Chemosphere 79 (2010) 570–576

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod

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ARTICLE INFO

Article history:
Received 3 October 2009
Received in revised form 19 January 2010
Accepted 28 January 2010
Available online 1 March 2010

Keywords:
Ecotoxicoproteomics
Soil invertebrates
Bisphenol A
Vinclozolin
Ecotoxicology

ABSTRACT

Endocrine disrupting compounds (EDCs) have been studied due to their impact on human health and increasing awareness of their impact on wildlife species. Studies concerning the organ-specific molecular effects of EDC in invertebrates are important to understand the mechanisms of action of this class of toxicants but are scarce in the literature. We have used a dose/response approach to unravel the protein expression in different organs of isopods exposed to bisphenol A (BPA) and vinclozolin (Vz) and assess their potential use as surrogate species. Male isopods were exposed to a range of Vz or of BPA concentrations. After animal dissection, proteins were extracted from gut, hepatopancreas and testes. Protein profiles were analysed by electrophoresis and differentially expressed proteins were identified by MALDI mass spectrometry. EDCs affected proteins involved in the energy metabolism (arginine kinase), proteins of the heat shock protein family (Hsp70 and GRP78) and most likely microtubule dynamics (tubulin). Different proteins expressed at different concentrations in different organs are indicative of the organ-specific effects of BPA and Vz. Additionally, several proteins were up-regulated at lower but not higher BPA or Vz concentrations, bringing new data to the non-monotonic response curve controversy. Furthermore, our findings suggest that some common responses to EDCs in both vertebrates and invertebrates may exist.

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1. Introduction

Bisphenol A (BPA) is a xenobiotic commonly employed in the manufacture of polycarbonate plastic and epoxy resins (Crain et al., 2007). Although the plastic industry still claims that BPA is safe, its endocrine disrupting (ED) properties raise concerns (Quitmeyer and Roberts, 2007). In fact, BPA is known to act as a teratogen (Crain et al., 2007) and may also lead to the alteration of sex determination and gonadal function (Crain et al., 2007). Low doses of BPA alter the gastrointestinal tract of rats, affecting the epithelial barrier function and enhancing the visceral nociceptive response by binding to estrogen receptors (Braniste et al., 2010). In invertebrates, BPA induces various adverse effects, such as on the reproduction of the water fleas *Ceriodaphnia dubia* and *Daphnia magna* (Tatarazako et al., 2002; Mu et al., 2005), and on the time to achieve sexual maturity of the copepod *Tigriopus japonicus* (Marcial et al., 2003). In isopods, BPA exposure causes sex-ratio shifts, and developmental and reproductive impairment (Lemos et al., in press, 2009a).

Vinclozolin (Vz) is a fungicide used mostly in turf grass and on vine plants. It is a proven ED with anti-androgenic effects mainly due to its active metabolites, M1 and M2 (Bursztyka et al., 2008). In *D. magna*, Vz induces a decrease in the number of newborn males (Haeba et al., 2008) and in molluscs, it induces the reduction of the number of ejaculated sperm cells, smaller testes, and disrupted male courtship behaviour (Baatrup and Junge, 2001). Vz has also been reported to cause female virilisation (imposex development) and reduction of accessory sex organ expression in prosobranch snails (Tillmann et al., 2001). Vz exposed isopods, show cases of incomplete ecdysis, and developmental and reproductive impairment (Lemos et al., in press, 2009a).

Both BPA and Vz were previously proven to be ED due to their ecdysteroidal activity in terrestrial isopods (Lemos et al., 2009b). The role of ecdysteroids in crustaceans enabled for a causal link to developmental a reproductive impairment to be established.

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doi:10.1016/j.chemosphere.2010.01.055

Despite the increasing amount of information concerning EDC effects on vertebrates and on (marine) invertebrates, to our knowledge the few data available on the toxicity of BPA and Vz to edaphic organisms is restricted to studies with isopods (Lemos et al., in press, 2009a). Furthermore, for these compounds the mechanisms of effects at the molecular level on invertebrates are still largely unknown. In fact, most studies of ED effects on invertebrates have been essentially focused in the higher levels of biological organization. However, ED may in some cases arise secondarily as a result of pathological processes (Depledge and Billinghurst, 1999). Therefore, detailed insights into the molecular effects of EDCs are necessary to understand the mode of action (MoA) of these compounds.

Woodlice are saprophytic detritivores, playing an important role in food webs, in the decomposition of organic material and in soil structuring (Arts et al., 2004). Their biology and physiology is relatively well-known and they have been shown to be suitable test organisms for monitoring studies and to acquire individual toxicity data (Drobne, 1997). Also, their suitability for EDCs effect assessment have also been shown (Lemos et al., 2009b).

