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Ecotoxicological assessment of phthalates in the freshwater gastropod *Physella acuta* and the sea urchin *Paracentrotus lividus*

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A mis padres, a Néstor y a Mirek

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Abbreviations

°C: Degrees Celsius

AChE: Acetylcholinesterase

AIF: Apoptosis-inducing factor

ANOVA: Analysis of variance

BBP: Benzyl butyl phthalate

bp: base pair

C. riparius: *Chironomus riparius*

CAS: Chemical Abstracts Service

CDD: Conserved Domains Database

CMR: Carcinogenic, Mutagenic or toxic for Reproduction

DBP: Dibutyl phthalate

DEHP: Bis(2-ethylhexyl) phthalate

DEP: Diethyl phthalate

DEPC: Diethylpyrocarbonate

DiBP: Di-isobutyl phthalate

DiDP: Di-isodecyl phthalate

DiNP: Di-isononyl phthalate

DNA: Deoxyribonucleic acid

cDNA: complementary DNA

DnBP: Di-n-butyl phthalate

DnOP: Di-n-octyl phthalate

dNTPs: Deoxyribonucleotide triphosphates

DTT: Dithiothreitol

EC_{50}: Median Effective Concentration

EC: European Commission

ECB: European Chemicals Bureau

ECHA: European Chemicals Agency

ECVAM: European Centre for the Validation of Alternative Methods

EDC: Endocrine Disrupting Chemical

EDs: Endocrine Disruptors

EDTA: Ethylenediaminetetraacetic acid

ER: Estrogen receptor

EtOH: Ethanol

GST: Glutathione S-transferase

HSP: Heat Shock Protein

sHSP: small Heat Shock Protein

hpf: hours post fertilization

LOEC: Lowest Observed Effect Concentration

M-MLV RT: Moloney Murine Leukemia Virus Reverse Transcriptase

MRP: Multidrug Resistance Protein

OECD: Organisation for Economic Co-operation and Development

P. acuta: *Physa acuta* or *Physella acuta*

P. lividus: *Paracentrotus lividus*

PCR: Polymerase chain reaction

PVC: Polyvinyl chloride

REACH: Registration, Evaluation, Authorization and Restriction of Chemicals

RNA: Ribonucleic acid

RT-PCR: Reverse transcription PCR

SSA: Swimming Speed Alteration

SOD: Superoxide dismutase

TBE: Tris-borate-EDTA

TBT: Tributyltin

UE: European Union

UNED: Universidad Nacional de Educación a Distancia

US EPA: U.S Environmental Protection Agency

ABSTRACT

1. ABSTRACT

Phthalates are a group of synthetic chemicals with a broad spectrum of uses, mainly employed as plasticizers in polyvinyl chloride production. Thus, these compounds are found in hundreds of food packaging, medical devices, paints, building materials, or cosmetics. Due to their massive production, phthalates are widely disseminated in the environment, including soil, air, sediment, and waters. Besides, its use has been restricted in certain products due to their potential endocrine-disrupting properties. However, studies on the effects of phthalates in invertebrates are scarce. This work assessed the impact of three phthalates in two aquatic invertebrates: *Physa acuta* and *Paracentrotus lividus*. The phthalates selected were butyl benzyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), and diethyl phthalate (DEP).

Juveniles and adults of the freshwater gastropod, *P. acuta*, were exposed to different exposure times and concentrations of BBP, DEHP, and DEP, and the changes in gene expression were analyzed. The genes studied are related to different physiological processes: endocrine system (*Hsp90*) and stress response (*sHsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4*, *Grp78*, *Cat*), oxidative stress response (*SOD Mn*, *SOD Cu/Zn*, and *HIF1α*), detoxification (*Cyp2u1, Cyp3a7, Cyp4f22, Cyp72a15, GSTk1, GSTm1, GSTo1, GSTt2,* and *MRP1*), DNA repair (*rad21* and *rad50*), apoptosis (*Casp3* and *AIF3*), epigenetics (*KAT6B*, *HDAC1*, and *DNMT1*), nervous system (*AChE*), immunity (*ApA*), energy reserves (*PYGL*), and lipid transport (*ORP8*). The GST enzymatic activity was also assessed in juveniles. Besides, the embryotoxic effects of BBP in *P. acuta* were evaluated.

The phthalates did not affect *P. acuta* survival, but BBP affected embryonic development. Regarding the gene expression analysis, the results show that while DEP and DEHP did not cause any alteration, BBP modulated almost all the analyzed genes after one-week exposure of *P. acuta* adults. However, after longer exposure times of juvenile individuals (15 and 30 days), BBP altered the expression of just three genes (*Hsp90*, *Hsc70-4*, and *GSTt2*).

Regarding *P. lividus,* an embryotoxicity test with the planktonic stages (embryos and larvae) was performed by exposing the embryos to several concentrations of BBP, DEHP, and DEP. Concentration-dependent morphological anomalies were observed in plutei formed after embryo exposure. Besides, their swimming speed was altered after exposure to the three phthalates.

This is the first work that evaluates the effects of the phthalates BBP, DEHP, and DEP at the transcriptional level of several genes in the mollusk *P. acuta* and the developmental and

behavioral effects on the early life stages of the sea urchin *P. lividus*, providing novel results on the differential effects of BBP, DEHP and DEP in the two species studied.

The results show that, unlike DEHP and DEP, BBP strongly alters different pathways involved in response to toxicants, like stress response and detoxification, DNA repair, apoptosis, epigenetic regulation, immunity, and energy metabolism, being toxic for *P. acuta* at the environmentally relevant concentrations used. It can be concluded that in *P. acuta*, the impact of BBP is extensive at the molecular level. Still, additional research is needed to elucidate the differences observed in the impact of these compounds on the gastropod. The developmental anomalies and swimming speed alterations of *P. lividus* early stages after exposure to BBP, DEHP, and DEP strengthen the known endocrine-disrupting character of this group of chemicals.

1. RESUMEN

Los ftalatos son un grupo de compuestos químicos sintéticos con un amplio espectro de aplicaciones, usados especialmente como plastificantes en la producción del policloruro de vinilo. Por ello, dichos compuestos se encuentran en cientos de envases alimentarios, dispositivos médicos, pinturas, materiales de construcción o cosméticos. Debido a su producción masiva, los ftalatos están ampliamente diseminados en el medioambiente, incluyendo suelos, aire, sedimentos y aguas. Además, se ha restringido su uso en ciertos productos, debido a sus potenciales propiedades como disruptores endocrinos. Sin embargo, los estudios acerca de los efectos de los ftalatos en invertebrados son escasos. Este trabajo ha evaluado el impacto de tres ftalatos en dos invertebrados acuáticos: *Physa acuta* y *Paracentrotus lividus.* Los ftalatos seleccionados fueron el butil benzil ftalato (BBP), el di(2-etilhexil) ftalato (DEHP) y el dietil ftalato (DEP).

Se expusieron individuos juveniles y adultos del gasterópodo de agua dulce, *P. acuta*, a distintos tiempos y concentraciones de BBP, DEHP y DEP y se analizaron los cambios en la expresión génica. Los genes estudiados están relacionados con distintos procesos fisiológicos: sistema endocrino (*Hsp90*) y respuesta al estrés celular (*sHsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4, Grp78* y *Cat*), respuesta al estrés oxidativo (*SOD Mn*, *SOD Cu/Zn* y *HIF1α*), detoxificación (*Cyp2u1*, *Cyp3a7*, *Cyp4f22*, *Cyp72a15*, *GSTk1*, *GSTm1*, *GSTo1*, *GSTt2* y *MRP1*), reparación del ADN (*rad21* y *rad50*), apoptosis (*Casp3* y *AIF3*), epigenética (*KAT6B*, *HDAC1* y *DNMT1)*, sistema nervioso (*AChE*), sistema inmune (*ApA*), reservas energéticas (*PYGL*) y transporte lipídico (*ORP8*). En los individuos juveniles, también se evaluó la actividad enzimática de la GST. Asimismo, se estudiaron los efectos embriotóxicos del BBP en *P. acuta.*

Los ftalatos no afectaron a la supervivencia de *P. acuta*, pero el BBP afectó al desarrollo embrionario. Respecto al análisis de la expresión génica, los resultados muestran que, mientras que DEHP y DEP no causaron ninguna alteración, el BBP moduló prácticamente todos los genes estudiados tras una semana de exposición de adultos de *P. acuta*. Sin embargo, tras la exposición de individuos juveniles a tiempos más largos (15 y 30 días), el BBP solo alteró la expresión de tres genes (*Hsp90*, *Hsc70-4* y *GSTt2*).

Con respecto a *P. lividus*, se llevó a cabo un ensayo de embriotoxicidad con los estadios planctónicos (embriones y larvas), exponiendo a los embriones a varias concentraciones de BBP, DEHP y DEP. En las larvas pluteus formadas tras la exposición de los embriones, se observaron

anomalías morfológicas dependientes de la concentración. Por otra parte, su velocidad natatoria se alteró tras la exposición a los tres ftalatos.

Este es el primer estudio que evalúa los efectos del BBP, DEHP y DEP a nivel transcripcional de distintos genes en el molusco *P. acuta* y los efectos en el desarrollo y comportamiento en los estadios tempranos del erizo de mar *P. lividus*, aportando resultados novedosos sobre los efectos diferenciales del BBP, DEHP y DEP en las dos especies estudiadas.

Los resultados obtenidos muestran que, a diferencia del DEHP y DEP, el BBP altera de forma significativa distintas vías involucradas en la respuesta a los tóxicos, como la respuesta al estrés y detoxificación, la reparación del ADN, apoptosis, regulación epigenética, inmunidad y metabolismo energético, resultando tóxico para *P. acuta* a las concentraciones ambientales usadas. Se puede concluir que el impacto del BBP a nivel molecular es extenso. Aun así, se deben realizar estudios adicionales que permitan dilucidar las diferencias observadas en el impacto de estos compuestos en *P. acuta*. Las anomalías en el desarrollo y las alteraciones en la actividad natatoria en los estadios tempranos de *P. lividus* tras la exposición a BBP, DEHP y DEP refuerzan el papel de este grupo de compuestos como disruptores endocrinos.

INTRODUCTION

2. INTRODUCTION

2.1. Environmental pollution

The overall growth in the global population and the industrial revolution during the last decades has raised the use and production of chemical products, which are widely disseminated in the environment. This systematic pollution of the environment poses a worldwide problem, being one of the main hazards that humanity faces nowadays since good water quality is essential for human well-being (OECD, 2017).

In recent years, water pollution prevention has gained increasing attention in OECD countries. The OECD Council Recommendation on Water calls for members to prevent, reduce and manage water pollution from all sources (OECD, 2016). Hence, the biodiversity of freshwater ecosystems has been strongly degraded. According to the Living Planet Index (LPI), it has declined overall by 81% between 1970 and 2012 due to pollution, overexploitation, and alteration of water bodies (WWF, 2016).

The discharge of pollutants into the aquatic environments is the outcome of countless anthropogenic activities, like the discharge of untreated municipal, industrial, and agricultural wastewaters directly into water bodies. The production, use, and trade of chemicals grow globally, and production is expected to double by 2030. Sectors like construction, automotive, and electronics are also growing, increasing the demand for chemicals (EC, 2020). Thus, aquatic ecosystems are susceptible, being the ultimate sinks for these pollutants. Besides, water bodies serve as transport pathways for contaminants, which reach other environmental compartments way from the source. Once in the water, pollutants become part of a cycle involving water bodies, sediments, and organisms. Many of these compounds can persist in the environment for decades, causing direct effects on the organisms or bioaccumulating in the food chain. Their presence in the aquatic environment has the potential to pollute drinking water resources and food supplies, damaging livelihoods dependent on the aquatic environment.

One of the types of toxicants that are gaining importance are phthalates, which are present in many plastic compounds. The growing plastic pollution can increase the presence of these toxicants in the environment as they are released during the plastic degradation process. With regard to the toxicity of these compounds, the main concern is their impact on organisms, since they are considered endocrine disruptors.

2.2. Phthalates

Phthalates, which are esters of phthalic acid, are a group of artificial chemical compounds synthesized with a broad spectrum of uses in industry, medicine, and personal care products. Phthalates are mainly used as plasticizers in polyvinyl chloride (PVC) production. They are found in hundreds of products such as food packaging, blood bags, and tubing, toys, paints, building materials, or cosmetics.

Due to their massive production, these compounds are widespread in different environments, including soil, air, sediment, and waters. Phthalates are not chemically bonded to the plastics when used as plasticizers, tending to migrate from the plastics and become a ubiquitous environmental pollutant (Herrero et al., 2015). For these reasons, humans and wildlife can be exposed to phthalates through ingestion, inhalation, or dermal absorption. On exposure, phthalates are generally rapidly metabolized and excreted in urine and feces (Hauser and Calafat, 2005).

Among others, in this group of chemicals we can find butyl benzyl phthalate (BBP), di (2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), dibutyl phthalate (DBP), di-isobutyl phthalate (DIBP), di-n-octyl phthalate (DnOP) di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP) and di-n-butyl phthalate (DnBP).

Benzyl butyl phthalate (BBP) and bis(2-ethylhexyl) phthalate (DEHP) are some of the phthalates which were identified as substances of very high concern (SVHCs) due to their endocrine-disrupting properties, with effects on human and wildlife health by the European Chemicals Agency (ECHA) in 2008 (ECHA, 2008a). As well as carcinogenic, mutagenic, or toxic to reproduction (CMR) according to REACH regulation. For this reason, the use of these two compounds is banned in toys and childcare articles in the EU.

2.3. Phthalates as endocrine disruptors

Endocrine-disrupting chemicals (EDCs) are exogenous chemicals in the environment, food, and consumer products that can interfere with the hormonal system and cause adverse health effects on organisms or their offspring. Exposure to these compounds can increase the risk of suffering various cancers (Sifakis et al., 2017; Giulivo et al., 2016), metabolic disorders (Cano-Sancho et al., 2017), and reproductive impairment (Johansson et al., 2017; Skakkebaek et al., 2016).

The mechanisms of actions of the EDCs can be:

- Mimicking the action of endogenous hormones and binding with endocrine receptors (agonistic effect)
- Binding to a receptor and blocking the endogenous hormone from binding (antagonistic effect)
- Altering the production, metabolism, transport, or secretion of natural hormones
- Altering hormone levels by interfering with endogenous hormone synthesis and degradation
- Modifying the gene transcriptional activity

The number of chemicals potentially able to alter the endocrine system has been increasing during the last years as studies analyzing different compounds are classifying new substances with this capacity. Currently, 564 chemicals have been suggested by different organizations and studies as suspected EDs (EC, 2000).

EDCs pose a severe threat to humans, animals, and ecosystems, being a very heterogeneous group with natural and synthetic origins. Natural hormones and phytoestrogens produced by some species can have a disruptive endocrine effect on other species. EDCs that are manufactured have multiple uses, including pharmaceuticals (hormonal contraceptives), pesticides, cosmetics, food additives, plastics, and plasticizers (phthalates), or industrial chemicals (PBC, Polychlorinated Biphenyls).

Humans are constantly exposed to these chemicals through the diet, skin, air, and water because of their wide distribution in the environment and their presence in plenty of products, such as plastics, personal care products, or pesticides.

