Analysis of the Impact of Three Phthalates on the Freshwater Gastropod Physella Acuta at the Transcriptional Level

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Abstract

Plastic pollution is one of the leading environmental problems. Phthalates are widely used plastic additives released into the environment. Although the phthalates' effects have been extensively studied on vertebrates, there is a gap in knowledge on their effects on invertebrates. This work analyzes the impact of three phthalates, diethyl phthalate (DEP), benzyl butyl phthalate (BBP), and bis-(2-ethylhexyl) phthalate (DEHP), on the gastropod *Physella acuta* at the molecular level to establish the putative pathways involved in its response to them. By real-time PCR, we obtained the expression profile of thirty genes in one-week exposed animals at 0.1, 10, and 1000 µg/L. The genes cover the DNA repairing mechanism, detoxification mechanisms, apoptosis, oxidative and stress responses, immunity, energy reserves, and lipid transport. The results show that while DEP and DEHP did not cause alteration of the mRNA levels, BBP modulates almost all the genes tested. It can be concluded that the impact of BBP is extensive at the molecular level. However, it cannot be dismissed that the increase in transcriptional activity is a general response due to this compound's well-known role as an endocrine disruptor. Additional research is needed to elucidate the differences observed in the impact of these compounds on this gastropod.

Introduction

Plastics are incredibly versatile materials useful for a wide range of applications. However, plastic production is under the scope of green policies to reduce the pollution of the environment. The two main processes used to produce plastics are polymerization and polycondensation, requiring specific catalysts and other additives. It has been estimated that 8300 million metric tons (Mt) of virgin plastics were produced in 2017. In 2015, approximately 6300 Mt of plastic waste were generated, with 79% accumulated in landfills or the natural environment[1]. Phthalates are esters of phthalic anhydride mainly used as plasticizers to increase the flexibility, transparency, durability, and longevity of plastics, mainly to soften polyvinyl chloride (PVC). As the phthalate plasticizers are not chemically bound to PVC, they can leach, migrate, or evaporate into indoor air and atmosphere, foodstuff, and other materials. The worldwide production of phthalates increased from 2.7 to nearly 6 million tons per year during the decade of 2007−2017[2], being now-ubiquitous environmental contaminants. They are released regularly from the containing products[2,3], and reach almost all the environment's compartments[4]. In German rivers, phthalates have been found in the range from 0.33 to 97.8 µg/L for bis-(2-ethylhexyl) phthalate (DHEP) and from 0.12 to 8.80 µg/L for dibutyl phthalate (DBP), while concentrations in sediment were from 0.21 to 8.44 µg/kg dw of DEHP and 0.06 to 2.08 µg/kg dw for DBP[5]. A more recent study found different phthalates in varying ranges in the Ganga river, such as dimethyl phthalate (DMP) 0.03−0.05 µg/L, diethyl phthalate (DEP) 0.04−2.14 µg/L, di-n−butyl phthalate (DnBP) non detected (ND)−2.27 µg/L, benzyl butyl phthalate (BBP) ND−0.13 µg/L, bis (2-ethylhexyl) adipate (DEHA) ND−0.19 µg/L, bis (2-ethylhexyl) phthalate (DEHP) 0.11−6.3 µg/L, and di-n-octyl phthalate (DnOP) ND−0.05 µg/L[6].

The impact of phthalates on the environment has been studied in the last few years, focusing mainly on vertebrates. It is known that phthalates act as endocrine-disrupting chemicals (EDCs), producing severe health effects[7−11] and even having a long-term impact on the epigenome[12]. Phthalates can alter an animal's metabolism, but there is still a lack of information about their effects on invertebrates. Although the studies often include analysis at the molecular level[13−15], the invertebrates' diversity demands additional studies involving other species and groups. One of the groups that has been poorly studied, especially in freshwater representatives, is the mollusk. Most of the studies focus on marine species, and the molecular analysis in freshwater species is basically absent. Furthermore, in gastropods, the studies are centered on the marine representative *Haliotis diversicolor*[16−18]. The freshwater snail *Physella acuta* (Draparnaud, 1805), also known as *Physa acuta*, belongs to the Physidae family. It is a cosmopolitan species living in lakes and ponds. It is easily cultured, so it could be a good representative of gastropods to assess the compounds' toxicity.

Toxicants can alter different physiological processes, such as growth and reproduction, reaching the effects by working in lower complexity levels such as the molecular and cellular processes. Therefore, it is essential to get information about the mode of action to define a compound's impact on an animal. The changes in mRNA levels of genes are useful tools to detect the putative pathways altered by a chemical, providing a picture of the damage that it can cause. Recently, we obtained the transcriptome of *Physella acuta* and identified genes to analyze the toxicity of the fungicide vinclozolin[19]. Taking advantage of the transcriptome and sequences in the database, we have identified new genes to study several pathways involved in response to phthalates. The genes are related to DNA repairing mechanisms, detoxification mechanisms (phase I, phase II, and phase III), oxidative stress, stress response, immunity, epigenetics, lipid transport, and energy reserve metabolism.

This work aims to offer some light on the impact, at the molecular level, that phthalates have on the gastropod *Physella acuta*. Two-month-old adult snails were exposed for one week to three phthalates, diethyl phthalate, benzyl butyl phthalate, and bis-(2-ethylhexyl)

phthalate. The gene expression profile of thirty genes was obtained for the first time in response to these compounds, which is a first step to assess the damage they can produce in a freshwater invertebrate that is pivotal on the food web of freshwater ecosystems.

Results

Gene identification

Eighteen genes were identified for the first time in this work for *P. acuta* (Table 1, Fig. 1). They all showed homology with genes in the database, mainly with those described in the freshwater snail *Biomphalaria glabrata*. In some of the genes, the homology was with *Aplysia californica* and *Aplysia kurodai*, and only one had homology with a gene of the marine bivalve *Mytilus coruscus*. The genes covered different pathways. Two of the genes coded for proteins homologous to RAD21 and RAD50 involved in repairing of DNA damage[20,21]. There were two other oxidative-stress-related genes, which code for catalase and Mn superoxide dismutase, enzymes involved in removing free radicals[22]. One of the sequences coded for acetylcholinesterase, an enzyme involved in nerve impulse transmission. The other genes coded for proteins related to apoptosis (AIF3, apoptosis inducible factor 3), stress (sHSP 17.9, small heat shock protein 17.9; Hsc70 (4), heat shock protein cognate 70 – 4; HIF-1α, hypoxia-inducible factor 1 α), histone modification (HDAC1, histone deacetylase 1; KATB6, histone acetyltransferase KAT6B), DNA methylation (DNMT1, DNA methyltransferase 1), immune system (ApA, L-amino acid oxidase Aplysianin-A), one cytochrome P450 (cyp72a15), energy reserves (PYGL, glycogen phosphorylase), and lipid transport (ORP8, oxysterol-binding protein-related protein 8).

