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Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of *Chironomus riparius* based on gene expression assays related to the endocrine system, the stress response and ribosomes

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ABSTRACT

In this work, the effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP), two of the most extensively used phthalates, were studied in *Chironomus riparius* under acute short-term treatments, to compare their relative toxicities and identify genes sensitive to exposure. The ecotoxicity of these phthalates was assessed by analysis of the alterations in gene expression profiles of selected inducible and constitutive genes related to the endocrine system, the cellular stress response and the ribosomal machinery. Fourth instar larvae, a model system in aquatic toxicology, were experimentally exposed to five increasing concentrations (0.01, 0.1, 1, 10, and 100 mg/L) of DEHP and BBP for 24 h. Gene expression was analysed by the changes in levels of transcripts, using RT-PCR techniques with specific gene probes. The exposures to DEHP or BBP were able to rapidly induce the *hsp70* gene in a concentration-dependent manner, whereas the cognate form *hsc70* was not altered by either of these chemicals. Transcription of ribosomal RNA as a measure of cell viability, quantified by the levels of *ITS2*, was not affected by DEHP, but was slightly, yet significantly, downregulated by BBP at the highest concentrations tested. Finally, as these phthalates are classified as endocrine disruptor chemicals (EDCs), their potential effect on the ecdysone endocrine system was studied by analysing the two genes, *EcR* and *usp*, of the heterodimeric ecdysone receptor complex. It was found that BBP provoked the overexpression of the *EcR* gene, with significant increases from exposures of 0.1 mg/L and above, while DEHP significantly decreased the activity of this gene at the highest concentration. These data are relevant as they show for the first time the ability of phthalates to interfere with endocrine marker genes in invertebrates, demonstrating their potential capacity to alter the ecdysone signalling pathway. Overall, the study clearly shows a differential gene–toxin interaction for these two phthalates and adds novel genomic tools for biomonitoring environmental xenobiotics in insects.

1. Introduction

Phthalates, diesters of benzenedicarboxylic acid, are a family of man-made chemicals typically used as plasticizers to increase the flexibility and durability of plastic polymers, mainly in polyvinylchloride (PVC) products. Phthalates are also used as solvents, lubricants and fixatives in a huge variety of industrial products, such as cosmetics, detergents, building materials, insecticides, pharmaceuticals and medical devices. Their high production, global consumption and widespread use in consumer products, mean that human and wildlife exposures to phthalates are virtually unavoidable (Schettler, 2006). These compounds are listed as priority substances in the European Union and are, therefore, subjected to an environmental risk assessment (ERA). Phthalates are suspected of possessing endocrine disrupting activity (Latini, 2005) and most of them are included in the European Catalogue of Endocrine Disrupting Compounds (EU-EDC website).

The adverse effects observed in rodent models raised concerns as to whether or not exposure to phthalates represents a potential health risk to humans (Howdesshell et al., 2008). At present, it has been shown that di(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) produce reproductive and developmental toxicities (Lyche et al., 2009). The adverse consequences due to phthalates depend upon the dose and timing of exposure. In laboratory animals, exposures to high concentrations were shown to induce liver and kidney injury, cancer, malformations and fetal death. In humans, particular concerns

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have been raised regarding adverse effects following exposure to phthalates during development. Phthalates are able to cross from maternal blood, via placenta or breast milk, reaching the fetus and neonates, affecting the developing endocrine system, which is essential for diverse biological functions, including sexual development and reproductive functions in adults (Latini, 2005). The high sensitivity of the early developmental stages for endocrine disruption has led to the hypothesis that increased incidence of human reproductive deficits may be produced by exposure to environmental chemicals during fetal and prepubertal life (Sharp and Irvine, 2004). The European Union has banned the use of DEHP (EU, 2008), DBP and BBP in children's products, and California and Canada have restricted their use. However, there is still some controversy regarding the causal links between exposure to EDCs and adverse human health effects (NRC, 2008).

Phthalates can be found at measurable concentrations in aquatic ecosystems throughout the world, due to their high volumes of production and almost continuous release into the environment (Oehlmann et al., 2008). As additive plasticizers, they are not covalently bound to the polymers and, therefore, they can leak out into the surrounding environment. Several recent studies have documented their constant environmental presence in river waters, sediments and home dust (Yuwatinni et al., 2006; Abb et al., 2009). DEHP is often found in freshwater systems and, because of its widespread use, dominated the phthalate concentrations evaluated (up to 8.44 mg/kg in sediments and 154 mg/kg in sewage sludge) in water samples from Germany (Fromme et al., 2002), with local PEC up to 219 μg/L in waters and up to 2045 mg/kg in sediments (EU, 2008). According to the half-life obtained experimentally, abiotic degradability for these two phthalates was 58–480 d for BBP and 390–1600 d for DEHP under sunlight irradiation, via photolysis plus hydrolysis (Lertsirisopon et al., 2009). Bioaccumulation of phthalates has been found in aquatic organisms, while the highest BCF values have been observed in invertebrates (EU, 2008).