Aquatic ecosystems are heavily affected by contamination of EDCs; sewage treatment plants represent the primary source of this pollution, as well livestock activity and industry, making the aquatic organisms inhabiting these ecosystems particularly vulnerable.

The hazardous effects of EDCs in aquatic invertebrates are well documented. For example, tributyltin (TBT), which is already banned and was used as a biocide in antifouling paints, has been shown to cause imposex in different marine gastropods, like *Nucella lapillus* and *Ocenebra erinacea* (Bettin et al., 1996; Gibbs et al., 1990), which leads to female infertility and may result in a population decline. Some UV filters' endocrine-disrupting potential has been reported, like the organic UV-filter BP3, which strongly compromised the fertility of the freshwater

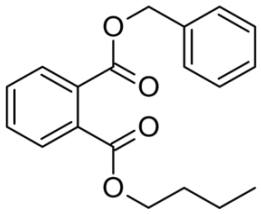
invertebrate, *Chironomus riparius,* altering the emergence rates and developmental times (Campos et al., 2019).

2.4. Benzyl Butyl Phthalate

Benzyl Butyl Phthalate (BBP) is a phthalate that appears as a clear, colorless liquid with a mild odor. In the EU, this phthalate is mainly used as a plasticizer for PVC production, especially for PVC for flooring, is also formulated as a component in paints, adhesives, or printing inks.

As previously mentioned, currently, BBP is not allowed in toys and childcare articles as it has been classified as an endocrine disruptor (ED), toxic for reproduction (CMR), Category 2, and as a Substance of very high concern (SVHC) (REACH Annex XIV). This substance may damage fertility and unborn child and is very toxic to aquatic life, causing long-lasting effects in the aquatic environment, according to ECHA.

Table 1. BBP physicochemical properties. Source: PubChem.

Benzyl Butyl Phthalate		
Synonyms	butyl benzyl phthalate, palatinol BB	
Abbreviation	BBP	
Molecular formula	$C_{19}H_{20}O_4$	
CAS N.	85-68-7	
Molecular weight (g/mol)	312.36	
Density (g/cm^3)	1.12	
Purity of commercial product	≥ 98.5%	
Solubility in water	2.7 mg/L	

The major releases are to air and wastewater. Release to the environment can occur during its manufacturing and the use of final products, with the washing of flooring as the largest single source, as BBP is not chemically bound in either preparations or articles containing it (ECHA, 2009; ECB 2007).

A recent study found different phthalates in the Ganga River; in the case of benzyl butyl phthalate, the concentrations ranged from ND (Non detected) to 0.13 µg/L (Chakraborty et al., 2021). The highest concentrations found on surface waters from different locations were 84 µg/L in Canada, 66 µg/L in the USA, and 13.9 µg/L in Europe. The highest concentrations of BBP measured in various wastewaters were 84 µg/L in Canada, 449 µg/L in the USA, and 30 µg/L in Europe, while the mean concentrations at the same locations were 3.05 µg/L, 299 µg/L, and 0.76 µg/L, respectively (Clark et al., 2003).

Several studies have proven the toxicity of this compound in fish. In zebrafish embryos (*Danio rerio*), BBP caused defects in caudal tail development (Roy et al., 2017). It also altered the expression of two genes related to heart development and caused growth inhibition (Sun & Liu, 2017). In the fish *Gasterosteus aculeatus,* BBP altered the feeding behavior and the shoaling and bottom-dwelling behavior (Wibe et al., 2002, 2004).

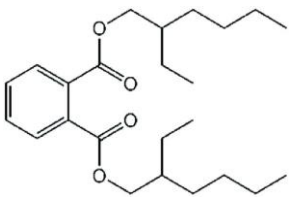
In the case of invertebrates, there are few scientific studies regarding the toxicity of BBP. An alteration of hormone-related genes in the insect *Chironomus riparius* and the expression inhibition of the vitellogenin gene in *Daphnia magna* have proven BBP an endocrine disruptor (Herrero et al., 2015; Li et al., 2021). In the marine gastropod *Haliotis diversicolor supertexta,* it caused embryonic abnormality and affected the larval stage (Liu et al., 2009). In the giant freshwater prawn *Macrobrachium rosenbergii* BBP significantly decreased phenoloxidase activity and expression of superoxide/reductase (Sung et al., 2003).

2.5 Bis(2-ethylhexyl) phthalate

Bis(2-ethylhexyl) phthalate (DEHP) appears as a colorless oily liquid with almost no odor and low solubility in water. It has been one of the most widely used plasticizers in medical devices such as blood bags, tubing, and catheters (ECB, 2008). In the EU, more than 95% of the total use of DEHP is as a plasticizer in polymer products, mainly in flexible PVC (ECHA, 2008).

Like BBP, Bis(2-ethylhexyl) phthalate is not allowed in toys and childcare articles as it has been classified as an endocrine disruptor (ED), toxic for reproduction (CMR), Category 2, and as a Substance of very high concern (SVHC) (REACH Annex XIV). This substance may damage fertility or the unborn child and is very toxic to aquatic life, according to ECHA.

Table 2. DEHP physicochemical properties. Source: PubChem.

Bis(2-ethylhexyl) phthalate		
Synonyms	Di(2-ethylhexyl) phthalate, Diethylhexyl phthalate	
Abbreviation	DEHP	
Molecular formula	$C_{24}H_{38}O_4$	
CAS N.	117-81-7	
Molecular weight (g/mol)	390.56	
Density (g/cm^3)	0.98	
Purity of commercial product	≥ 99.7%	
Solubility in water	0.27 mg/L	

The major releases are to soil and wastewater. It can occur during its manufacturing as well as during the use of end products such as the washing of flooring, releases from underground cables or pieces lost in the environment, as DEHP is not chemically bound in either preparations or articles, the potential for release and subsequent exposure is high (ECHA, 2009).

Recently DEHP was found at concentrations ranging from 0.11 to 6.3 µg/L and from ND to 0.007 µg/mL in Ganga River and two Nigerian rivers, respectively (Chakraborty et al., 2021; Ogunwole et al., 2021), while in surface water of various German rivers it was found ranging from 0.33 to 97.8 µg/L (Fromme et al., 2002).

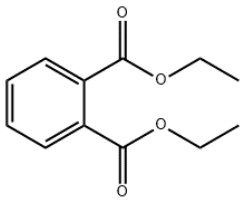
In Canadian surface waters, the highest concentrations measured of DEHP were 336 µg/L (for surface water with substantial industrial sources); in the USA and Europe, the highest concentrations found were 137 µg/L and 50 µg/L. In the case of wastewaters, the highest concentrations measured were 336 µg/L, 4400 µg/L, and 1800 µg/L in Canada, the USA, and Europe, respectively, while the mean concentrations at the same locations were 5.72 µg/L, 27 µg/L, and 34.4 µg/L (Clark et al., 2003).

It has been proved that DEHP significantly reduced body length in a dose-dependent manner in guppy fish, *Poecilia reticulata*, affecting the development in fish (Zanotelly et al., 2010) and the capacity of these compounds to alter spermatogenesis and affect reproduction in zebrafish at environmentally relevant concentrations (Corradetti et al., 2013). In the marine medaka fish (*Oryzias melastigma*), the exposure to DEHP from hatching to adulthood accelerated the spawning start time. It also decreased the fecundity of exposed females paired with unexposed males (Ye et al., 2014).

When it comes to invertebrates, in the clam *Venerupis philippinarum*, DEHP altered gene expression in immune-related genes (Lu et al., 2013). In other invertebrates such as *Chironomus riparius*, *Drosophila melanogaster*, and *Caenorhabditis elegans* DEHP has been proved to alter endocrine pathways (Herrero et al., 2017; Chen et al., 2019; How et al., 2019). In the nematode *Caenorhabditis elegans*, exposure to environmentally relevant concentrations of DEHP altered the expression of genes involved in DNA repair during meiosis (Cuenca et al., 2020).

2.6. Diethyl phthalate

Table 3. DEP physicochemical properties. Source: PubChem.

Diethyl phthalate		
Synonyms	Phthalic acid diethyl ester	
Abbreviation	DEP	
Molecular formula	$C_{12}H_{14}O_4$	
CAS N.	84-66-2	
Molecular weight (g/mol)	222.24	
Density (g/cm^3)	1.12	
Purity of commercial product	≥ 99.7%	
Solubility in water	1,08 mg/L	

Diethyl phthalate (DEP) appears as a clear colorless liquid and practically odorless. Besides its use as a plasticizer for manufacturing plastic products such as food packaging, this phthalate is mainly used as a solvent and softener in fragrances and, therefore, is common as an ingredient in scented mixtures and articles, such as cleaning and washing products, having a wide dispersive use (ECHA, 2015).

DEP is suspected of being CMR and ED and is currently under assessment by ECHA to evaluate its potential endocrine-disrupting character. The release to the environment of this substance can occur during its manufacturing and industrial use and the use of final products such as perfumes and other cosmetics.

Like DEHP, DEP has been recently found at concentrations from 0.04 to 2.14 µg/L in the Ganga River and from ND to 0.00003 µg/mL in two Nigerian rivers (Chakraborty et al., 2021; Ogunwole et al., 2021). The maximum concentrations of this compound found on surface waters from different locations were 55 µg/L in Canada, 55 µg/L in the USA, and 4 µg/L in Europe. For wastewaters, the maximum concentration of 1220 µg/L was reported in the USA, which was more than an order of magnitude higher than the ones in Canada and Europe; 55 µg/L 76.9 µg/L, while the mean concentrations at same locations were much lower and comparable, being 3.04 µg/L in Canada, 6.24 µg/L in the USA and 4.73 µg/L in Europe (Clark et al., 2003).

In the freshwater fish *Cirrhinus mrigala,* brain AChE activity was significantly decreased in DEP-treated fish compared to control (Ghorpade et al., 2002). In zebrafish embryos, the exposure to DEP lead to the induction of antioxidant enzyme activities (Xu et al., 2013).

Researchers have previously described that DEP can alter the physiology of invertebrates. In the mosquito, *Chironomus circumdatus* DEP upregulated the gene expression of *hsp70* and *EcR*

(Shaha et al., 2020). In the nematode *Caenorhabditis elegans,* alterations of genes involved in lipid metabolism and stress response were observed (Pradhan et al., 2018).

It is worth mentioning that in the abalone *Haliotis diversicolor supertexta,* DEP displayed higher toxicity than other phthalates tested, such as DEHP, reducing embryo hatchability and increasing developmental malformations (Zhou et al., 2011), serving as evidence of its potential endocrine-disrupting character.

2.7. Toxicological assays with aquatic invertebrates

Benthic fauna has gained relevance regarding aquatic pollution, as they can significantly impact the fate and mobility of pollutants. Contaminants associated with sediment often enter the food chain through these organisms, which serve as a food source for higher trophic levels. Therefore, to study the effects of different pollutants in these organisms must be addressed. The toxicity tests are a helpful tool that allows assessing the risk that chemicals can pose for the environment.

In recent years invertebrates have become one of the most used organisms in monitoring aquatic environments and in ecotoxicological assays; due to their widespread distribution and abundance, their short life cycles compared to other organisms such as fish, which reduce the time to obtain results, as well as the ease to culture and maintain them under laboratory conditions and their sensitivity to a wide range of toxicants. Besides, the lack of bioethical implications makes invertebrates an excellent alternative to vertebrate testing. Moreover, invertebrates play a crucial ecological role in the trophic chain, both in terrestrial and aquatic ecosystems, as they constitute an essential food source for the higher trophic levels. For all the reasons mentioned above, invertebrates serve as ideal organisms for ecotoxicological assays.

The OECD Test Guidelines for Chemicals assess the potential effects of chemicals on different organisms and the environment. Despite no guidelines designed for *Physa acuta*, there are assays for two freshwater gastropods: *Potamorpyrgus antipodorum* and *Lymnaea stagnalis* (a hermaphrodite snail very similar to *Physa acuta*) that can be applied, where parameters such as mortality-survival rates, reproduction, and development are analyzed. Similarly, there are no guidelines designed for the sea urchin *Paracentrotus lividus* either, but sea urchins are used by international agencies for toxicity studies and monitoring coastal sea environments (ASTM, 2004; US EPA, 2002). Besides, different laboratories have proposed these organisms as good alternative models for the standardization of ecotoxicological tests (Buznikov et al., 2001; Qiao et al., 2003; Aluigi et al., 2008). In this study, *Physa acuta* embryos, juveniles, and adults,

Paracentrotus lividus embryos, and plutei (larval stage) were used to assess the effects of BBP, DEHP, and DEP through different molecular and physiological methods.

2.7.1. *Physa acuta* and *Paracentrotus lividus* in ecotoxicological assays

Freshwater snails have a ubiquitous distribution and are an essential component of aquatic ecosystems worldwide. They constitute a vital energy source for different consumers, such as birds and fish, being a vital part of the food chain (Gondal et al., 2020). Moreover, these organisms are known to be sensitive to several EDCs and other substances (Morales et al., 2018; Das & Khangarot, 2011). For all the previously mentioned reasons, it has been concluded that there is a need for mollusk-based lifecycle tests, from what the OECD has included among their standardized bioassays with aquatic organisms, two guides with the freshwater snails: *P. antipodorum* and *L. stagnalis* (OECD, 2016a; OECD, 2016b).

Physella acuta (*Physa acuta*) (Draparnaud, 1805) is a freshwater hermaphrodite gastropod native to North America. Currently occurs in all the continents except Antarctica (Ebbs et al., 2018), being among the most cosmopolitan freshwater snails. This is facilitated by its capacity of self-fertilization, although it prefers outcrossing (Jarne et al., 2000). Its life cycle is composed of 3 main stages, egg masses (embryos), juvenile stage, and adult stage. Under culture conditions, they reach sexual maturity (first oviposition) at eight weeks, approximately after hatching.

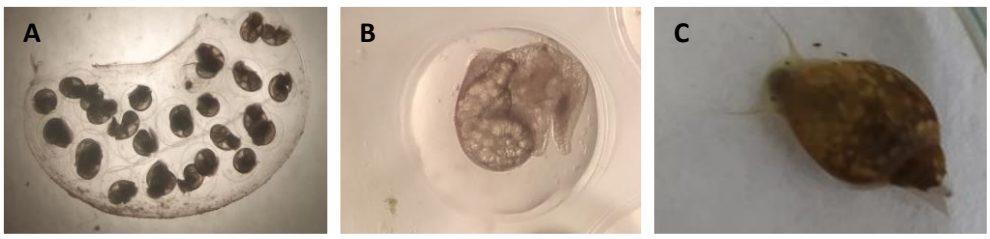


Figure 1. Different life stages of *Physa acuta.* (A) Egg mass. (B) Embryo at the 14-day stage. (C) Adult.

Echinoderms are key species in coastal environments. These organisms inhabit hard bottoms where they graze and prune the algae, like *Posidonia*, and therefore, can remodel the bottom. Besides, during the planktonic stages (embryo and larva), they constitute an essential food source for other organisms (Gambardella et al., 2016).

The purple sea urchin, *Paracentrotus lividus* (Lamarck, 1816), is frequently used as a model organism to assess shallow marine waters. This species is widely distributed around the

Mediterranean and North-Eastern Atlantic benthic littoral; areas strongly affected by human activities and pollution (Boudouresque & Verlaque, 2001). In fact, its early developmental stages are recommended by ECVAM protocols for testing the toxicity of chemical compounds (ECVAM, 2019), as they are susceptible to the presence of pollutants in the water.