Table 1
Accession numbers and homologies of the newly identified genes in *Physella acuta*

Accession number	Gene	ORF size (aa)	Similarity	Identity-Homology
MW456925	rad21	678	PREDICTED: double-strand-break repair protein rad21 homolog - *Biomphalaria glabrata*	85% – 92%
MW456929	rad50	1200	PREDICTED: DNA repair protein RAD50-like - *Biomphalaria glabrata*	68% – 84%
MW456922	AChE	548	PREDICTED: cholinesterase 1-like - *Biomphalaria glabrata*	56% – 69%
MW456920	Catalase	508	PREDICTED: catalase-like - *Biomphalaria glabrata*	82% – 90%
MW456928	AIF3	591	PREDICTED: apoptosis-inducing factor 3-like - *Biomphalaria glabrata*	77% – 88%
MW456924	Cyp72a15	486	cytochrome P450 72A15 - *Aplysia californica*	68% – 82%
MW456930	DNMT1	212	DNA methyltransferase 1, partial - *Aplysia californica*	88% – 91%
MW456923	KAT6B	1277	PREDICTED: histone acetyltransferase KAT6B-like - *Biomphalaria glabrata*	49% – 62%
MW456919	HDAC1	527	probable histone deacetylase 1-B - *Aplysia californica*	88% – 94%
MW456918	sHSP17.9	159	PREDICTED: heat shock protein Hsp-12.2-like - *Biomphalaria glabrata*	52% – 71%
MW456926	ApA	522	Aplysianin-A; Precursor - *Aplysia kurodai*	45% – 65%
MW456921,	ORP8	955	PREDICTED: oxysterol-binding protein-related protein 8-like - *Biomphalaria glabrata*	74% – 83%
MW456927	PFKFB2	234	PREDICTED: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-like - *Biomphalaria glabrata*	84% – 89%
Suplementary material	SOD Mn	219	superoxide dismutase - Mn, mitochondrial-like - *Biomphalaria glabrata*	80% – 88%
	Hsc70-4	648	PREDICTED: heat shock 70 kDa protein cognate 4 - *Biomphalaria glabrata*	98% – 98%
	HIF1α	716	PREDICTED: hypoxia-inducible factor 1-alpha-like, partial - *Biomphalaria glabrata*	64% – 73%
	PYGL	847	glycogen phosphorylase, brain form - *Aplysia californica*	89% – 97%
	ACTB_G1	376	ACTB_G1 - *Mytilus coruscus*	99%-100%

Expression profile

The adult snails were exposed for one week to each phthalate. The mRNA analysis showed that DEP and DEHP did not modify the mRNA quantity of any of the genes and the concentrations tested (Figs. 2–6). However, the BBP was strikingly very effective and altered almost all the genes tested but with some differences. It is worth clarifying that statistical analysis without Bonferroni correction rendered significant differences for all the BBP treatments in all genes except for the higher concentration in *GSTm1*. However, applying Bonferroni's correction, the results showed that for *rad21*, *AChE*, *SOD CuZn*, *SOD Mn*, *Casp3*, *AIF3*, *Cyp2u1*, *Cyp3a7*, *Cyp72a15*, *GSTk1*, *HDAC1*, *sHSP17.9*, *HSP60*, *Grp78*, *HSP90*, *HIF1a*, *ApA*, *PYGL*, and *ORP8*, there was an increase of transcriptional activity at all the concentrations tested. Although there is an increasing trend in the rest of the genes, some results did not show a statistical significance to be considered different from the control. The genes which showed statistical significance for the two lower concentrations but not at the highest were *rad50*, *DNMT1*, *KAT6B*, *sHSP16.6*, and *HSC70.4* (Figs. 2, 5, and 6). *Cat* and *Cyp4f22* were altered for the concentration of 1 µg/L but not for the other two (Figs. 2 and 3). *MRP1/ABCC1* mRNA levels were increased for 1 and 100 µg/L, while *GSTo1* and *GSTt2* were significantly upregulated for 0.01 µg/L only (Fig. 4). Finally, *GSTm1* did not show statistically significant alterations, although a trend to have a higher mean than the control can be observed (Fig. 4).

Discussion

The development of massive sequencing has provided a relatively cheap method to obtain the transcriptome of a species. Taking advantage of it, we have used a previously obtained transcriptome to identify eighteen genes related to different pathways of interest in ecotoxicology. With others yet described, the genes identified allow us to analyze these pathways' response to different chemicals. The genes identified cover processes of interest in ecotoxicology such as DNA repair mechanisms, stress, detoxification, apoptosis, immunity, energy reserves, and lipid transportation. There is a growing interest in combining ecologically relevant endpoints with biochemical and molecular parameters to seek a more integrative analysis. In this sense, to increase the number of genes described will allow for the design of standard arrays that could be used in combination with toxicity tests. In this way, initiatives such as the Adverse Outcome Pathway wiki[23] will increase its relevance in assessing old and new compounds and provide putative mechanisms of action to explain the differences to the animals' specific physiology. Furthermore, increasing knowledge at the molecular level in this species supports its use as a representative of freshwater gastropods in toxicity analysis. There is a lack of model organisms in freshwater mollusk, being one of the animal groups whose pollution response is currently less known.

The eighteen genes evaluated in this work show homology with those previously described in other species, as expected. *Rad21* and *rad50* are both genes involved in DNA repairing mechanisms. The first one is an essential gene encoding a DNA double-strand break (DSB) repair protein[20]. In contrast, the second is a member of the protein complex MRN (including Mre11, Rad50, and Nbs1) that functions in DNA double-strand break repair to recognize and process DNA ends as well as a signal for cell cycle arrest[24]. Practically, there is no information about these genes in mollusks, with only one report in *Carssostrea gigas* for *rad50*[25]. The relevance of these genes is that they can be combined with other methodologies, such as the comet assay, to perform an integrated study to know if a compound is genotoxic and if the organism has the ability to compensate for the damage.

The catalase and the Mn superoxide dismutase genes allow us to analyze the impact of oxidative stress. Usually, oxidative stress analysis is focused on biochemical parameters, such as the enzyme's activity. However, it should also include a transcriptional activity study since it can provide additional information about the mid- and long-term response. The protein turnover can also be relevant in the response, especially in chronic exposure to toxicants. It is similar to that concerning detoxification mechanisms. The GST activity is one of the most used methods to assess detoxification[26], but it does not differentiate between the members involved. A similar situation is found with the cytochromes P450, which show high diversity with many roles in the cell[27]. The Cyp72a15 gene increases the number of Cyp450s described in *P. acuta* and helps to elucidate how the organism can process the toxicants.

The small heat shock gene and the heat shock cognate gene extend the battery of genes available to assess this species' stress response. The small heat shock gene is difficult to match with other species' genes because the alpha-crystallin domain characterizes them, but there is no other sequence that, presently, allows for the homology to be established. Additional functional studies will help search for it. However, it is worth mentioning that *HIF1a* offers a new aspect of stress related to hypoxia[28]. The stress response is mainly focused on the canonical heat shock proteins, so other mechanisms involved in specific stresses, such as hypoxia, usually are neglected. The description of the factor inducible by low oxygen levels will help to know the effect of the toxicant on oxygen intake.

Similarly, the rest of the genes identified allow for the analysis of additional pathways that can also be altered by toxicants, like apoptosis (*AIF3*), the immune system (*ApA*), energy reserves (*PYGL*), and lipid transport (*ORP8*). To our knowledge, these genes are analyzed for the first time concerning pollution in freshwater mollusks. The last three genes, *DNMT1*, *KATB6*, and *HDAC1*, are involved in epigenetic

mechanisms. Epigenetic regulation is arising as one of the long-term effects of toxicants. However, the genes involved in invertebrates are still poorly represented in toxicity analysis. The description of these three genes opens the possibility of analyzing their role in the epigenetic response and the relevance that they have in the transgenerational effects that have started to be described with different toxicants[29–31].