Despite the recent increase in research on the ecotoxicity of phthalates, ecotoxicological studies in aquatic wildlife invertebrates and, particularly, benthic biota are limited and highly scarce in comparison to the research into human risks. Chironomid larvae are one of the most ubiquitous freshwater benthic invertebrates, and are abundant and ecologically relevant to the aquatic food chain. As they live in the sediments and feed on sediment particles, they are directly exposed to toxicants. Sediments are the ultimate repository of anthropogenic contaminants entering into water resources and the likely final sink of phthalates, which are generally insoluble in water (Petrovic et al., 2001). The midge *Chironomus riparius* is widely used as test organism in environmental toxicology (EPA, 1996; OECD, 2001). The study of the taxonomic composition of chironomid larvae and the percentage of occurrence of deformities in mouthparts, mainly in the mentum, are used in biomonitoring programmes to obtain information on the levels of organic and chemical pollutions of aquatic ecosystems (Martinez et al., 2003). *Chironomus* has recently been selected as a reference organism for investigations of the potential endocrine disrupting effects of chemicals (OECD, 2006; Taenzler et al., 2007). It was also chosen as the model species for a European IDEA project to evaluate the endocrine disrupting effects in aquatic invertebrates (Segner et al., 2003). To date, most studies carried out in *Chironomidae* regarding the effects of EDCs have mainly focused on developmental or reproductive alterations. Mouthpart deformities have been found in contaminated sediments, and have been associated with physiological alterations induced by these chemicals during moulting, causing mandibular deformities and asymmetries (Watts et al., 2003; Servia et al., 2006; Park and Kwak, 2008a). Delays in emergence, changes in the length of the life cycle and in the sex ratio are other effects associated with EDC treatment (Watts et al., 2001). Research into molecular parameters is an important approach, because these parameters could be used as biomarkers in larger-scale tests for the toxicological evaluation of these chemicals. Chironomids are increasingly being used for toxicity testing, using molecular biomarkers such as enzyme and gene activity, even though DNA sequence information in these species is still scarce (Gopalakrishnan Nair et al., 2011). The objective of this study was to analyse the early effects of two of the most extensively used phthalates catalogued as EDCs, di(2-ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBP), at the level of gene activity in the aquatic larvae of *C. riparius*. Our study focused on the transcriptional profile of selected genes related to the endocrine system (the ecdysone receptor complex), the cellular stress response (the 70-kDa HS-family) and the protein production machinery (ribosomal RNA). The study had two aims: firstly, to attempt to identify suitable genes related to endocrine disrupting activity, in order to develop a screening system for ecotoxicity monitoring of EDCs and, secondly, to find out whether or not these two related compounds, generally assumed to have similar effects, elicit a similar cellular response. The identification and validation of possible marker genes are gaining acceptance, because they can provide a fast, sensitive and high throughput assay to test compounds improving, at the same time, our understanding of the molecular mechanism underlying toxicity and the mode of action of xenobiotics.

2. Materials and methods

2.1. Animals and treatments

The experimental animals were the aquatic larvae from the midge *C. riparius*. Stock cultures are maintained in the laboratory of Biology and Environmental Toxicology (UNED) from natural populations of midge larvae originally collected in a non-polluted area of Valencia (Spain). Larvae were reared under standard laboratory conditions, according to toxicity testing guidelines (US-EPA, 1996; OECD, 2001). They were grown from egg masses in aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, and 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue in polyethylene tanks (500 mL). Cultures were maintained under constant aeration at 20 °C and under standard light–dark periods (16L:8D). For experimental treatments, fourth instar larvae, 10–15 days old, were exposed to the chemicals diluted in culture medium for 24 h with constant aeration at 20 °C in glass recipients. No food or substrate was provided during exposure. Dose selection was based on the results from previous studies in environmental samples (Petrovic et al., 2001; Fromme et al., 2002; Ogunfokan et al., 2006; EU, 2008). The larvae were submitted to butyl benzyl phthalate (BBP) (Aldrich) and di(2-ethylhexyl) phthalate (DEHP) (Sigma) for 24 h at 0.01, 0.1, 1, 10 and 100 mg/L, in all cases nominal concentration. Ethanol was used as solvent. Three independent experiments were carried out in each concentration for each phthalate, using 10 larvae arising from three different egg masses (same age or days after hatching), and each sample consisted of at least three replicates ($n = 9$). The control larvae used in each case were exposed to the same concentration of solvent as the corresponding treatment and were also measured in triplicate. Larvae were stored at −80 °C until RNA isolation was carried out.

2.2. Survival test

For the acute toxicity test, three independent experiments were carried out in which groups of 15 larvae proceeding from three different egg masses were randomly mixed and exposed to the five selected concentrations of BBP and DEHP. After a 24-h exposure, the number of dead individuals was determined and compared to control groups.

2.3. RNA isolation

Total RNA was extracted from control and exposed fourth instar larvae (ten animals for each experiment) using a guanidine isothiocyanate based method, performed with a commercial kit (Trizol, Invitrogen) according to the manufacturer's protocol. Briefly, frozen larvae were homogenized in one volume of Trizol and left for 5 min at room temperature. Then, 0.2 volumes of chloroform were added to each sample, mixed and left for 5 min at room temperature. Subsequently, the samples were centrifuged for 15 min at 4 °C and 15,000 × *g*. Following transfer of the aqueous phase, the RNA was finally recovered by isopropyl alcohol precipitation (0.5, v/v), washed with 70% ethanol, and resuspended in DEPC water. DNase treatment using 1 μL RNase-free DNase (Roche) per 50 μL sample was carried out, with subsequent phenol/chloroform extractions. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf), and purified RNA was finally stored at −20 °C.

2.4. Reverse transcription

After checking RNA integrity in 1.5% agarose gels, reverse transcription was undertaken using 1 μg of total RNA. Either an oligo dT primer (Invitrogen) or random hexamers (Invitrogen) were used with M-MLV enzyme (Invitrogen) following the manufacturer's instructions. The cDNAs obtained were stored at −20 °C until used as templates for PCRs.

