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The ribosome biogenesis pathway as an early target of benzyl butyl phthalate (BBP) toxicity 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.
- rDNA and genes encoding ribosomal proteins were specific targets of the xenobiotic.
- A general decrease in the expression pattern of polytene chromosomes was observed.
- BBP altered the puffing patterns of the Balbiani rings.

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ABSTRACT

Butyl benzyl phthalate (BBP) is a ubiquitous contaminant whose presence in the environment is expected for decades, since it has been extensively used worldwide as a plasticizer in the polyvinyl chloride (PVC) industry and the manufacturing of many other products. In the present study, the interaction of BBP with the ribosome biogenesis pathway and the general transcriptional profile of *Chironomus riparius* aquatic larvae were investigated by means of changes in the rDNA activity (through the study of the internal transcribed spacer 2, ITS2) and variations in the expression profile of ribosomal protein genes (*rpL4*, *rpL11*, and *rpL13*) after acute 24-h and 48-h exposures to a wide range of BBP doses. Furthermore, cytogenetic assays were conducted to evaluate the transcriptional activity of polytene chromosomes from salivary gland cells, with special attention to the nucleolus and the Balbiani rings (BRs) of chromosome IV. BBP caused a dose and time-dependent toxicity in most of the selected biomarkers, with a general depletion in the gene expression levels and the activity of BR2 after 48-h treatments. At the same time, decondensation and activation of some centromeres took place, while the activity of nucleolus remained unaltered. Withdrawal of the xenobiotic allowed the larvae to reach control levels in the case of *rpL4* and *rpL13* genes, which were previously slightly downregulated in 24-h tests. These data provide the first evidence on the interaction of BBP with the ribosome synthesis pathways, which results in a significant impairment of the functional activity of ribosomal protein genes. Thus, the depletion of ribosomes would be a long-term effect of BBP-induced cellular damage. These findings may have important implications for understanding the adverse biological effects of BBP in *C. riparius*, since they provide new sensitive biomarkers of BBP exposure and highlight the suitability of this organism for ecotoxicological risk assessment, especially in aquatic ecosystems.

1. Introduction

The machinery for protein biosynthesis is essential for cell survival (Chen and Ioannou, 1999) and ribosome biogenesis is the most demanding energetic and metabolic expenditure of the cell, playing a central role in the growth and development of eukaryotic cells and organisms (Moss et al., 2007). Four rRNAs (45S, 32S, 28S, and

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5S) and numerous ribosomal proteins (RPs) are involved in the complex task of coordinating ribosome synthesis to maintain the cell homeostasis. This critical role is reflected in the high similarity between the amino acid sequences of equivalent RPs from different and distant organisms (Draper and Reynaldo, 1999).

The nucleolus is a nuclear compartment where ribosomal DNA (rDNA) transcription, maturation and assembly into ribosome subunits take place. Transcription of rDNA depends on multiple signalling pathways that respond to external environmental factors, such as stress, hormones, and nutrients, among others (Moss et al., 2007). Three of the four rRNAs are transcribed as a single precursor. Through a series of cleavages, the external and internal transcribed spacers (ETS, ITS) are removed from the precursor to liberate the mature rRNAs (Gerbi and Borovjagin, 2000).

The transcription process is highly coordinated with ribosome biogenesis and, in this context, RPs play a critical role. However, in recent years a wide variety of secondary functions have been described for RPs, related to the regulation of development, cell proliferation and apoptosis, DNA repair, and malignant transformations (de Las Heras-Rubio et al., 2014; Lindström, 2009; Wool, 1996). Furthermore, they are also differentially regulated under stress conditions (Nair et al., 2011; Planelló et al., 2008). Despite that more than 80 different RPs have been characterized in Eukarya to date, only a few of them are well described in some species of *Chironomus*: RPL8, RPL11, RPL13, RPL15, RPS3 and RPS6 (Govinda et al., 2000; Martínez-Guitarte et al., 2007; Nair and Choi, 2011; Park and Kwak, 2012; Planelló et al., 2007). Nevertheless, due to the high degree of sequence similarity between RPs from different species, *Chironomus riparius* could be a good model organism for making predictions based on comparative genomics approaches (Martínez-Guitarte et al., 2007).

Chironomids, a large group of non-biting midges, 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, 2000; OECD, 2004a, 2004b). Their use in toxicological analysis by means of changes in molecular endpoints, such gene activity, has gained relevance in recent years because several molecular biomarkers have demonstrated to be responsive against different xenobiotics, including those for RPs, heat-shock proteins (HSPs), nuclear receptors, and detoxication pathways, among others (Herrero et al., 2015; Martínez-Paz et al., 2014; Morales et al., 2014; Nair and Choi, 2011; Ozáez et al., 2014, 2013; Park and Kwak, 2010, 2012; Park et al., 2010; Planelló et al., 2010, 2008, 2007, 2011). The use of these rapid-response molecular targets is particularly relevant in the case of aquatic organisms, which are routinely exposed to fast (and often severe) changes in the chemical composition of their habitats that constitute a serious threat to them, by altering biochemical, physiological and behavioural parameters (Scott and Sloman, 2004).

Butyl benzyl phthalate (BBP) is an ubiquitous environmental pollutant extensively used as a plasticizer in the PVC industry, although it is also present in the composition of many other commercial products, such as food conveyor belts, artificial leather, automotive trim, traffic cones, vinyl gloves and adhesives (CPSC, 2010). The slow migration of BBP out of the plastics and its release into the aquatic environments have allowed for its detection in influents and effluents from sewage treatment plants (STP), surface water and seawater samples, and even in drinking water (Domínguez-Morueco et al., 2014; ECB, 2007; Liu et al., 2015). Its presence may cause adverse effects at different levels in aquatic organisms (Clark et al., 2011; Laughlin et al., 1978), and recently it has been described that BBP alters the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms in *C. riparius* larvae (Herrero et al., 2015; Planelló et al., 2011). Compared to other phthalates studied in chironomids, the compound has proved to be more toxic by reducing survival rates (Call et al., 2001; Planelló et al., 2011). However, information on the potential effects that this toxicant may cause on the protein synthesis machinery is still scarce.

