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The plasticizer benzyl butyl phthalate (BBP) alters the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms in *Chironomus riparius* larvae

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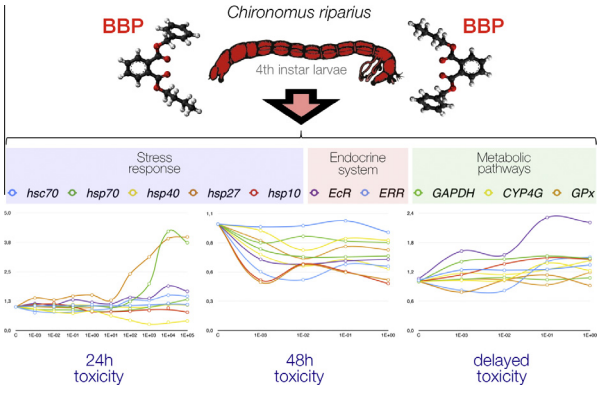
HIGHLIGHTS

- The genomic toxic effects of butyl benzyl phthalate were evaluated in *C. riparius*.
- BBP induced time and dose-dependent changes in the transcription levels analysed.
- *hsp70*, *hsp40* and *hsp27* heat-shock genes were specific targets of the xenobiotic.
- Alterations of hormone-related genes confirm BBP as an endocrine disruptor in insects.
- BBP caused delayed toxic effects, not detected in conventional acute exposures.

GRAPHICAL ABSTRACT



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ABSTRACT

Butyl benzyl phthalate (BBP) has been extensively used worldwide as a plasticizer in the polyvinyl chloride (PVC) industry and the manufacturing of many other products, and its presence in the aquatic environment is expected for decades. In the present study, the toxicity of BBP was investigated in *Chironomus riparius* aquatic larvae. The effects of acute 24-h and 48-h exposures to a wide range of BBP doses were evaluated at the molecular level by analysing changes in genes related to the stress response, the endocrine system, the energy metabolism, and detoxication pathways, as well as in the enzyme activity of glutathione S-transferase. BBP caused a dose and time-dependent toxicity in most of the selected biomarkers. 24-h exposures to high doses affected larval survival and lead to a significant response of several heat-shock genes (*hsp70*, *hsp40*, and *hsp27*), and to a clear endocrine disrupting effect by upregulating the ecdysone receptor gene (*EcR*). Longer treatments with low doses triggered a general repression of transcription and GST activity. Furthermore, delayed toxicity studies were specially relevant, since they allowed us to detect unpredictable toxic effects, not immediately manifested after contact with the phthalate. This study provides novel and interesting results on the toxic effects of BBP in *C. riparius* and highlights the suitability of this organism for ecotoxicological risk assessment, especially in aquatic ecosystems.

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1. Introduction

Butyl benzyl phthalate (BBP) is a man-made phthalate ester mainly used as a plasticizer in the polyvinyl chloride (PVC) industry, although it can also be found in a variety of other products such as food conveyor belts, artificial leather, automotive trim, traffic cones (CERHR, 2003), vinyl gloves and adhesives (CPSC, 2010). Because BBP is an external plasticizer, which softens the resins without binding chemically to the final product, it tends to migrate slowly out of the plastic, becoming a ubiquitous environmental pollutant (Laughlin et al., 1978; Clark et al., 2011) and entering into the food chain (CERHR, 2003). BBP is included in the list of Substances of Very High Concern (SVHC) by the European Chemicals Agency (ECHA, 2008), and in the Substance Priority List (SPL) by the Agency for Toxic Substances and Disease Registry (ATSDR, 2014). The compound is classified as a reprotoxic substance in the CLP Regulation 1272/2008 (EU, 2008), and its use is prohibited in the production of toys and childcare articles (EU, 2006), cosmetics (EU, 2004), and materials intended to come into contact with food (EU, 2007). According to industry, the high production registered in the mid- and late-1990s (e.g. 45 000 tonnes in Europe) has declined over the recent years due to its toxic properties and labelling (ECB, 2007). However, despite all restrictions, migration of this compound from products containing it will ensure its presence in many environmental compartments (air, water, sediment, soil and sludge, and biota) (Staples et al., 1997; Fromme et al., 2002; ECB, 2007) for decades, thus being particularly relevant to know its effects on human health and ecosystems.

In comparison with more exhaustively investigated phthalates, such as di(2-ethylhexyl) phthalate (DEHP) or di-*n*-butyl phthalate (DBP), there are not many specific epidemiological studies about the toxic effects of BBP in humans, although there have been reported irritating effects (Mallette and Von Haam, 1952), asthma and allergic symptoms (Bornehag et al., 2004; Jaakkola and Knight, 2008) and, specially, associations with reproductive and developmental alterations (Lyche et al., 2009), including the decrease in anogenital distance (AGD) (Swan et al., 2005), the alteration of endogenous reproductive hormones (Main et al., 2006), and the gestational age reduction and preterm delivery (Huang et al., 2014).

Phthalates have produced a variety of adverse effects in animal studies, including hepatic peroxisome proliferation and cancer, and changes in kidney and thyroid (Kavlock et al., 2006), although the most severe adverse effects observed appear to be related to reproductive and developmental effects (Lyche et al., 2009). At present, the general consensus is that DEHP, DBP, and BBP have potential to disrupt normal development and reproduction, effects observed in one or more animal species (Fabjan et al., 2006).

Studies on the environmental toxicity of BBP have particularly focused on fish models, in which the compound has demonstrated to cause mortality (Chen et al., 2014), to be oestrogenic (Jobling et al., 1995; Harris et al., 1997; Chen et al., 2014), to induce vitellogenin synthesis (Christiansen et al., 1998), to inhibit growth, to disturb the gonadal differentiation process, to delay testicular development and to induce the feminisation process (Jarmołowicz et al., 2014). Recent studies have also described that BBP modulates lipid peroxidation and expression of androgen receptors and genes related with xenobiotic metabolism (Mankidy et al., 2013), alters enzymatic antioxidant activities (Qu et al., 2014), modifies individuals behaviour (Wibe et al., 2002, 2004; Kaplan et al., 2013), and protects sperm quality under oxidative stress conditions (Oehlmann et al., 2009), possibly through the interaction of the compound with the PPAR receptor (Corton and Lapinskas, 2005).