2.5. Polymerase chain reaction (PCR)

Multiplex reactions were carried out using gene-specific primers, with actin as reference gene. *Actin*, *hsp70* and *EcR* gene primers are described in Martínez-Guitarte et al. (2007), Planelló et al. (2008) and Morales et al. (2011). Gene-specific primers for *hsc70* and *usp* were designed from DNA sequences present in databases, following a search in the FASTA invertebrate database to detect conserved regions. The *hsc70* sequence from *Chironomus yoshimatsui* (accession # AF448434) was compared with sequences from *Chironomus tentans* (AF448433), *Ceratitis capitata* (U20256), *Drosophila melanogaster* (AY084193), *Manduca sexta* (AF194819), *Locusta migratoria* (AY299637), *Bombyx mori* (AB084922), *Chilo suppressalis* (AB206478), *Trichoplusia* spp. (DQ845103), *Cotesia rubecula* (AY150371) and *Mamestra brassicae* (AB251896) and a highly conserved region was defined and used to design a set of primers. The *usp* sequence from *C. tentans* (accession # AF045891) was compared with sequences from *Aedes aegypti* (AF305213), *D. melanogaster* (NM_057433), *Tribulium castaneum* (XM_970162), *Leptinotarsa decemlineata* (AB211193), *Xenos peckii* (AY8271559) and *L. migratoria* (AY348873) and primers were obtained from highly conserved regions. The rDNA internal transcribed spacer 2 (ITS2) primers were designed from *Chironomus thummi* DNA sequence present in the databases (accession # AJ296807). The primers were designed to produce different-sized PCR products to allow simultaneous multiplex analysis with actin as inner control. The amplification of ITS2 fragment was carried out with 26s as internal control. Sequences and fragment size of each gene-specific pair of primers are shown in Table 1. *C. riparius* gene fragments were cloned and sequenced to ensure that they matched the selected genes.

PCR was performed in 20 μL with 2 mM of $MgCl_2$, 0.2 mM dNTPs (Biotools, Spain), 0.4 μM of each primer and 0.2 u of Taq polymerase (Biotools, Spain) under the following conditions: denaturation for 30 s, annealing for 30 s and elongation for 40 s. A MiniOpticon Thermocycler (Bio-Rad) was used. After several trials to ensure log-phase amplification, 28 cycles were carried out, with an annealing temperature of 52 °C to amplify the fragments corresponding to *EcR* and 54 °C for *usp*, *Hsp70* and *Hsc70* and ITS2. The amplified PCR products were run in a 9% acrylamide gel at 60 V for 3 h in 1× TGE buffer (40 mM Tris–Cl (pH 8.5), 200 mM glycine, and 2.5 mM EDTA), visualized after ethidium bromide staining and quantified with Chemigenius3 (Syngene), using GeneSnap 6.05 and GeneTools 3.06 software. Values of density across the whole bands were normalized against the actin or 26s standards, and the relative expression levels were then calculated. The level of expression in non-exposed control larvae was considered as background level or 1. To verify reproducibility, the analysis was repeated three times in independent experiments, with three replicates for each sample. The data were statistically analysed using SPSS software 19. The significant differences between groups were determined using the analysis of variance (ANOVA), with the Games–Howell procedure as the post hoc test. A 95% significance level ($p < 0.05$) was established for all statistical analyses carried out.

Table 1
Primers used and fragment sizes obtained in PCRs.

Gene	Primer	Fragment size
EcR forward	5′-AGACGGTTATGAACAGCC-3′	240 bp
EcR reverse	5′-CGAGCCATGCGCAACATC-3′	
Usp forward	5′-CCGCCCAATCATCC-3′	121 bp
Usp reverse	5′-CTGTGCGTTTGAAGAATCC-3′	
Hsp70 forward	5′-CATGTGAACGAGCCAAGAGA-3′	274 bp
Hsp70 reverse	5′-TTGCCACAGAAGAAATCTTG-3′	
Hsc70 forward	5′-CGTGCTATGACTAAGGACAA-3′	239 bp
Hsc70 reverse	5′-GCTTCATTGACCATACGTTC-3′	
Actin forward	5′-GATGAAGATCCTCACCGAACG-3′	201 bp
Actin reverse 2	5′-CGGAAACGTTCATTACCG-3′	
ITS2 forward	5′-TCATCAAAGCCGTTGTCT-3′	243 bp
ITS2 reverse	5′-AATCGAATTGCAAACACC-3′	
26s forward	5′-TTCGCGACCTCAACTCATGT-3′	220 bp
26s reverse	5′-CCGCATTCAAGCTGGACTTA-3′	

3. Results

3.1. Effects of BBP and DEHP on larval survival

Larval mortality was not found under any of the 24 h DEHP exposures (0.01, 0.1, 1, 10, and 100 mg/L), reaching 100% survival even at the highest concentration tested, although the larvae appeared to be affected, with a decrease in mobility and loss of colour. At the same concentrations and time intervals, BBP treatments were more toxic to the fourth instar larvae, reaching a significant mortality at the highest concentration tested (100 mg/L) (Fig. 1).