The aim of this study was to determine at the molecular level the early toxic effects of BBP on the two main components of the ribosome biosynthesis (RPs and rDNA) 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 the transcriptional profile of the genes coding for some RPs (*rpL4*, *rpL11*, and *rpL13*) and the expression levels of ribosomal genes (estimated through *its2* values). Additionally, polytene chromosomes were used for the screening of BBP-induced alterations in the general expression pattern of exposed larvae and the transcriptional activity of important regions such as BRs and nucleolus.

2. Materials and methods

2.1. Test animals

The experimental animals were aquatic larvae from the midge *C. riparius*. They were obtained from laboratory cultures, reared under standard laboratory conditions according to toxicity testing guidelines (EPA, 2000; OECD, 2004a, 2004b). 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. Experiments were carried out using exclusively fourth instar larvae, and the larval stage was determined based on the size of head capsule (EPA, 2000).

2.2. Exposure conditions

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}, which represent a broader range than those minimum and maximum values described in the literature for aquatic ecosystems and drinking water samples.

Expression studies were carried out in a wide range of BBP concentrations (10^{-3} to 10^5 μg L^{-1}) for 24-h exposures, and at the four lowest doses (10^{-3} to 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 fourth instar 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. Immunocytochemistry

For each experimental condition, a total of 200 cells from

salivary glands of the larvae were used for microscopic analysis. The detection of DNA/RNA hybrids was carried out as described in previous works (Martínez-Guitarte et al., 2012; Planelló et al., 2007). Isolated salivary glands were fixed in ethanol:acetic acid (3:1) and squashed in 50% acetic acid. After freezing the slides on dry ice, the coverslip was removed and the material was dehydrated in absolute ethanol for at least 10 min. Slides were then rehydrated with 1× PBS and incubated in a moist chamber at room temperature overnight with an anti-DNA/RNA IgG antibody (provided by Dr. JL Díez, CSIC, Spain) diluted 1:20 in blocking buffer 1× PBS, 1% blocking reagent (Roche). Slides were washed three times with 1× PBS, 0.1% Tween 20 (PT) and detection was performed with anti-goat IgG secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma) diluted 1:80 in blocking buffer 1× PBS, 1% blocking reagent (Roche), for 1 h at room temperature. After two washes with PT, slides were counterstained with Hoeschst 33258 (5 μg L^{-1}) (Riedel-de Haen) and mounted in ProLong (Invitrogen) anti-fading.

2.4. RNA extraction

Total RNA from frozen larvae was extracted according to the protocol described in Herrero et al. (2015). Purified RNA was finally stored at −20 °C.

2.5. 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, PCR amplification and products quantification were performed according to Herrero et al. (2015). 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 SM1. Fragments corresponding to *actin*, *26s*, *its2*, *rpL4*, *rpL11 and rpL13* were amplified under 24 and 34 cycles with an annealing temperature of 55 °C. A total of twenty larvae in four independent experiments (each with five larvae) were used for gene expression analysis, with three replicates in each sample.

2.6. Sequence alignment, primer design and phylogenetic trees

A selection of *rpL4* gene sequences from different organisms was analysed through the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Sequence alignments of different *rpL4* genes were performed with the Clustal X program Version 2 (Larkin et al., 2007) and the MAFFT software Version 7 (Katoh and Standley, 2013). A partial clone was obtained by using PCR primers designed from the conserved insect sequences: *Pediculus humanus* XM_002428638.1; *Tribolium castaneum* XM_970086.1; *Nasonia vitripennis* XM_001599942.2; *Apis mellifera* XM_624907.3; *Armigeres subalbatus* EU205741.1; *Aedes aegypti* XM_001657831.1; *Culex quinquefasciatus* XM_001848899.1; *Anopheles gambiae* XM_321619.4; *Drosophila erecta* XM_001972575.1; *Drosophila willistoni* XM_002062057.1; *Ixodes scapularis* XM_002433676.1.

Phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA) software Version 6 (Tamura et al., 2013) through the *UPGMA* method (Sneath and Sokal, 1973). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 2010) and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965).

2.7. Image capture and processing

Slides containing polytene chromosomes were examined under a Leica DMI300B microscope equipped with a Leica DFC310FX digital camera or, in some cases, a Zeiss Axiohot photomicroscope with a Photometrics® CoolSNAP CCD camera. Image processing was performed using Leica Application Suite 3.5.0 software and Adobe® Photoshop® CS6 software.

The transcriptional activities of the nucleolus and the Balbiani rings (BRs) of the polytene chromosome IV were analysed following the model described by Beermann (1971), wherein three categories were established based on the expansion grade of the puffs (diffuse uncoiled regions that are sites of RNA transcription). Thus, nucleoli and BRs were semi-quantitavely classified as high-expanded, semi-expanded, or collapsed, the latter being the group in which the transcriptional activity is lower.

2.8. Data analysis

Probit and statistical analysis were performed by means of IBM® SPSS® Statistics 19 software. Normality and homoscedasticity of data were tested using the Shapiro–Wilk and Levene tests, respectively. The normalized levels of the specific gene transcripts were analysed with ANOVA, followed by Games Howell's or Bonferroni's post Hoc tests, when appropriate. 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. Sequence and phylogenetic analyses of the CrRPL4 cDNA/mRNA

In the present work, a 149-bp-long mRNA fragment encoding the *C. riparius* ribosomal protein L4 (ChRPL4), showing 82% base sequence identity with the *A. gambiae rpL4* gene, was amplified and identified by the authors.