In this work we developed a simple method to extract proteins from the organs of a terrestrial isopod. Also, we evaluated the protein expression of the gut, hepatopancreas and testes of organisms exposed to BPA and Vz. Proteomic methodologies can be used to determine which molecules underlie toxicological effects of stressors (Dowling and Sheehan, 2006; López-Barea and Gómez-Ariza, 2006). Herein lies the major difference between traditional experimental biology and biochemical analysis and proteomics: the latter is not hypothesis-driven. Hence, the possibility of measuring (and identifying) several proteins without any prior assumption on biomarker or mechanisms of action as well as revealing associations between proteins and toxicant exposure that have not been described earlier, is indubitably the major advantage of proteomics (Lemos et al., 2010).

1.1. Chemicals and preparation of soil

A natural soil, from an agricultural field kept in fallow from central Portugal, was oven dried at 60 °C for 48 h and immediately weighed. Soil was spiked with a range of chemical concentrations, chosen taking into account the concentrations previously tested which caused ecdysteroidal activity (Lemos et al., 2009b).

BPA (Merck Schuchardt, Germany, 2,2-bis-(4-hydroxyphenyl)-propane, purity >99%) was dissolved in methanol and mixed with the soil at concentrations of 10, 30, 100, 300 and 1000 mg kg^{-1} dry soil. Afterwards, the solvent was allowed to evaporate under a fume hood for 12 h. Subsequently, soil moisture content was adjusted to 20% (v/w) with distilled water.

Vz (Ronilan 50% active ingredient; BASF AG, Germany; 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione) dissolved in water, was mixed in with the soil at concentrations of 10, 30, 100, 300 and 1000 mg a.i. (active ingredient) kg^{-1} dry soil. Soil moisture content was adjusted to 20% (v/w) with distilled water.

Controls were made by adjusting water content to the soil as described above. A solvent control (0^+) was also prepared, adding the same volume of methanol but without BPA to the soil and following the treatment above mentioned.

Chemical analyses of test concentrations were made at the Terracon Laboratorium für Umwelt und Pestizidanalytik GmbH (Jüterborg, Germany). Vz contaminated soil samples (10 g) were extracted using acetonitrile at 40 °C for 30 min and determined by GC-MS (Varian, USA). For BPA contaminated soil samples with methanol, the extract was evaporated to 10 mL by rotary vacuum evaporator (40 °C). BPA concentrations were determined by UV detection at 230 nm using a HPLC–PDA system (Shimadzu, Japan) equipped with a C_{18} column (5 μm). Analytical values were within ±5% of the nominal concentrations. Results are presented as nominal values.

1.2. Organism exposure

Male adult *P. scaber* were exposed to Vz and BPA for 15 d. Animals were randomly and individually placed in polyethylene terephthalate (PET) boxes (Ø100 mm × 50 mm) filled with 60 g of moist spiked soil and four Ø10 mm alder leaf discs.

1.3. Organism dissection

At the end of the experiments, five animals per toxicant concentration undergoing intermolt, were dissected for gut, hepatopancreas and testes, on a frozen stainless steel plate in the presence of 100 mM Tris buffer pH 8.0 with PMSF (0.5 mM, Sigma). Each organ was immediately frozen in liquid nitrogen and kept at −80 °C until protein extraction.

1.4. Protein extraction and quantification

Each organ was suspended in buffer (8 M urea, 2% SDS, 100 mM Tris/Bicine) and homogenized. After centrifugation the supernatant was collected and kept at −20 °C. Samples were kept on ice throughout the extraction process. Protein concentration was determined using the BCA kit from Thermo Scientific, according to the manufacturer's instructions.

1.5. SDS–PAGE

Proteins were denaturated and separated (25 μg protein/lane) by SDS–PAGE according to (Laemmli, 1970). The separation was performed in the Mini-PROTEAN 3 (Bio-Rad) with lab casted SDS polyacrylamide gels (15%). Gels ran for 2.5 h or 3 h, depending on the organ, at 125 V. Proteins were visualized by Coomassie Brilliant Blue staining. Each gel image was acquired using the GS-710 calibrated imaging densitometer (Bio-Rad). Apparent molecular weights and band intensities were determined using the Quantity One v4.1 software (Bio-Rad). The apparent molecular weight of the proteins was determined using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standard, from Bio-Rad). In each gel, three lanes were loaded with the molecular mass standard and the molecular weights of the proteins were calculated using data from all standard lanes on each gel. Band optical density was determined as (OD) mm^{-2}, subtracted for background and corrected for OD differences between gels.