3.2. Effects of BBP and DEHP on ribosomal DNA transcription

Ribosomal genes are considered as housekeeping genes; their transcription products are essential for cellular maintenance as they constitute the ribosome, the machinery for the synthesis of all cellular proteins. Ribosomes are basic for cellular viability; therefore, analysis of ribosomal gene transcription was selected as a measure of cell injury provoked by the exposure to the chemicals. Ribosomal transcription represents over 80% of total transcriptional activity in cells, with the rRNAs being highly abundant and stable. To evaluate the changes in the transcription profile, it is necessary to differentiate between the newly synthesized rRNA and the bulk of mature 28s, 18s and 5.8s rRNAs. To this end, alterations in rDNA gene expression were evaluated by analysing the levels of recent transcripts by RT-PCR, using a probe to the internal transcribed spacer 2 (ITS2) (Gorab et al., 1995). ITS2 is present in the first immature transcription product of the rDNA gene and absent in the mature processed 28s and 18s rRNAs. As shown in Fig. 2A, when

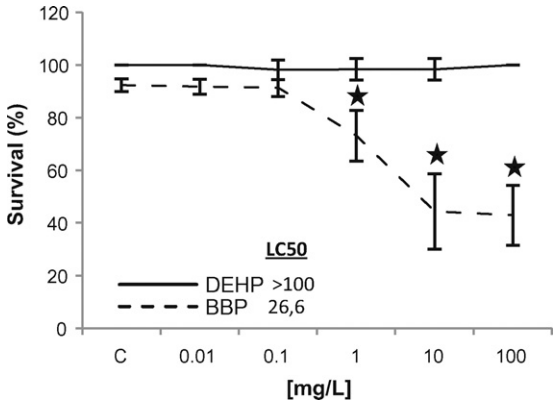

Fig. 1. Dose-dependent effect of BBP and DEHP treatments on the survival of fourth instar *Chironomus riparius* larvae. The values represent mean and SE from data obtained in three independent experiments. *Significant differences ($p \leq 0.05$). Median lethal concentration (LC50) was derived through Probit analysis. Twenty-four hour LC50 was estimated as 26.6 mg for BBP and >100 mg for DEHP.

compared to controls, the different DEHP treatments (24 h—0.01, 0.1, 1, 10, and 100 mg/L) did not alter the levels of the ribosomal precursor in any of the concentrations assayed. For BBP at the same concentrations and time exposures, the results showed that ribosomal transcription was not affected at the lower concentrations, but a slight, yet significant, decrease (around 15% inhibition at 1 mg/L)

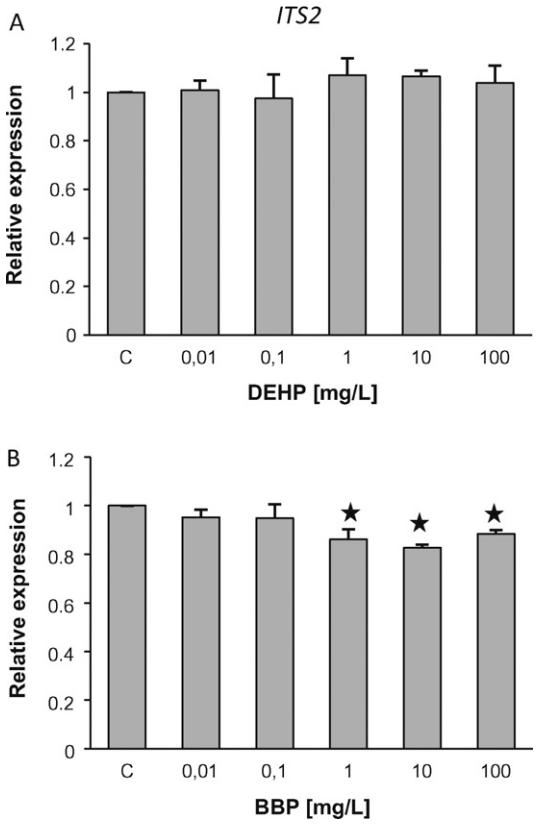

Fig. 2. Effect of (A) DEHP and (B) BBP treatments (24 h) on rDNA transcription in *C. riparius* larvae. Levels of newly synthesized rRNA were obtained by RT-PCR using primers to the internal transcribed spacer 2 (ITS2). Values were normalized relative to 26s rRNA transcript levels and shown as mean ± SE of three independent experiments, each with three replicates. Data are presented as fold change relative to the transcript expression in the control group. *Significant difference ($p < 0.05$) as compared to control cultures.

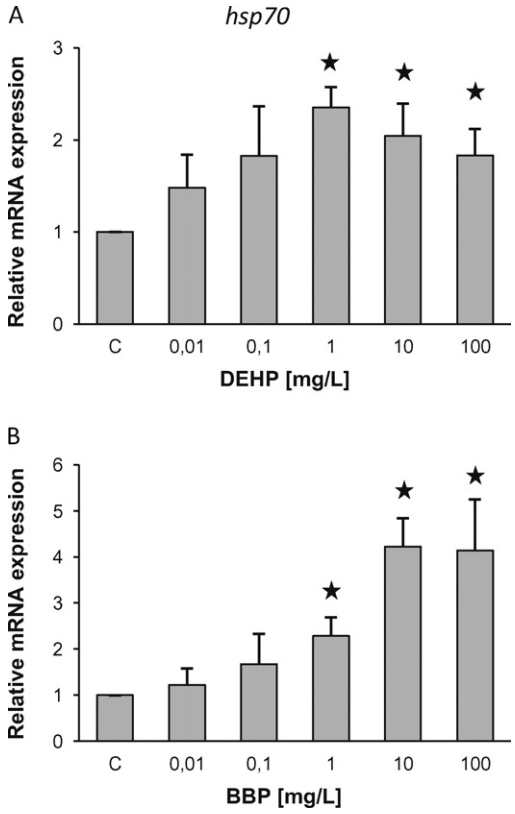

Fig. 3. Changes in the expression of the *hsp70* gene after 24-h treatments with (A) DEHP and (B) BBP in *C. riparius* larvae. The mRNA level values were calculated relative to *actin* gene expression. Each bar is the mean ± SE obtained from three independent experiments, each with three replicates. Values are expressed as fold changes with respect to the control. *Significant difference ($p < 0.05$) as compared to control cultures.

in the levels of immature rRNA (still containing ITS2) was provoked by exposure to BBP at the higher concentrations tested, from 1 mg/L and above (Fig. 2B). Therefore, BBP exposures were more toxic to the fourth instar larvae than were DEHP exposures, as measured by the impairment of rDNA gene activity, which could lead to potential cell injury.