The comparative analysis by BLAST in the genome databases let us to find a full length 1453-bp-long mRNA that shared 100% base sequence identity to our fragment which was recently uploaded to the Transcriptome Shotgun Assembly (TSA) Database (Gene Bank Accession Number KA185462.1), though still unidentified. This mRNA proceeds from the assembly of various clones within the *C. riparius* SRA BioProject PRJNA167567 (*C. riparius* transcriptome sequencing SRR496839) (Marinković et al., 2012). The 1453-bp-long cDNA included a 1236-bp open reading frame encoding a putative protein of 412 amino acids. An 82-bp 5-untranslated region and a polyadenylation signal (AATAAA) were also present (Fig. SM1).

The comparison between the ChRPL4 amino acid sequence and homologous sequences from other insects allowed us to describe two highly conserved domains (Fig. 1A). As an example of the alignment performed for this work four of these sequences are shown in Fig. 1B, where the first domain (aa V25-D265 in CrRPL4) corresponds to the ribosomal protein L4/L1 superfamily domain and shows a 61% identity in the four proteins, while the second domain (aa K278–K350 in CrRPL4) shows a 41% identity and corresponds to the 60S ribosomal protein L4 C-terminal domain.

A phylogenetic study was conducted to evaluate the relationship between ChRPL4 and protein homologues in other insects (Table SM2). Taking into account the presence of a ribosomal RPL4/RPL1 domain in the sequence isolated in our laboratory, our analysis also included the comparison with RPL1 in order to confirm the

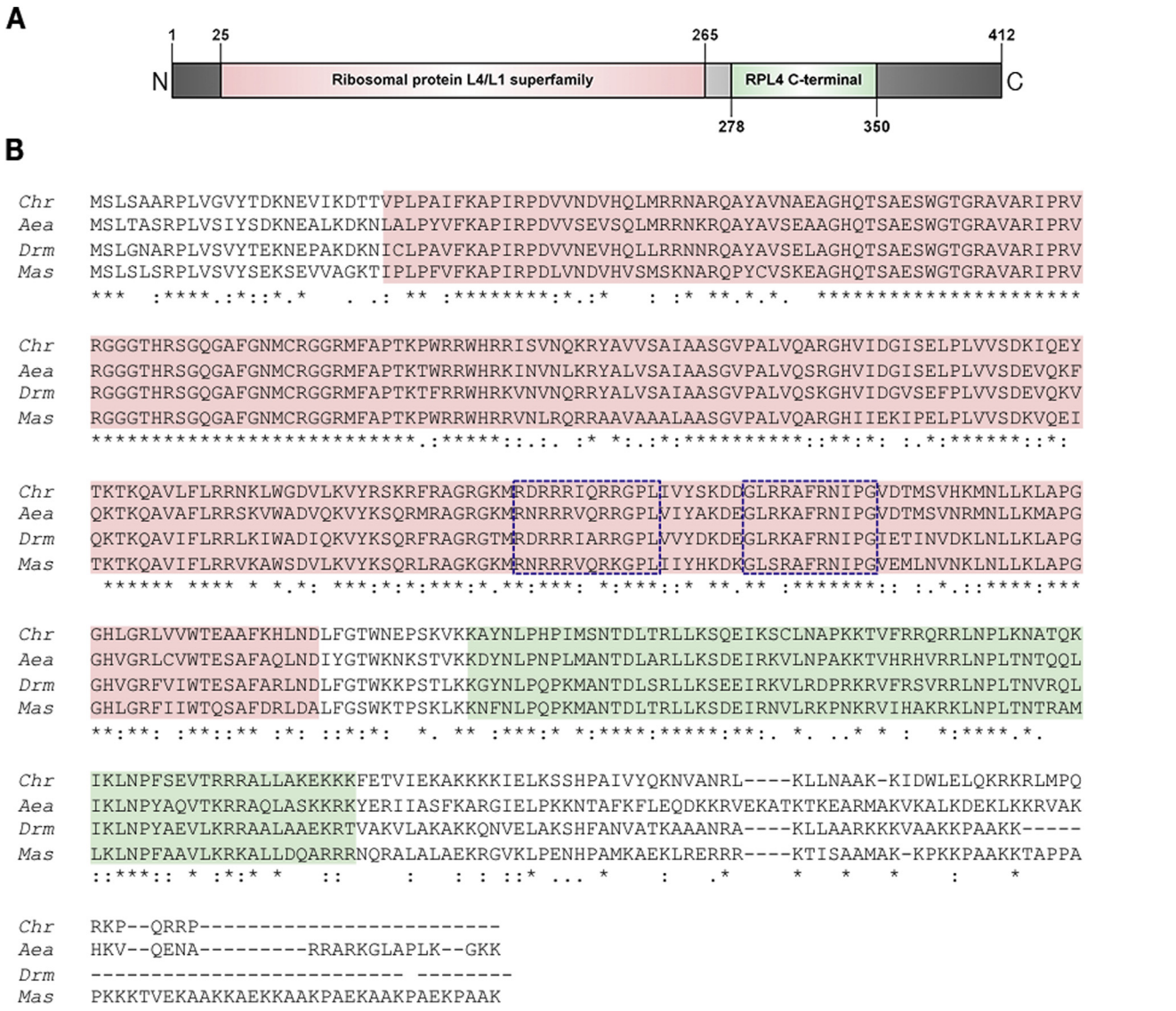

Fig. 1. (A) Schematic representation of the protein domains of *C. riparius* RPL4 showing a ribosomal protein L4/L1 superfamily domain (aa 25–265) and a 60S ribosomal protein L4 C-terminal domain (aa 278–350). (B) Comparison of the homologous amino acid sequences of RPL4 from *C. riparius* (Chr), *A. aegypti* (Aea), *D. melanogaster* (Drm), and *M. sexta* (Mas). Asterisks, double dots, and single dots denote fully conserved, strongly conserved and weakly conserved amino acid residues, respectively. The two conserved regions selected for primer design are framed in dashed blue boxes. The two mentioned domains of RPL4 are marked in red and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identity of both the fragment and the gene reconstructed *in silico*. As shown in Fig. 2, the isolated fragment of ChRPL4 was clearly grouped with other RPL4 sequences, in a different cluster than RPL1. The protein sequence was more closely related to other dipterans, such as *C. quinquefasciatus* or *A. aegypti*, than with other insect groups.