Plastics in the environment are a growing problem that involves the release of polymers themselves and the compounds used as catalysts and additives during the degradation process. Phthalates are such additives that are increasing their presence in the environment[5,32,33]. Three phthalates, BBP, DEP, and DEHP, have been analyzed in this work, which showed a differential impact in *Physella acuta*. Two of the compounds, DEP and DEHP, did not show any change in the genes' mRNA levels. It has been described previously that both phthalates can alter the physiology of invertebrates[15,34–37], including mollusks[38–40]. The differences observed here can be assigned to the type of analysis (molecular vs. physiological), the exposure time (one week vs. a few hours or days), the concentration used (μg/L vs. mg/L), and evidently, the species used. Additional research will help elucidate the putative relationship between the different data obtained and those from other organisms. However, it is essential to highlight that the obtained results suggest that *Physella acuta* can manage the environmentally relevant doses used in this work for DEP and DEHP. This species may be less sensitive to these phthalates, but this eventually will require further research, including the use of other methodological approaches, to confirm it.

On the other hand, BBP showed a very different picture, with almost all genes increasing the mRNA levels in response to the compound. Previous studies in other organisms have confirmed that BBP can induce different types of damage such as apoptosis[41], genotoxicity[42], oxidative stress[43], stress response activation[44], or endocrine disruption[13]. Although there are studies in invertebrates showing the impact on development and other physiological processes[38,45], most of them did not focus on the putative mode of action, with only a few of them trying to delve into the mechanisms of response. BBP can affect very diverse processes in the organisms, and here we have found that all the pathways analyzed are modulated. The results can be considered in two ways. The first is that the changes are reflecting specific alterations on these pathways. It would mean that BBP is the most active phthalate in *P. acuta*, with a broad spectrum of action and a potential activity on many pathways. The second possibility is that BBP induces some change in other non-tested processes, like the hormonal system, that increases the transcriptional rate of the genes because of its regulatory role in the cell. It is relevant to keep in mind its known role as an endocrine disruptor of BBP[46]. However, a recent study in *Daphnia magna* can help elucidate between both possibilities since, by RNA-Seq, it has been observed that genes involved in signal transduction, cell communication, and embryonic development were significantly down-regulated, while those related to biosynthesis, metabolism, cell homeostasis, redox homeostasis were remarkably upregulated upon BBP exposure[47]. Although the organism and the stage analyzed are different than in our study, those results support the idea that BBP can have multiple effects on the cell metabolism, altering the pathways analyzed.

It is also essential to consider that most of the studies on invertebrates that involve transcriptional activity analysis use short exposure times and use arthropods[13,44,47]. Limited data are available on mollusks and, usually, they are on the marine representatives[39,46]. To our knowledge, this is the first study on a freshwater snail that shows that BBP can produce a substantial effect on cell metabolism. Several of the altered pathways can explain, in some way, the effects observed in other organisms, like the DNA repairing mechanisms, which are related to DNA damage, or the alteration of the genes involved in histone and DNA modification, which are related to epigenetic regulation. However, other genes open new possibilities, such as *ApA* and *ORP8*, involved in pathways not studied in other organisms. The changes in genes involved in oxidative stress, stress response, and detoxification back previous analysis, adding new light about the mechanisms involved in modulating these processes. The absence of changes in *GSTm1* supports a differential role for each GST family in response to toxicants. On the other hand, the differences concerning concentrations for some of the genes suggest subtle differences requiring additional kinetic analysis to elucidate early and late activated genes.

As stated before, the results obtained in this work show that DEP and DEHP have no apparent effect in a week exposure at environmentally relevant concentrations. However, BBP shows a strong effect in comparison. It could be due to several reasons that need to be explored in future works. One possibility is the structure of each compound. In this sense, BBP has two rings while DEP and DEHP have only one. This factor could determine the biological activity that they have. Another possibility is that DEP and DEHP have effects earlier than the time studied, and the cell returned to the basal state, being able to process and remove the compounds. Finally, it cannot be dismissed that DEP and DEHP are not toxic for *P. acuta*, at least at environmentally relevant concentrations. In any case, BBP can alter the metabolism of this species. Additional research should be done in *P. acuta* and other freshwater species to know the impact on organisms based on freshwater ecosystems' food web.

Conclusions

An analysis of the transcriptional activity of thirty genes in adults exposed to three phthalates has shown that DEP and DEHP have no apparent effect on the organism, so the mRNA levels were similar to those observed on controls. However, the third compound analyzed, BBP, strongly affects almost all the genes with an extensive action that alters DNA repairing, apoptosis, epigenetic regulation, stress response, immunity, and energy metabolism. BBP is toxic for *P. acuta* at the environmentally relevant concentrations used. However, additional research is needed to elucidate the kinetics of the response and how extent is it. Additional research is also required at different time points with DEP and DEHP to confirm that they cannot induce responses at the concentrations used. Finally, eighteen of the genes have been used for the first time to describe *P. acuta*. They increase the number of pathways that can be analyzed and support the use of this species in assessing toxicants in freshwater mollusks.

Materials And Methods

Chemicals and reagents

Diethyl phthalate 99.5% (DEP), benzyl butyl phthalate 98% (BBP), and bis-(2-ethylhexyl) phthalate 98% (DEHP) were acquired from Sigma. Stock solutions for each compound were prepared for a concentration of 10.78 mg/mL BBP, 11.44 mg/mL DEP, and 965.3 mg/ml DEHP. The stock solutions were prepared in acetone for BBP and DEP, while DEHP was diluted in ethanol. Exposure solutions were prepared by 1:10,000 dilution of these stocks in the artificial pond water (see *Treatment* section below).

TRIzol and M-MLV enzyme were obtained from Invitrogen (Germany), oligonucleotide dT18 primer and gene-specific primers were supplied by Macrogen (Korea), RNase-free DNase was purchased from Sigma, DNA polymerase and dNTPs were obtained from Biotools (Spain), and EvaGreen was purchased from Biotium (USA).

Animals

The animals exposed were adults of the freshwater snail *Physella acuta*. The origin and the maintenance of the animals have been described previously[48]. The exposures were carried out in glass vessels filled with artificial pond water (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.77 mM $NaHCO_3$, and 0.08 mM KCl). The animals were fed twice a week, once with a 1:1 mixture of Sera Micron and Sera Shrimp Natural (Sera) and once with Sera Shrimp Natural.

Treatment

Each compound was tested in three concentrations, and six animals were exposed for each concentration and experiment. A glass vessel with 300 mL of artificial pond water was used for each concentration and compound; in the control, the same amount of solvent (30 µL) was added. The artificial pond water was replaced after three days, and the animals were fed with 20 mg of Sera Shrimp Natural per recipient. The concentrations used were 0.1078 µg/L (0.345 nM), 10.78 µg/L (34.5 nM), and 1078.0 µg/L (3.45 µM) for BBP; 0.1114 µg/L (0.501 nM), 11.14 µg/L (50.1 nM), and 1114.0 µg/L (5.01 µM) for DEP; and 0.0965 µg/L (0.247 nM), 9.65 µg/L (24.7 nM), and 965.3 µg/L (2.47 µM) for DEHP. Three experiments were performed for each compound, and the animals were exposed for seven days. At the end of the experiment, three of the animals were frozen in separate tubes for RNA extraction. The concentrations have been named as 0.1, 10, and 1000 µg/L to simplify the labeling in the figures.