3.3. Effects of BBP and DEHP exposures on the expression of the 70-kDa heat-shock gene family

The 70-kDa heat-shock protein family is an ancient and conserved group of proteins present in all species and every cell type analysed to date, and plays a basic role in cells as molecular chaperones. The family includes the cognate proteins (HSC70), highly abundant in normal cellular conditions, as well as inducible members (HSP70) present in stressed conditions provoked by a broad spectrum of physical and chemical insults. To analyse if BBP and DEHP have effects on the expression of the *hsp70* and *hsc70* genes encoding for these proteins, the levels of their respective mRNA were measured by RT-PCR using specific probes in exposed fourth instar larvae and, then, compared to those measured in the corresponding control groups of untreated larvae exposed to the solvent. As shown in Fig. 3, exposures to DEHP or BBP for 24 h were able to induce the *hsp70* gene in a concentration-dependent manner, with significant increases of up to twofold above control levels in the case of DEHP and fourfold for BBP, at the highest concentrations tested. In contrast, analysis of the expression of the constitutive *hsc70* gene showed that DEHP or BBP exposures did not affect the mRNA levels, which remained similar to those found in untreated

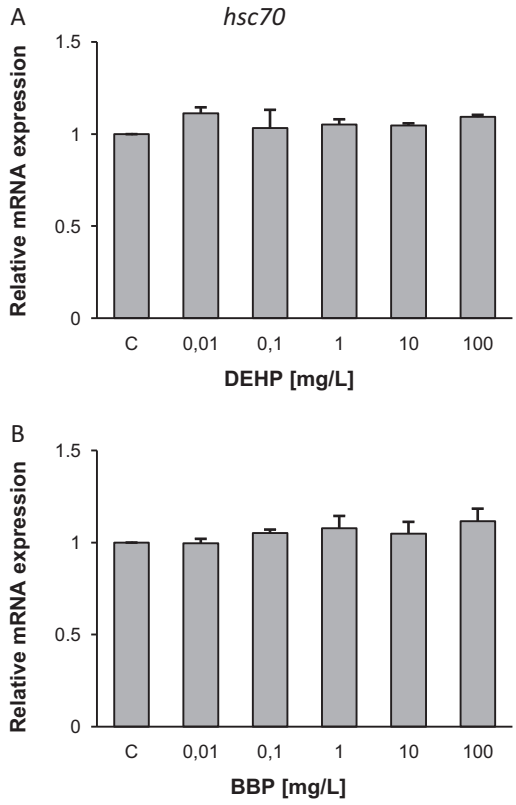

Fig. 4. RT-PCR of *C. riparius hsc70* expression after 24-h treatments with (A) DEHP and (B) BBP. The *hsc70* mRNA expression level values were calculated relative to *actin* gene expression and shown as mean ± SE of three independent experiments, each with three replicates. No significant differences from the control larvae ($p \leq 0.05$).

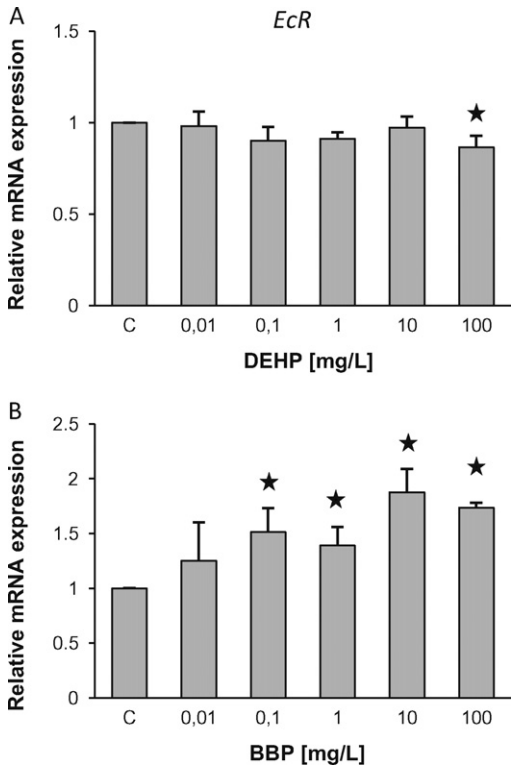

Fig. 5. Relative expression of the *EcR* gene under control and 24-h (A) DEHP and (B) BBP treatments in *C. riparius* larvae. RT-PCR experiments were carried out with the primers listed in Section 2. Transcript levels were normalized against *actin* gene expression and presented in relation to the values for the non-exposed control larvae. The mean ± SE are shown of measurements taken in three independent biological samples, each with three replicates. Densitometric values were normalized using those for *actin* mRNA and represented in relation to the values for control larvae. *Significant differences ($p \leq 0.05$).

control groups of larvae for all the concentrations assayed (Fig. 4).