3.2. Effects of BBP on ribosomal DNA transcription

Alterations in rDNA gene expression were assessed by RT-PCR analysis of the gene encoding the internal transcribed spacer 2 (*its2*), which represents newly synthesized transcripts. As shown in Fig. 3A, BBP triggered a clear effect on the levels of immature rRNA (still containing ITS2) in 24 h treatments. Thus, the *its2* gene showed a slightly and dose-dependent significant repression (about 20% below control levels) at the highest concentrations tested (10^3–10^5 μg L^{-1}), while no effects where detected for lower ones. Longer exposures had no noteworthy effects, except for the significant repression detected when larvae were exposed to 10^{-2} μg L^{-1} BBP for 48 h (Fig. 3B). Delayed toxicity experiments showed a tendency towards gene overexpression, highlighting a 40% increase for a dose of 10^{-1} μg L^{-1} (Fig. 3C).

3.3. Transcriptional changes of ribosomal protein genes after exposures to BBP

The effects of BBP on the transcriptional activity of *rpL4, rpL11* and *rpL13* genes are represented in Fig. 3D-L. While 24-h treatments induced a clear and statistically significant decrease in the transcript levels of *rpL4* (Fig. 3G) and produced a mild reduction in the activity of *rpL13* (Fig. 3D), no effects were detected for the gene encoding RPL11 (Fig. 3J). However, a clear and significant down-regulation (about 30% below control) of these three genes confirmed BBP's ability to attenuate the larval transcriptional

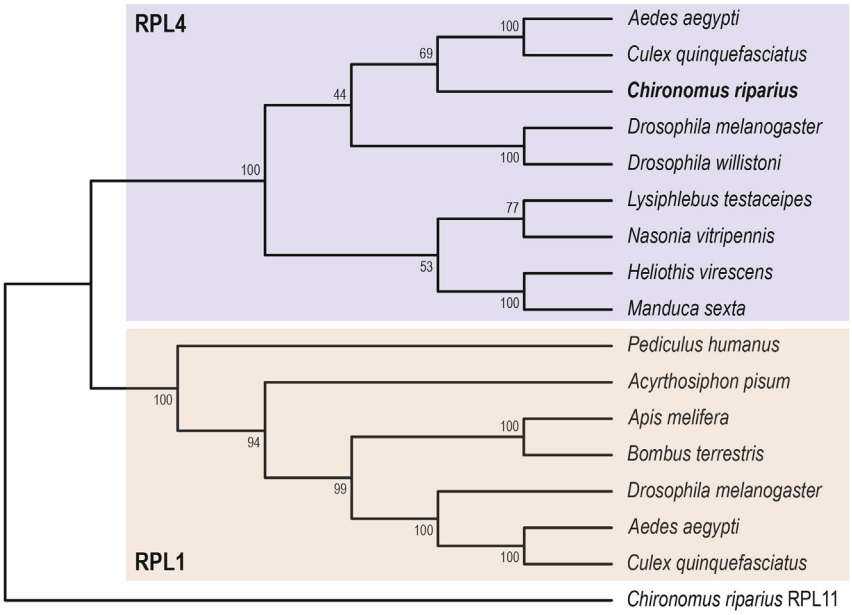

Fig. 2. Evolutionary relationships of the taxa included in the analysis of the amino acid sequences of RPL4 and RPL1 from *C. riparius* and other insects. GenBank Accession numbers are indicated in Section 2.6. The evolutionary history was inferred using the UPGMA method, and the bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 116 positions in the final dataset.

activity after prolonged exposures (48 h), as shown in Fig. 3E, H and K. Removal of the compound boosted the recovery of *rpL4* normal transcriptional levels after 24 h (Fig. 3I), while *rpL13* and *rpL11* remained unchanged at control levels (Figs. 3F and L).

3.4. Changes in the general expression pattern of polytene chromosomes

Polytene chromosomes from control and BBP-exposed larvae were analysed by indirect immunofluorescence after incubation with a fluorescein-labelled secondary antibody. As shown in Fig. 4D–F, under control culture conditions many active loci could be visualized as fluorescent bands along the three larger chromosomes characteristic of this species. While 24-h exposures to 1 μg L^{-1} BBP exhibited no significant changes in the banding pattern of chromosomes I, II and III (Figs. 4J–L), 48-h treatments led to a significant reduction of most of the signals associated with transcriptionally active sites (Figs. 4P–R), which correlated with the downregulation detected for all the RP genes under the same experimental conditions. In contrast, after 48 h, an increase in the fluorescence signal was clearly evident at the centromeres of chromosomes II and III (Figs. 4Q–R).

3.5. Structural alterations of the nucleolus and the Balbiani rings

Untreated cells showed a high degree of decondensation in both the nucleolus and the BRs (Fig. 5A and D), which was evidenced by a strong fluorescent signal of the anti-DNA/RNA antibody. However, although nucleoli did not appear to be affected in BBP-exposed cells, the activity of BRs seemed to be notably altered after 24-h and 48-h exposures to 1 μg L^{-1} BBP, as shown in Fig. 5B, C, E and F. These changes were statistically significant and showed a 2-fold activation of BR1 after 24-h treatments (Fig. 6B) and a 4-fold repression of BR2 in both 24-h and 48-h scenarios (Fig. 6C), which correspond in each case with an increase in the number of high-expanded or collapsed puffs.