1.6. Protein differential expression – statistical analysis

Results are presented as mean ± SE. All data were checked for normality and homoscedascity. One way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means were employed to determine significant differences relatively to control treatment. For all statistical tests the significance level was set at $P < 0.05$. Calculations were performed with SigmaStat (Systat Software Inc., California, USA). Protein bands with a significantly different expression from the control (in the case of Vz) and solvent control (in the case of BPA) were selected for identification.

1.7. Protein identification – reduction and alkylation

Bands of interest were manually excised, reduced (10 mM dithiothreitol in 7 M GuHCl/0.3 M Tris, pH 9.0, 45 min at 55 °C) and alkylated (55 mM iodoacetamide in 200 mM NH_4HCO_3 (pH 7.0),

45 min in the dark at room temperature) as described (Samyn et al., 2006). Removal of the excess iodoacetamide was accomplished by washing the gel pieces twice with 150 μL 50% acetonitrile/ultra-pure water (ACN/MQ). Finally, the gel plugs were dried (Speedvac, Eppendorf).

1.8. Protein identification – trypsin digestion

To the dried gel plugs, buffer (50 mM ammonium bicarbonate, pH 7.8) containing modified trypsin μL^{-1} (Promega, Madison, WI) were added, and kept on ice for 45 min. Digestion was performed overnight at 37 °C, the supernatant was recovered, and the resulting peptides were extracted twice with 60% ACN/0.1% N,N-diisopropyletylamine (DIEA). The extracts were pooled, dried in a SpeedVac and were redissolved in 0.1% TFA (trifluoroacetic acid). One μL of sample was mixed with 1 μL matrix solution (7 mg mL^{-1} α-cyano-4-hydroxycinnamic acid solution in 0.1% TFA/50% acetonitrile) and mixture was spotted on a MALDI-plate. The plates were allowed to air-dry at room temperature, and were then inserted in the mass spectrometer and subjected to mass spectrometric analysis.

1.9. Protein identification – matrix-assisted laser desorption/ionization TOF/TOF mass spectrometry

The Applied Biosystems 4800 Proteomics Analyzer with TOF/TOF optics was used in this study for reflectron analysis and MALDI MS/MS applications (Applied Biosystems, Foster City, CA, USA). The mass spectrometer uses a 200-Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm.

Prior to analysis, the mass spectrometer was externally calibrated with a mixture of Angiotensin I, glu-fibrino-peptide B, adrenocorticotropic hormone (ACTH) (1–17), and ACTH (18–39). For MS/MS experiments, the instrument was externally calibrated with fragments of glu-fibrino-peptide. MS and MS/MS data were further processed using DataExplorer 4.0 (Applied Biosystems) or by manual interpretation.

2. Results

2.1. Protein extraction from hepatopancreas, testes and gut

Although BPA and Vz induce no significant lethality to isopods after 15 d of exposure at these concentrations (LC_{50} is >1000 mg kg^{-1} of soil), exposure to the chemical elicited a protein response in all organs.

The extraction method was adequate as protein profiles were similar within each organ/toxicant concentration. After image analysis of each organ protein profiles, several bands, with clearly different intensity compared to controls were selected (Figs. 1–3). The intensities of these proteins were analysed in all gels and average intensities are shown in Figs. 1a–3a (for Vz exposed organisms) and in Figs. 1b–3b (for BPA exposed organisms).

2.2. Exposure to bisphenol A

In the BPA exposed isopods' hepatopancreas, two proteins were significantly over-expressed by the highest and lowest concentration tested (10 and 1000 mg kg^{-1} of soil; Fig. 1b) (ANOVA, Dunnett's test, $F_{5.25} = 3.703$, $P = 0.012$ and $F_{5.24} = 5.024$, $P = 0.003$ for 68.5 and 38.8 kDa proteins, respectively).

The gut protein profiles showed that from the bands detected, one (74.9 kDa) was up-regulated with increasing concentrations of BPA (Fig. 2b) with a LOEC of 300 mg kg^{-1} of soil (ANOVA, Dunnett's test, $F_{5.80} = 5.049$, $P = 0.009$).

