): 17.87 ± 4.29 and sexual maturation index (SMI): 3.20 ± 1.37. Mussels samples in September (when mussels are expected to be in a resting stage, RS-2) showed length: 45.81 ± 2.02 mm, mussel dry mass: 327.63 ± 50.05 mg, CI: 9.29 ± 1.81, GSI: 10.52 ± 3.74 and SMI: 0.40 ± 0.83. Biological indices were calculated as described elsewhere (González-fernández et al., 2016b). Briefly, CI = (total dry mass/shell dry mass) × 100; GSI = (mantle dry mass/total dry mass of the soft tissues) × 100 and SMI was calculated as the average gonadal development stage (González-fernández et al., 2016b). Sex and reproductive state of the mussels was determined using histology as described in González-fernández et al. (2016b). For analysis, five reproductive stages were considered (Kim et al., 2006): Stage 0, inactive gonad; Stage 1, gametogenesis has begun yet no ripe gametes are visible; Stage 2, ripe gametes are present and gonia occupy about one-third of the section area; Stage 3, gonia occupy half of the section area; Stage 4, gonad fully ripe.

After acclimatization, mussels were exposed to two nominal concentrations of fluoranthene (FLU), 3 μg L^{-1} (Low) and 60 μg L^{-1} (High) using acetone as a carrier, for 3 weeks as described elsewhere (González-fernandez et al., 2016b). Briefly, mussels were randomly divided between 30 L tanks and the toxicant was added daily. Water of each tank was renewed every 24 h during exposure and re-dosed with appropriate quantities of FLU stock solutions. FLU stock solutions were prepared at concentrations of 60 and 1200 μg FLU mL^{-1} acetone, respectively for Low and High exposures. 2 mL of stock solution was mixed with the microalgae culture (containing 60 mm^3 of algal cells) and incubated in agitation with light, for 45 min. After that, the contaminated microalgae were distributed within each tank following the protocol described in González-Fernandez et al. (2015b). After exposure, mussels were dissected, gills of 5 mussels were quickly flash-frozen in liquid nitrogen and stored individually at −80 °C until analyses.

2.1.2. Experiment B: effects of the mussels' nutritive state on biomarker expression

Wild mussels, *Mytilus galloprovincialis*, were collected in April of 2013 from an unpolluted site of Galicia (Santa María de Oia, NW Spain), and transported within 24 h under cool humid conditions to the IEO. Mussels (mean ± s.d) of 44.81 ± 2.02 mm length, total dry mass: 192.06 ± 38.79 mg, CI: 6.55 ± 0.70, GSI: 15.03 ± 4.42 and SMI: 0.39 ± 0.93 were acclimated to standardized laboratory conditions for 1 week (0.5 μm filtered seawater at 15 ± 1 °C, 36 salinity in an aerated closed system) and fed daily with *I. galbana*, clone T-ISO (0.17% microalgal organic matter per mussel live mass and day). After the preliminary acclimation, mussels were conditioned to three different rations of the microalgae *I. galbana*, clone T-ISO, for a

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