Its life cycle comprises a planktonic stage (embryos and larva) and an adult benthic stage. Gametes are released into the water column, and eggs are fertilized. The embryos reach the larval stage, known as pluteus, after 48 hours, which grows and ultimately metamorphoses into adults (Morgana et al., 2016).

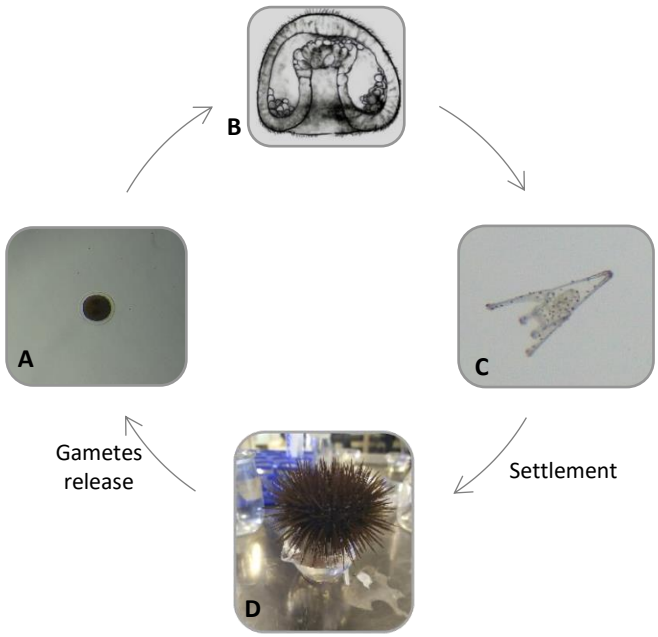


Figure 2. Different life cycle stages of *P. lividus*. (A) Fertilized egg. (B) Gastrula. (C) Pluteus. (D) Adult.

2.8. Molecular biomarkers

Over the last decades, biomarkers have become an essential tool in toxicology to assess the health of the organisms and the ecosystems, as biomarkers can help identify a substance's degree of toxicity and the mechanisms by which that substance causes toxic effects. Biomarkers can be defined as inner indicators in biological systems of measurable changes at the molecular, biochemical, cellular, physiological, or behavioral levels in response to xenobiotics (Gupta, 2019). Molecular biomarkers can serve as early-warning indicators after contaminant exposure, allowing remedial or preventative actions to be taken (Livingstone, 1993).

2.8.1. Endocrine system biomarkers

Recently, there has been a raised concern regarding the potential effect at an endocrine level caused by chemicals on aquatic invertebrates. These toxicants, such as plasticizers, cosmetics, or pesticides, usually enter the aquatic ecosystems through sewage treatment plants or agricultural and industrial runoff. The endocrine system regulates the development, growth, and reproduction processes in vertebrates and invertebrates. There is a wide variety when it comes to the invertebrate endocrine system, presenting distinct differences depending on the biological taxa, with characteristic events like the presence of one or more larval stages, followed in some groups by pupation, metamorphosis, or diapause, which do not occur on vertebrates (Jeon, 1996). Within invertebrates, the endocrine system of insects is the best characterized due to their economic and ecological importance, while less is known about other groups such as crustaceans and mollusk.

Insect hormones play a crucial role through the insect life cycle, regulating specific functions such as molting and metamorphosis. In holometabolous insects, the juvenile hormone (JH) and the ecdysone, known as the molting hormone, coordinate the hormonal regulation. Each stage is preceded by peaks of ecdysone release, which along with the JH, determine molting and metamorphosis (Berger et al., 1992; Tassou & Schulz, 2009).

One of the best-documented examples of the high sensitivity of mollusks to EDCs is the development of imposex or intersex, yet the mollusk endocrine system is poorly understood (Matthiessen, 2008). In vertebrates, estrogens are regulated through nuclear receptors such as estrogen receptor (ER), essential for the reproductive cycle and development. ER gene has been identified in several mollusks, including *Physa acuta*, like *Aplysia californica* or *Bithynia tentaculata* (Martínez-Paz., 2017; Thornton et al., 2003; Hultin et al., 2014). However, it is unknown whether these estrogen receptors play a role in reproduction regulation.

As in mollusks, there is limited knowledge on the endocrine physiology of echinoderm species, which are essential components of marine ecosystems. The synthesis of vertebrate-type sex steroids, progesterone, estrogens, and androgens by echinoderms has been reported in several studies (Wasson & Watts, 2013). Still, estrogen receptors have not yet been identified in echinoderms (Sugni et al., 2007).

2.8.2. Cellular stress response biomarkers

The response to cellular stress involves the induction of a set of proteins known as heat-shock proteins (HSP) that counteract cellular stress and its associated damage. This family of proteins is highly conserved and serves as an early bioindicator of possible environmental hazards, thanks to their high sensitivity to changes in cellular homeostasis (Morales et al., 2011). Besides heat stress, HSPs are expressed during other stressful conditions such as exposure to xenobiotics, hypoxia, etc. Under normal conditions, these proteins function as molecular chaperones and play an essential role in protein transport and folding. According to their molecular weight, sequence homology, and function, the HSPs are categorized into six major families, namely: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock proteins (sHSP).

Hsp70 family is one of the most conserved protein families in evolution and serves critical protein homeostasis roles. These proteins unfold misfolded or denatured proteins and control the proper folding of nascent proteins (Murphy, 2013). Its expression can be induced by stimuli other than thermal stress, including xenobiotic exposure, thus serving as biomarkers for exposure to toxicants. A member of this family is the Heat shock 70 kDa protein cognate 4 (Hsc70-4) is a constitutively expressed molecular chaperone that cooperates with co-chaperones to carry out multiple cellular functions such as promoting membrane protein folding and polypeptide translocation (Liu et al.,2012). Another member of the Hsp70 family is the Glucose-regulated protein 78k Da (Grp78), which is one of the initial components in the signaling cascade that results in the unfolded protein response (UPR) (Quinones, 2008). Hsp60 and its co-chaperone Hsp10 are located in the mitochondria, maintaining its integrity and function. Hsp90 is involved in activating and stabilizing a range of proteins, including steroid hormone receptors (Pearl, 2016). Besides its link to steroid receptors, Hsp90 is required for cellular stabilization under stress conditions (Holzbeierlein et al., 2010).

The small HSPs have a molecular weight between 10 and 30 kDa and constitute the most diverse family of HSPs. The sHSPs participate in different biological processes like cell differentiation, growth, apoptosis, etc. Under cellular stress, sHSPs act as molecular chaperones binding to denatured proteins (Tsvetkova et al., 2002; Martín-Folgar & Martínez-Guitarte, 2017).

2.8.3. Cellular detoxification system biomarkers

To prevent the possible toxic effects and damage that xenobiotics cause when entering an organism, cellular detoxification is triggered to favor the metabolism and/or elimination of the compound. The detoxification mechanisms are divided into three phases: I, II, and III.

The detoxification process involves a first phase to decrease xenobiotic toxicity. Some chemical reactions include oxidation, reduction, and hydrolysis reactions during this phase. Among the enzymes involved in phase I, the cytochrome P450 superfamily stands out. These enzymes have low specificity for the substrate, allowing them to metabolize a wide range of chemically different xenobiotics. The purpose of phase II is to perform a conjugation reaction with molecules such as glutathione (GSH) to increase the compound's solubility, easing its excretion. The glutathione S-transferases are the best-known phase II group of enzymes, which, apart from glutathione conjugation, are also involved in cellular protection against oxidative stress (Enayati et al., 2005). At last, the phase III transporters, primarily the ATP-binding cassette (ABC) proteins, transport the unwanted molecules outside the cell for excretion. The multidrug resistance proteins (MRPs) are a group of the ABC transporters (Trujillo et al., 2020).

Enzymatic activities are a frequent tool used to analyze xenobiotic toxicity. GST is an important part of the cellular detoxification system. Thus, the study of the expression analysis of different genes belonging to the three detoxification phases together, with the evaluation of the GST enzymatic activity, offers a more global perspective of the cellular detoxification response after exposure to xenobiotics.

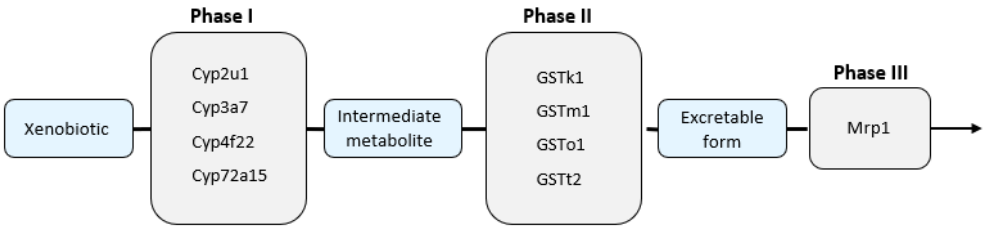


Figure 3. Detoxification pathway scheme.

2.8.4. DNA repair

Genotoxicity is one of the most critical parameters in environmental risk assessment and chemical analysis. The DNA is subject to constant harmful effects of different chemical and physical agents, leading to DNA damage. The most common DNA lesions are simple base modifications or mismatches, single and double-strand breaks (SSB and DSB, respectively), and DNA-protein cross-links.

To compensate for the different types of DNA damage, cells have developed multiple repair mechanisms such as base excision repair, mismatch repair, nucleotide excision repair, and double-strand break repair. Besides these repair mechanisms, different genes involved in response to DNA damage have been identified. Thus, the expression profile of these genes could help to clarify the mechanisms that cause this damage. In this study, the expression profile of several genes related to double-strand break repair and apoptosis has been analyzed.

Rad21 is a protein involved in repairing DNA double-strand breaks and is a structural component of the highly conserved cohesin complex, involved in chromatid cohesion during mitosis. *Rad50* codes for a protein involved in DNA double-strand break repair and forms a complex with MRE11 and NBS1 (MRN complex) (Watrin & Peters, 2006; Cheng et al., 2020). When DNA damage is too severe, cells may utilize apoptosis as a final security mechanism (Hoeijmakers, 2001); for this reason, two apoptosis-related genes have been studied, the executor caspase *Casp3* and the apoptosis-inducing factor 3 (*AIF3*).

2.9. Swimming behavior and morphological features as ecotoxicological sub-lethal endpoints

The study of behavioral parameters is a valuable tool to assess the effects of exposure to environmental stressors. Motility is an essential behavioral determinant for most aquatic organisms, and therefore, changes in locomotor behavior can be used as a stress indicator in ecotoxicological studies (Garaventa et al., 2010). Swimming response is increasingly used as a behavioral parameter to evaluate environmental pollutant effects. Various studies have proved its sensitivity in terms of swimming behavior to several stressors using different marine organisms such as fish (*Dicentrarchus labrax*), crustaceans (*Artemia sp*, *Amphibalanus amphitrite*), or sea urchin larvae (*Paracentrotus lividus*) (Gravato & Guilhermino, 2009; Mesarič et al., 2015; Morgana et al., 2016).

The study of morphological features in an organism during development is a valuable tool to assess the toxicity and impact of contaminants. Xenobiotics and other environmental stressors can affect the biological processes of organisms, like the sea urchin development, which may vary depending on the developmental stage which is exposed (gametes, embryos, or larvae) (Carballeira et al. 2012; Gambardella et al., 2021). These anomalies can ultimately affect the population viability, preventing further development and impairing reproductive success.

After assessing different pollutants, the sensitiveness of *P. lividus* developmental malformations has been proved, like chemicals, nanoparticles, heavy metals, or plastic leachates (Carballeira et al., 2012; Gambardella et al., 2013; Gharred et al., 2016; Oliviero et al., 2019).

The present study aims to analyze the impact of phthalates as contaminants of deep concern. Their growing presence in the environment and the scarce information about the damage produced on invertebrates demands a deeper analysis. In this sense, the study focuses on elucidating the putative mechanism involved in the response by combining the traditional toxicity test approach with molecular data. Furthermore, the analysis performed in freshwater and marine species tries to find putative similarities and differences in response to these chemicals.

OBJECTIVES

3. OBJECTIVES

This study aims to address the toxicity that three phthalates, BBP, DEHP, and DEP, have on two aquatic invertebrates, the gastropod *Physa acuta* and the sea urchin *Paracentrotus lividus.* The studies focus on the effects at the molecular and cellular levels, although other endpoints are assessed. The effects of this group of chemicals on invertebrates are scarce, especially at a molecular and cellular level. There is no previous data on the effects of phthalates in *P. acuta* and *P. lividus.*

Specific aims are:

- To investigate the effect of phthalates on *P. acuta* juveniles and adults at the molecular and cellular level by analyzing the gene expression profile for specific target genes involved in different pathways of interest and the enzymatic activity. The processes of interest include the stress response, oxidative stress, detoxification, DNA repair, apoptosis, epigenetic regulation, immunity, energy reserves, and lipid transport.

- To evaluate the behavioral effects through the Swimming Speed Alteration (SSA) and the larvae morphological anomalies in the sea urchin *P. lividus* upon exposure to phthalates.

- To assess the potential phthalate-induced embryotoxicity in *P. acuta* and *P. lividus.*

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Biological materials

4.1.1. *Physa acuta*

Physa acuta is a freshwater pulmonated gastropod. The snails used in this work were established from animals provided by the National Institute for Agricultural and Food Research and Technology (INIA) and maintained at the Biology and Environmental Toxicology Group laboratory of the *Universidad Nacional de Educación a Distancia* (UNED) under constant conditions during several generations. The cultures are maintained at a temperature-controlled camera of 19 ±1 °C with constant aeration and a light-dark period of 16:8 hours. There are no standardized test guidelines for this mollusk, but there are for similar freshwater gastropods such as *Lymnaea stagnalis* (OECD, 2016b). The cultures start by collecting the gelatinous egg masses and the subsequent culture in glass vessels with culture medium (see section 4.2 reactives). After hatching, the juvenile individuals are fed with commercial fish food Sera Micron® (Sera), which afterward is substituted for adult food; Sera Shrimp Natural (Tetramin). Once reached the adult stage, the organisms are moved to new aquariums for oviposition.

4.1.2. *Paracentrotus lividus*

Paracentrutus lividus is an echinoderm commonly known as purple sea urchin. The adult individuals of *P. lividus* used in this work were supplied from the Aquarium of Genova (Italy), maintained in 0.22 µm filtered seawater (FSW) at 18 ± 1 °C. Once collected, the mature specimens were induced to spawn, and gametes were collected for the embryotoxicity test. Once the gametes were obtained, the adult individuals were moved back to the aquarium tanks.

4.2. Reactives

TRIzol and M-MLV enzyme were obtained from Invitrogen (Germany), 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).

- Electrophoresis loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol

- *Physa acuta* culture medium: $CaCl_2$ 2 mM, $MgSO_4$ 0.5 mM, $NaHCO_3$ 0.77 mM, KCl 0.08 mM
- Protein extraction buffer: 10mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EGTA pH 8, 0.5 Mm DTT, 5% glycerol and 0.5 mM PMSF
- TBE Buffer: Tris-Borate 40 mM, EDTA 0.5 M

4.3. Chemical compounds

The chemical compounds used in the present study were three phthalates: benzyl butyl phthalate 98% (BBP), bis(2-ethylhexyl) phthalate 98% (DEHP), and diethyl phthalate 99.5% (DEP) (Sigma-Aldrich).