Gene identification

Thirty-four genes were used. Sixteen have been previously described (Table 1)[19,48]. From the other eighteen, two are reference genes (actin beta/gamma 1 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase) while the other sixteen belong to DNA repairing mechanisms (Double-strand-break repair protein rad21, *rad21*; DNA repair protein rad50, *rad50*), nervous system (acetylcholinesterase, *AChE*), oxidative stress (catalase *Cat*; Mn superoxide dismutase, *SOD Mn*), apoptosis (apoptosis-inducing factor 3, *AIF3*), detoxification mechanisms (cytochrome P450 72A15, *Cyp72a15*), epigenetics (DNA methyltransferase 1, *DNMT1*; lysine acetyltransferase 6B, *KAT6B*; histone deacetylase 1B, *HDAC1*), stress response (sHeat shock protein 17.9, *sHSP17.9*; heat shock cognate protein 70 4, *Hsc70 (4)*, hypoxia-inducible factor-1 alpha, *HIF1a*), immune system (Aplysianin-A, *ApA*), energy metabolism (Glycogen phosphorylase, *PYGL*), and lipid transport (Oxysterol-binding protein-related protein 8, *ORP8*).

The sequences were obtained following the procedure described in Aquilino et al.[19], from the same transcriptome and the sequences obtained by Romiguier et al.[49]. The transcriptome sequences were deposited in GenBank with the accession numbers indicated in Table 2, while those from Romiguier et al. are included in Supplementary Material.

Table 2
Primer sequence and efficiency of the primer set for each gene

Primer	Primer sequence	Efficiency (%)	Primer	Primer sequence	Efficiency (%)	Reference
rad21 F	CCGGCCAATGTCTGATGACT	96.9	rpL10 F	TGCACGTGAGGCTGATGAAA	102.3	Aquilino et al., 2019
rad21 R	GCAATTGCTTGCTGGCATCT		rpL10 R	GTGGCCACTTTGTGAAACCC		
rad50 F	AGGCAAGGAGGAGCTACAAC	98.7	GAPDH F	ATACATCAGGAACAGGGACTC	93.9	
rad50 R	TTCAGCCAATGCTAAGCGGA		GAPDH R	GACTTATGACAACCGTGCA		
AChE F	AGTGTCCCGTCGTGGATTTC	89.8	Casp3 F	GTCTGTGTAATTCTCACCCATG	107.2	
AChE R	CACGACCTCGATCTCGTAGC		Casp3 R	AGTTCAGTGCCTCTGCAAGC		
Catalase F	CCCAGTCAGTGGTGATGTCC	98.8	Hsp16.6 F	GCATGAGGAGAAGCAAGACA	96.4	
Catalase R	TTCAGGTGCCCGACAATGTT		Hsp16.6 R	CAGTACACCATGGGCATTCA		
SOD Mn F	TCGAATTGCTACCTGTGCCA	105.4	MRP1 F	CAGGGGCAGGTAAGTCATCC	94.5	
SOD Mn R	ATTCACGTAGTCAGCTCGCA		MRP1 R	AGTGAGCCTTGATCGCACAT		
AIF3 F	ACCACAAGATGCCAACGCTA	102.0	Hsp90 F	GTTTGTGTCACTAAAGAAGGCC	91.8	
AIF3 R	ACTGGCAGCCTTATCAGCAA		Hsp90 R	TGTCACTAGCCTATTTGATACAACC		
Cyp72a15 F	AGGGAAGTGGCTTGAGTGAC	91.9	cyp2U1 F	GTGCATCCTCTACGCGATCA	102.1	
Cyp72a15 R	GGTGCTCAGCCAGCATAAGA		cyp2U1 R	GGCTAGTTTGGGCCTGTCTT		
DNMT1 F	GACGCCATGTCCGATTTACCT	93.3	cyp3a7 F	ACGGCTTGGCCTCTCAATAC	84.8	
DNMT1 R	TCATCCGCGCTGCCACCAG		cyp3a7 R	CGGTTTCTTTCTCGGCGTTC		
KAT6B F	CTTCCATGGGGATGACGAGG	95.1	cyp4f22 F	AGCAGAAAAAGCTCAGCCCT	87.2	
KAT6B R	AAGCTTTGAACGTTTGCCCC		cyp4f22 R	CTTGGTTTTGGCAGCCAGTC		
HDAC1 F	CCCATCAAACATGGCCAACC	90.8	GSTo1 F	CCACCTGGCAACTTGGTTTG	92.8	
HDAC1 R	GTGCATGTGGCAACATTCTGA		GSTo1 R	GCTTGCCAGATGCGTAAGAC		
sHSP17.9 F	TTCACGCGTTGGTGAATCAG	102.5	GSTt2 F	TCGATCTTCTATCGCAGCCG	86.0	
sHSP17.9 R	TTAGCAGCTACAGTCAGCGT		GSTt2 R	TTCTGAGCGCAACAGGTTTG		
Hsc70-4 F	TGGTGTGCCCCAGATTGAAG	107.8	SOD CuZn F	AGAAAGCTGGTGCTGCAACTA	104.9	
Hsc70-4 R	TCCTCTTTGGACAGACGACC		SOD CuZn R	AGGATTAAAGTGGCCTCCAGC		
HIF1a F	AGGATAGATGCTGGCACACC	81.4	GSTk1 F	TGAGCAGAGTAGTTTGGCTGC	96.7	Alonso-Trujillo et al., 2020
HIF1a R	CATAGACACGGTCCTCCCCT		GSTk1 R	ATGCCCCTAATTCTGTGGCT		

Primer	Primer sequence	Efficiency (%)	Primer	Primer sequence	Efficiency (%)	Reference
ApA F	GTGCCGGGAAAGTGATTTTAG	99.6	GSTm1 F	ATTGGGCCATTAGAGGGCTT	93.1	
ApA R	CAGCCACCAGGGTCGCGA		GSTm1 R	GTTGGACCATCTCCTTGCAC		
PYGL F	ACTGACCCCTGCGTGTAAAG	104.2	Hsp60 F	ATTGCTTATCGTGGCTGAGG	82.0	
PYGL R	TGGAGGCAGGGTTGATCTTG		Hsp60 R	TGGCAATAGCCATATCCTGC		
ORP8 F	GCTGGACGGACATCACTTGT	98.9	Grp78 F	TGGTGGCTCAACCCGTATTC	96.8	
ORP8 R	TGGATGTCTACCACTCGGGA		Grp78 R	CCCCACTCAAAACACCAGCT		
ACT F	GAAGAGCTACGAGCTTCCCG	102.1				
ACT R	CATGGATACCGGCAGACTCC					
PFKFB2 F	AGCGCACTATCCAAACTGCT	110.6				
PFKFB2 R	TCTGCAAAGTCCTGGGGGTA					

RNA extraction

The frozen snails were used to extract the RNA using TRIzol (Invitrogen, Germany). The manufacturer's instructions were followed. The animals were homogenized in 300 µL of TRIzol, including the shell. Once the RNA was isolated, it was treated with RNAse-free DNAse for 45 minutes at 37 ºC to remove the rest of the DNA. Afterward, a phenol: chloroform extraction was performed with Phase Lock tubes (5 prime) to remove any DNAse. The RNA was precipitated and resuspended in 100 µL of diethylpyrocarbonate treated water (DEPC water). After checking the integrity by agarose gel electrophoresis and the quantity by a spectrophotometer (Biophotometer, Eppendorf), the samples were stored at -80 ºC until the next step.