Although invertebrates constitute a critical part of the food chain and therefore are essential to maintain ecosystems stability and homeostasis, there are very few studies on the toxicity of BBP in these organisms. It is known that the BBP increases mortality in the amphipod crustacean *Hyalella azteca*, the freshwater oligochaete *Lumbriculus variegatus* and the midge *Chironomus tentans* (Call et al., 2001a,b), induces cell death and causes significant decreases in phenoloxidase activity and superoxide production in the giant freshwater prawn *Macrobrachium rosenbergii* (Sung et al., 2003), produces adverse effects on embryogenesis and larval development in the marine univalve *Haliotis diversicolor* (Liu et al., 2009), and affects markedly life expectancy at birth, net reproductive rate and generation time in the freshwater cladoceran *Moina macropopa* (Wang et al., 2011).

Chironomids are among the most ubiquitous and abundant freshwater benthic invertebrates, and some of its species are widely used as test organisms in standard freshwater and whole sediment toxicity tests for ecological risk assessment (EPA, 1996a,b, 2000; OECD, 2004a,b, 2010, 2011). The midge *Chironomus* is also considered to be an appropriate test species for research about potential endocrine disrupting substances (OECD, 2006; Taenzler et al., 2007) and is the focus of numerous research and regulatory initiatives to evaluate the endocrine disrupting effects in aquatic invertebrates (Segner et al., 2003). Moreover, *Chironomus riparius* is increasingly being used for toxicity testing using molecular endpoints such as enzyme and gene activity, which constitute an important approach given that these parameters could be used as biomarkers in larger-scale tests for the toxicological evaluation of chemicals. Although there is still insufficient DNA sequence information for these aquatic species (Nair and Choi, 2011a), in the last few years several molecular biomarkers have demonstrated to be responsive against different xenobiotics, including those for heat-shock proteins (HSPs), ribosomal proteins, nuclear receptors and detoxication pathways, among others (Planelló et al., 2007, 2008, 2010, 2011; Park and Kwak, 2008, 2009, 2010, 2012; Park et al., 2009, 2010; Morales et al., 2011, 2013, 2014; Nair and Choi, 2011a,b, 2012; Nair et al., 2011, 2012; Martínez-Paz et al., 2012, 2014; Ozáez et al., 2013, 2014).

The group of heat-shock proteins (HSPs) includes ancient and conserved protein families present in all species and every cell type analysed to date, named according to their molecular weight. The most prominent members of this group play a basic role in cells as molecular chaperones, involved in the folding and unfolding of other proteins, but HSPs also participate in processes such as thermotolerance, inhibition of apoptosis, regulation of cell development, cell differentiation, and signal transduction, among others. The increased expression of heat-shock genes can be triggered by exposure to different kinds of environmental stress conditions, although some of these proteins are constitutively highly abundant in normal cellular conditions, like the 70 kDa heat-shock cognate protein (HSC70).

Steroid hormones are transported in the plasma circulation to their target organs and exert their effects after gaining entry to the cell, acting as ligands for nuclear receptors and altering gene transcription. The ecdysone action inside the cell has a direct influence on insect moulting and metamorphosis and it is mediated through the interaction with the ecdysone receptor (EcR). Estrogen-related receptors (ERRs) share structural and functional attributes with the estrogen receptors (ERs) and perform critical roles in the hormone system, functioning as transcription factors that control essential developmental and physiological pathways.

Over all metabolic activities that take place in an organism, there are two particularly relevant for survival against exposure to toxic compounds: obtaining the necessary energy to maintain all cellular processes and transforming and/or excreting the toxin through the detoxication mechanisms. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) plays a key role in energy production throughout glycolysis, although it is also involved in other functions at multiple subcellular compartments. At the same time,

cytochrome p450 (CYP4G) and glutathione enzymes such as glutathione peroxidase (GPx) and glutathione S-transferase (GST) are involved respectively in phase I and phase II detoxication reactions, in which a toxin is metabolised to reduce its toxicity and facilitate excretion.

The aim of the present study was to evaluate the early effects of butyl benzyl phthalate (BBP), an extensively used plasticizer with known toxic and endocrine disrupting properties, in the aquatic larvae of *C. riparius*. Our work tested a wide range of BBP concentrations and several exposure scenarios (including delayed toxicity conditions), and focused on larval survival and the transcriptional profile of selected genes related to the cellular stress response (*hsc70*, *hsp70*, *hsp40*, *hsp27*, *hsp10*), the endocrine system (*EcR*, *ERR*) and the energy (*GAPDH*) and detoxication metabolisms (*CYP4G*, *GPx*). In addition, the enzyme activity of glutathione S-transferase was analysed, as it is also related to detoxication pathways.

2. Materials and methods

2.1. Test animals

The experimental animals were the aquatic larvae from the midge *C. riparius*. Larvae used were reared under standard laboratory conditions, according to toxicity testing guidelines (EPA, 2000; OECD, 2004a,b). They were grown from egg masses in polyethylene tanks (500 mL) with 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, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue. Cultures were maintained under constant aeration at 20 °C and standard light–dark periods 16:8.

2.2. Exposure conditions and survival test

Solutions of butyl benzyl phthalate (BBP, CAS No. 85-68-7, Aldrich) were dissolved in analytical grade ethanol to provide a stock concentration of 10^7 μg L^{-1}. The test solutions were constructed in culture medium at 0.01% ethanol. This was the final percentage of ethanol present in the solvent controls used in the experiment and preliminary tests demonstrated that induced no effects on the organisms in any of the selected endpoints. The nominal concentrations of BBP were 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^2, 10^3, 10^4, and 10^5 μg L^{-1}.