The three phthalates were selected due to their widespread presence in the aquatic environment and their proven toxic effects observed in different organisms. As mentioned above, two of these three compounds, BBP and DEHP, have been classified as ED and CMR, while DEP is under assessment by ECHA to evaluate its potential endocrine-disrupting character.

4.4. Toxicological assays with *Paracentrotus lividus*

The toxicity of DEP, DEHP, and BBP in the sea urchin *P. lividus* was evaluated using embryo and larval stages. Considering previous studies and the sensitivity for these early stages, the studied concentrations for BBP, DEHP, and DEP to assess the developmental anomalies were 0.01, 0.1, 1, 10, 100, 1000 µg/L. For the SSA test, the concentrations tested were 0.01, 0.1, 1, 10, 100 µg/L.

For stock solutions, the three compounds were diluted in acetone. The controls had a final solvent concentration of 0.1% and 0.01%, corresponding to the two highest solvent concentrations in the treatments (1000 and 100 µg/L, respectively). All experiments were carried out in triplicate.

Table 4. Concentrations used in *P. lividus* treatments

Compound	Concentrations	
BBP	0.01078 µg/L	0.0345 nM
	0.1078 µg/L	0.345 nM
	1.078 µg/L	3.45 nM
	10.78 µg/L	34.5 nM
	107.8 µg/L	345 nM
	1078.0 µg/L	3.45 µM

DEP	0.01114 µg/L	0.0501 nM
	0.1114 µg/L	0.501 nM
	1.114 µg/L	5.01 nM
	11.14 µg/L	50.1 nM
	111.4 µg/L	501 nM
	1114.0 µg/L	5.01 µM
DEHP	0.00965 µg/L	0.0247 nM
	0.0965 µg/L	0.247 nM
	0.965 µg/L	2.47 nM
	9.65 µg/L	24.7 nM
	96.5 µg/L	247 nM
	965.3 µg/L	2.47 µM

4.4.1. Embryotoxicity test

As previously mentioned, mature specimens of *P. lividus* provided by the Aquarium of Genoa were induced to spawn by administering 0.5 ml 0.5 M KCl solution into the coelomic cavity. Gametes were collected from three males and three females. While eggs were collected in 0.22 µm ultrafiltered seawater (FSW), sperm was collected and stored directly "dry" from the genital pores, as it has been proved that the motility of sea urchin spermatozoa is initiated within a brief period after contact with seawater. Samples were brought to the laboratory in a refrigerated bag, and sperm samples were maintained in aliquots of 200 µL at 4 °C. The FSW containing eggs was changed three times to remove any debris.

Sperms from the three different specimens were mixed, and 5 µl volume of sperm suspension was added for fertilization into a 300 ml egg suspension containing approximately 1000 eggs/ml in standard FSW. After successful fertilization (40 minutes), samples aliquots were observed with a microscope to evaluate if a good percentage (>80%) of eggs had successfully been fertilized (Beiras et al., 2003). The embryotoxicity test was performed in 6 multi-well plates. 1 ml of fertilized egg suspension was added to each well containing 9 mL of 6 different BBP, DEHP, and DEP concentrations (0.01, 0.1, 1, 10, 100, and 1000 µg/L). Controls had a final solvent concentration of 0.1% and 0.01%, corresponding to the two highest solvent concentrations in the treatment (1000 and 100 µg/L, respectively).

All plates were incubated for 72 hours in a dark thermostatic chamber at 18 ± 2 °C. All the experiments were carried out in quadruplicates. The anomalies were observed according to Gambardella et al. (2021) and quantified by means EC_{50}, which is the effective concentration resulting in 50% pluteus that present developmental anomalies exposed to phthalates.

4.4.1.1. Swimming Speed Alteration (SSA)

Swimming behavior was assessed as an ecotoxicological sub-lethal endpoint by evaluating the Swimming Speed Alteration (SSA). The swimming speed was measured using the automatic recording system SBR (Swimming Behavioral Recorder). The SBR system is a video camera-based system (Kenko, Japan) with a macro-objective, developed at CNR-IAS, that records the paths of a sample of swimming larvae in a small recording chamber. The apparatus is located inside a black box (60x 60x 100 cm) to block any external light sources (Faimali et al., 2006). The camera was manually focused on the sample, with a sample-camera distance of approximately 20 cm, and the volume of interest was illuminated from below using an infrared light source. Before video-recording, the sea urchin plutei were dark-adapted for 2 min. Swimming behavior was digitally recorded for 3 s at 25 frames/sec. To obtain the individual path-tracks and measure the average swimming speed (mm/sec) for each sample image was analyzed using advanced image analysis software (BIOMONITOR software developed by On Air srl, Genova, Italy).

After 72 hours of incubation, the swimming speed of *P. lividus* pluteus was recorded under dark conditions for 5 s by SBR and compared to controls. To record the swimming behavior, 1 mL per well of the different phthalates concentrations was added into 25 multi-well plates, each one containing approximately 15-20 pluteus. Organisms were considered motionless when the larvae did not change their barycenter position nor move any appendages in 5 s, as described in Morgana et al. (2016). The swimming speed of embryos and larvae was calculated in mm/sec.

4.4.1.2. Developmental anomalies

After 72 hours incubation period and recording SSA, approximately 100 larvae (pluteus) for each replicate were fixed with 4% paraformaldehyde. To analyze the morphological abnormalities, the state of the plutei, including the formation of skeletal rods, was checked under a Leica light microscope (DM3000B, Leica, Germany). The larvae were mounted on slides, and characteristics were compared among the different concentrations of the three phthalates and the controls. Effective Concentration resulting in 50% anomalies in the exposed organisms (EC_{50}) was calculated.

The classification was performed according to the specific anomalies identified and described by Carballeira et al. (2012) and Gambardella et al. (2013). The acceptability of the results was fixed at a percentage of normal development >80% in control tests.

4.5. Toxicological assays with *Physa acuta*

The toxicity of DEP, DEHP, and BBP was evaluated through different time exposures and life cycle stages of *P. acuta*, embryos, juveniles, and adults. Considering previous studies and the different sensitivities for each stage the studied concentrations of BBP were 0.01, 1 and 100 µg/L for embryos; 0.01, 1, 10, 100, 1000, 10000 µg/L for juveniles and 0.1, 10, 100, 1000, 10000 µg/L adults; for DEP and DEHP the studied concentrations were 0.1, 10, 100, 1000 and 10000 µg/L for adults.

Due to these xenobiotics' low solubility in water, the stock solutions were prepared in acetone for BBP and DEP. At the same time, DEHP was diluted in ethanol, always at a final concentration of 0.01%. Alongside the concentrations of the toxic studied, the controls also had a final solvent concentration of 0.01%. All the experiments were carried out under the same laboratory conditions, as detailed in section 4.1.1. Three replicates were performed for each treatment.

Table 5. Concentrations used in *P. acuta* treatments**.**

Compound	Concentration	
BBP	0.01078 µg/L	0.0345 nM
	0.1078 µg/L	0.345 nM
	1.078 µg/L	3.45 nM
	10.78 µg/L	34.5 nM
	107.8 µg/L	345 nM
	1078.0 µg/L	3.45 µM
	10780 µg/L	34.5 µM
DEP	0.1114 µg/L	0.501 nM
	11.14 µg/L	50.1 nM
	111.4 µg/L	501 nM
	1114.0 µg/L	5.01 µM
	11140 µg/L	50.1 µM
DEHP	0.0965 µg/L	0.247 nM
	9.65 µg/L	24.7 nM
	96.5 µg/L	247 nM
	965.3 µg/L	2.47 µM
	9654 µg/L	24.7 µM

4.5.1. Survival analysis

A survival test was performed to study the lethal effects of the selected xenobiotics. Juvenile individuals of *P. acuta* were exposed to BBP, and adult individuals were exposed to BBP, DEHP, and DEP for 96 hours at 10, 100, 1000, 10000 µg/L. Death organisms were counted every 24 hours. Three independent experiments were performed with 30 individuals for each studied concentration in a 200 mL culture medium final volume. The medium was renewed after 48 hours.

4.5.2. Embryotoxicity test

An embryotoxicity test was performed to assess the hatching percentages of offspring after exposing the embryos to 0.01, 1, and 100 µg/L of BBP at 19 °C from the moment of the oviposition until hatching. The experiment was performed for 21 days with constant aeration and a light-dark period of 16:8 hours.

Egg masses were immediately harvested after oviposition and divided into two halves, one used as control and another for the corresponding treatment. The egg masses were placed individually in a petri dish with 12 mL of culture medium, renewed twice a week.

4.5.2.1. Hatching success

The egg masses were monitored twice a week, and the number of eggs inside each egg mass was counted with a stereomicroscope (Olympus SZX12). The hatching percentage was calculated through direct observation comparing the total number of hatched individuals with the total number of embryos. The tests were performed in triplicates per treatment.

4.5.3. Gene expression analysis

Three experiments were performed in which juvenile and adult individuals were exposed to different concentrations of BBP, DEHP, and DEP. Once exposures ended, each animal was individually frozen and stored at -80 °C in separate tubes for RNA extraction.

The adult individuals were exposed to several concentrations of BBP, DEHP, and DEP for one week in glass vessels with a final volume of 300 mL culture medium. Each compound was tested at three concentrations; 0.1, 10, and 1000 µg/L, and three animals were exposed for each replicate (n=9). The culture medium with the corresponding concentration was renewed after

three days, and the animals were fed with 20 mg Sera Shrimp Natural (Tetramin, Germany) per recipient.

The juveniles were exposed for 15 days and one month to three different concentrations of BBP; 0.01, 1, and 100 µg/L in glass vessels with a final volume of 200 mL culture medium, and three animals were exposed for each concentration (n=9). The medium was renewed every three days, and the animals were fed twice a week with 8, 10, 12, and 14 mg per recipient Sera Micron® (Sera), along the first, second, third, and fourth week respectively.

Table 6. Exposure conditions summary for each experiment**.**

Exp	Time (days)	Compound	Concentrations (µg/L)	Life cycle stage
1	7	BBP, DEHP, and DEP	0.1, 10 and 1000	Adult
2	15	BBP	0.01, 1 and 100	Juvenile
3	30	BBP	0.01, 1 and 100	Juvenile

4.6. Molecular methods

4.6.1. RNA extraction

Once frozen at -80 °C degrees, the snails, including the shell, were homogenized in 200 µL TRIzol (Invitrogen, Germany) following the manufacturer's instructions and were immediately frozen in dry ice. Afterward, samples were let unfreeze, and 40 µL of chloroform were added. After mixing the samples by inversion for 15 seconds followed by 3 minutes incubation at room temperature, samples were centrifuged at 10,000 g for 15 min at 4 °C. The remaining upper aqueous phase, which contains the RNA, was recovered. Subsequently, 140 µL of 2-propanol were added to precipitate the RNA and maintained at room temperature for 10 minutes. The samples were centrifuged again at 10,000 g for 15 min at 4 °C. The supernatant was discarded, and RNA pellets were then washed with one volume of ethanol 75% and centrifuged at 10,000 g for 5 min at 4 °C. The RNA pellets were resuspended in diethylpyrocarbonate (DEPC) treated water.

Once the RNA was isolated, to remove the possible rest of DNA, it was treated with 1 µL RNAse-free DNAse I, 90 µL DEPC water, and 10 µL of 10X DNAase buffer for 45 min at 37 °C. Afterward, a phenol: chloroform extraction was performed with Phase Lock tubes (5 prime, USA) to remove any DNAse remaining. The RNA was precipitated and resuspended in 100 µL of DEPC treated water. The samples were centrifuged at 10,000 g for 5 min at 4 °C. Finally, the RNA was

precipitated with one volume of 2-propanolol and washed with ethanol 75%. RNA was resuspended in 40 µL DEPC water and stored at -80 °C for later use.

The RNA quality was analyzed by agarose gel electrophoresis at 1.5% dissolved in TBE buffer. Gels were dyed with 0.5 µg/m ethidium bromide (Sigma-Aldrich) and visualized using the Chemigenius 3 Imaging System (SynGene). The RNA extracted from each sample was quantified by spectrophotometry (BioPhotometer® 6131 Eppendorf). Optical density values 260/280 between 1.80-2.00 and DO values 260/230 equal to or higher than 1.80 were taken as reference.

4.6.2. Reverse transcription

The retrotranscription of RNA to complementary DNA was performed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme. This enzyme is an RNA-dependent DNA polymerase that allows cDNA synthesis with long messenger RNA (mRNA) templates which can then be used as a template for PCR.

For each sample, a mixture of 5 µg RNA, 1 µL oligonucleotide (poliT), 1 µL 10mM deoxynucleotides triphosphate (dNTPs) and DEPC treated water until 40 µL was used and incubated for 5 minutes at 65 °C and immediately afterward kept in ice. Then, 4 µL 5x First-Strand Buffer, 2 µL DTT 10 mM, and 1 µL M-MLV enzyme were added. The samples were incubated for 50 minutes at 37 °C, followed by a thermal shock for 15 minutes at 70 °C to inactivate the reaction. cDNA samples were stored at -20 °C.

4.6.3. Sequence identification

The sequences were obtained from a transcriptome obtained in our laboratory (Aquilino et al., 2019) and the sequences published by Romiguier et al. (2014).

The sequences obtained were identified using the Blast2GO tool (Conesa et al., 2005) by blasting them to the database using the txblast. The identified sequences were translated with Snapgene software (GSL Biotech LLC, USA), and the proteins were compared with the GenBank protein database to confirm the identity. Then, the sequences identified as proteins of interest were used for primer design.

Heat shock protein cognate 70-4 (Hsc70-4), actin beta/gamma 1 (ACTB_G1), Mn superoxide dismutase (SOD Mn), glycogen phosphorylase (PYGL), and hypoxia-inducible factor 1α (HIF1α) were identified from sequences published by Romiguier et al. In contrast, double-strand-break repair protein rad21 (rad21), DNA repair protein rad50 (rad50), acetylcholinesterase (AChE),

catalase (Cat), apoptosis-inducing factor 3 (AIF3), cytochrome P450 72a15 (Cyp72a15), DNA methyltransferase 1 (DNMT1), lysine acetyltransferase 6B (KAT6B), histone deacetylase 1 (HDAC1), small heat shock protein 17.9 (sHSP17.9), aplysianin-A (ApA), oxysterol binding protein-related protein 8 (ORP8) and 6-phosphofructo-2-kinase/fructose2,6-bisphosphatase (PFKFB2) were obtained from the transcriptome.