3.4. Effects of BBP and DEHP on the expression profiles of the ecdysone receptor genes

Two main hormones, ecdysone and the juvenile hormone, control the development of insects. Ecdysone, produced by the prothoracic gland, has a direct influence on insect moulting and metamorphosis. The ecdysone action inside the insect cell is mediated through the interaction with the ecdysone receptor, a heterodimer comprised of two elements: the ecdysone receptor (EcR) and the ultraspiracle (USP). The genes, *EcR* and *usp*, coding for these two proteins were selected to evaluate if the phthalates can alter the activity of specific endocrine-related genes. RT-PCR analysis was used to evaluate the levels of the transcripts of these genes, after 24-h exposures at the five different concentrations of BBP and DEHP selected in this study. Following normalization to avoid random effects on sampling data, the expression of mRNA was analysed in multiplex RT-PCR in relation to actin mRNA levels. In each case, gene expression patterns were compared to those obtained from control cultures exposed to the same concentration of solvents. To avoid differences being caused by experimental procedures or those intrinsic to the larvae being sampled, for each condition, three independent experiments from different cultures, as well as three replicates, were carried out. As shown in Fig. 5, BBP treatments provoked a clear increase in the level of *EcR* mRNA when compared to control untreated larvae, with significant overexpression of the *EcR* gene from exposures of 0.1 mg/L and above. On the contrary, DEHP treatments (Fig. 5A) provoked a slight decrease, statistically significant at the highest dose tested. In the case of the *usp* gene, in contrast to that found for its partner *EcR*, expression levels remained unaffected in all the different DEHP and BBP concentrations tested (Fig. 6A and B), although a slight increase, not statistically significant, was observed at the higher BBP treatments. These results provide the first demonstration of PE-induced alterations of the ecdysone signalling pathway in arthropoda.

4. Discussion

Gene expression analysis is being increasingly used in the diagnosis of environmental contamination, as it might be more sensitive and is less time-consuming than conventional toxicology endpoints. In addition, it offers mechanistic values and provides a more comprehensive insight into toxicity (Ankley et al., 2006; Steinberg et al., 2008). The present study was designed to compare the effects of DEHP and BBP, two of the most extensively used phthalates, in the aquatic larvae of *C. riparius* at the cellular level. Acute short exposures were selected, in the range 0.01–100 mg/L for both chemicals, aimed to detect the early responses to and/or primary effects of these chemicals not derived from complex cellular toxicity processes. By monitoring the expression profile of different model genes, we found rapid genomics effects of these environmental contaminants in the three different gene families analysed: stress-related genes (*hsp70*/*hsc70*), hormonal-related genes (*EcR* and *usp*) and housekeeping genes (*rDNA*). Notable differences were found, not only in the response of the different genes to these phthalates but also among the distinct phthalates, in their ability to alter a particular gene. This is discussed below and compared with the information available from other experimental systems,

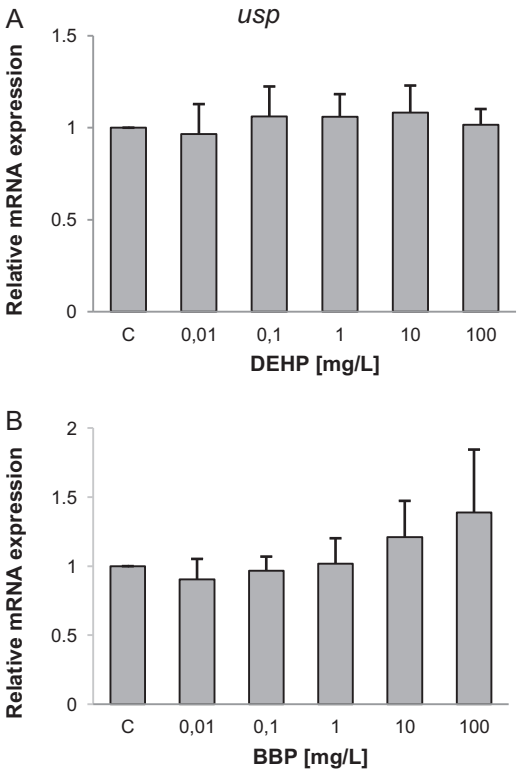

Fig. 6. *usp* mRNA levels in fourth instar midge larvae exposed to (A) DEHP and (B) BBP treatments. Densitometric analysis of *usp* mRNA values was normalized against actin and presented in relation to the values for the non-exposed control larvae. Means and standard error are shown from three independent experiments, each with three different samples. No significant differences from the control larvae ($p \leq 0.05$).

mainly vertebrates, where most research on the cellular effects of phthalates has been carried out.

DEHP, BBP and DBP are the most frequently studied phthalates, and they were found to produce almost identical responses in rodent models, with a relative toxic potency of DEHP > DBP > BBP (Foster, 2005). In contrast, BBP was found to be more toxic than DEHP in *C. riparius*, as shown by the larval survival test at the times and concentrations assayed. Moreover, BBP at concentrations of 1 mg/L and above provoked a significant inhibition in ribosomal gene transcription, while in the case of DEHP it was not affected even at the highest doses tested. It is tempting to speculate that the inhibitory effect of BBP on ribosomal gene activity could be correlated with the higher larval mortality rates provoked by this compound. As ribosomal transcription is essential for ribosomal production, and ribosomes are essential for the synthesis of all the cellular proteins, depletion in cellular proteins in our study led to cellular failure. Ribosome biogenesis is a major cellular undertaking, and is a diagnostic indicator for the general metabolism of the cell. Indeed, almost any disturbance that slows down cell growth or protein synthesis, such as nutrient or growth factor starvation, senescence, toxic lesion, viral infections, leads to decrease in rDNA transcription (Grummt, 2010). In *Chironomus*, cadmium exposure, but not that to bisphenol A, inhibited transcription of ribosomal genes (Planelló et al., 2007, 2008).

