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iTRAQ analysis of hepatic proteins in free-living *Mus spretus* mice to assess the contamination status of areas surrounding Doñana National Park (SW Spain)☆

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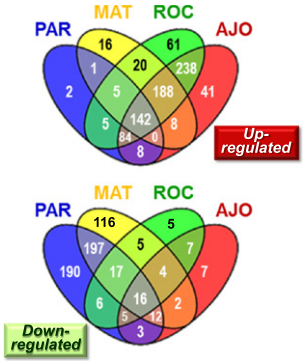
HIGHLIGHTS

- iTRAQ quantitation was used for the first time to monitor a wildlife reserve
- Over 2,000 proteins with altered expression were identified in problem Doñana sites
- Of them, 118 changed over 2.5-fold in, at least, two problem sites
- Upregulation of protective proteins prevails over downregulation of the harmful ones

GRAPHICAL ABSTRACT



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ABSTRACT

This work aims to develop and integrate new *-omics* tools that would be applicable to different ecosystem types for a technological updating of environmental evaluations. We used a 2nd-generation (iTRAQ-8plex) proteomic approach to identify/quantify proteins differentially expressed in the liver of free-living *Mus spretus* mice from Doñana National Park or its proximities. Mass spectrometry was performed in an LTQ Orbitrap system for iTRAQ reporter ion quantitation and protein identification using a *Mus musculus* database as reference. A prior IEF step improved the separation of the complex peptide mixture. Over 2000 identified proteins were altered, of which 118 changed by ≥2.5-fold in mice from at least two problem sites. Part of the results obtained with

Abbreviations: ACN, acetonitrile; AJO, *Ajoli* bridge; AhR, aryl hydrocarbon receptor; CID, collision-induced dissociation; CYPs, cytochrome P450s; 2-DE, two-dimensional electrophoresis; DNP, Doñana National Park; ECM, extracellular matrix; ER, endoplasmic reticulum; FDR, false discovery rate; FWHM, full-width at half-maximum; IEF, isoelectrofocusing; iTRAQ, isobaric tags for relative and absolute quantification; HCD, high energy collision dissociation; HdAc, histone deacetylase; IPG, immobilised pH gradient; LP, *Lucio del Palacio*; LTQ, linear ion trap quadrupole; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight; MAT, *Matochal*; MS/MS, tandem mass spectrometry; MMTS, methylmethane-thiosulfonate; OT, Orbitrap; PAR, *Partido* stream; ROC, *Rocina* stream; ROS, reactive oxygen species; RP-HPLC, reversed-phase high pressure liquid chromatography; SDS, sodium dodecyl sulfate; TF, transcription factor; TFA, trifluoroacetic acid.

☆ Animal rights. The work was performed in accordance with EU Directive 2010/63/EU for animal experiments and was approved by the Ethical Committees of Cordoba and Huelva Universities.

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the iTRAQ analysis was confirmed by Western blot. Over 75% of the 118 proteins were upregulated in animals captured at polluted sites and only 16 proteins were downregulated. Upregulated proteins were involved in stress response; cell proliferation and apoptosis; signal transduction; metastasis or tumour suppression; xenobiotic export or vesicular trafficking; and metabolism. The downregulated proteins, all potentially harmful, were classified as oncoproteins and proteins favouring genome instability. The iTRAQ results presented here demonstrated that the survival of hepatic cells is compromised in animals living at polluted sites, which showed deep alterations in metabolism and the signalling pathways. The identified proteins may be useful as biomarkers of environmental pollution and provide insight about the metabolic pathways and/or physiological processes affected by pollutants in DNP and its surrounding areas.

1. Introduction

Doñana National Park (DNP), established in 1969 and declared a World Heritage Site in 1981, is an area of marsh, shallow streams and sand dunes located at the mouth of the Guadalquivir River (SW Spain), which shelters millions of migratory birds and endangered species (Grimalt et al., 1999). The DNP marshes are fed by the *Rocina*, *Partido*, and *Guadiamar* streams and by the Guadalquivir River. However, DNP is threatened by areas of intense agriculture that are located nearby, and by industries located at Huelva Estuary (40 km west). The presence of contaminants at the DNP core raised a great concern (Bonilla-Valverde et al., 2004; Ruiz-Laguna et al., 2001).

Environmental studies typically rely on the study of well-established bioindicator species in areas of high pollutant burden (Lopez-Barea, 1995). The mouse is the favourite model organism for generating knowledge useful to humans and *Mus spretus*, the best characterised aboriginal species, is an excellent pollution bioindicator (Bonilla-Valverde et al., 2004; Ruiz-Laguna et al., 2001). *M. spretus* diverged from the lab mouse *Mus musculus* 1.6 million years ago and both species show high gene sequence homology, allowing the use of *M. musculus* databases in the molecular studies with *M. spretus* (Abril et al., 2011, 2014; Ruiz-Laguna et al., 2006).