Larval survival was studied in a wide range of BBP concentrations (10^{-3}–10^5 μg L^{-1}) for 24-h exposures, and at the four lowest doses (10^{-3}–1 μg L^{-1}) for 48-h exposures and delayed toxicity studies (24 + 24 h), in which larvae were exposed 24 h to BBP and then were maintained 24 h in fresh culture medium. Groups of 20 larvae proceeding from different egg masses were randomly mixed and exposed to BBP, and larval mortality was recorded after 24 or 48 h, according to the treatments. Experiments were performed in triplicate and the median lethal concentration (LC50) was determined by Probit analysis (Finney, 1971). For further analysis, RNA and protein extraction were performed from surviving larvae, which were frozen and stored at −80 °C.

2.3. RNA extraction

Total RNA was extracted from frozen larvae using a guanidine isothiocyanate based method, performed with a commercial kit (TRIzol, Invitrogen) according to the manufacturer's protocol. Briefly, frozen material was homogenated 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 (0.5v/v), washed with 70% ethanol, and resuspended in DEPC water. The RNA was then treated with RNase-free DNase (Roche). The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Nanodrop1000, Thermo), and the purified RNA was finally stored at −20 °C.

2.4. Reverse transcription polymerase chain reaction (RT- PCR)

RT-PCR was used to evaluate the mRNA expression profiles of the different selected genes in treated and unexposed larvae. Reverse transcription was performed with 1 μg of the isolated RNA. An oligo dT primer (Invitrogen) was used with the M-MLV enzyme (Invitrogen) following the manufacturer's instructions. The cDNAs obtained were stored at −20 °C until being used as templates for PCR reactions. Amplification reactions were carried out using gene-specific primers, with actin and 26s as reference genes. Sequences, fragment size and appropriate references 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 MgCl2, 0.2 mM dNTPs (Biotools, Spain), 0.4 μM of each primer and 0.2 μL 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, 24 and 34 cycles were carried out depending on the gene, with an annealing temperature of 55 °C to amplify the fragments corresponding to *actin*, *26s*, *hsc70*, *hsp70*, *hsp40*, *hsp27*, *hsp10*, *EcR*, *ERR*, *GAPDH*, *CYP4G and GPx genes*. The amplified PCR products were run in a 9% acrylamide gel at 60 V for 3 h in 1x 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 median values of *actin* and *26s* standards, and the relative expression levels were then calculated. A total of twenty larvae were used for gene expression analysis. To avoid differences being caused by experimental procedures or by those intrinsic to the larvae being treated, four independent experiments (each with five larvae) were carried out for each exposure condition, with three replicates in each sample.

2.5. Protein extraction

Frozen larvae were homogenized in 0.5 mL of Tris–EDTA buffer (40 mM Tris, 1 mM EDTA, pH 7.8) with 7x complete EDTA-Free protease inhibitor (Roche), with a pellet mixer (VWR). Crude homogenate was then centrifuged for 15 min at 500 rpm at 4 °C. Subsequently, the supernatant was centrifuged for 30 min at 8000 g at 4 °C. Total protein was quantified with BCA Protein Assay Reagent (Thermo Scientific) and 25 μg of total protein was used for the enzymatic assay.

2.6. Glutathione S-transferase (GST) activity

Glutathione-S-transferase activity was evaluated at 1 and 10^3 μg L^{-1} BBP. Five control larvae and five treated larvae for each exposure time were collected after treatments. Three independent experiments were carried out, three replicates were performed in each experiment and each sample was run in duplicate wells. Protein extraction from larvae was performed as described above. Total protein was quantified with BCA Protein Assay Reagent (Thermo Scientific) and 25 μg of total protein were used for the

Table 1
Primers used for RT-PCR amplification of the genes studied in *C. riparius*. Forward (F) and reverse (R) sequences, base pair (bp) length of the obtained fragments and origin of sequences are provided.

Gene	DNA sequence (5′–3′)	Fragment size	References
Actin	**F** GATGAAGATCCTCACCGAACG **R** CGGAAACGTTCATTACCG	201 bp	Martínez-Guitarte et al. (2007)
26S	**F** TTCGCGACCTCAACTCATGT **R** CCGCATTCAAGCTGGACTTA	220 bp	Planelló et al. (2011)
hsc70	**F** CGTGCTATGACTAAGGACAA **R** GCTTCATTGACCATACGTTC	239 bp	Planelló et al. (2011)
hsp70	**F** CATGTGAACGAGCCAAGAGA **R** TTGCCACAGAAGAAATCTTG	274 bp	Morales et al. (2011)
hsp40	**F** TACGTGACGCTAGAGGAAA **R** TTCCAGCCCGGCTT	131 bp	Planelló et al. (2010)
hsp27	**F** TCCTCGTGCTTGCC **R** CAAGGATGGCTTCCA	202 bp	Martínez-Paz et al. (2014)
hsp10	**F** GAGGAATTGTGATTCCAGA **R** TGCCACCGTATTCAGG	151 bp	Unpublished data
EcR	**F** AGACGGTTATGAACAGCC **R** CGAGCCATGCGCAACATC	240 bp	Planelló et al. (2008)
ERR	**F** CTCAGCAAGTAAGGAGGAG **R** CGTCTAATAATGTGATCGG	222 bp	Park and Kwak (2010)
GAPDH	**F** GGTATTTCATTGAATGATCACTTTG **R** TAATCCTTGGATTGCATGTACTTG	110 bp	Park and Kwak (2009)
CYP4G	**F** GACATTGATGAGAATGATGTTGGTG **R** TAAGTGGAACTGGTGGGTACAT	340 bp	Martínez-Paz et al. (2012)
GPx	**F** AAGTGTGGTTACACAGCTAAGCATT **R** GATATCCAAATTGATTACACGGAAA	112 bp	Nair et al. (2012)

enzymatic assay. The GST activity was assessed spectrophotometrically with the kit GST (Sigma) in a Jasco V-530 spectrophotometer. This kit uses 1-Chloro-2, 4-Dinitrobenzene (CDNB), which is suitable for the broadest range of GST isozymes. Conjugation of the thiol group of the glutathione to the CDNB substrate produces an increase of absorbance at 340 nm. The change in absorbance at 340 nm is used to calculate GST activity.