The cellular response to stress is characterized by the activation of a set of genes to counteract the physiological disturbance induced by physical or chemical agents. Among these genes, the 70-kDa heat-shock gene family appears extraordinarily well conserved throughout evolution from bacteria to man, encoding proteins that may be responsible for survival and adaptation under heat-shock conditions, and also during xenobiotic exposure of cells (Gupta et al., 2010). In this study, following different exposures to BBP and DEHP, remarkable differences were found in the transcriptional response of heat-shock protein 70 gene and heat-shock cognate protein 70 gene (*hsp70*/*hsc70*) in *C. riparius*. Both phthalates exert upregulatory effects on the *hsp70* gene, following a concentration–response relationship, with BBP being a stronger inducer (up to fourfold increase). However, neither phthalate, at any of the concentrations tested, altered the counterpart *hsc70* constitutively expressed under control conditions, whose levels of mRNA remained nearly identical to those found in the corresponding control samples. These results suggest that the *hsc70* gene seems to have a robust resistance to the effects of these toxicants, and appears to be differentially regulated than *hsp70*. Nevertheless, other authors have reported that *hsc70* is activated as well as *hsp70* under exposure to different metals and pollutants in *C. tentans* (Karouna-Renier and Zehr, 2003; Lee et al., 2006; Karouna-Renier and Rao, 2009). Our results indicating that the stress gene *hsp70* could be a sensitive molecular biomarker for these phthalates are an addition to the growing body of literature that connects the HSP70 protein to different chemical stressors. A number of recent studies have shown the potential of these genes in pollution monitoring. The inducible form of the *hsp70* gene from the midge *Chironomus* has been described as being activated by different environmental toxicants, including pesticides (Yoshimi et al., 2002), metals (Karouna-Renier and Rao, 2009; Planelló et al., 2010) and bisphenol A (Planelló et al., 2008). Currently, the potential use of heat-shock proteins for predicting the toxicity of chemicals is being actively investigated (Gupta et al., 2010). Although HSP70 is a defence protein, often mentioned as a broad range sensor of cellular stress, it is worth pointing out that other compounds, such as pentachlorophenol and tributyltin, are unable to activate this gene even though they are potent biocides (Morales et al., 2011). The induction patterns of different heat-shock genes exhibit variations based on the type of inducer, and are potentially useful as biomarkers of early cellular stress-sensing events provoked by a particular chemical. Genes encoding for other stress-related proteins, such as HSP90 and HSP40, were also found to be activated by DEHP in *Chironomus* (Park and Kwak, 2008a), while other stress genes, such as *hsp16.1* and *hsp16.2*, were found to be downregulated by DEHP in the nematode *Caenorhabditis elegans* (Roth et al., 2007). Apart from heat-shock genes, other genes have been reported to be altered by DEHP, such as the haemoglobin genes in *C. tentans* (Lee et al., 2006) and serine-type endopeptidase and alcohol dehydrogenase genes in *C. riparius* (Park and Kwak, 2008b, 2009).

Experimental evidence for adverse effects on reproduction and development has identified phthalates as endocrine active chemicals in humans and also in mammalian models (Lyche et al., 2009). Although there have been a number of studies examining the effects of phthalates on steroid hormones, mainly androgens and estrogens, there is limited information on the direct effects of these compounds on invertebrate hormones and, up to now, no information available about interactions with insect ecdysteroid hormones. In this study, the effects of DEHP and BBP on hormonal endpoints have been assessed by measuring the potential effect on the activity of the genes coding for the hormone ecdysone receptor in *C. riparius* larvae. In insects, development of both larval and imaginal tissues is controlled by the steroid hormone ecdysone that triggers the changes that result in metamorphosis. Ecdysone responsiveness of cells is mediated by two members of the superfamily of hormone nuclear receptors: ecdysone receptor EcR and ultraspiracle USP. The functional complex is a heterodimer EcR–USP (Lezzi et al., 1999, for a review) that, once activated by the hormone, becomes a transcription factor acting as a molecular switch, which turns on the gene programmes that allow developmental changes and

lead to the progression of moulting and metamorphosis. Our data revealed that BBP, but not DEHP, is able to upregulate the expression of the *EcR* gene, while neither of them alters the expression of the *usp* gene. In contrast, DEHP displayed a tendency to decrease the activity of this *EcR* gene, which was statistically significant at the highest concentration. These results clearly show that each phthalate had a differential selectivity for the ecdysone receptor gene *EcR*. As the nuclear receptor is a key factor in hormone signal transduction inside the cells, our results provide the first evidence that phthalates are able to directly interact at the cellular level with the insect endocrine pathway. *EcR* is induced directly by ecdysone, and provides an autoregulatory loop that increases the level of the receptor protein in response to the hormone ligand (Koelle et al., 1991). Therefore, our data demonstrate that BBP has an ecdysone-mimetic action and is able to upregulate the levels of *EcR* in *C. riparius* cells, whereas DEHP might act as an antagonist. In contrast to that found for its functional partner, *usp* gene expression was not affected by the presence of the phthalates at the time and concentrations tested. Although this differential behaviour could, at first sight, be surprising it does resemble the effect of the natural hormone ecdysone, which increases EcR concentrations. In contrast, USP (constitutively expressed) remains constant, although phosphorylation is enhanced (Yao et al., 1993; Rauch et al., 1998). Interestingly, the effects of BBP on the hormone receptor gene are in agreement with those previously reported in *C. riparius* for other well-known endocrine disruptors, such as bisphenol A and cadmium, which also upregulated the *EcR* gene (Planelló et al., 2008, 2010) differing from the behaviour found for DEHP. Our results reinforce the potential of this gene to be assayed as a biomarker for the assessment of EDCs in aquatic insects, and suggest a possible mechanism for the role of endocrine disruptors in invertebrates. It is worth mentioning that the estrogen-related receptor (ERR), a member of the orphan nuclear-receptor family, appeared also activated in the presence of BPA, nonylphenol and DEHP (Park and Kwak, 2010).