4.6.4. Primer design

The mRNA levels of the selected genes were studied through the RT-PCR technique, for which a primer pair was designed for the amplification of specific fragments of each gene, according to the following criteria:

- Proximity of the sequences to the 3´ mRNA extreme
- Annealing temperature near 58 °C, to simultaneously amplify all the genes
- Primer length between 100–200 bp

Primers were designed using the NCBI primer designing tool Primer-Blast (Ye et al., 2012). Most of the primer sequences used in this work were specifically designed in the laboratory: *rad21*, *rad50*, *AChE*, *Catalase*, *SOD Mn*, *AIF*, *Cyp72a15*, *DNMT1*, *KAT6B*, *HDAC1*, *sHSP17.9*, *Hsc70-4*, *HIF1α*, *ApA*, *PYGL*, *ORP8*, *ACT*, and *PFKFB2*. The rest were obtained from previous works: *rpL10, GAPDH*, *Casp3*, *Hsp16.6*, *MRP1*, *Hsp90*, *Cyp2u1*, *Cyp3a7*, *Cyp4f22*, *GSTo1*, *GSTt2*, and *SOD CuZn* (Aquilino et al., 2019); *GSTk1*, *GSTm1*, *Hsp60*, and *Grp78* (Alonso-Trujillo et al., 2020).

4.6.5. Primer efficiency

Because some genes were in low quantities, efficiency curves could not be prepared with the cDNA. Hence, an alternative approach was used. A PCR with the same conditions as RT-PCR for each studied 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 until reaching 50 µL (1:50 dilution). From this 1:50 dilution, 1 µL was taken and diluted in 500 µl, obtaining a 1:25,000 final dilution. Then, five serial dilutions (1:2) were prepared for the efficiency curve. The efficiency of each primer pair was calculated from the slope of the standard curve obtained after performing the serial dilutions of PCR products and amplifying the genes under the same PCR conditions used in the study. Primer sequence and efficiency for each evaluated gene are shown in table 7.

Table 7. Primer sequences and efficiencies for each studied gene.

Gene	Forward	Reverse	Efficiency (%)
rad21	CCGGCCAATGTCTGATGACT	GCAATTGCTTGCTGGCATCT	96.9
rad50	AGGCAAGGAGGAGCTACAAC	TTCAGCCAATGCTAAGCGGA	98.7
AChE	AGTGTCCCGTCGTGGATTTC	CACGACCTCGATCTCGTAGC	89.8
Catalase	CCCAGTCAGTGGTGATGTCC	TTCAGGTGCCCGACAATGTT	98.8
SOD Mn	TCGAATTGCTACCTGTGCCA	ATTCACGTAGTCAGCTCGCA	105.4
AIF	ACCACAAGATGCCAACGCTA	ACTGGCAGCCTTATCAGCAA	102.0
Cyp72a15	AGGGAAGTGGCTTGAGTGAC	GGTGCTCAGCCAGCATAAGA	91.9
DNMT1	GACGCCATGTCCGATTTACCT	TCATCCGCGCTGCCACCAG	93.3
KAT6B	CTTCCATGGGGATGACGAGG	AAGCTTTGAACGTTTGCCCC	95.1
HDAC1	CCCATCAAACATGGCCAACC	GTGCATGTGGCAACATTCTGA	90.8
sHSP17.9	TTCACGCGTTGGTGAATCAG	TTAGCAGCTACAGTCAGCGT	102.5
Hsc70-4	TGGTGTGCCCCAGATTGAAG	TGGTGTGCCCCAGATTGAAG	107.8
HIF1α	AGGATAGATGCTGGCACACC	CATAGACACGGTCCTCCCCT	81.4
ApA	GTGCCGGGAAAGTGATTTTAG	CAGCCACCAGGGTCGCGA	99.6
PYGL	ACTGACCCCTGCGTGTAAAG	TGGAGGCAGGGTTGATCTTG	104.2
ORP8	GCTGGACGGACATCACTTGT	TGGATGTCTACCACTCGGGA	98.9
ACT	GAAGAGCTACGAGCTTCCCG	CATGGATACCGGCAGACTCC	102.1
PFKFB2	AGCGCACTATCCAAACTGCT	TCTGCAAAGTCCTGGGGGTA	110.6
rpL10	TGCACGTGAGGCTGATGAAA	GTGGCCACTTTGTGAAACCC	102.3
GAPDH	ATACATCAGGAACAGGGACTC	GACTTATGACAACCGTGCA	93.9
Casp3	GTCTGTGTAATTCTCACCCATG	AGTTCAGTGCCTCTGCAAGC	107.2
Hsp16.6	GCATGAGGAGAAGCAAGACA	CAGTACACCATGGGCATTCA	96.4
MRP1	CAGGGGCAGGTAAGTCATCC	AGTGAGCCTTGATCGCACAT	94.5
Hsp90	GTTTGTGTCACTAAAGAAGGCC	TGTCACTAGCCTATTTGATACAACC	91.8
Cyp2u1	GTGCATCCTCTACGCGATCA	GGCTAGTTTGGGCCTGTCTT	102.1
Cyp3a7	ACGGCTTGGCCTCTCAATAC	CGGTTTCTTTCTCGGCGTTC	84.8
Cyp4f22	AGCAGAAAAAGCTCAGCCCT	CTTGGTTTTGGCAGCCAGTC	87.2
GSTo1	CCACCTGGCAACTTGGTTTG	GCTTGCCAGATGCGTAAGAC	92.8
GSTt2	TCGATCTTCTATCGCAGCCG	TTCTGAGCGCAACAGGTTTG	86.0
SOD CuZn	AGAAAGCTGGTGCTGCAACTA	AGGATTAAAGTGGCCTCCAGC	104.9
GSTk1	TGAGCAGAGTAGTTTGGCTGC	ATGCCCCTAATTCTGTGGCT	96.7
GSTm1	ATTGGGCCATTAGAGGGCTT	GTTGGACCATCTCCTTGCAC	93.1
Hsp60	ATTGCTTATCGTGGCTGAGG	TGGCAATAGCCATATCCTGC	82.0
Grp78	TGGTGGCTCAACCCGTATTC	CCCCACTCAAAACACCAGCT	96.8

4.6.6. Amplification by Real-Time PCR

The gene expression levels of the selected genes were assessed through Real-Time PCR, known as quantitative PCR (RT-PCR), a variation of the polymerase chain reaction (PCR) where the RNA template is converted into cDNA that later is amplified and quantified.

The final reaction volume of the RT-PCR was 10 µl, made up of gene-specific primers, at a final concentration of 2.5µM each, and a master mix composed of cDNA, 0.5X EvaGreen, 20 µl of Taq DNA polymerase, 0.4 mM dNTPs, and 2 mM Cl_2Mg. The fluorophore used in this study, EvaGreen, emits fluorescence when it bounds to double-stranded DNA and the intensity of fluorescence varies proportionally to the concentration of PCR product. Therefore, the

fluorescence emitted can be used to calculate the generation rate of the specific product (Heidi et al., 1996; Jalali et al., 2017).

The amplification program was the following: an initial denaturation for 30 seconds at 95 °C followed by 40 cycles which included a denaturation at 95 °C for 15 seconds, 58 °C annealing for 15 seconds, and 72 °C elongation for 30 seconds. In the end, a melting curve from 65 to 95 °C for each 0.5 °C increase was generated to confirm the specificity of the primers and the presence of a single peak. The reactions were performed in 96 multiwell plates (BioRad) with a CFX96 thermocycler (Bio-Rad, USA). Each RT-PCR was performed running duplicate wells for each sample, and two independent replicates were done for each experiment.

4.6.7. Quantification

To quantify the expression of the study genes, the cycle threshold values (Ct) (also known as quantification cycle (Cq)) were used, which correspond to the amount of fluorescence detected in the exponential phase of the reaction. The mRNA levels of each sample were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L10 (rpL10), actin (act), and 6-phosphofructo-2-kinase (PFKFB2) as reference genes. Bio-Rad CFX Maestro software was used to analyze and determine total mRNA levels of normalized gene expression ($2^{-\Delta\Delta Cq}$).

4.6.8. Enzymatic activity

4.6.8.1. Protein extraction

Protein extraction was performed following a protocol described for *Drosophila* (Zhong et al., 1996) adapted for *P. acuta.* Once frozen at -80 °C, each juvenile snail, including the shell, was homogenized in 200 µL protein extraction buffer (see section 4.2 reactives), frozen in dry ice, thawed, and frozen once again. After that, the samples were kept on ice for 15 minutes, followed by centrifugation at 10,000 g and 4 °C for 10 minutes. At last, the supernatant was recovered and stored at -80 °C for further analysis. To quantify protein concentration, the Bradford method (Bradford, 1976) in a microplate reader (Multiskan Go, ThermoUSA) was used, using the specific reactive, Bradford reactive (BioRad).

4.6.8.2. Glutathione S-Transferase activity

The previously extracted proteins for each treatment were used to assess the glutathione S-Transferase (GST) enzymatic activity. 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) were used as substrates. The reaction was developed by mixing 188 µL of the cocktail with 12 µL of protein. Absorbance was read at 340 nm in a plate reader (Multiskan, Thermo USA) for 5 min, and the activity was calculated according to:

$$(\Delta A340)/\min = \frac{A340\ (\text{final read}) - A340\ (\text{initial read})}{\text{reaction time}\ \ (\text{min.})}$$

$$(\Delta A340)/\min = \frac{(\Delta A340)/\min \text{ x V (ml) x dil}}{\varepsilon \text{mM x } V_{\text{enz}}\ (\text{ml})}$$

Where V is the reaction volume, dil is the dilution factor of the original sample, ƐmM is the extinction coefficient for CDNB conjugate at 340 nm, and V_{enz} is the volume of the protein added.

4.7. Statistical analysis

Statistical analysis was performed using SPSS 25 (IBM, USA). The Shapiro–Wilk and Levene tests, respectively, were tested for normal distribution and variance homogeneity. For *P. acuta*, the normally distributed data were analyzed by analysis of variance (ANOVA) using Dunnet´s post-hoc test. The nonparametric Kruskal–Wallis test with Bonferroni´s correction was used for the not normally distributed data. Regarding *P. lividus*, all data are expressed as means ± standard error of the 4 replicates and as effect percentage. Sea urchin development assays were considered acceptable if the control developed larvae were higher than 80%. Effective concentration (EC_{50}) and related 95% confidence limits were calculated using Trimmed Spearman Karber analysis (Finney, 1978). Significant differences between controls and treated samples were determined using ANOVA. When data failed to meet the assumption of normality, Kruskal- Wallis test and Mann-Whitney test were used to compare individual treatments. In all cases, the significance level was set at $p \leq 0.05$. Figures were prepared using Excel 365 and Powerpoint 365 (Microsoft, USA).

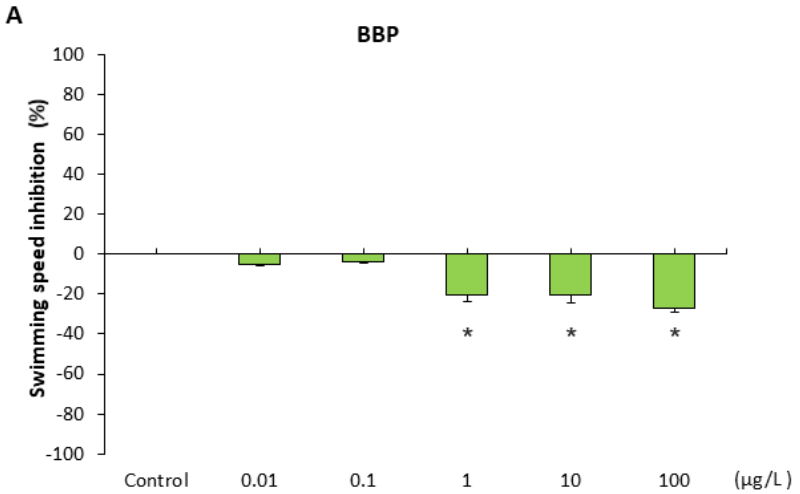
RESULTS

5. RESULTS

5.1. Effects of BBP, DEHP, and DEP on *Paracentrus lividus*

5.1.1. Swimming speed alteration (SSA)

The behavioral endpoint is referred to as swimming speed alteration (SSA). As stated in 4.4.1.1. section, swimming speed (movement of sea urchin larvae) values measured for each concentration of phthalates were normalized to the average swimming speed of the control (mm/sec). Exposure to BBP displayed the lowest toxicity out of the three tested compounds. However, a -27% effect was observed at the highest concentration (100 µg/L), significantly different from the controls. Specimens of 72 hpf *P. lividus* exposed to DEHP showed a concentration-dependent swimming inhibition (see figure 4). A maximum effect of -40.28% was observed at the highest concentration tested (100 µg/L); therefore, the EC_{50} was not calculable. Swimming activity was also inhibited in plutei exposed for 72 hours at DEP. The highest SSA, equal to -40.34%, was found at the maximum concentration tested (100 µg/L). The same phthalate-dose dependent effect found for DEHP was also observed after DEP exposure. For both DEP and DEHP, the LOEC was observed at 0.1 µg/L, while for BBP, the LOEC value was 1 µg/L.



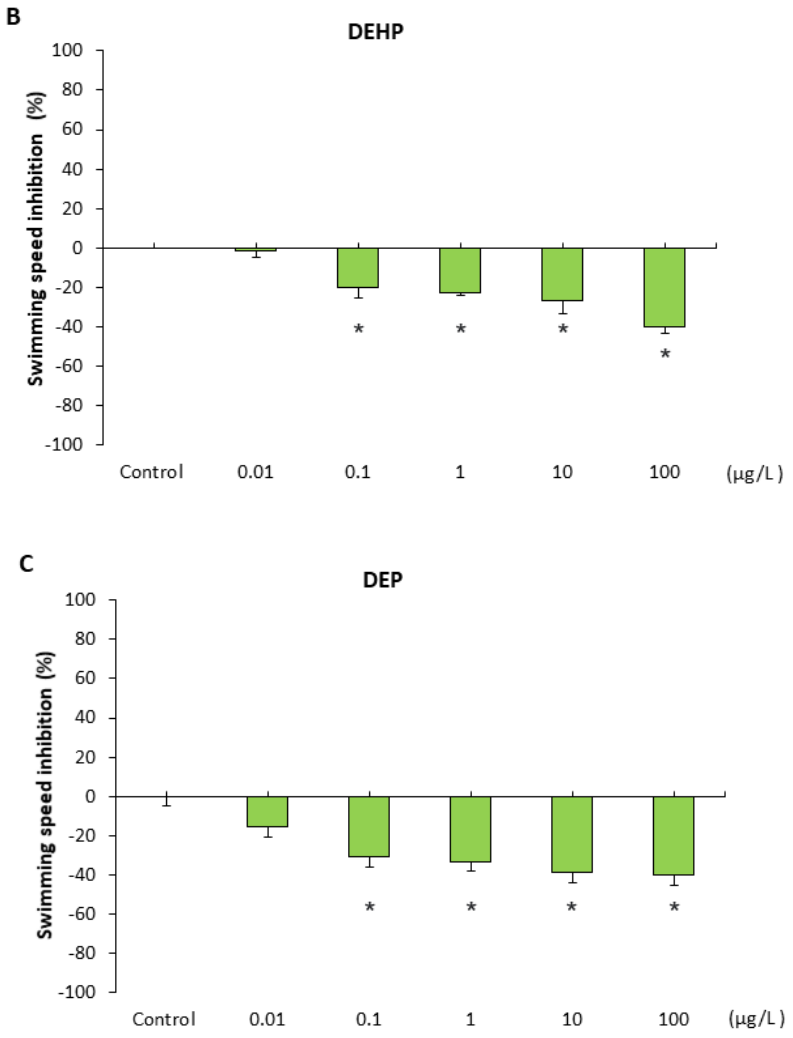


Figure 4. Percentage of swimming speed inhibition of *P. lividus* plutei after 72 h embryo exposure to BBP (A), DEHP (B), and DEP (C). Significant differences to controls (asterisk) are indicated (*p < 0.05).