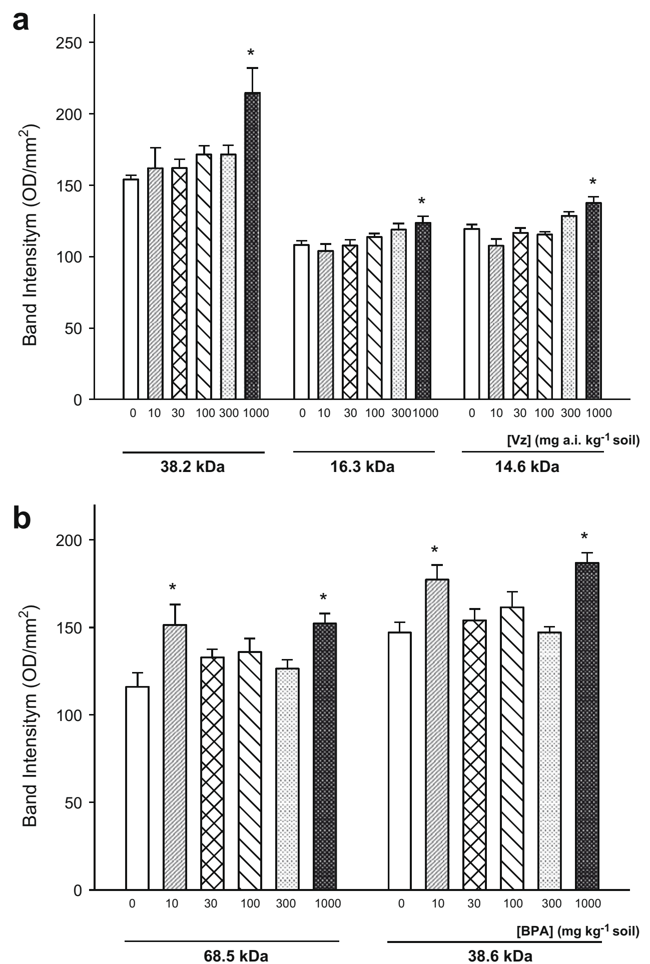

Fig. 1. Effect of toxicant exposure on protein expression in the hepatopancreas of *Porcellio scaber*. (a) Expression of proteins with molecular weights of 38.2, 16.3 and 14.6 kDa of the hepatopancreas of isopods exposed to vinclozolin contaminated soil, expressed as intensity of bands. (b) Expression of proteins with molecular weights of 68.5 and 38.6 kDa of the hepatopancreas of isopods exposed to bisphenol A contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control (Vz) or solvent control (BPA) at $P \leqslant 0.05$ (ANOVA, Dunnett's test).

Considering the testes of BPA exposed organisms, three proteins (molecular weights of 75.8, 73.0 and 53.8 kDa) were significantly over-expressed (Fig. 3b). Heavier proteins (75.8 and 73.0 kDa) were up-regulated at concentrations of 30 and 100 mg kg^{-1} of soil (ANOVA, Dunnett's test, $F_{5.20} = 4.057$, $P = 0.011$ and $F_{5.19} = 5.483$, $P = 0.006$, respectively). The 53.8 kDa protein was over-expressed with increasing concentrations with a NOEC of 100 mg kg^{-1} of soil (ANOVA, Dunnett's test, $F_{5.19} = 4.646$, $P = 0.006$).

2.3. Exposure to vinclozolin

After exposure to Vz, differences in the isopods' testes, hepatopancreas and gut protein expression were observed. Both testes and hepatopancreas showed three proteins up-regulated (Figs. 1a and 2a). Nevertheless, while in the hepatopancreas up-regulation had a LOEC of 1000 mg a.i. kg^{-1} of soil for all three proteins, in the testes up-regulation occurred and was significantly different at the lowest concentrations tested, 10 and 30 mg a.i. kg^{-1} of soil for the 75.8 kDa protein (ANOVA, Dunnett's test, $F_{3.12} = 5.285$, $P = 0.015$) and 10 mg a.i. kg^{-1} of soil for the 73.0 and 62.6 kDa proteins (ANOVA, Dunnett's test, $F_{3.13} = 8.3$, $P = 0.002$ and $F_{3.14} = 12.496$, $P < 0.001$, respectively) (Fig. 1a). In the gut one protein with a molecular weight of 74.9 kDa was over-expressed,