2.7. Data analysis

Probit and statistical analysis were performed by means of IBM® SPSS® Statistics 19 software. The normalized levels of the specific gene transcripts and GST enzyme activity were analysed with ANOVA, followed by Games Howell's or Bonferroni's post Hoc tests, when appropriate. Normality and homoscedasticity of data were tested using the Shapiro–Wilk and Levene tests, respectively. If data were not homogeneous or normally distributed the Kruskal–Wallis test was used, and the differences between pairs were analysed with Mann–Whitney U tests. Probabilities were adjusted using the Bonferroni correction. The differences were considered significant at $p < 0.05$.

3. Results

3.1. Effects of BBP on larval survival

Our first approach to the toxic effects of BBP treatments in *C. riparius* was the analysis of the survival of fourth instar larvae (Fig. 1). A wide range of concentrations (10^{-3}–10^{5} μg L^{-1}) was selected to evaluate the mortality after 24 h exposure to BBP. This range included the maximum and minimum values found in different natural exposure scenarios (Fromme et al., 2002; Clark et al., 2003; ECB, 2007), and also exposure concentrations tested in laboratory studies (Call et al., 2001a; Morales et al., 2011; Planelló et al., 2011). BBP showed a dose-dependent toxic effect with a significant mortality at concentrations over 10^{3} μg L^{-1}. The LC50 was determined by Probit analysis and was $2.7 \cdot 10^{4}$ μg L^{-1}. For longer treatments, the lowest concentrations were selected (10^{-3}–1 μg L^{-1}) and no mortality was detected neither after 48 h of continuous exposure to BBP nor in the delayed toxicity studies (24 + 24 h).

3.2. Transcriptional changes of heat-shock genes after exposure to BBP

In the assessment of the toxic properties of BBP in *C. riparius*, we analysed the expression profile of constitutive (*hsc70*) and inducible (*hsp70*, *hsp40*, *hsp27*, and *hsp10*) heat-shock genes under the same experimental conditions tested in the survival assays, as shown in Fig. 2.

Semiquantitative RT-PCR results showed that the BBP triggered a clear dose-dependent effect on the transcriptional levels of *hsp70*, *hsp40*, and *hsp27* after 24 h exposures, but in different ways. On the one hand, the highest concentrations produced a significant overexpression of *hsp70* (Fig. 2D) and *hsp27* genes (Fig. 2J), reaching values above 4-fold as compared to the controls. On the other hand, the gene coding for the 40 kDa protein showed a remarkable inhibition even at the lowest concentrations tested, with a strong repression (70% below control) at high doses of BBP (Fig. 2G). No significant changes were detected for the constitutive gene *hsc70* (Fig. 2A) and the mitochondrial chaperone gene *hsp10* (Fig. 2M) in the same conditions.

Longer experiments had two clear opposite effects. Prolonging BBP exposures to 48 h caused widespread inhibition of all the genes studied (up to 50%), except for *hsc70* (which remained stable). In contrast, *hsp70*, *hsp40*, and *hsp10* inducible genes tended towards significant overexpression after the toxin removal, in the delayed toxicity experiments, showing effects not detected in the acute 24-h expositions.

3.3. Effects of BBP on the expression profile of hormone-related genes

Relative expression levels of both genes encoding nuclear receptors after BBP treatments in *C. riparius* are shown in

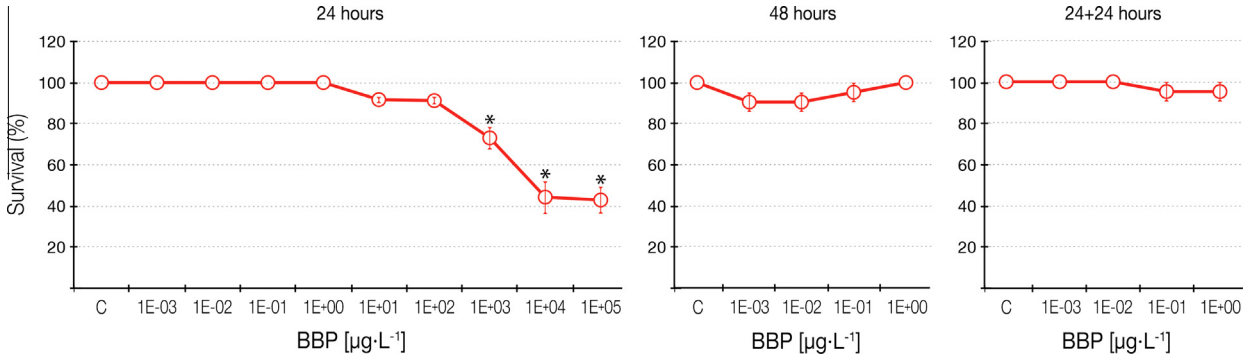

Fig. 1. Dose and time-dependent effects of benzyl butyl phthalate treatments on the survival of *Chironomus riparius* fourth instar larvae. The values represent mean and SE from data obtained in three independent experiments. *Significant differences ($p \leqslant 0.05$).

Fig. 3(A–F). Exposure to BBP for 24 h caused a similar dose-dependent overexpression of *EcR* (Fig. 3A) and *ERR* (Fig. 3D) at higher concentrations and were statistically significant for *EcR*. Their responses were also similar in longer experiments, with a moderate decrease for all concentrations after 48 h exposure (Fig. 3B and E) and a marked upregulation in the delayed toxicity tests (Fig. 3C and F). It is noteworthy that the removal of BBP let us detect important disrupting effects on both hormone-related genes, which were unpredictable with just 24-h treatments.