5.1.2. Morphological development of plutei

One parameter to assess the toxicity is the morphological development of plutei, which can provide information concerning developmental toxicity. The plutei formed after embryos' exposure to BBP, DEHP, and DEP were observed after 72hpf and showed abnormalities in the skeletal architecture and larval shape. All compounds yielded significant anomalies if compared to controls. A dose-dependent effect was observed after exposure to all three phthalates.

BBP displayed the highest toxicity among the three tested phthalates, reaching over 90% of anomalous pluteus at the highest concentration (1000 µg/L) The EC_{50} was 15.25 (6.74 – 34.49) ug/L while the LOEC was observed at the lowest BBP concentration (figure 5A). Regarding DEHP, the percentage of anomalous plutei increased markedly with exposure to the increasing concentrations of DEHP, reaching its maximum (about 80%) at the highest concentration tested

(1000 µg/L). Thus, the EC_{50} value was 13343 (79.35 – 224.38) µg/L, while the LOEC value was observed at 10 ug/L (figure 5B). DEP was less toxic than the other phthalates, with similar values to control at the lowest concentration (7% anomalies at 0.01 µg/L) and a maximum of 56% anomalous plutei at the highest concentration (1000 µg/L). The EC_{50} value was 758.58 (338.28 – 1791.99) µg/L and the LOEC value was 100 ug/L (see figure 5C).

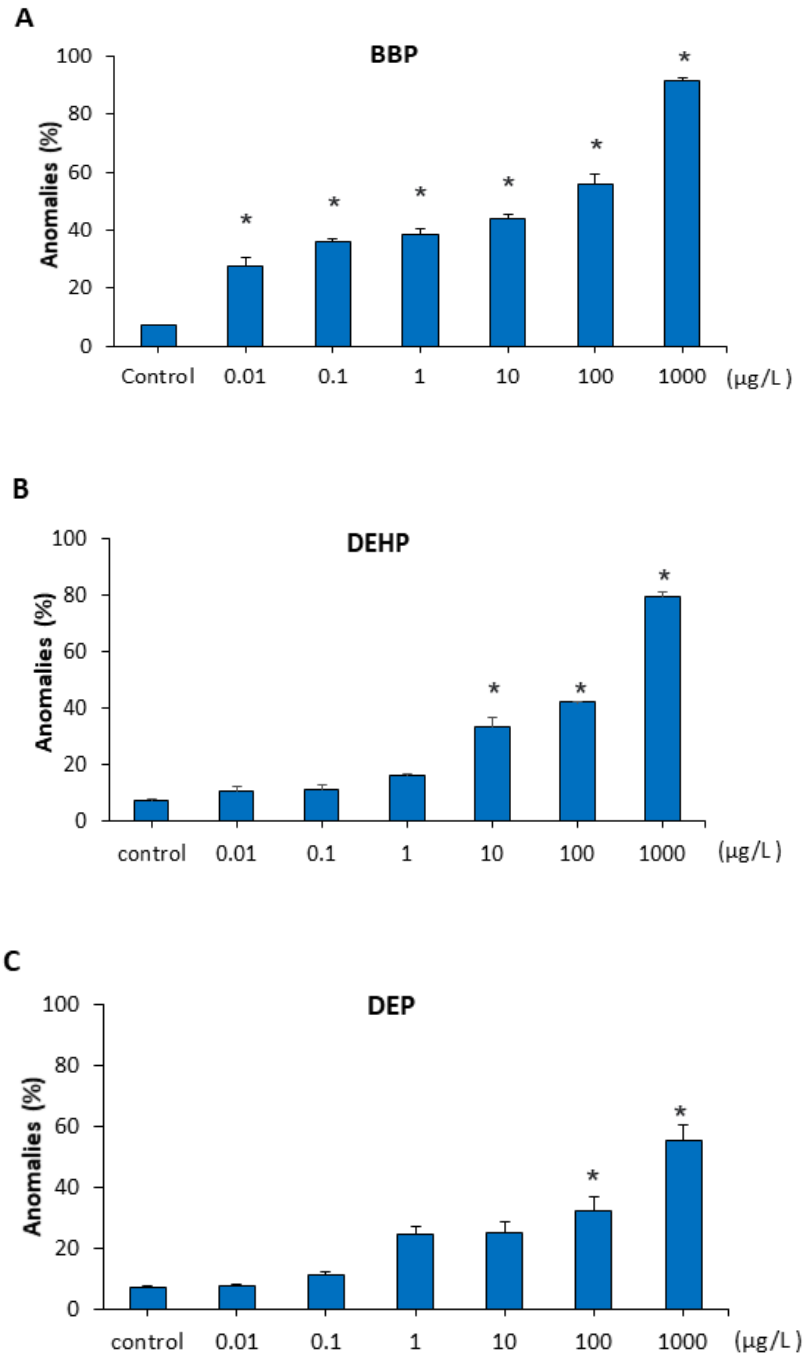


Figure 5. Percentage of anomalous plutei of *P. lividus* after 72 h embryo exposure to BBP (A), DEHP (B) and DEP (C). Significant differences to controls (asterisk) are indicated (*$p < 0.05$).

The most common anomalies found in the 72h plutei stage after phthalates exposure were fused anterior arms; crossed tips at the apex; supernumerary rods; short plutei, with distant symmetrical anterior arms not oriented to the mouth; larvae with incomplete or absent skeletal rods and delayed or incomplete migration of the mesenchyme cells, forming a ring with irregular skeletal spines. See figure 6.

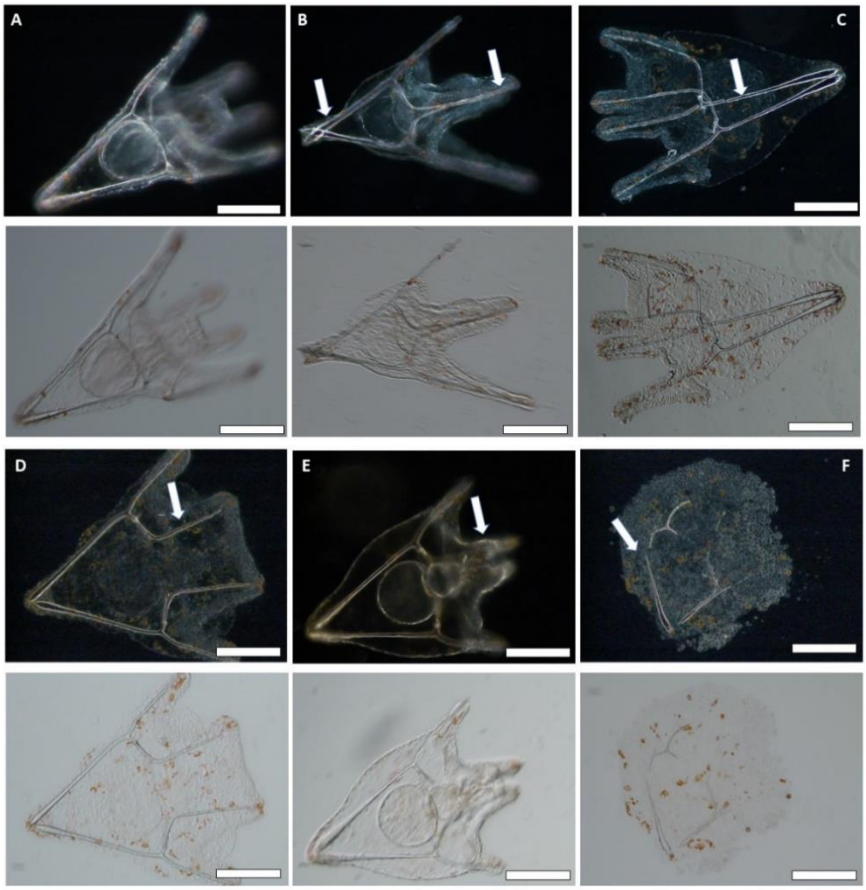


Figure 6. Main anomalies found at 72 h pluteus stage: (A) control. Pluteus showing the correct position of skeletal rods and a complete development and arrangement of the arms; (B) pluteus showing crossed skeletal tips at the hood apex and with joined anterior arms; (C) supernumerary rods; (D) slightly smaller plutei, with distant symmetrical anterior arms not oriented to the mouth; (E) larvae with incomplete or absent skeletal rods (F) delayed or incomplete migration of the mesenchyme cells, forming a ring with irregular skeletal spines. Bar equals 100 µm.

5.2. Effects of BBP, DEHP, and DEP on *Physa acuta* adults

5.2.1. Survival

As a first approach to evaluate the potential toxicity of the studied phthalates and select a concentration range for subsequent tests, adult individuals of *Physa acuta* were exposed to 10, 100, 1000, and 10000 µg/L of DEP, DEHP, and BBP and the possible lethal effects were observed every 24 hours for 96 hours. As shown in figures 7, 8, and 9, none of the DEP, DEHP, and BBP concentrations tested affects adult *P. acuta* survival, observing a 100% or near 100% survival in all treatments. Thus, it can be concluded that the selected phthalates at the time and concentrations tested do not affect *P. acuta* survival. Therefore, according to these results, the concentrations of 0.1, 10, and 1000 µg/L of DEP, DEHP, and BBP were selected for gene expression analysis.

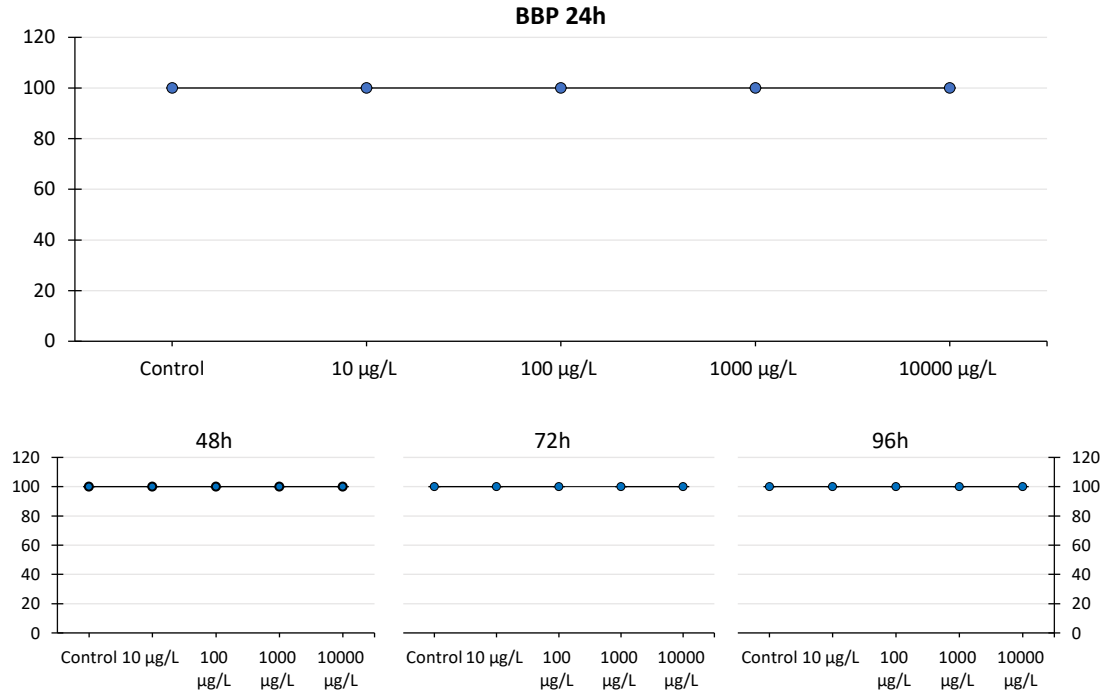


Figure 7. Survival analysis of *P. acuta* adults exposed to 10, 100, 1000, and 10000 µg/L of BBP after 24, 48, 72, and 96 hours.

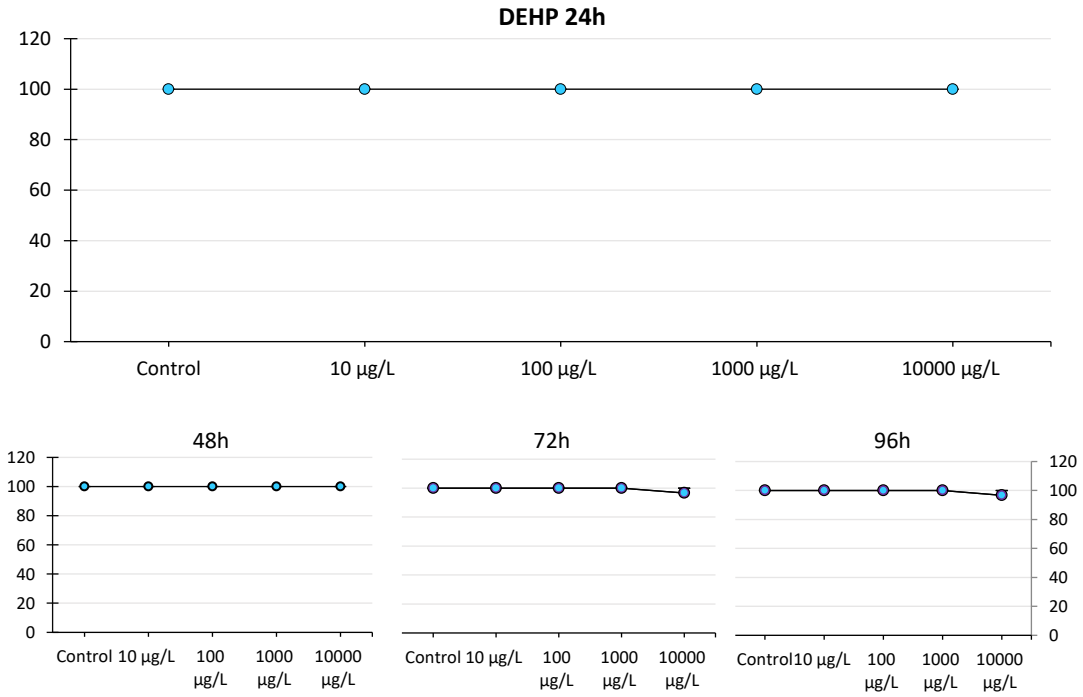


Figure 8. Survival analysis of *P. acuta* adults exposed to 10, 100, 1000, and 10000 µg/L of DEHP after 24, 48, 72, and 96 hours.