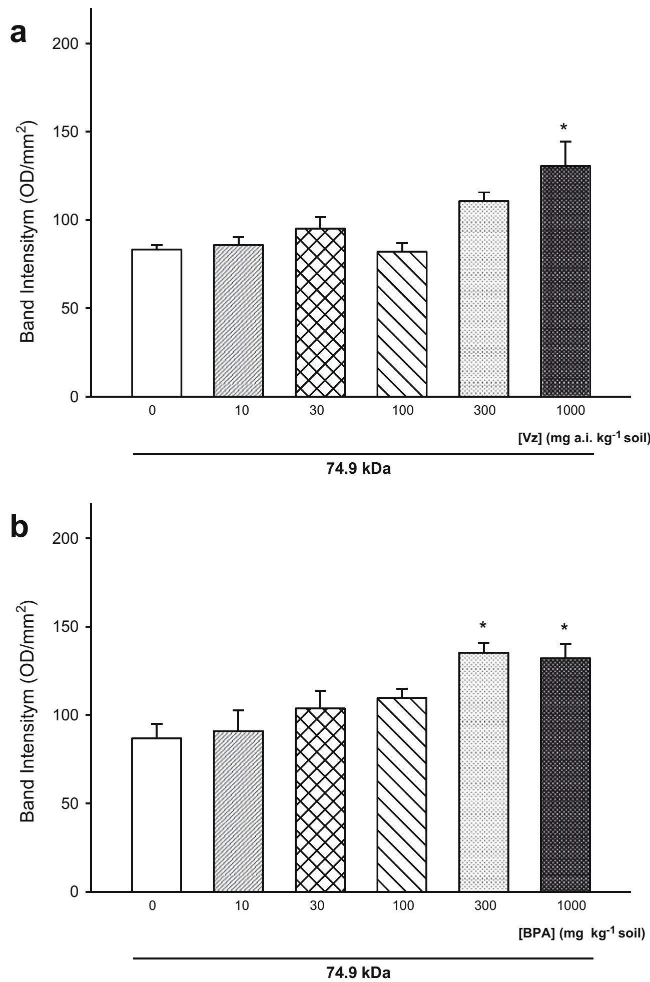

Fig. 2. Effect of toxicant exposure on protein expression in the gut of *Porcellio scaber*. (a) Expression of hepatopancreas proteins with molecular weight of 74.9 kDa of isopods exposed to vinclozolin contaminated soil, expressed as intensity of bands. (b) Expression of the protein with molecular weight of 74.9 kDa of the gut of isopods exposed to bisphenol A contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control (Vz) or solvent control (BPA) at $P \leqslant 0.05$ (ANOVA, Dunnett's test).

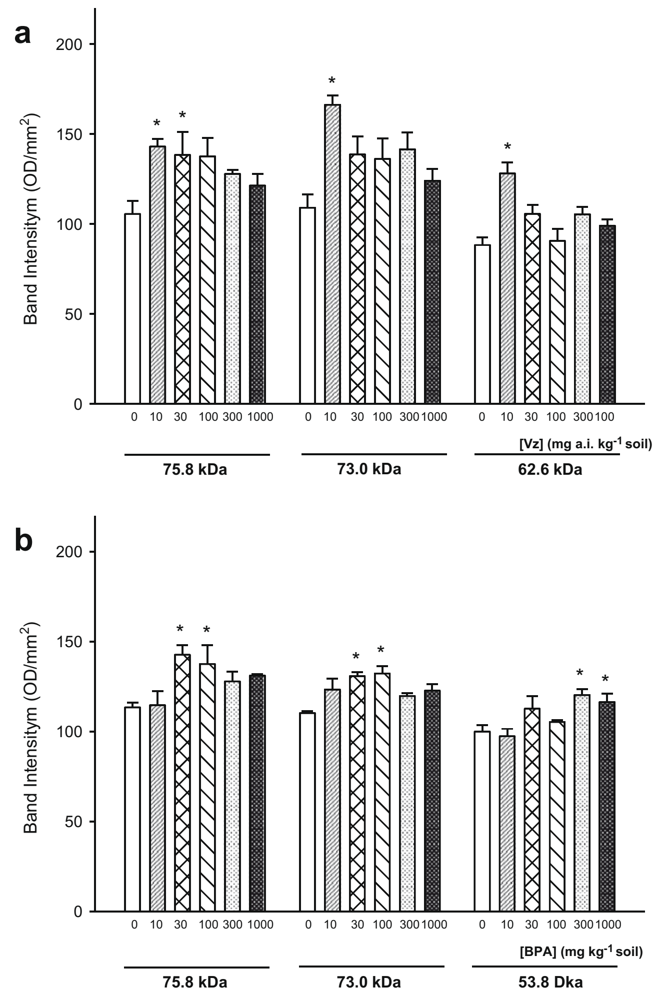

Fig. 3. Effect of toxicant exposure on protein expression in the testes of *Porcellio scaber*. (a) Expression of proteins 75.8, 73.0 and 62.6 kDa of the testes of isopods exposed to vinclozolin contaminated soil, expressed as intensity of bands. (b) Expression of proteins with molecular weights of 75.8, 73.0 and 53.8 kDa of the testes of isopods exposed to bisphenol A contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control (Vz) or solvent control (BPA) at $P \leqslant 0.005$ (ANOVA, Dunnett's test).

being statistically significant comparing to control at the concentration of 1000 mg Vz kg^{-1} of soil (Fig. 3a) (ANOVA, Dunnett's test, $F_{5.19} = 5.918$, $P = 0.002$).