Although the endocrine systems of invertebrates differ drastically from those of vertebrates, it is of interest to note that the ecdysteroid hormones in insects belong to the family of steroid hormones. Moreover, the ecdysone receptor belongs to the superfamily of nuclear hormone receptors that includes estrogens, androgens, thyroid hormone, retinoic acid and glucocorticoid receptors, among others. Several studies have demonstrated that some phthalates are capable of interacting with estrogen receptors (ERs) and induce ER-mediated responses (Jobling et al., 1995). It has been reported that BBP binds to ERα and enhances the transcriptional activity of ERα (Fujita et al., 2003). Our pioneering results in invertebrates are in accordance with experimental evidence in vertebrates, which highlights that the nuclear-receptor superfamily is a molecular target for endocrine disruptors (Diamanti-Kandaris et al., 2009 for review). Interestingly, also the differences we found in relation to EcR between BBP and DEHP have been reported for ER in other experimental systems. *In vitro* assays demonstrated that BBP exhibits an estrogenic effect through estrogen receptor transactivation, whereas DEHP did not elicit any agonistic ER activity (Ghisari and Bonefeld-Jorgensen, 2009). BBP was estrogenic in yeast screen and human breast cancer cells, while DEHP showed no estrogenic activity in these *in vitro* assays (Harris et al., 1997). More recently, a toxicogenomic study of phthalate–gene interactions in humans showed that BBP and DBP interact with estrogen receptor 1, estrogen receptor 2 and androgen receptor genes, while DEHP and MEHP did not, although they did interact with the peroxisome proliferator-activated receptor PPAR (Singh and Li, 2011). In male zebrafish, exposure to DEHP (5000 mg/kg) increased the levels of two peroxisome proliferator-activated receptor-responsive genes (Uren-Webster et al., 2010). In rodents, DEHP has been reported to inhibit the enzyme aromatase, which converts testosterone to estradiol and, thereby, plays an important role in sexual differentiation (Andrade et al., 2006). The phthalates discussed here (DEHP and BBP) both interfere with the synthesis of testosterone, as well as with insulin-like growth factor 3 (IGF-3). They are being considered to be anti-androgenic phthalates, whereas their effects on female sexual differentiation have been less studied (Howdeshell et al., 2008).

To sum up, the effects of DEHP and BBP on the expression of genes related to the insect endocrine system do share some similarities with those encountered in the vertebrate estrogenic system. The data obtained in our study provide the first insights into the potential ability of phthalates to have an impact on the gene expression of the ecdysone receptor and, consequently, on the ecdysone-mediated responses in insects. The ecological relevance of these genomic effects, especially the capability to interact with the hormonal system in insects, clearly shown for the first time in our study, merits further research. The potential effects at lower doses, but over longer periods of exposure, should be considered particularly regarding the bioaccumulation of phthalates. Overall, our study improves the knowledge of gene–toxin interactions and adds new genomic endpoints for further monitoring phthalates, as well as other aquatic pollutants, in natural populations under environmentally relevant scenarios (lower concentrations and chronic exposures as well as combined exposures to mixtures of phthalates). Most of the physiological, organismal and population responses to the environment, natural or polluted, have their origin and explanation at the genomic level, by turning genes up and down.

5. Conclusions

Chironomids are sentinel organisms in the monitoring of the health of aquatic systems and constitute an attractive model of ecotoxicological relevance for the study of pollutant-induced genomic-level responses. There is little DNA sequence information in *Chironomus*, due to the lack of a genome-sequencing programme. This reinforces the need to identify specific and sensitive genes in these species, to be able to develop screening systems for ecotoxicity monitoring. Our results clearly show that, following DEHP and BBP exposures, rapid and differential changes in gene expression take place in *C. riparius*, among the group of genes that encode proteins belonging to three different metabolic pathways: stress-related (*hsp70*/*hsc70*), endocrine-related (*EcR*/*usp*) and housekeeping genes (*rDNA*). The data suggest that short acute exposures to these phthalates are able to specifically alter the stress response and the ecdysone signalling pathway, and that long-term chronic exposures may alter development and reproduction. Our results show for the first time that hormone-mimicking chemicals in vertebrates, such as phthalates, can also have hormonal activities in invertebrates. Moreover, the results suggest that the behaviour of BBP and DEHP in relation to the steroid hormone ecdysone signalling pathway resembles that of steroid hormones, particularly sex steroids, in vertebrates. Although further research is needed, these findings are of considerable biological relevance. Such research should study the potential of the ecdysone receptor genes, and/or proteins, for assessing interference by endocrine disrupting chemicals in natural ecologically relevant scenarios, and should provide better understanding of endocrine physiology and of the role of invertebrate steroids in these processes.

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