Reverse transcription

The reverse transcription was performed with the M-MLV enzyme (Invitrogen, Germany). The manufacturer's instructions were followed. For each sample, 5 µg of RNA were used for a final 40 µL reaction. The mix was carried out with poly-dT(18) and 200 units of M-MLV. The mixture was incubated for 50 minutes at 37 ºC and stopped at 65 ºC for 15 minutes. The samples were stored at − 80 ºC until use.

Real-time PCR

The primers were poured into the wells, and a master mix of cDNA (0.2 µL/well), 0.5X EvaGreen, 20 units of Taq polymerase, 0.4 mM dNTPs, and 2 mM Cl_2Mg was prepared. The reaction's final volume was 10 µL and was performed with a CFX96 thermocycler (Bio-Rad, USA). The thermal cycling program included an initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C denaturation for 15 s, 58°C annealing for 15 s, and 72°C elongation for 30 s. Finally, a melting curve was generated to confirm the presence of a single peak. Reference genes were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L10 (*rpL10*), actin (*act*), and 6-phosphofructo-2-kinase (*PFKFB2*). Because some of the genes were in low quantities, the efficiency curves cannot be prepared with the cDNA. An alternative approach was used. A PCR with the same conditions as RT-PCR for each gene was carried out, and electrophoresis was run to ensure that single products were obtained. One microliter of each gene PCR was mixed in a tube, and water was added to 50 µL. From this 1:50 mixture was taken 1 µL and used to obtain a 1:25000 final dilution. Then, 1 µL was used to do the first concentration of the five 1:2-dilution series prepared to perform the efficiency curve. Primers and efficiencies are listed in Table 2. The RT-PCR was done running duplicate wells for each sample, and two independent replicates were used for each experiment. Bio-Rad CFX Maestro software was used to analyze and determine total mRNA levels of normalized gene expression ($2^{-\Delta\Delta Cq}$).

Statistical analysis

Statistical analysis was done using SPSS 25 (IBM, USA). Normal distribution and variance homogeneity were tested by the Shapiro-Wilk and Levene tests, respectively. Because the data was not normally distributed, they were analyzed with the nonparametric Kruskal-Wallis test with the Bonferroni correction. Statistical significance was set at $p \leq 0.05$.

Declarations

Acknowledgments

Author contributions (names must be given as initials)

J-L. M-G.conceived and designed the work and wrote the manuscript text and prepared the figures 1-6, tables, and supplementary material. M. P-A. and P. C. collected the data and contributed to data analysis and figure preparation. All the authors reviewed the articles.

Competing Interests Statement

The authors declare no competing interests.