Responses at molecular, cellular or organismal levels are used in sentinel organisms as biomarkers of pollutant exposure and/or effects. "Conventional" biomarkers, such as the levels of biotransforming or antioxidative enzymes, are suggested "*a priori*" by their biological roles, but they are quite biased for pollution assessment because they concentrate in few proteins but exclude others whose relationship with pollution is still unknown (Lopez-Barea and Gomez-Ariza, 2006). In contrast to this type of hypothesis-driven approach, direct *-omic* approaches are becoming a powerful strategy in environmental studies. Environmental Proteomics studies the relationship between pollution and protein expression changes (Lopez-Barea and Gomez-Ariza, 2006), whereas Ecotoxicoproteomics studies the proteins involved in toxic responses.

Until recently, most proteomic analyses have been performed using two-dimensional electrophoresis (2-DE) followed by MS/MS-based protein identification (Lopez-Barea and Gomez-Ariza, 2006). We have previously assessed the suitability of these high-throughput proteomic methods to monitor terrestrial and aquatic DNP ecosystems by using as bioindicator the mouse *M. spretus* and the crayfish *Procambarus clarkii*, respectively, captured at DNP and neighbouring sites (Montes-Nieto et al., 2007; Pueyo et al., 2011). These proteomics studies are hindered by several problems: i) only a few proteins show clear expression changes, ii) they are difficult to identify by MS/MS analysis, and iii) quantitative results are needed, distinct of those derived from image analysis. The *i*sobaric *t*ags for *r*elative and *a*bsolute *q*uantification (*iTRAQ*) has become popular for protein identification and quantification. Each iTRAQ reagent has a reporter group and an amino-reactive group, spaced by a mass-balancing group. The N-terminal amino group and the ε-amino group of the Lys of each peptide can be derivatized via succinimide chemistry (Ross et al., 2004). Because the tags are isobaric, the peptides from different experimental groups, with different labels, co-elute and appear in the MS spectra at the same m/z value. Relative quantitation of the differentially labelled peptides is achieved with their fragmentation products: the peak ratios of all reporter ions reflect the peptide ratios. Reporter ions are generated via fragmentation by collision-induced dissociation (CID) (Ross et al., 2004). The LTQ-Orbitrap combines the fast duty cycle of the linear ion trap quadrupole (LTQ) for MS/MS and the high resolution and mass accuracy of the Orbitrap (OT). The combined benefits of high energy collision dissociation (HCD), in the octopole collision cell, and CID, in the LTQ, greatly enhance its capability to analyse iTRAQ-labelled peptides.

Currently, our work focused in Doñana National Park aims to develop and integrate new *-omics* tools that would be applicable to different ecosystem types for a technological updating of environmental evaluations and the identification of responsive proteins that may serve as biomarkers of environmental pollution. A preliminary working scheme combining transcriptomics and proteomics for monitoring DNP has been published (Abril et al., 2011). Here, we report the results of an iTRAQ-based proteomic study in *M. spretus* from DNP areas with different pollution levels as indicated by previous studies (Lopez-Barea and Gomez-Ariza, 2006; Pueyo et al., 2011; Ruiz-Laguna et al., 2001; Vioque-Fernandez, 2009).

2. Materials and methods

2.1. Animal capture and sample preparation

Mice were captured from November to December 2009 at five DNP sites (SW Spain, Fig. 1). Those from the Lucio del Palacio (LP), a clean site at the core of DNP, were used as reference. Mice from the other four sites MAT, ROC, PAR and AJO, were the problem samples. The Matochal (MAT) site, next to the Guadiamar stream, is affected by rice growing fields and suffered the input of pesticides and metals used as algaecides in the growth of rice. In the upper part of the Rocina stream (ROC) converges several aquifers coming from large land extension covered with strawberry greenhouses. The Partido (PAR) site, located at the upper part of El Partido stream, is under the influence of citrus fruit and grape fields and several urban enclaves. The Ajoli (AJO) site, downstream of PAR, is also affected by urban wastes and strawberry growing fields. Mice were captured with live traps and transported alive to a close lab, where their gender and weight were determined before being sacrificed by cervical dislocation and dissected. Livers were individually frozen in liquid N_2 and taken to Cordoba University, where they were kept frozen at −80 °C. Each organ was individually pulverised under liquid N_2 in a SPEX SamplePrep 6770 Freezer/Mill (Metuchen, NJ, USA). Eight male mice per site were selected by their similar average weights (LP mice 11.9 ± 0.77 g; MAT mice 12.3 ± 1.27 g; ROC mice 13.4 ± 0.64 g; PAR mice 12.5 ± 0.64 g; AJO mice 12.2 ± 0.77 g) to be included in the study.

2.2. Cell-free extract preparation and protein assay

For each sampling site, one pool was prepared by mixing 50-80 mg aliquots of frozen liver powder from each of eight male mice collected per site. Pools were homogenised with a plastic pestle in Eppendorf tubes using 3 mL/g of 20 mM phosphate buffer (pH 7) containing 10 mM NaCl, 10 mM KCl and 0.1 μL of protease inhibitor cocktail (P2714, Sigma, Barcelona, Spain) per μL. Cell debris was cleared by