2.4. Protein identification

The proteins which showed statistically different expressions among treatments were selected for identification. Each band

Table 1
Identification of proteins differentially expressed in the isopod *Porcellio scaber* after 15 d of exposure to vinclozolin and bisphenol A in soil. The apparent molecular weight of proteins was determined by SDS–PAGE. Protein identification was achieved by mass spectrometry.

Toxicant	Organ	Apparent MW (kDa)	Identification (FASTS)	Analysis method	Peptides	FASTS/ *E*-score	BlastP/ *P*-score	Organism
Vinclozolin	Hepatopancreas	38.2	Arg kinase	PMF, MS/MS and *de novo*	4	2.40E−06	0.069	*Homarus gammarus* (Crustacea; Malacostraca)
		16.3	*n.i.*	–				
		14.6	*n.i.*	–				
	Testes	75.8	*n.i.*	–				
		73.0	Hsp70	PMF, MS/MS	*	*	*	*
		62.6	*n.i.*	–				
	Gut	74.9	*n.i.*	–				
BPA	Hepatopancreas	68.5	*n.i.*	–				
		38.6	Arg kinase	PMF, MS/MS and *de novo*	6	4.40E−22	0.0023	*Homarus gammarus* (Crustacea; Malacostraca)
	Testes	75.8	GRP78	PMF, MS/MS and *de novo*	2	1.50E−10	0.9996	*Rattus norvegicus*
		73.0	*n.i.*	–				
		53.8	Beta-tubulin	PMF, MS/MS and *de novo*	5	4.20E−15	0.27	*Trypanosoma brucei rhodesiense*
	Gut	74.9	*n.i.*					

n.i.: Protein not identified; *no *de novo* identification was performed. Significant Mascot values were achieved.

was manually excised from the gel, trypsin digested and analysed by MALDI-TOF MS. Only the PMFs (peptide mass fingerprint) that were confirmed by MS/MS were considered as a positive identification. Identifications were performed by homology search. The *de novo* determined peptide sequences were used for similarity searches using the FASTS and BlastP algorithms. On-line submissions were performed at the EBI server (EMBL-EBI) (http://www.ebi.ac.uk/Tools/similarity.html). Searches were performed against the Uniprot Knowledgebase using standard settings (Samyn et al., 2007). FASTS search results were considered significant only if the *E*-value was below 1.0e−04.

An obvious limitation of working with non-model organisms is that since their genome is not known, protein sequences are absent from the databases and only some proteins can be positively identified. Therefore, whenever necessary, *de novo* sequencing of proteins was performed. From the 12 potential proteins that were significantly over-expressed we were able to make a positive identification of five proteins (Table 1).

3. Discussion

Endocrine, immune and neuronal systems as well as reproductive organs are frequently pointed out as targets of EDCs in both vertebrates and invertebrates (vom Saal and Welshons, 2006). Nevertheless, EDCs MoA and concentrations at which organisms' responses are observed have not yet been completely clarified and remain a fundamental issue for toxicologists (Yang et al., 2008).

Protein profile modifications in response to specific chemicals can provide useful marker proteins against specific chemicals, and have been detected in organisms exposed to chemical stressors (vom Saal and Welshons, 2006). This technique has primarily benefited the research on well-characterised species such as human, mouse and yeast. Unfortunately, these species are inappropriate from an ecotoxicological perspective (Hogstrand et al., 2002). The use of proteomics for studies of ecologically relevant species is not impossible, but since there are no genome sequencing data available (Monsinjon and Knigge, 2007), it entails more work and more money for less information. The lack of sequenced genomes increases the complexity of protein identification by MS technologies. In fact, contrasting with the early start of studies with non-model organisms exposed to selected pollutants, and the ensuing studies in model organisms, very few proteomic studies exist yet in animals from natural environments (López-Barea and Gómez-Ariza, 2006). But, as Hutchinson (Hutchinson et al., 2006) stated, "the practical science (ecotoxicoproteomics) is still in its infancy" and studies like the present one should broaden our knowledge on the effects of toxicants at the protein level of these organisms (Lemos et al., 2010).

In this work, we searched for proteins that are differentially expressed in particular organs of *P. scaber* after exposure to Vz and BPA. Furthermore, we have established a dose–response correlation, which is, to the best of our knowledge, one of the first reports that show a proteome statistically valid concentration–response relationship. The terrestrial isopod gut is where digestive processes mainly take place, whereas the hepatopancreas (digestive gland) secretes digestive fluids into the hindgut and is involved in the absorption of digestively released nutrients and is the main factory of proteins (Zimmer and Brune, 2005), having intestinal, hepatic, and pancreatic functions (Leser et al., 2008). Significant changes in protein profiles of each of these organs may lead to effects at the individual level but also at the population level being especially relevant for gonads. If chemically exposed isopods show gonad alterations, it may affect breeding and most probably affect isopods' population dynamics.