3.4. Energy metabolism and detoxication in larvae exposed to BBP

The effects of BBP on the transcriptional activity of *GAPDH*, *CYP4G*, and *GPx* are represented in Fig. 3(G–O). Fig. 4 shows the variations in GST enzyme activity in larvae exposed to two selected concentrations of BBP (1–10^3 µg L^{-1}). *GAPDH* transcriptional levels suffered no noteworthy changes at the conditions analysed, except for a significant overexpression at the highest BBP concentration after 24 h (Fig. 3G) and a slight widespread repression after 48 h (Fig. 3H). No effects were detected in either *GYP4G* or *GPx* gene neither in 24-h acute exposures (Fig. 3J and M) or delayed toxicity tests (Fig. 3L and O), with the exception of a significant increase of the CYP4G level after removing 1 µg L^{-1} BBP. However prolonged exposures up to 48 h resulted in a marked repression of both detoxication genes (Fig. 3K and N).

Two concentrations were selected for the analysis of the enzymatic activity of GST: 1 and 10^3 µg L^{-1}. Even at the lowest dose, GST activity was reduced by 25% in the first 24 h in the presence of BBP. This effect was emphasized after 48 h, reaching a significant reduction of 40% at the lowest concentration and 70% at the highest one. It should be noted that the two doses of BBP provoked significant delayed inhibitions, with more severe effects after toxin removal than those detected in the first 24 h.

4. Discussion

Despite being considered a ubiquitous pollutant and having been classified by international regulations as toxic to the environment, specially to aquatic ecosystems, ecotoxicological information about BBP is still scarce. The main objective of the present work has been to characterise molecular toxic endpoints of this pollutant in the model species *C. riparius*, in order to find accurate responsive biomarkers that could help in the assessment of ecosystems health.

This work reveals novel and significant dose and time-dependent BBP-induced alterations of genes related to important cell events, such as hormone pathways, energetic metabolism, detoxication routes, and stress response. These results add new information about the mode of action of this contaminant in cellular processes, which to date has received little attention in invertebrates.

On the basis that any change caused by a toxin in an organism usually begins with altered transcriptional profiles, we have focused mainly in potential target genes selected for their involvement in metabolic and hormonal pathways of special interest. We have studied the toxic effects of BBP in 24 and 48 h acute exposures, and also the delayed toxicity of the xenobiotic in treatments that consisted of 24 h exposure followed by toxin free culture for 24 additional hours.

4.1. Effects of short-term (24-h) exposures to BBP

A wide range of concentrations was analysed in 24-h exposures (10^{-3}–10^5 µg L^{-1}), where BBP showed a clear toxic effect on larval survival from 10^3 µg L^{-1}. A LC50 value of $2.7 \cdot 10^4$ µg L^{-1} allowed us to confirm that the chemical was less toxic in our experimental conditions than in previous studies with other invertebrates, such as *H. azteca*, *L. variegatus*, *C. tentans* (Call et al., 2001b), and *M. macropopa* (Wang et al., 2011), or with zebrafish embryos (Chen et al., 2014). That range was narrowed (10^{-3}–1 µg L^{-1}) for longer treatments, in order to reduce cellular and molecular alterations associated with death processes that could cover up the effects of BBP on the molecular targets analysed. No mortality was detected in such conditions.

Heat-shock genes encode for proteins involved in cellular adaptive processes and cell survival under stress conditions (Gupta et al., 2010). In recent years, there have been significant advances in the understanding of the cellular functions of these genes, which include signalling pathways and protein homeostasis (Henderson, 2010). In addition, there have been much progress in the evaluation of the responses of these genes in different species under diverse environmental stresses, including exposure to xenobiotics capable of altering the production of some HSPs (Karouna-Renier and Rao, 2009; Rhee et al., 2009; Dang et al., 2010; Ming et al., 2010; Simoncelli et al., 2010; Sinha et al., 2010; Waagner et al., 2010; Zhang and Denlinger, 2010; Luo et al., 2014; Martínez-Paz et al., 2014; Morales et al., 2014).

Short-term exposures to BBP significantly upregulated *hsp70* and *hsp27*, confirming the ability of these heat-shock genes to be induced upon exposure to xenobiotics (Morales et al., 2011; Martínez-Paz et al., 2014), and strongly repressed *hsp40*. The activation/repression appears to be specific and independent of *hsc70* and *hsp10* transcriptional activity, which remained unaltered in the phthalate treatments assayed in this study. This response clearly differs from the coordinate expression of heat-shock genes fairly well demonstrated under temperature shifts (Morimoto et al., 1992). Results obtained in this work are consistent with the fact that HSPs contribute to normal cell physiology under stress