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Figures

Figure 1
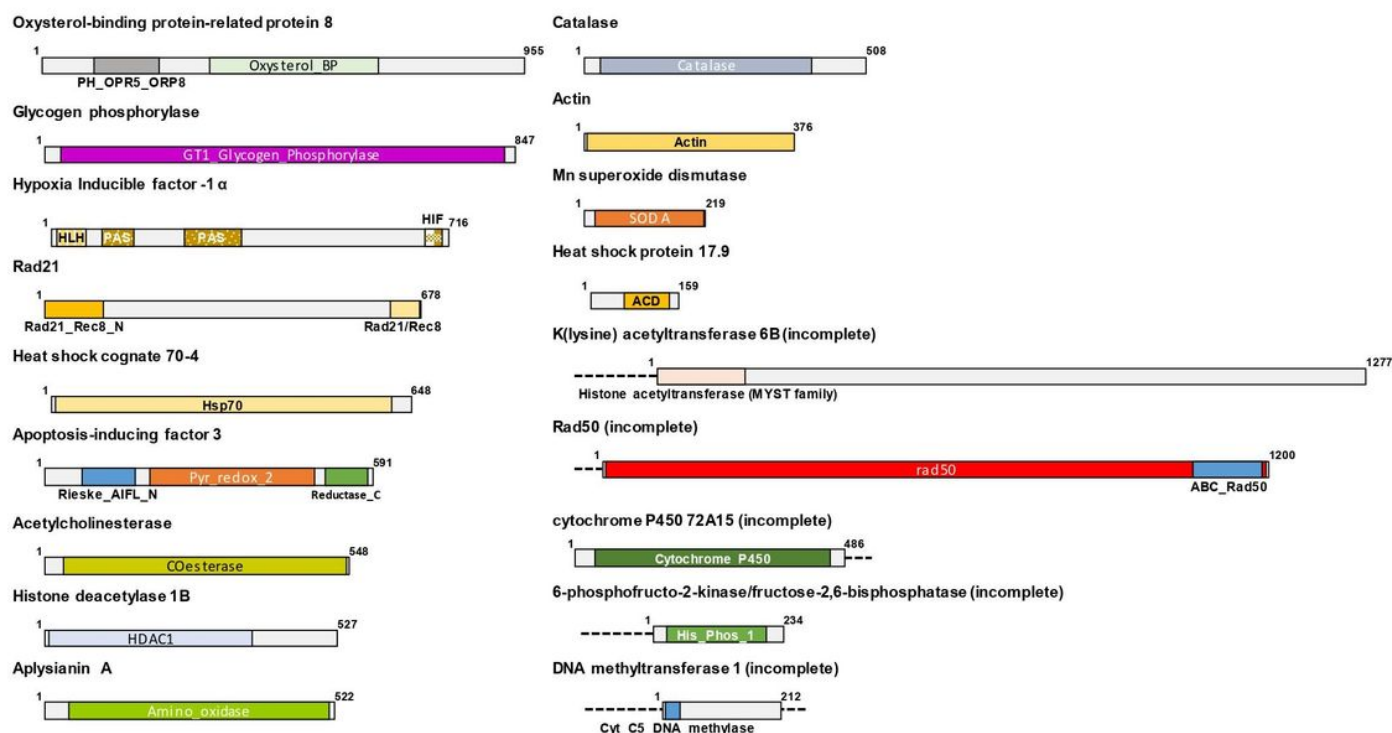
Figure 1

Structure and conserved domains of the identified Physella acuta proteins. Each protein corresponds to an open reading frame from the sequences used in the study. The proteins are shown with the different motifs that characterize them. The domains are defined according to the CCD functional classification of proteins. Some of the genes were not complete, and the discontinuous line indicates the unknown up- and down-stream regions.
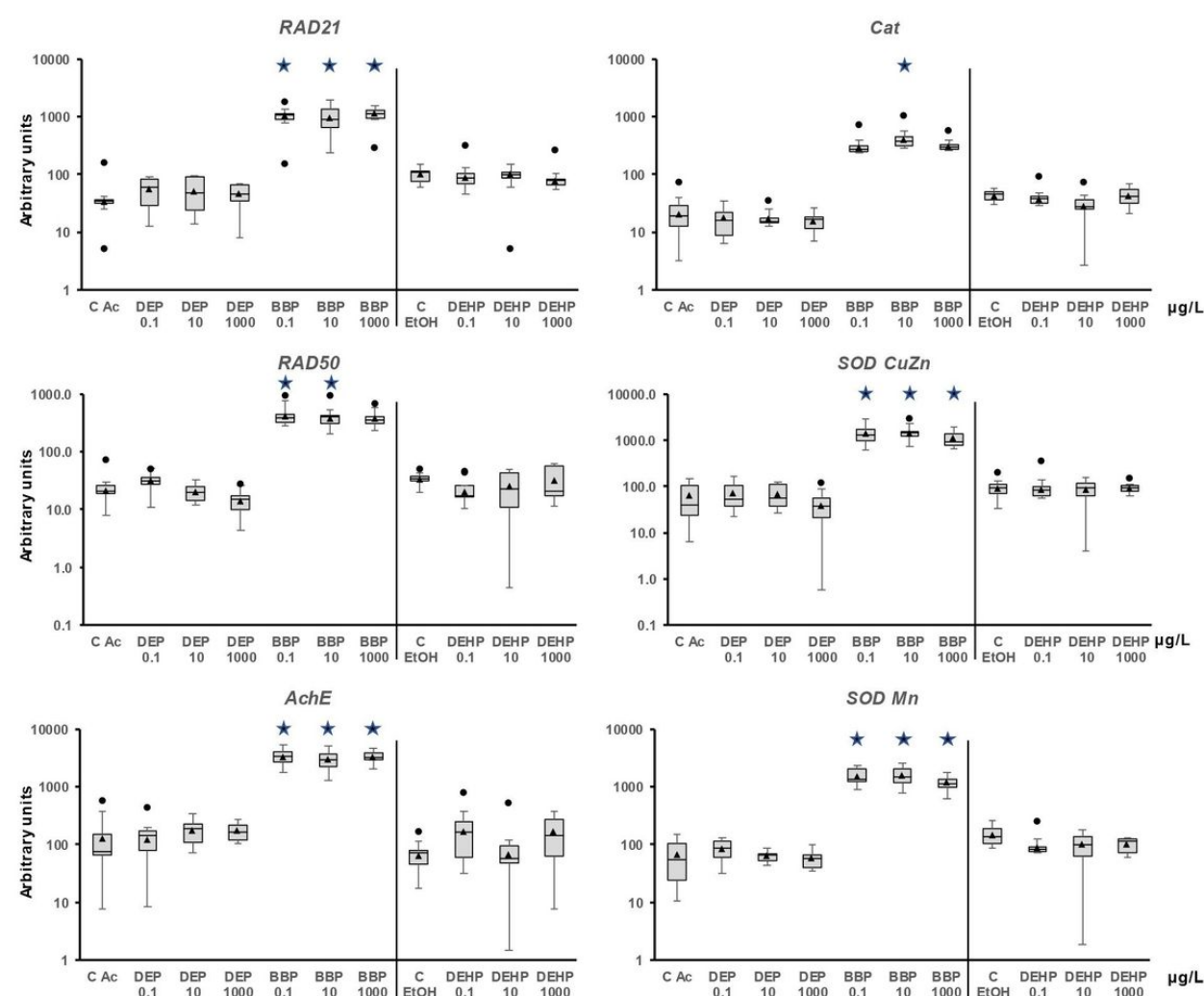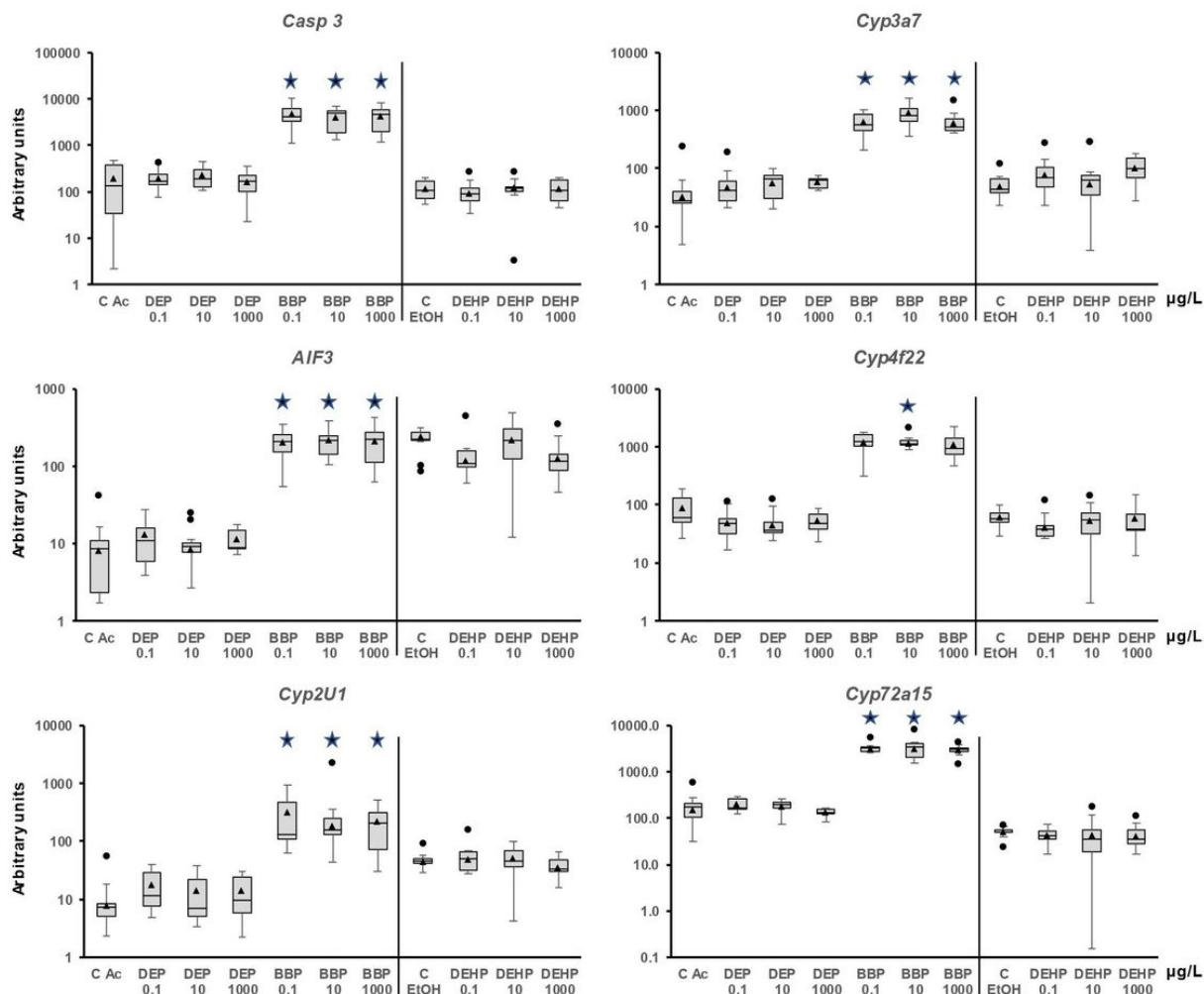
Figure 2