This study revealed significant differences in all tested organs of *P. scaber* after BPA and Vz exposure. However, the sensitivity of each organ was not the same. In *P. scaber* testes it was possible to detected up-regulated proteins at Vz concentrations as low as 10 mg a.i. kg^{-1} soil while for hepatopancreas this was only possible at concentrations equal to 1000 mg a.i. kg^{-1} soil. A similar pattern of gonad sensitivity was found for BPA exposed organisms, while for gut and hepatopancreas altered protein profiles were respectively detected only at BPA concentrations higher than 300 and 1000 mg kg^{-1}. Testes showed increased protein expression at concentrations as low as 30 mg BPA kg^{-1} soil. This suggests that testes are more susceptible to these compounds than other organs, showing that the male isopod reproductive traits may therefore be especially susceptible and sensitive to this class of chemicals. Previous studies have also stressed the gonads and reproductive traits as preferential targets by EDCs in vertebrates (Navas and Segner, 2006). An important aspect concerning the ecological relevance of EDC studies on invertebrates is the possibility of establishing useful biomarkers (Rodriguez et al., 2007). The low concentrations at which protein profile alterations were detected in the testes (Figs. 3a and b) not only are far from those lethal to *P. scaber*, but also were observed earlier than reproductive, developmental and molting effects were (Lemos et al., in press, 2009a). On the other hand, effects at low concentrations, as seen in our investigation, are in agreement with the literature that has shown that some of these EDCs produce effects with humped dose–response curves, with tests conducted at high doses missing biological effects that are induced by lower doses (Kaiser, 2000; Lemos et al., 2009a,b).

Our data shows an up-regulation of Hsp70 proteins (known as ubiquitous stress response proteins) in the testes of organisms exposed both to Vz (around 160% increase after exposure to 10 mg a.i. kg^{-1} and around 130% at 30 mg a.i. kg^{-1}) and BPA (around 120% increase at 30 and 100 mg kg^{-1}). The induction of Hsp70 proteins has been reported in a wide range of organisms, from microorganisms to humans, upon exposures to various kinds of chemical, physical and biological stressors (Yoshimi et al., 2002). Hsp70 proteins are anti-apoptotic proteins, protecting cells from cytotoxicity and inhibiting cell death induced by several agents (Arya et al., 2007). Increases in Hsp70 protein concentration aid refolding damaged proteins during stress (Ma and Hendershot, 2001).

The fact that Hsp70 was induced by both toxicants suggests that it could be used as a biomarker of exposure to low concentrations of chemicals in testes. Its response is not stressor specific but should be further investigated and validated as a general biomarker of environmental quality. Such a suggestion has also been made for *Mytilus* sp., where a significant up-regulation of Hsp70 after exposition to BPA was observed (Jonsson et al., 2006). BPA also induced an increase of Hsp70 mRNA in the aquatic larvae of *Chironomus riparius* (Planello et al., 2008).

In BPA exposed testes, the Hsp70 protein was identified as glucose-regulated protein 78 (GRP78), which is known for being induced by stress (Liu et al., 1997). An up-regulation of this protein seems to be related to the accumulation of unfolded proteins in the ER (Ma and Hendershot, 2001) protecting cells against toxic insults (Liu et al., 1997). It has been shown that GRP78 plays important roles in the survival of cells during calcium stress, being induced by depletion of stored calcium. An elevated intracellular calcium concentration induced by BPA in mouse hippocampal neuronal cells was shown previously (Lee et al., 2008), as a mobilization of intracellular calcium induced by BPA leading to ER stress (Tabuchi et al., 2006). Furthermore, GRP78 is induced by natural estrogens in the mouse uterus via the estrogen receptor (ER)-independent mechanism (Ray et al., 2007) and that it is involved in the control of ER gene expression, able to amplify estrogenic potency of weak xenoestrogens.

Therefore, this up-regulation of GRP78 in isopod testes is an indication that in invertebrates similar molecular events might occur as in vertebrate cells exposed to BPA.