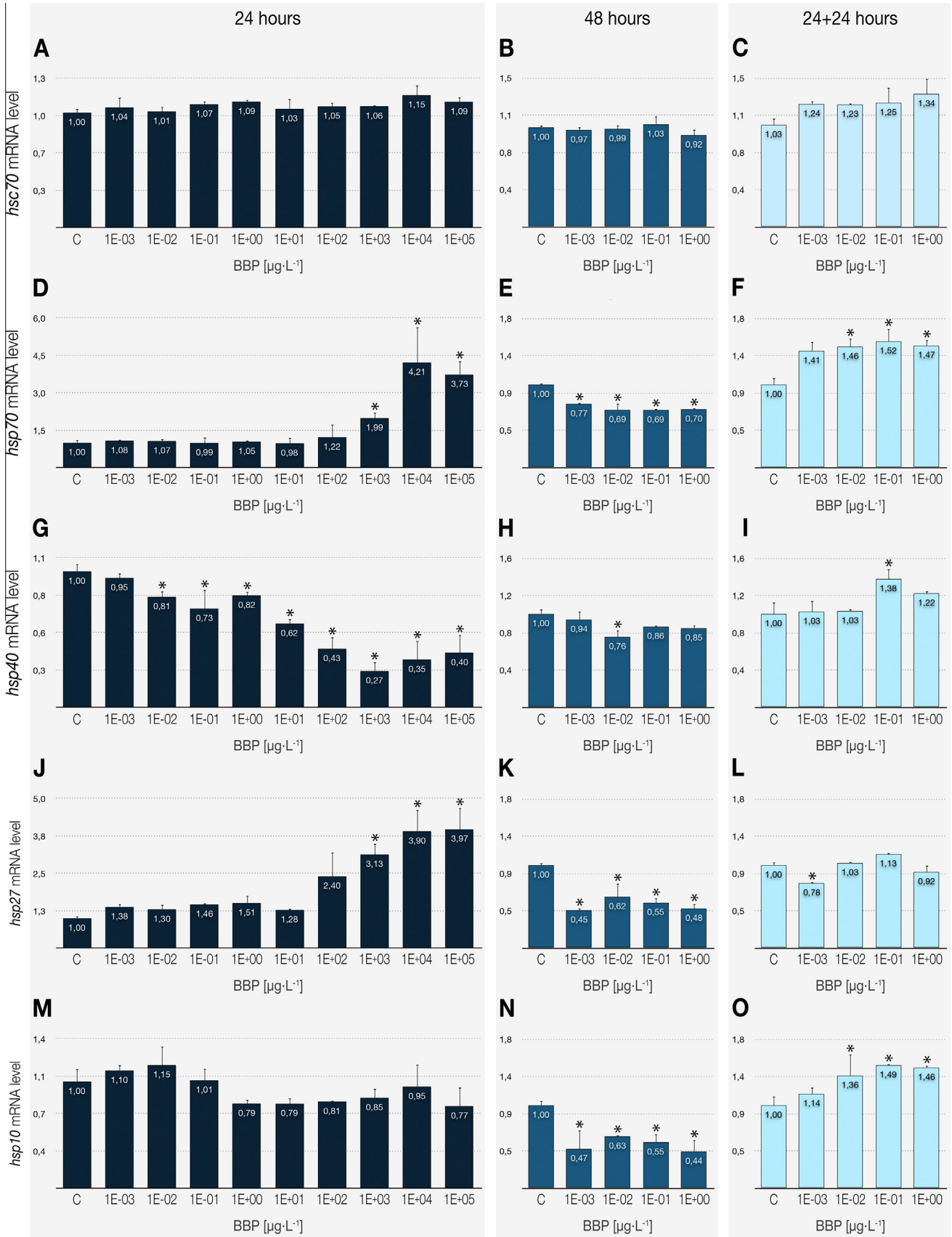

Fig. 2. Effects of benzyl butyl phthalate treatments on the relative expression of different heat-shock genes in *Chironomus riparius* fourth instar larvae: *hsc70* (A–C), *hsp70* (D–F), *hsp40* (G–I), *hsp27* (J–L), and *hsp10* (M–O). The mRNA level values were calculated relative to actin gene expression. 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 differences ($p \leqslant 0.05$) as compared to control cultures.

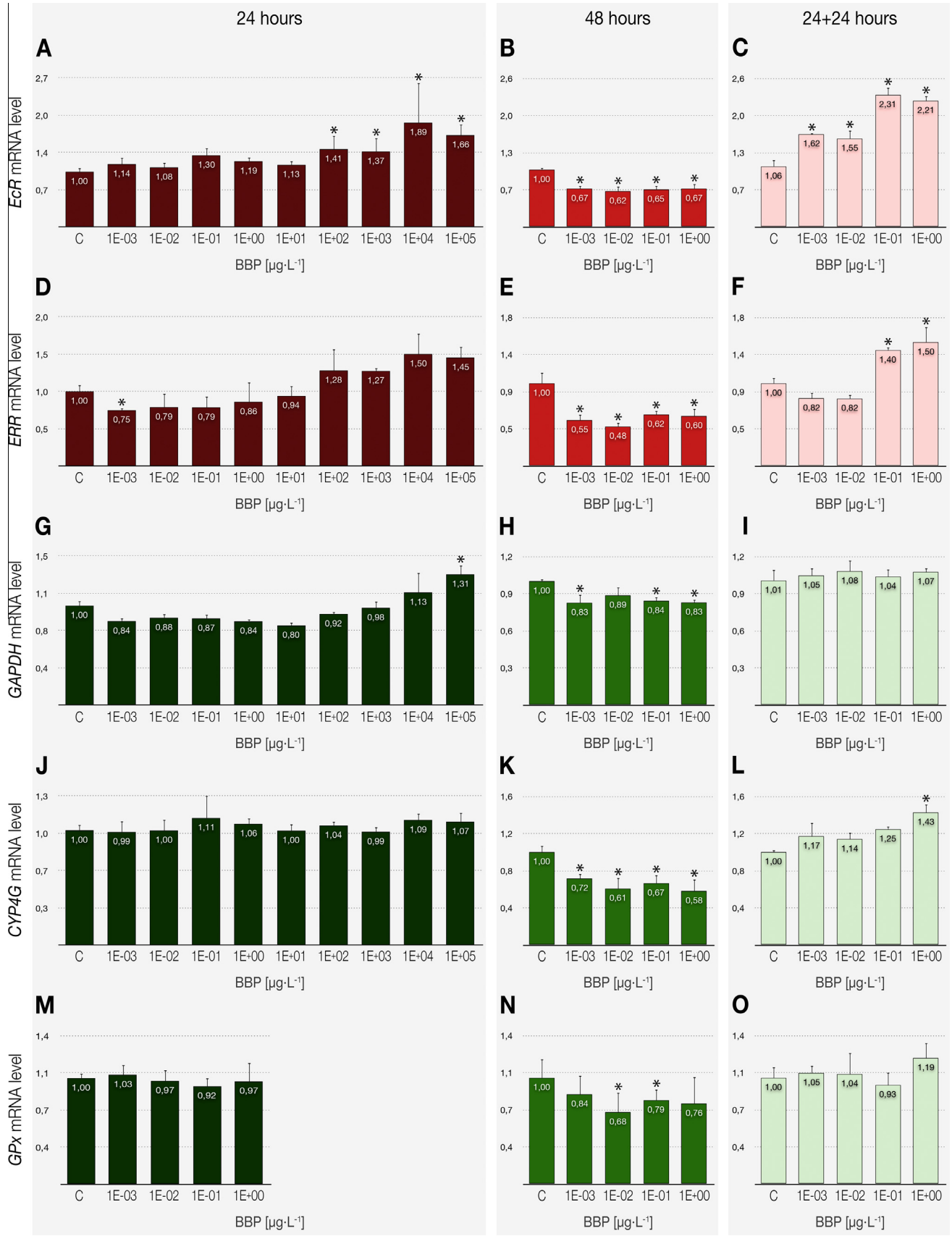

Fig. 3. Effects of benzyl butyl phthalate treatments on the relative expression of genes involved in hormonal pathways (*EcR*, A–C; and *ERR*, D–F) and in the detoxication metabolism (*GAPDH*, G–I; *CYP4G*, J–L; and *GPx*, M–O) in *Chironomus riparius* fourth instar larvae. The mRNA level values were 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 differences ($p \leqslant 0.05$) as compared to control cultures.