Figure 2

Transcript levels of DNA repairing mechanisms (RAD21 and RAD50), nervous system (AChE), and oxidative stress (Cat, SOD CuZn, and SOD Mn) genes in Physella acuta adults after in vivo exposure to diethyl phthalate (DEP), benzyl butyl phthalate (BBP), and bis-(2-ethylhexyl) phthalate (DEHP) for seven days at 19 °C. Transcriptional activity was quantified by RT-PCR using rpL10, Act, PFKFB2, and GAPDH as reference genes. The comparison was performed with the solvent-exposed controls. Whisker boxes are shown. Each box corresponds to nine individuals. The median is indicated by the horizontal line within the box, and the 25th and 75th percentiles are indicated by the boundaries of the box. The highest and lowest results are represented by the whiskers. The small triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences to respective controls (asterisk) are indicated ($p < 0.05$).
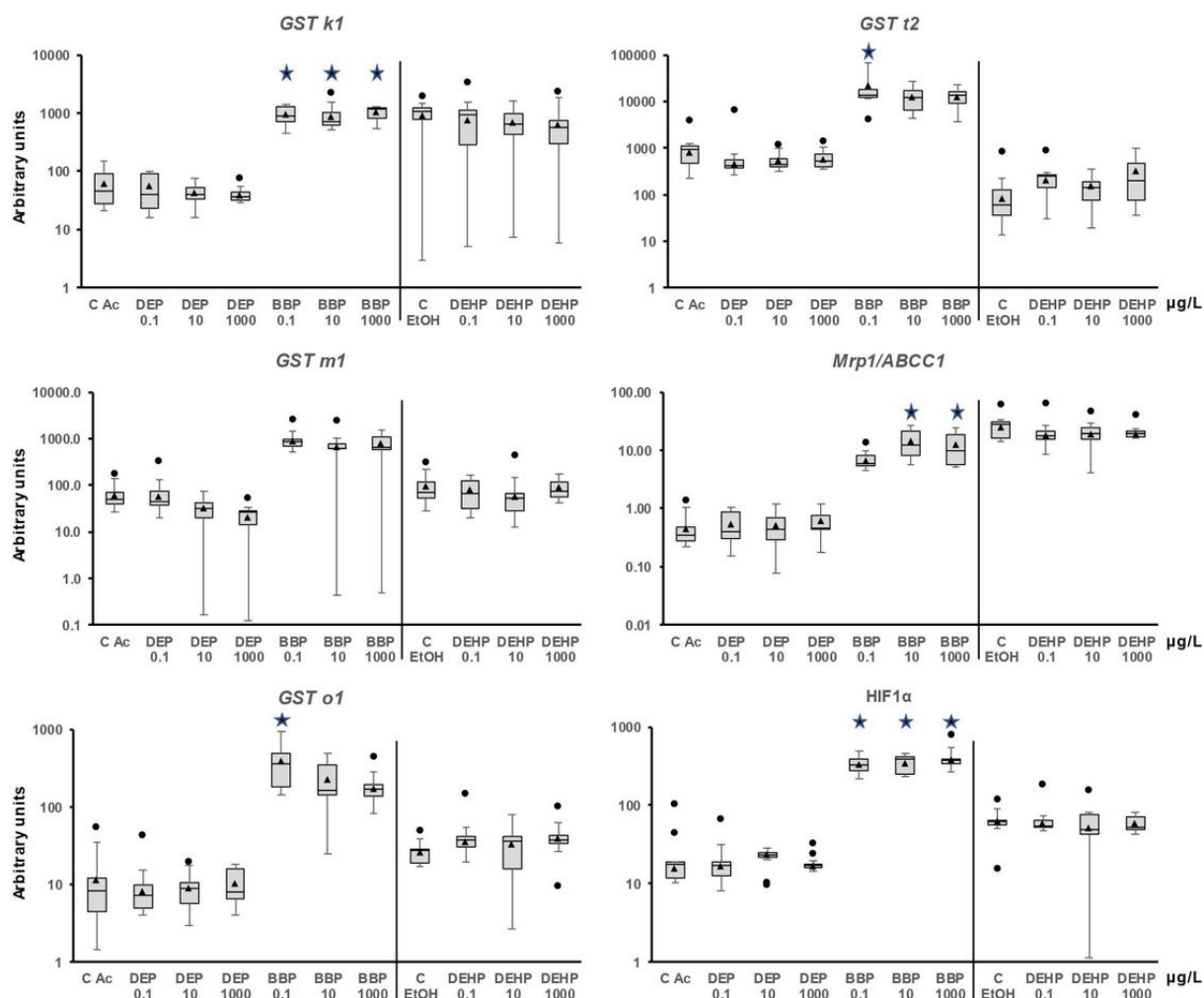
Figure 3

Figure 3

Transcriptional activity of apoptosis (Casp3 and AIF3) and phase I related genes (Cyp2u1, Cyp3a7, Cyp4f22, and Cyp72a15) in adult Physella acuta after in vivo exposure to three phthalates at 19 °C for seven days. Levels of mRNA were normalized using rpL10, Act, PFKFB2, and GAPDH as reference genes. The comparison was performed with the solvent-exposed controls. Whisker boxes are shown. The n for each box is nine. The median is indicated by the horizontal line within the box, and the 25th and 75th percentiles are indicated by the boundaries of the box. The highest and lowest results are represented by the whiskers. The small triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences to respective controls (asterisk) are indicated (p < 0.05).