For BPA exposed isopod testes, there was also an increase of β-tubulin with increasing concentrations (NOEC 100 mg kg^{-1} soil). β-tubulin and α-tubulin are the components of microtubules in eukaryotic cells (Wade, 2007) playing a central role in many aspects of cell function, including cell motility and division (Moores, 2008). During cell division (mitosis or meiosis) microtubule dynamics play an essential role in the proper orientation and segregation of chromosomes. Impairment in the functioning of microtubules leads to an abnormal morphology of the cells and may lead to apoptosis (Singh et al., 2008). An interaction between tubulin and BPA in eggs of the urchin (*Lytechinus pictus*) was reported earlier (George et al., 2008). These authors have identified tubulin as a direct target of BPA, affecting microtubule assembly, suggesting that BPA induces the *de novo* formation of ectopic asters. These results are in agreement with our data that show an over-expression of tubulin in the testes. Such up-regulation may cause mitotic or meiotic aneuploidy (George et al., 2008). Exposure of mouse oocytes to BPA affected spindle formation, distribution of pericentriolar material and chromosome alignment on the spindle, and caused a significant meiotic arrest (Eichenlaub-Ritter et al., 2008). Taken together, one can postulate that BPA may be considered as a reproductive toxin both for vertebrates and invertebrates, sharing similar MoA. In isopod testes this could have implications for gametogenesis and therefore have a major impact on isopod reproduction and most certainly lead to effects at the population level. In fact, we have seen that the same BPA concentration induces the abortion rate of exposed isopods (Lemos et al., 2009a) which could be explained by an increase of defective male reproductive cells caused by BPA interaction with tubulin.

Arginine kinase (AK) is widespread in invertebrates, where it serves a function analogous to that of creatine kinase in vertebrates (Blethen and Kaplan, 1968). This enzyme is involved in the cellular energy metabolism, catalysing the reversible formation of arginine phosphate and adenosine diphosphate from ATP and L-arginine (Blethen and Kaplan, 1968) and therefore responsible for ATP buffering in the cytosol and energy shuttle between mitochondria and the cytosol (Silvestre et al., 2006). This buffering function of the phosphagen kinases seems to be characteristic in tissues with short bursts of energy demand (Kotlyar et al., 2000). After Vz and BPA exposure we detected an up-regulation of AK in isopod hepatopancreas (around 150% at 1000 mg a.i. Vz kg^{-1} and approximately 120% and 130% in isopods exposed to 10 and 1000 mg BPA kg^{-1}). The hepatopancreas not only is the major digestive organ of isopods, as it also is the main site for synthesis and secretion of digestive enzymes, absorption of nutrients, storage of metabolic reserves and excretion of wastes (Leser et al., 2008). The up-regulation of AK may be due to the stress response to toxicant exposure, with the activation of metabolic processes related to detoxification and metabolization of energy reserves to provide for those energy-demanding processes (Jones and Hopkin, 1996). In fact, *P. scaber* exposed to high concentrations of Vz and BPA grew less and had lower reproductive allocation (Lemos et al., in press, 2009a) compared to control organisms. Because AK plays an important role in energy metabolism, its up-regulation seems to indicate that there is a substantial energetic cost in terms of hepatopancreas ATP demand after Vz and BPA exposure with a clearly visible effect at physiologic level: organisms grew less than controls.

The understanding of mechanisms that develop in cells due to toxic effects of EDCs is far from being complete. The approach developed in the present study provides evidence that Vz and BPA can alter the expression of different protein profiles in different organs of a soil invertebrate. We were also able to demonstrate the existence of low-dose effects. Further investigations should also consider the use of a lower range of EDCs concentrations than the one tested here in order to determine NOEL values. To our knowledge, this is the first demonstration of such effects on the isolated organs of terrestrial arthropods. In this work, the use of MS/MS and *de novo* sequencing enabled the identification of several proteins that were over-expressed.

Certainly, other protein expression changes occurred in our experiments, but they were either too small to be detected or concerned less abundant proteins. To obviate such an issue, fluorescent 2D differential in gel electrophoresis (DIGE) should be addressed. Also, the correlation between the expression of these proteins and the internal toxicant concentrations should result in fruitful information regarding the ED subject.

Further studies are needed and we are still far away from attaining full knowledge on how endocrine disruptors exert their action in living organisms. Research focusing on the effects of these compounds in the proteome of terrestrial organisms is essential and will increase our knowledge of how endocrine disruptors may affect wildlife and ecosystems.

Acknowledgments

This work was supported by fellowships provided to MFL Lemos (BD/13868/2003) and AC Esteves (BPD-38008/2007) by the Fundação Portuguesa para a Ciência e Tecnologia. B. Samyn is a postdoctoral fellow of the Fund for Scientific Research-Flanders (F.W.O.-Vlaanderen, Belgium). Parts of this work were supported by FCT Project Grant reference PTDC/BIA-BDE/75690/2006.

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