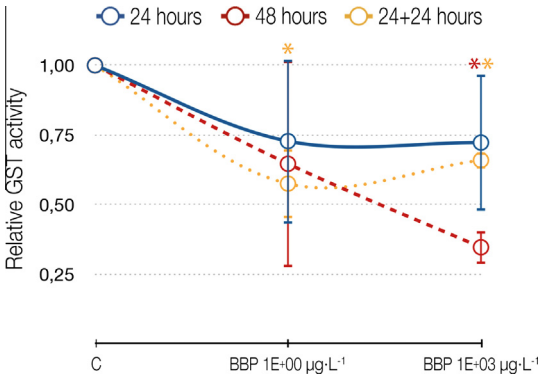

Fig. 4. Glutathione S-transferase activity in *Chironomus riparius* fourth instar larvae after exposure to benzyl butyl phthalate (1 and 10^3 µg L^{-1}) for 24, 48, and 24 + 24 h. Each bar is the mean ± SE obtained from three independent experiments, each with three replicates. Values are expressed as fold changes with respect to the control. *Significant differences ($p \leqslant 0.05$) as compared to control cultures.

conditions (Welch, 1993; Concannon et al., 2003) and, since overexpression took place in conditions in which there was a significant mortality, reflect a survival response. It has been described the role of *hsp27* gene in toxin resistance and stress response (Feder and Hofmann, 1999; Martínez-Paz et al., 2014) and, according to that, *hsp27* dose-dependent overexpression observed in our study could be part of this protective role, through which cells try to trigger mechanisms to mitigate damage.

Surprisingly, although HSP40 is known to act as a co-chaperone to HSP70 and also to co-localize with HSP70 in the nucleus during stress condition (Michels et al., 1997), BBP affected differently the genes coding for these two heat-shock proteins, with a strong downregulation of *hsp40* activity at low doses and reaching up to 70% reduction at the highest ones. Given that the major function of HSP40 proteins is to regulate adenosine triphosphate (ATP)-dependent polypeptide binding by HSP70 protein (Fan et al., 2003), the strong repression of *hsp40* gene could compromise the effective course of *hsp70* role. It is important to note that the expression profile of *hsc70* gene remained unchanged under our experimental conditions, reinforcing the idea about the constitutive presence of the 70 kDa heat-shock cognate protein and confirming that the observed effects were specifically due to the toxic properties of the phthalate.

Our data reflect that some genes coding HSPs are differentially activated, compared to other environmentally regulated heat-shock genes, and constitute potential biomarkers of exposure to BBP.

One of the main concerns about BBP is its capacity to mimic the action of steroid hormones, like ecdysone or estrogen, which can be responsible for major endocrine disorders in different organisms. This study provides new evidences about endocrine disrupting effects of BBP on insects under different exposure conditions. BBP clearly behaved like an ecdysone hormone agonist at high doses and 24-h exposures, leading the ecdysone receptor gene (*EcR*) to a significant overexpression, as the hormone would (Dubrovsky, 2005). Interestingly, our results show a time-dependent and concomitant response of the hormone-related genes *EcR* and *ERR*, which confirms the disrupting activity of acute doses of BBP previously described (Planelló et al., 2011). Thus, the interaction between BBP and the ecdysone receptor might be due to the ability of BBP to mimic the role of 20-hydroxyecdysone (20E), the active form of the hormone.

In ecotoxicological studies, classical targets of toxicity such as survival, growth or reproduction may ultimately reflect alterations in energy metabolism of individuals (Servia et al., 2006). Furthermore, the metabolic ability of an organism to carry out detoxication processes is directly related to the toxic effects that xenobiotics can exert on it. Alteration of enzyme systems that protect cells from exposure to harmful substances can determine the proper biotransformation and excretion processes and the ability of the compound to bioaccumulate. Alterations in these systems are associated with cell damage to which an organism can adapt or not, depending on whether the injury is minor/temporary or cell homeostasis is severely affected. Regarding this, apart from a slight increase in the expression of the *GAPDH* gene at the highest dose of BBP, no important metabolic changes were detected after 24-h exposure in any of the analysed genes, although the enzyme activity of GST was reduced at both 1 and 10^3 µg L^{-1}, the two representative concentrations selected for this study. Our results show that BBP had no significant early effects on the metabolic pathways analysed. Thus, this may allow *C. riparius* larvae to launch adaptive mechanisms to increase survival rates (Chaty et al., 2004; Vasseur and Leguille, 2004; Giraudo et al., 2010; Trevisan et al., 2014). In addition, given that *GAPDH* is usually considered a good reference gene for normalization in quantitative RT-PCR studies (Chervoneva et al., 2010), it is important to note that the slight overexpression caused by BBP highlights the need to carefully check the stability of this gene in each test condition, as reported in previous research (Schmittgen and Zakrajsek, 2000; Goidin et al., 2001; Wang and Xu, 2010).

4.2. Effects of long-term (48-h) exposures to BBP

In contrast to the observed absence of effects in 24-h exposures to low doses of BBP, prolonged contact with the toxin caused a general and significant decline in the transcriptional activity of most of the analysed genes, with the only exception of *hsc70*, which remained unchanged and, once again, confirms the stability of the 70 kDa heat-shot cognate gene. The stability of the *hsc70* gene reinforces the idea that altered genes are specific targets of the early effects of BBP, although these variations could also be due to a toxic effect on the energy metabolism of the larvae that lead to a decline in the rate of cellular transcriptional machinery. Further long-term studies are needed to establish a potential energy damage/failure.