4. Discussion

BBP is a ubiquitous anthropogenic pollutant that has been classified by different international regulations as toxic to the environment, specially to aquatic ecosystems. Regarding invertebrates, the compound has demonstrated to exert toxic effects by reducing survival and reproductive rates of the cladoceran *Moina macrocopa* (Wang et al., 2011), altering embryogenesis and larval development of the marine univalve *Haliotis diversicolor supertexta* (Liu et al., 2009), damaging hemocytes and further influencing the defence mechanism of the giant freshwater prawn *Macrobrachium rosenbergii* (Sung et al., 2003), and affecting survival, transcriptional rates and enzyme activity of chironomids (Call et al., 2001; Herrero et al., 2015; Martínez-Guitarte et al., 2012; Morales et al., 2011; Planelló et al., 2011). However, ecotoxicological information about the toxic effects and the mode of action of this chemical is still scarce. The main objective of the present work has been to characterise molecular toxic endpoints of this pollutant by analysing its effects on the ribosome biosynthesis machinery of the model species *C. riparius*, in order to find accurate responsive biomarkers that could help in the assessment of ecosystems health.

Ribosomal genes are considered as housekeeping genes that are constitutively expressed because their transcription products are indispensable for cell metabolism, as they constitute the ribosome, the necessary machinery for the synthesis of all cellular proteins. In this sense, regulation of ribosome biogenesis is a key element of cell biology, not only because ribosomes are directly required for growth, but also because ribosome production monopolizes nearly 80% of the global transcriptional activity (Laferté et al., 2006). The constitutive presence of RPs, even under different stress conditions (Govinda et al., 2000; Martínez-Guitarte et al., 2007; Park and Kwak, 2012), has made suitable their precursors as reference genes in transcriptional studies (Martínez-Guitarte et al., 2012; Martínez-Paz et al., 2014; Morales et al., 2014; Ozáez et al., 2013; Yang et al., 2015). However, recent studies have demonstrated that the expression of RP genes could be altered by environmental

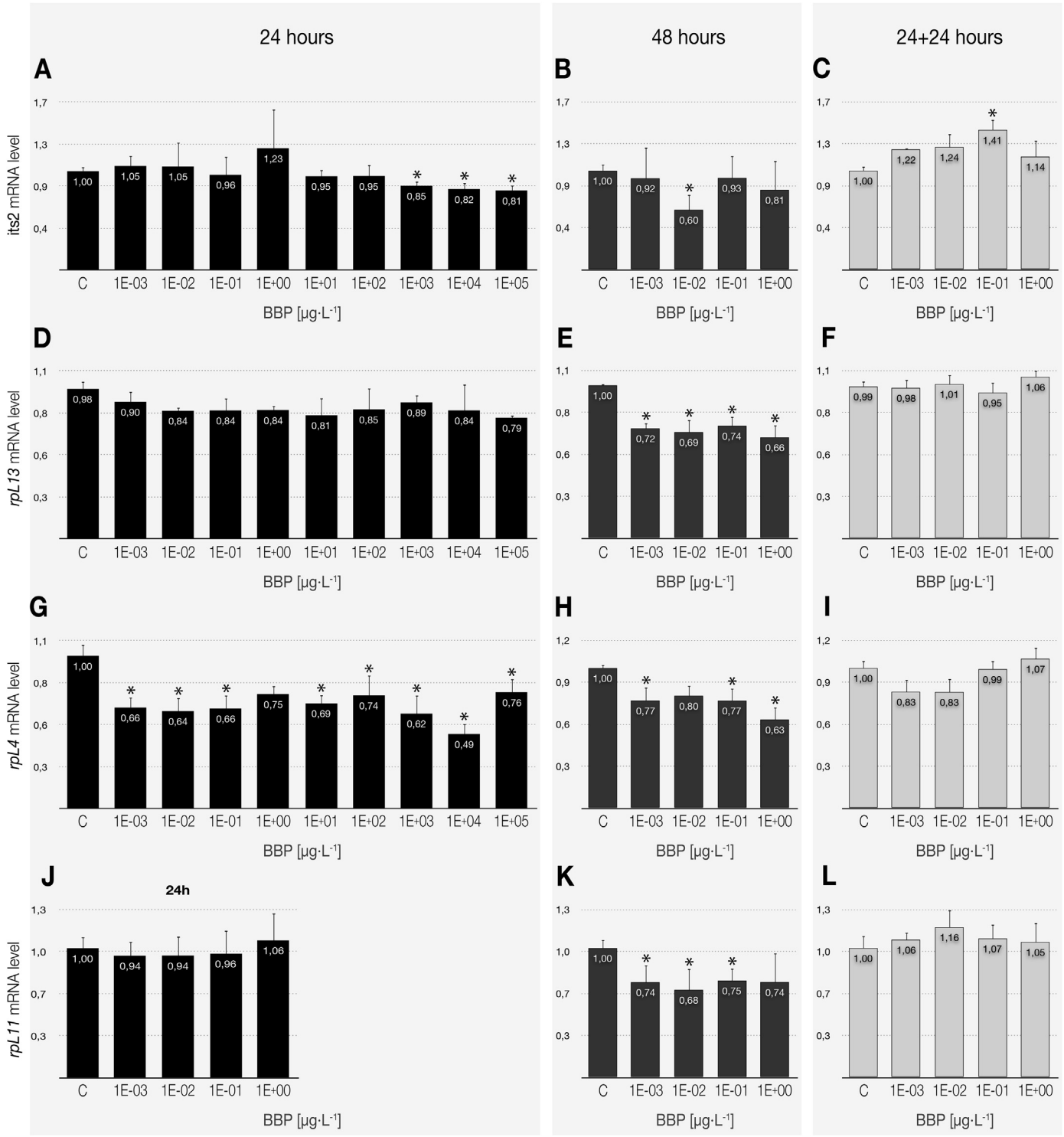

Fig. 3. Effects of BBP treatments on the relative expression of rDNA (*its2*) (A–C) and different ribosomal protein genes in *C. riparius* fourth instar larvae: *rpL13* (D–F), *rpL4* (G–I), and *rpL11* (J–L). Values were normalized relative to 26s rRNA transcript levels in the case of *its2*, or to *actin* gene expression for the genes encoding ribosomal proteins. 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 ($p \leq 0.05$) as compared to control cultures.

stressors, like exposure to heavy metals (Achard-Joris et al., 2006; Aliza et al., 2012; Ludwig and Tenhaken, 2001; McIntosh and Bonham-Smith, 2005; Nair and Choi, 2011; Nair et al., 2011; Planelló et al., 2007; Wang and Crowley, 2005; Yamanaka et al., 2005) or heat (Volkov et al., 2003). Moreover, the expression of these genes appears to be deregulated in several human disorders, playing novel roles in tumorigenesis (De Las Heras-Rubio et al., 2014). All these evidences make necessary selecting the right reference gene for each particular assay, rather than using a empirically-determined or pre-validated one (Ling and Salvaterra, 2011).