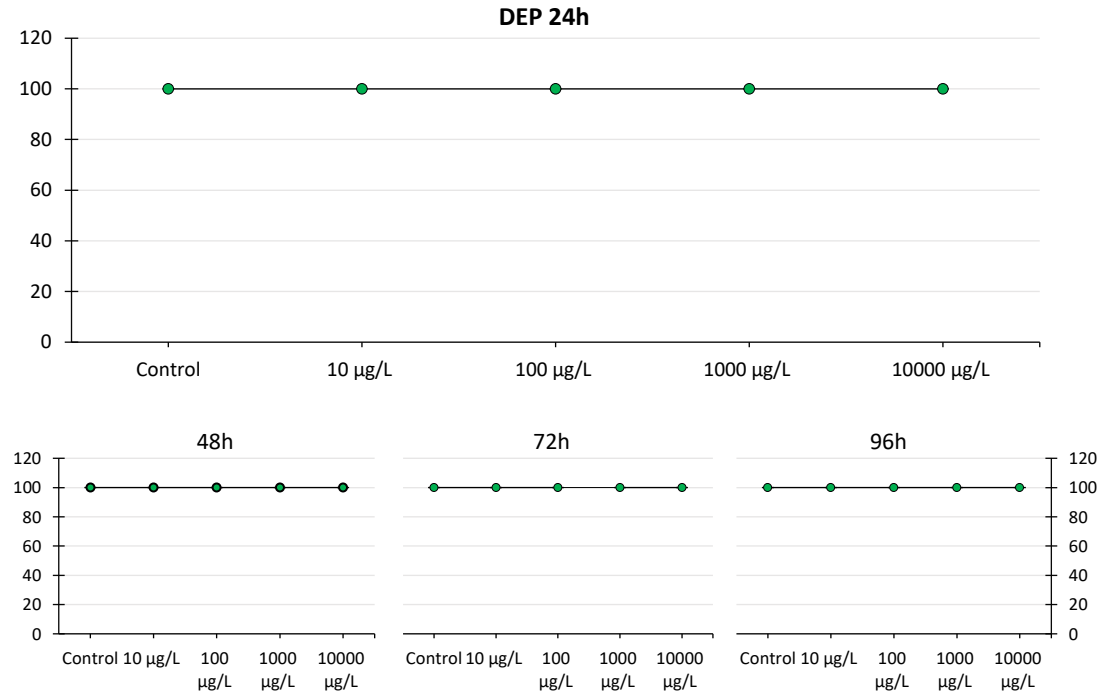


Figure 9. Survival analysis of *P. acuta* adults exposed to 10, 100, 1000, and 10000 µg/L of DEP after 24, 48, 72, and 96 hours.

5.2.2. Gene identification

In the present study, eighteen genes were identified for the first time for *P. acuta* (figure 10). A comparison of the sequences to the GenBank database using the Blast2GO software was carried out. They all showed homology with genes in the database, most of them with those described in the freshwater snail *Biomphalaria glabrata*. For the rest, the homology was with the sea slug *Aplysia californica.* Only one had homology with a gene of the marine bivalve *Mytilus coruscus* and another with the sea slug *Aplysia kurodai.*

The genes covered several pathways. Two genes code for proteins homologous to rad21 and rad50, which are involved in DNA damage repair. Two other oxidative-stress-related genes code for the enzymes catalase and superoxide dismutase Mn, involved in removing free radicals. One sequence codes for acetylcholinesterase, an enzyme involved in nerve impulse transmission. The other genes code for proteins related to apoptosis (apoptosis-inducing factor 3 and caspase 3); stress (sHsp 17.9, Hsc70-4, hypoxia-inducible factor 1α); histone modification (histone deacetylase 1, Lysine Acetyltransferase 6B); DNA methylation (DNA methyltransferase1); the immune system (Aplyasinin A); phase I detoxification mechanism (Cytochrome P450 72a15), energy reserves (Glycogen Phosphorylase L), and lipid transport (Oxysterol-binding protein-related protein 8).

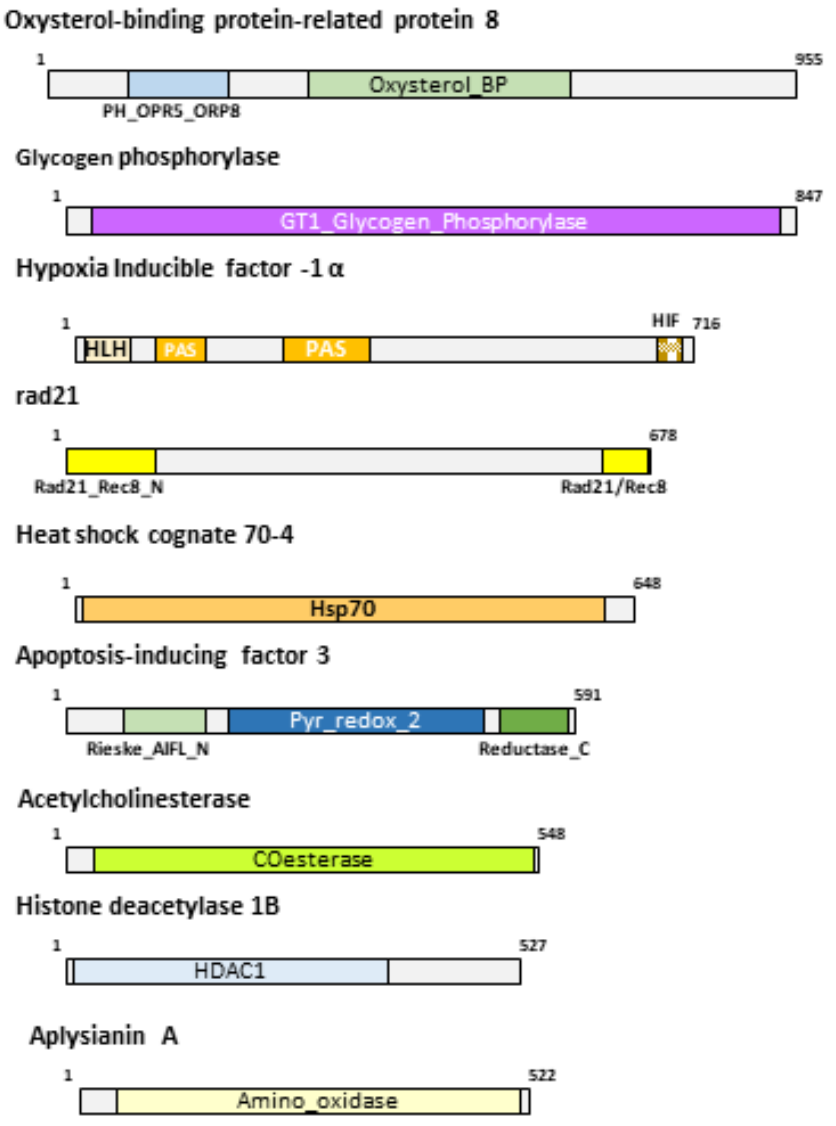
Three stress-related sequences were identified. Two of them code for members of the small heat shock proteins and HSP70 families of proteins: Hsp17.9 and Hsc70-4. Hsp17.9 shared 52% identity with Hsp12.2 of *B. glabrata* and presented the ORF with the characteristic alpha-crystallin domain, while for Hsc70-4, the protein presented the characteristic Hsp70 domain. HIF-1α sequence shared 64% identity with *B. glabrata* and presented the basic helix-loop-helix domain, followed by two distinct PAS domains.

One detoxification-related sequence was identified. It codes for the Cyp450 superfamily of proteins: Cyp72a15, which presented the characteristic Cyp450 domain. It exhibited more than 60% sequence identity with *A. californica.* Another group of identified sequences was related to oxidative stress. Cat and SOD Mn shared a high identity (greater than 80%) with catalase and SOD Mn of *B. glabrata*, respectively. Two DNA repair-related sequences were identified. Both ORFs showed identity with rad21 and rad50 from *B. glabrata* and presented the characteristic domains associated with those proteins.

The sequence coding for AIF3 presented the characteristic N-terminal Rieske domain and shared an identity higher than 70% with AIF of *B. glabrata*. Regarding glycogen metabolism, the PYGL ORF shared a high identity (around 90%) with the PYGL brain isoform of *A. californica.*

DNMT1, HDAC1, and KATB6, involved in epigenetic regulation, were identified. KATB6 sequence coded for a 1277 aa ORF and shared an identity lower than 60% to the histone acetyltransferase of the snail *B. glabrata.* Both DNMT1 and HDAC1 sequences showed around 90% homology with the DNA methyltransferase and the histone deacetylase of *A. californica.* The nervous system related gene AChE shared identity of 56% with *B. glabrata.* ApA presented the amino-oxidase characteristic domain, and ORP8 presented the conserved domains PH_OPR5_ORP8 and Oxysterol_BP. The identity with another mollusk was high for ORP8 (around 80%), while it was weaker in the case of ApA (around 50%).

The sequences for two reference genes were also identified. ACTB_G1 coded for a 376 aa protein. The protein presented the characteristic domain of this enzyme. It showed high identity with ACTB_G1 from *M. coruscus.* PFKFB2 coded for a 234 aa protein. The presence of the His_Phos_1 domain and its homology with PFKFB2 of *B. glabrata* confirmed the identity.



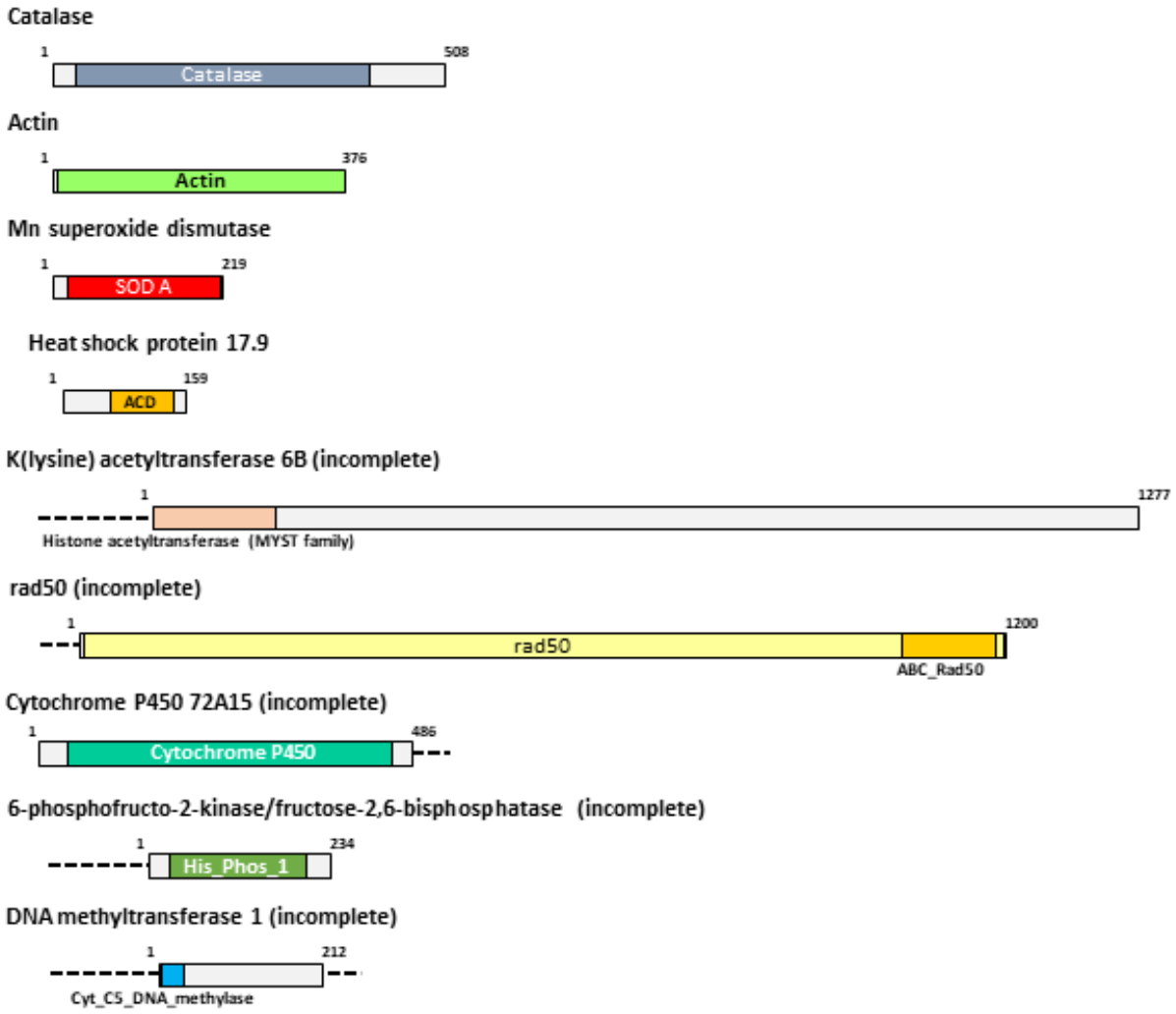


Figure 10. Structure and conserved domains of the identified *P. acuta* proteins. Each protein corresponds to an open reading frame (ORF) from the sequences used in the study. The proteins are shown with the different motifs that characterize them. The domains are defined according to the CDD functional classification of proteins. Some of the genes were not complete, and the discontinuous line indicates the unknown up- and downstream regions.

5.2.3. Gene expression effects

The changes in mRNA levels of 30 genes were analyzed through RT-PCR to study several pathways involved in response to the three phthalates. The selected genes are involved in different biological pathways, endocrine system, cellular stress response and detoxification process, oxidative stress, DNA repair, apoptosis, immunity, epigenetics, lipid transport, and energy reserve metabolism.

The gene expression analysis was performed exposing *Physa acuta* adults for one week to DEP, DEHP, and BBP to 0.1, 10, and 1000 µg/L, which did not have any lethal effects. In addition, the analysis revealed that DEP and DEHP did not modify the mRNA levels of any of the genes at the concentrations tested.

5.2.3.1. Effects in endocrine system related genes

To evaluate the effects of these compounds in the endocrine system, the gene that codes for Hsp90 was analyzed, as this protein participates in the maturation of steroid receptors (Eckl & Richter, 2013). As shown in figure 11, DEP and DEHP do not alter the values of the gene at the concentrations tested after one-week exposure, while for BBP the transcription of the gene increases at all tested concentrations. Besides, this protein is also involved in the stress response.