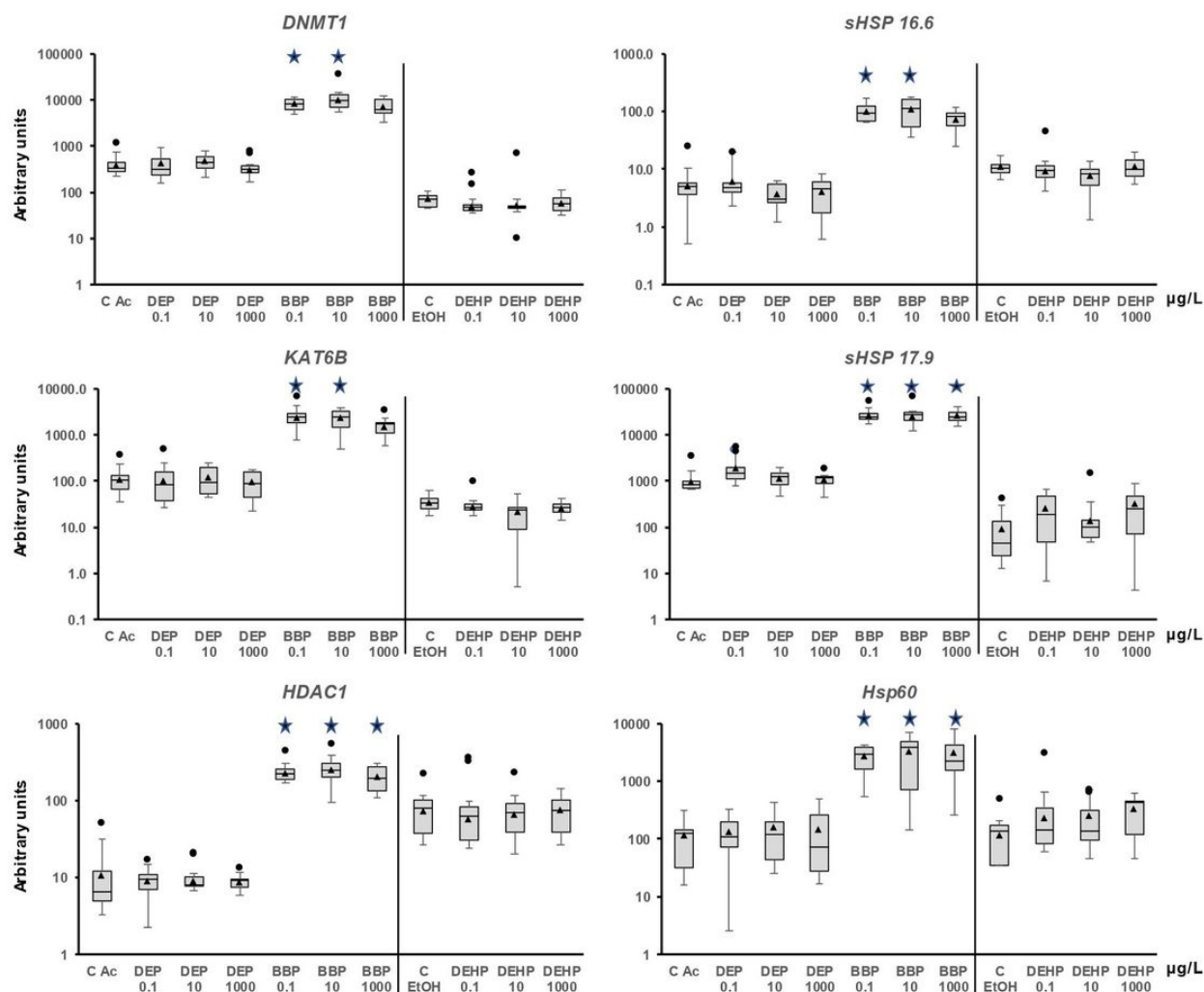# Figure 4

Figure 4

The mRNA levels of genes related to phase II (GSTk1, GSTm1, GSTo1, and GSTt2) and phase III (MRP1/ABCC1) of detoxification and hypoxia (HIF1α) in adult Physella acuta after in vivo exposure to DEP, BBP, and DEHP at 19 °C for seven days. As reference genes for normalization, rpL10, Act, PFKFB2, and GAPDH were used. The comparison was performed with the solvent-exposed controls. Whisker boxes are shown. The n for each box is nine. The median is indicated by the horizontal line within the box, and the 25th and 75th percentiles are indicated by the boundaries of the box. The highest and lowest results are represented by the whiskers. The small triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences to respective controls (asterisk) are indicated (p < 0.05).
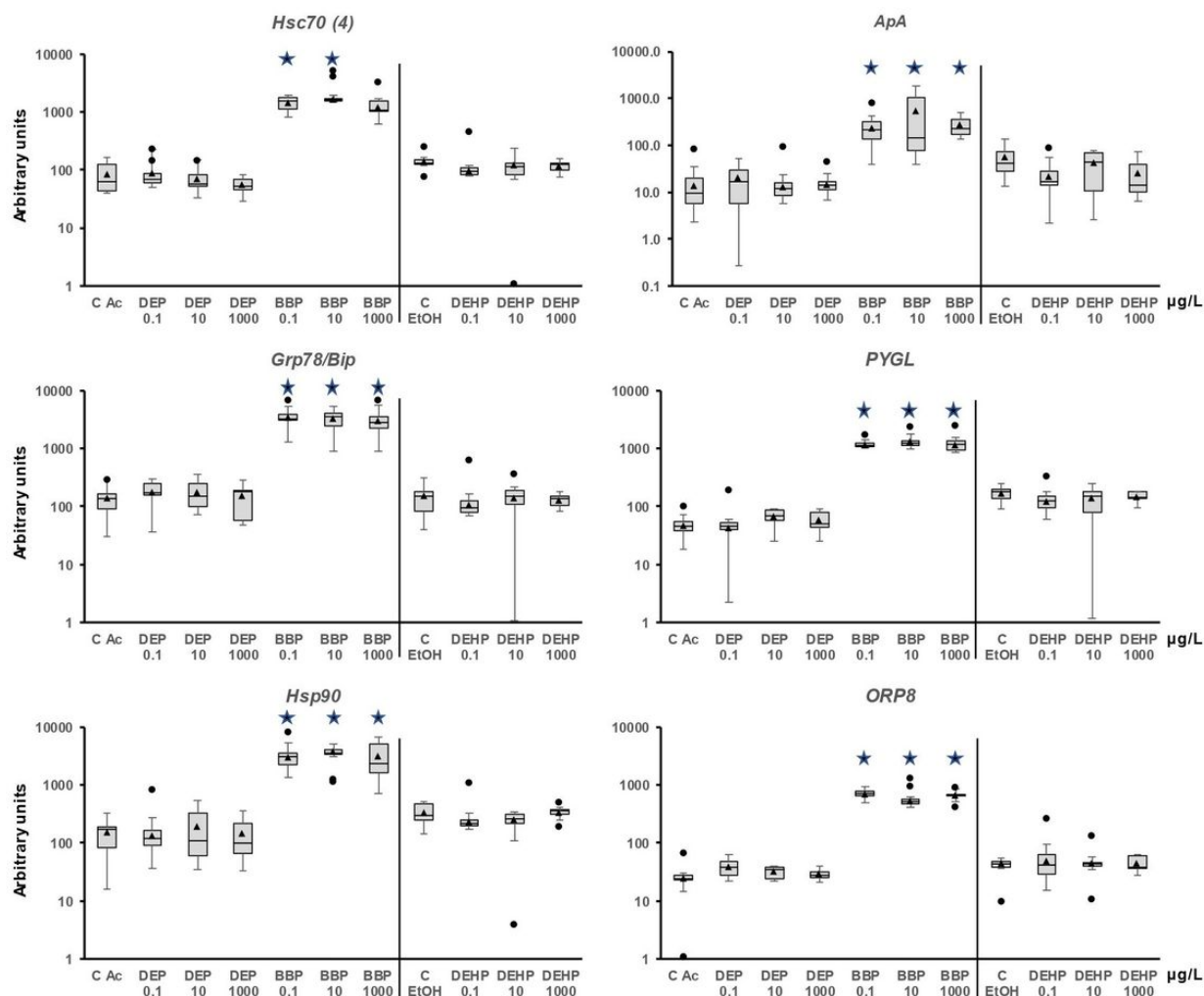
Figure 5

Figure 5

Response of the genes involved in epigenetic regulation (DNMT1, KAT6B, and HDAC1) and stress response (sHsp 16.6, sHsp 17.9, and Hsp60) in adult Physella acuta after in vivo exposure to the phthalates DEP, BBP, and DEHP at 19 °C for seven days. Normalization was performed by using rpL10, Act, PFKFB2, and GAPDH as reference genes. Treated animals were compared to solvent-exposed controls. Whisker boxes are shown. Each box corresponds to nine individuals. The horizontal line within the box indicates the median, and the box's boundaries indicate the 25th and 75th percentiles. The whiskers represent the highest and lowest results. The small triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences to respective controls (asterisk) are indicated (p < 0.05).

Figure 6

Figure 6

Modulation of genes related to stress (Hsc70 (4), Grp78/BiP, and Hsp90), immune system (ApA), energy metabolism (PYGL), and lipid transportation (ORP8) in adult Physella acuta after in vivo exposure to DEP, BBP, and DEHP at 19 °C for seven days. Levels of mRNA were normalized using rpL10, Act, PFKFB2, and GAPDH as reference genes. Treatments were compared to respective solvent-exposed controls. Whisker boxes are shown. The n for each box is nine. The median is indicated by the horizontal line within the box, and the 25th and 75th percentiles are indicated by the boundaries of the box. The highest and lowest results are represented by the whiskers. The small triangle inside the box denotes the mean and the outliers are shown (circles). Significant differences to respective controls (asterisk) are indicated ($p < 0.05$).

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