To date, only three genes encoding RPs have been identified in

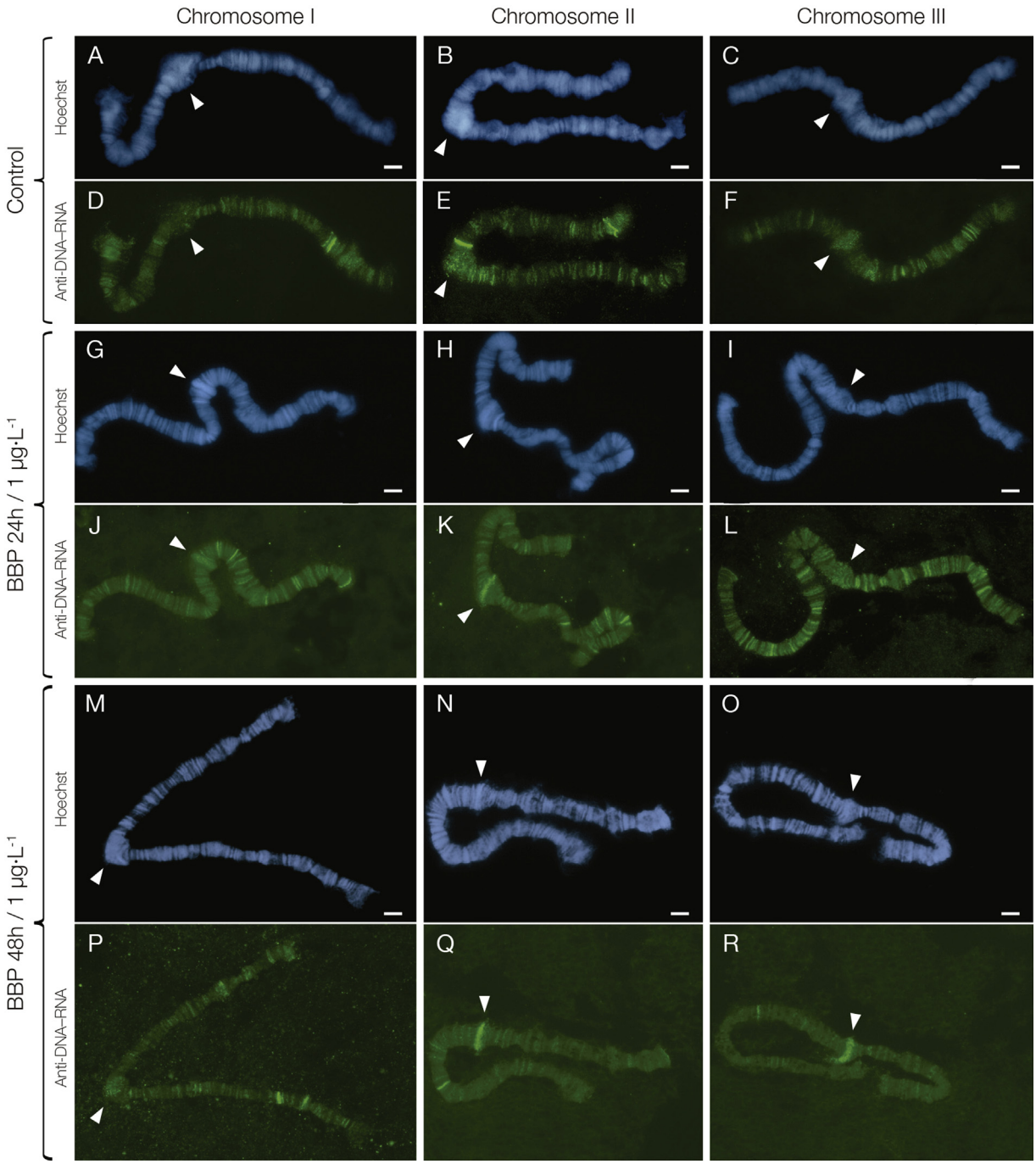

Fig. 4. Representative images of polytene chromosomes in salivary gland cells of *C. riparius* fourth instar larvae. Chromosomes I, II and III are presented showing Hoechst staining and the pattern of labelling of DNA/RNA hybrids from untreated larvae (A–F), and from larvae exposed to 1 µg L^{-1} BBP during 24 h (G–L) or 48 h (M–R). Arrowheads point to the centromeric regions. Bar, 10 µm.

C. riparius and used in ecotoxicity studies: *rpL8* (Govinda et al., 2000), *rpL15* (Nair and Choi, 2011), *rpL11*, *rpL13* (Martínez-Guitarte et al., 2007), *rpS3* and *rpS6* (Park and Kwak, 2012). In the present study, the cDNA for the ribosomal protein gene L4 (*rpL4*) from this species was identified and characterized, thus providing the scientific community a new potential biomarker that could help in the assessment of the early toxic effects of environmental stressors. A comparative analysis of the CrRPL4 protein with equivalent RPL4 sequences from other insects showed highly conserved features of the rpL4 ribosomal protein genes between different species.