Among all the inducible heat-shock genes, *hsp27* and *hsp10* were specially repressed after 48 h, which may have important consequences for cells. Like most of the small heat-shock proteins (sHsp), HSP27 possesses chaperone-like activity, preventing aggregation of improperly folded or partially denatured protein, although it also plays an important role in cell death regulation (Feder and Hofmann, 1999; Acunzo et al., 2012). Increased expression of this protein also has cytoprotective effects, modulating oxidative stress and regulating the cytoskeleton (Concannon et al., 2003). On the other hand, HSP10 is the co-chaperone for HSP60 inside mitochondria and participates in the folding of proteins in this organelle, once they have been imported from cytoplasm through pores in the mitochondrial membrane (Feder and Hofmann, 1999; Henderson, 2010). Besides mitochondria, mounting evidence demonstrates that HSP10 can also be located in cytosol, cell membrane, intercellular space, and periphery (Czarnecka et al., 2006; Corrao et al., 2010), playing roles probably independent of Hsp60 (Corrao et al., 2014).

The hormonal response was opposite for longer exposures to that observed in the 24-h treatments. In contrast to the dose-dependent induction observed in 24-h exposures, this work reveals a time-dependent response of the nuclear receptors, with a strong and significant repression of transcription levels after 48-h exposures to BBP. Therefore, BBP acts as a hormone antagonist in such conditions, reducing the transcription of *EcR* and *ERR* genes by 40% and 50%, respectively. These inhibitory effects appear to be BBP-specific, given that the stability of our reference genes and

hsc70 remained unchanged under the same experimental conditions.

Our results highlight the possible relationship between the repression of ecdysone receptor and the concomitant inhibition detected in the expression of *CYP4G* under the same experimental conditions. Some xenobiotics are able to repress the expression of different genes in the family CYP4 in *Drosophila melanogaster* and *Spodoptera littoralis* (Davies et al., 2006). Other genes are overexpressed or suppressed by the effect of ecdysteroids, suggesting some hormonal regulation for them. In addition, several of these genes are involved in the metabolism of hormone development (Aragon et al., 2002). Therefore, this suggests the existence of interactions between exposure to certain chemicals and the endocrine function, referring to the response of some cytochromes (Le Goff et al., 2006). A significant downregulation was also detected in *GPx* gene expression, concomitantly with a drop in GST enzyme activity (up to 70%). These results showed a clear inhibition of phase I and phase II detoxication reactions due to BBP exposure and could lead to a decreased ability of the larvae to metabolize and thereby minimize its toxic effects in even longer treatments.

Taking into account that subtle changes in mRNA abundance may be related to a dramatic impact on protein activity (Jolly et al., 2005), data obtained suggest that a general decrease in protein activity could be taking place as an adaptive mechanism to cellular injury, in which the cell decreases protein synthesis until the damage is over. This would be consistent with the absence of mortality in these experimental conditions.

4.3. Delayed toxicity studies

Some of the most remarkable results of this study came from the experiments carried out to analyse the delayed toxicity of BBP. Our goal with this approach was to determine whether a short acute exposure to the compound was able to induce effects on the targets studied after the larva has stopped being in contact with the toxin.

Although no effects were detected on the survival rate 24 h after withdrawal of the drug, interesting changes were observed in transcript levels of most analysed genes.

In general, all stress-responsive genes analysed were lead to a greater or lesser overexpression 24 h after the BBP removal, with the sole exception of *hsp27*. Given that stress proteins, such as chaperones, are involved in protein folding processes, this general response would be in concordance with a greater need for these proteins in response to increased rate of protein synthesis, in order to reach the cell homeostasis after a sublethal injury. On the other hand, it has been reported that the induction of HSP27 is transient and the protein returns to basal levels after removal of the stress event (Concannon et al., 2003), which may explain the results obtained for this gene.

Specially relevant are the effects of BBP on both hormone-related genes, which were unpredictable with just 24 h treatments. It is worth noting that toxin removal caused a significant activation of both *EcR* and *ERR*, allowing us to detect clear endocrine disruption effects of BBP in concentrations that showed no effects on 24-h exposures and seemed to be harmless. This induction was even higher than that detected at the highest concentrations tested in this study, revealing a strong delayed character as hormone agonist in these experimental conditions.

Finally, once the compound was removed from culture medium, it was possible to detect the upregulation of *CYP4G* gene and a decrease in GST enzyme activity. GST activity values were similar to those obtained in 24-h studies, which reflects a sustained toxic effect of BBP on this detoxication pathway. However, *CYP4G* was significantly activated after BBP removal, while this gene was previously not affected in the presence of the compound. This effect could not be directly related to the detoxication or biotransformation of the xenobiotic, but may be due to the fact that cytochrome P450-dependent monooxygenases are also important in regulating levels of endogenous compounds such as hormones, fatty acids and steroids (Scott, 1999) and, in addition, they are found in the biosynthetic pathways of ecdysteroids and juvenile hormones, which are central to insect growth, development, and reproduction (Feyereisen, 1999). Thus, taking into account the relation between P450 activity and ecdysteroids metabolism (Guittard et al., 2011), increased levels of *CYP4G* may be directly related to the marked increase in the levels of genes coding for hormone receptors and the obvious behaviour of BBP as a hormone agonist.

5. Conclusions

This work is an in depth study focused on the acute and delayed toxicity of BBP exposures in *C. riparius*, a model organism in ecotoxicology studies. Results reveal novel and significant dose and time-dependent alterations in the expression profile of genes involved in important cell processes, such as stress response, hormone pathways, energy metabolism, and detoxication activities. The phthalate confirms its endocrine disrupting capacity in insects, with the ability to act as an ecdysone agonist. Furthermore, our data demonstrate that some toxic effects are not revealed immediately in larvae right after the compound withdrawal, and emphasize the partial knowledge that is derived from conventional ecotoxicological testing. Understanding the correlation between chemical exposure and gene expression could lead to additional markers that provide mechanistic information regarding the type of damage.

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