The first approach to the sublethal toxic effects of BBP on the ribosome biogenesis pathway was to evaluate the alterations on the levels of newly synthetized rRNA. To this end, alterations in rDNA gene expression were estimated by RT-PCR analysis of the levels of recent transcripts of the *its2* gene (Gorab et al., 1995), given that ITS2 is present in the first immature transcription product of the rDNA gene and absent in the mature 28S and 18S rRNAs. We also tested the transcriptional activity of genes encoding the ribosomal proteins L4, L11, and L13. In a recent study from our laboratory the authors demonstrated the ability of this chemical to modify the expression pattern of genes related to the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms (Herrero et al., 2015). Those

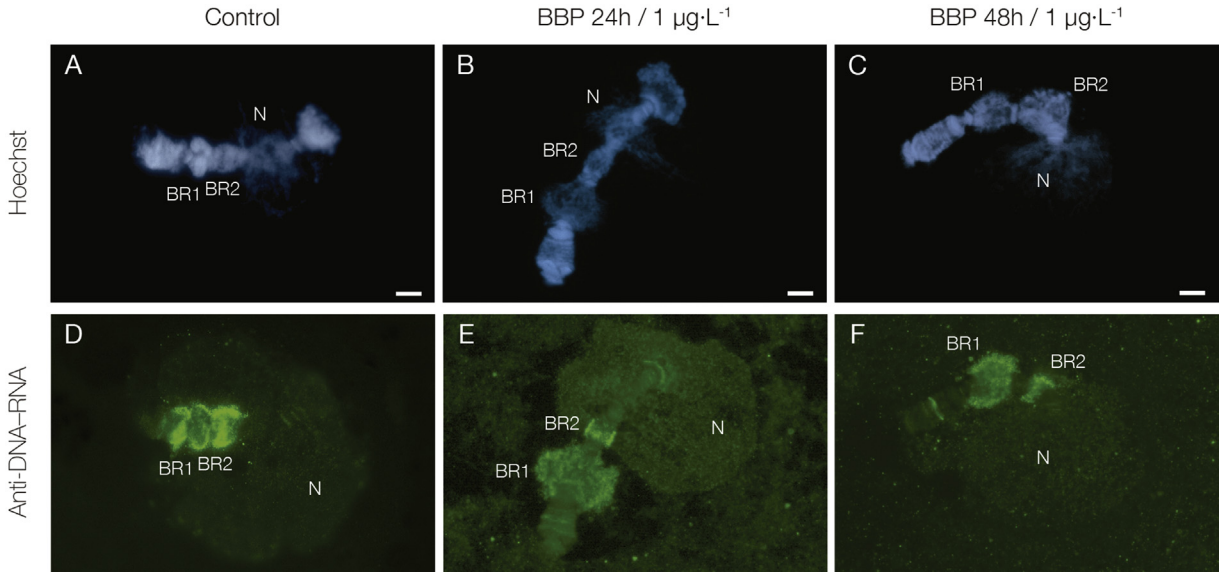

Fig. 5. Hoechst staining and *in situ* detection of DNA/RNA hybrids in polytene chromosome IV of untreated *C. riparius* fourth instar larvae (A and D), and from larvae exposed to 1 µg L^{-1} BBP during 24 h (B and E) or 48 h (C and F). Nucleolus (N) and Balbiani rings (BR1, BR2) are identified. Bar, 10 µm.

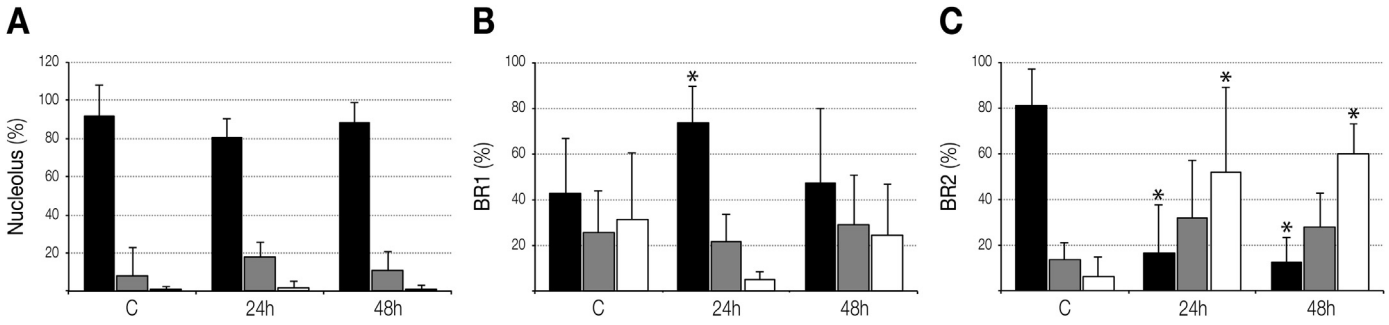

Fig. 6. BBP-induced alterations in the nucleolus and the puffing pattern of the Balbiani rings (BR1, BR2) in polytene chromosomes of *C. riparius* larvae exposed to 1 µg L^{-1} BBP during 24 h or 48 h. The expansion grade of the puffs where divided into three categories: high-expanded (black bars), semi-expanded (grey bars), or collapsed (white bars). Each bar represents the mean ± SD of about 200 nuclei. *Significant differences ($p \leq 0.05$) as compared to control conditions.

results, in which it was noticeable a general decline in gene activity in 48-h exposures to BBP, correlate well with the clear and significant decrease in the transcriptional activity described in this manuscript for all the RP genes under the same experimental conditions. Among all these genes, the newly characterized *rpL4* proved to be the most sensitive, as it was affected in the first 24 h and even at the lowest BBP concentrations tested. In the case of *its2*, the gene also had an early response to the xenobiotic, but at higher doses. In all cases, removal of the compound favoured the recovery of transcriptional control levels, thus confirming a reversible toxic damage.

To date, only a few studies have focused on the nucleolar region as a potential target of exposure to xenobiotics, and data are particularly scarce in invertebrates. Structural changes in the nucleolus have been described in plant cells after cadmium (Marcano et al., 2002) and copper (Bellani et al., 2014) exposures, altering the biosynthesis of the preribosomal RNA precursor and affecting root cell division, respectively. It has also been described the curcumin-mediated decrease in the expression of nucleolar organizer regions (Lewinska et al., 2014) and the inhibition of rDNA transcription and the reduction of the total RNA content in response to acute oxidative stress (Mironova et al., 2014) in HeLa cells. In *C. riparius* larvae, cadmium exposures led to a significant reduction of the 32S and the 45S rRNA precursors (Planelló et al., 2007). Additionally, it is well known that the depletion RPL11 inhibits ribosome biogenesis (Donati et al., 2013; Fumagalli et al., 2012) through the inhibition of rRNA transcription and processing, import of RPs, and export of immature ribosomal subunits (Bursac et al., 2014). Additionally, the temporary downregulation of another RP gene, such as *rpL2*, is followed by a transient block in the synthesis of new proteins (Ludwig and Tenhaken, 2001). On the knowledge that cell growth is a pre-requisite for cell proliferation, and ribosome biogenesis is a limiting factor for cell growth (Donati et al., 2012), the impairment of the ribosome synthesis pathway could slow down the metabolism of exposed organisms and lead to a faster adaptation of the cellular protein inventory to the new environmental conditions. In our study, the significant decline in the transcriptional activity of genes encoding rRNA or ribosomal proteins in larvae exposed to BBP as a possible adaptive cell response to the injury caused by the toxin is consistent with the fact that withdrawal of the compound allowed the larvae to recover control transcript levels. Thus, once the stressor disappears cells would return to their normal activity, necessary to achieve and maintain homeostasis.

In order to assess if the transcriptional deregulation of these genes were due to a specific toxic effect of BBP or, on the contrary, was part of a general cellular response, cytological analyses of polytene chromosomes from the salivary gland cells were carried

out, given that in these interphase chromosomes it is possible to visualize the most active loci as decondensed puffs and therefore evaluate the overall pattern of gene activity and the state of the nuclear function. After 48-h exposures, detection of DNA/RNA hybrids, which recognize transcriptional active sites, showed a drastic reduction in the fluorescent signals in all long polytene chromosomes and an increase in the centromeric signals of chromosomes II and III. This centromeric activation is particularly interesting, given that these are heterochromatic regions containing repetitive sequences of non-coding DNA, and reflects the ability of BBP to induce decondensation of these specific regions. This type of response has been described previously in *C. riparius* under different stress conditions, such as acute exposures to cadmium and bisphenol A (Martínez-Guitarte et al., 2012; Planelló et al., 2007), aluminium (Michailova et al., 2003) and copper (Michailova et al., 2006). However, other heterochromatin-rich regions such as telomeres did not show modifications in any experimental condition tested. Despite the increasing number of references on the ability of certain compounds to influence these heterochromatic regions, the physiological significance of this response remains unknown.

Analysis of the chromosome IV showed that the nucleolus, a very dynamic structure that functions primarily as a ribosome factory, controlling eukaryotic cell cycle progression, was not significantly altered by BBP, which is consistent with the lack of effect of the compound on new synthetized rRNA. This is remarkable, since recent works evidenced that this region forms a crucial stress-sensing structure, playing a central role in the coordination of cellular stress response (Pederson and Tsai, 2009).

Also in this chromosome IV, the puffing pattern of the Balbiani rings (BRs) was studied. The visible aspect of a puff is essentially the complex of RNA and protein that accumulates as a result of vigorous transcriptional activity. Among the gene products encoded by the BRs are the glue proteins, required to attach the midge pupa to its substrate and essential to the formation of the larval tube, where the larva feed, breathe (Rydlander and Edström, 1980) and find protection against predators (Hershey, 1987) or environmental stressors, such as toxicants (Halpern et al., 2002). There is little preliminary information on the activity of the BRs in response to toxic chemicals. Among the heavy metals, cadmium does not affect any of the BR genes (Planelló et al., 2007), while copper suppresses the activity of BR1 but not BR2 (Aziz et al., 1991). Long-term effects on the size of BRs have also been described in Chironomus eggs exposed to aluminium (Michailova et al., 2003).

With respect to our findings, the significant increase (about 2-fold) detected in the expression of BR1 after 24-h exposure to BBP could be due to a defensive response of the larvae against the toxic effects of the phthalate. However, this effect differed with the drastic decrease in the degree of condensation of BR2 (about 80% below control) in both 24-h and 48-h treatments. The significant repression detected in BR2 may be related to the ability of BBP to alter the ribosomal expression pattern, probably leading to an altered assembly function and a disturbed nucleocytoplasmic traffic of transcriptional products (Baurén and Wieslander, 1994; Daneholt, 2001; Kiesler et al., 2002), ultimately affecting the DNA translation process and cell homeostasis.

The inhibitory effects that certain xenobiotics cause on rDNA transcription should not be ignored because their consequences may result in deregulation of vital physiological processes of organisms, such as growth and development. Our data reinforce the idea of ribosomal genes as an early target of BBP and suggest the need for further research to assess the implication of these changes in the potential disruption of *C. riparius* normal growth and development.

5. Conclusions

This work provides new information about the significant dose and time-dependent alterations induced by BBP on genes related to the ribosomal biogenesis in *C. riparius*, a model organism in ecotoxicology studies, and complete our previous study in which this phthalate induced changes in the ecdysone hormone pathway, the cellular response to stress, the energy metabolism, and several detoxication mechanisms. Acute exposures to BBP, at lower concentrations than those which affected other authors' endpoints and/or those detected in aquatic environments, modulate the nucleolar function by altering the expression pattern of rDNA and genes coding for ribosomal proteins. These changes are particularly reflected in the cytological analyses, which show changes in the puffing pattern of the nucleolus and the Balbiani rings. Our results demonstrate the sensitivity of the selected biomarkers and may help in the development of new procedures for the characterization of the toxic effects and the mode of action of BBP, especially in invertebrates.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.10.051.

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