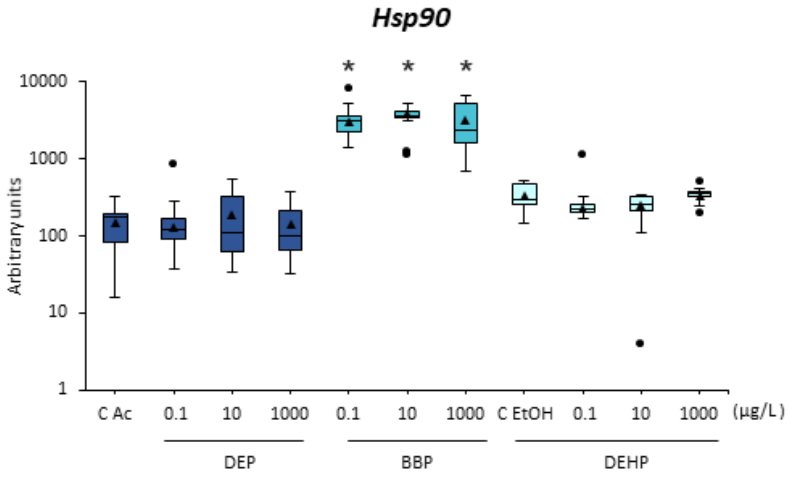
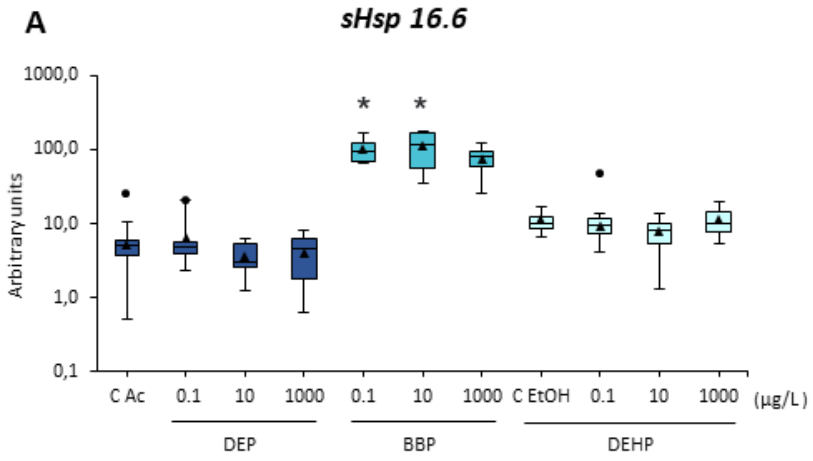


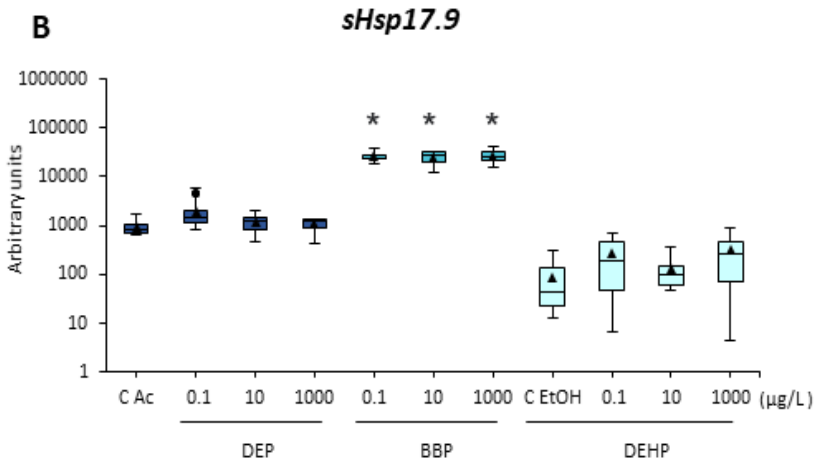
Figure 11. Expression of *Hsp90* in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and the box's boundaries indicate 25th and 75th percentiles. The whiskers represent the highest and lowest results. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.2.3.2. Effects in cellular stress response-related genes

To evaluate the stress response after exposing the adults to the three phthalates, the gene expression profile of the following genes was analyzed: *sHsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4*, and *Grp78*. The small heat shock proteins are the largest and most heterogeneous family of HSPs, which function as molecular chaperones and respond to cellular stress against environmental pollutants. Hsp60 is a mitochondrial chaperone involved in folding and preserving newly imported and stress-denatured proteins (Xu et al., 2014). Hsc70-4 is a cytoplasmatic chaperone that belongs to the Hsp70 family and is involved in different cellular functions (Liu et al., 2012). Finally, Grp78 also referred to as BiP, is an endoplasmic reticulum chaperone that belongs to the Hsp70 family, which ensures the quality of proteins of the endoplasmic reticulum and activates transmembrane signaling pathways that maintain homeostasis under stress (Trujillo et al., 2020).

Compared to control, *sHSP16.6* and *Hsc70-4* showed statistical significance for the two lower concentrations (0.1 and 10 µg/L) of BBP but not for the highest. On the other hand, the transcription of *sHSP17.9*, *HSP60*, and *Grp78* increased significantly at the three tested concentrations after exposure to BBP.

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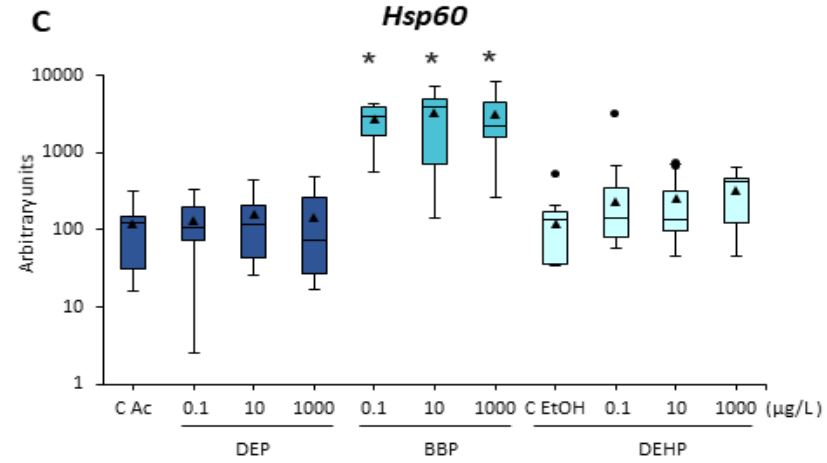
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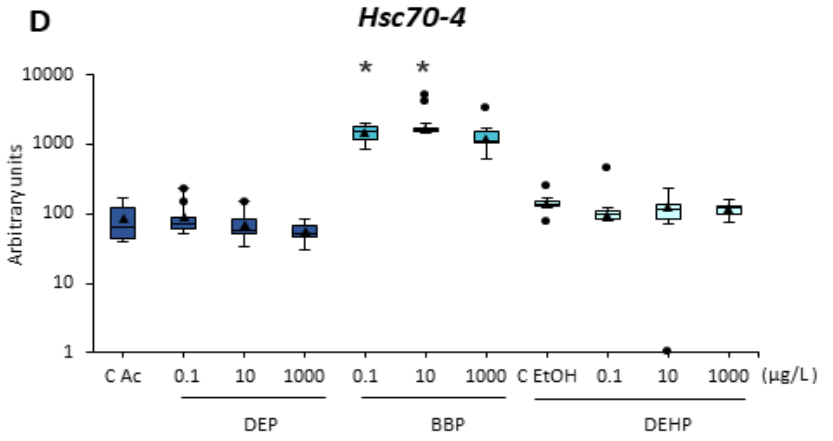
BBP
DEHP


D
Hsc70-4
Arbitrary units
10000
1000
100
10
1
C Ac
0.1
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP

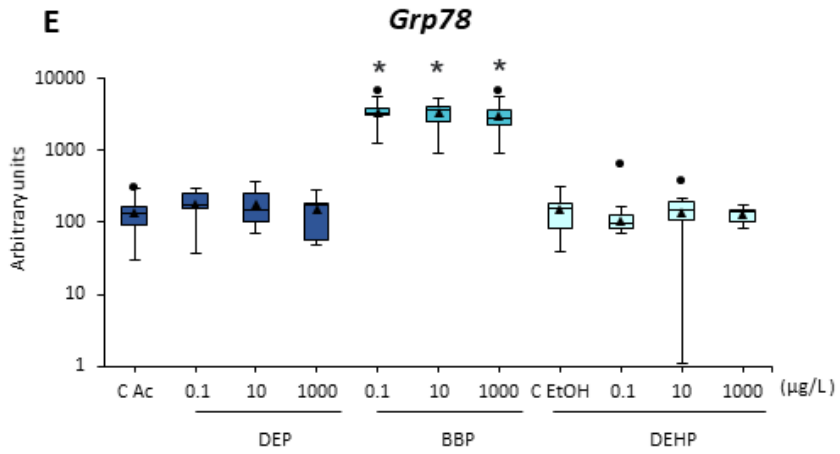
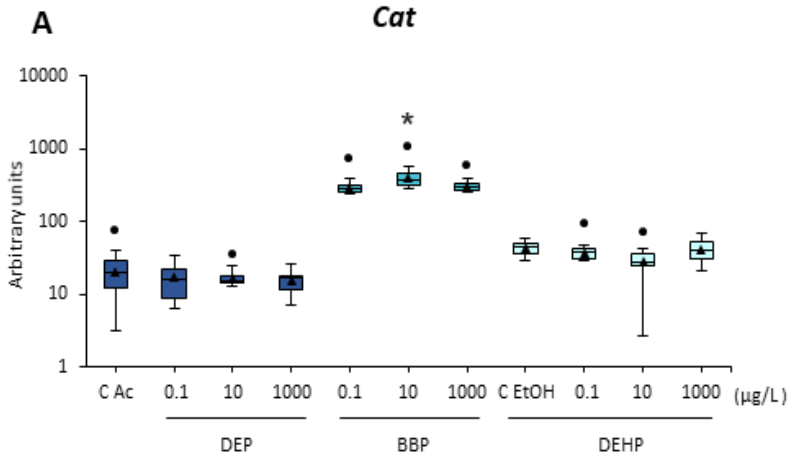


Figure 12. Expression of genes related to stress (*Hsp16.6*, *sHsp17.9*, *Hsc70-4*, *Hsp60*, and *Grp78*) in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and the box's boundaries indicate 25th and 75th percentiles. The whiskers represent the highest and lowest results. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

To test the ability of the phthalates to activate the response to oxidative stress, *Cat*, *SOD Mn*, and *SOD Cu/Zn* expression was analyzed. These oxidative-stress-related genes code for catalase (Cat), manganese superoxide dismutase (SOD Mn), and copper-zinc superoxide dismutase (SOD Cu/Zn), enzymes involved in removing free radicals (Bhagat et al., 2016). *Cat* was altered for the concentration of 1 µg/L but not for the other two, while the expression of *SOD Mn* and *SOD Cu/Zn* increased at the three tested concentrations after one-week exposure to BBP (figure 13). Ultimately, the response to stress related to hypoxia was analyzed through the expression of the hypoxia-inducible factor-1 alpha (*HIF1α*), whose transcriptional activity was significantly altered at the three BBP tested concentrations.



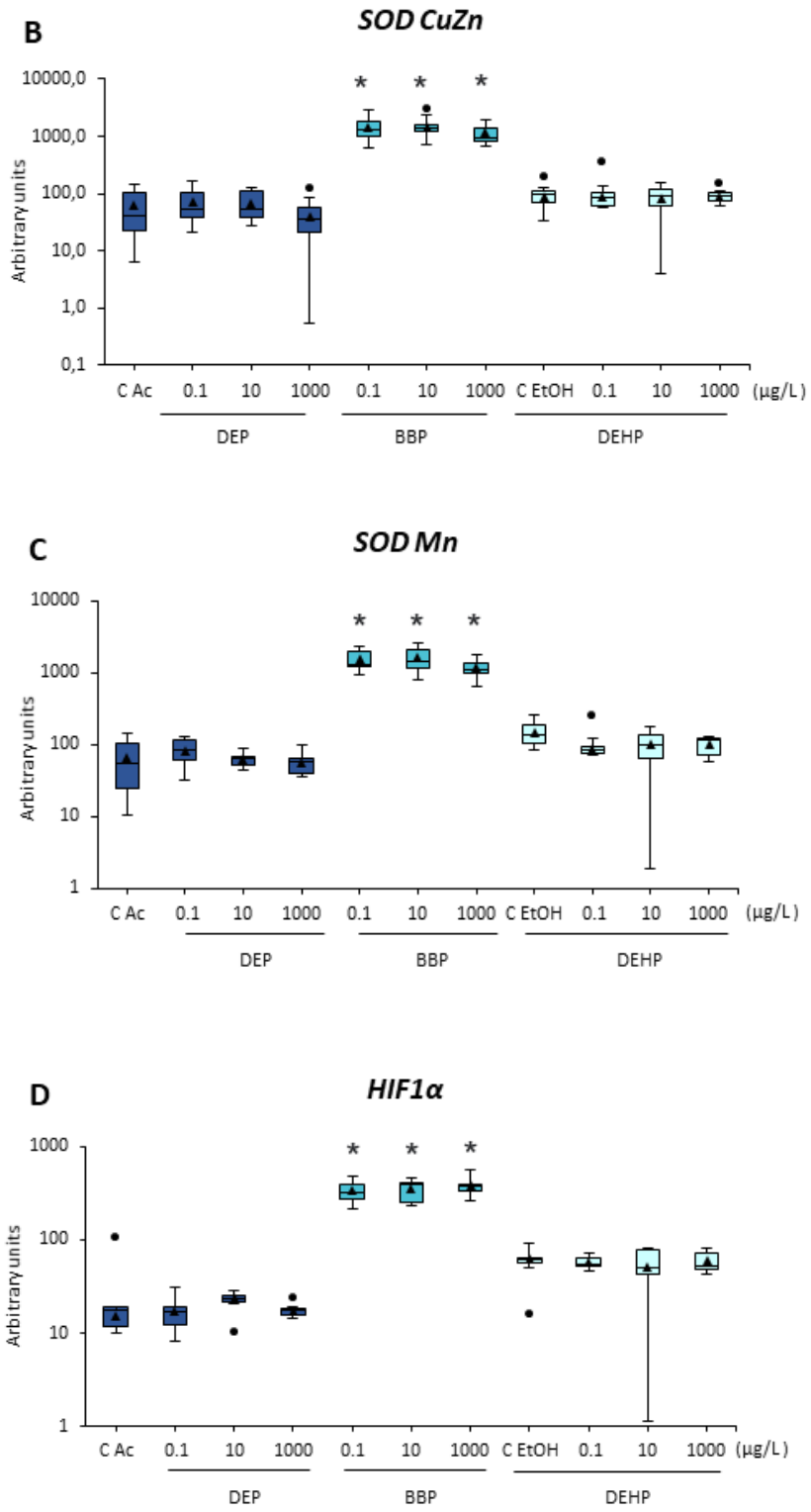


Figure 13. Expression of genes related to oxidative stress (*Cat*, *SOD Cu/Zn*, and *SOD Mn*) and hypoxia (*HIF1α*) in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

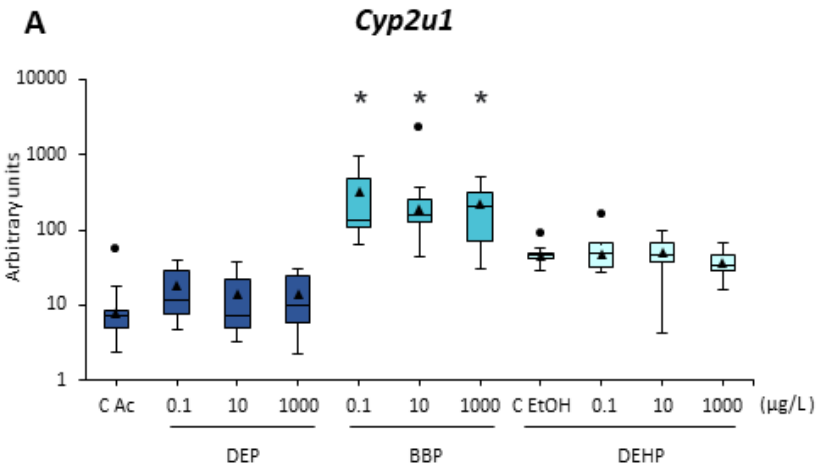
In compliance with the data obtained, it can be concluded that BBP could alter the cellular stress response increasing the activity of the heat shock protein genes *Hsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4*, and *Grp78*, the oxidative-stress-related genes *Cat*, *SOD Mn*, and *SOD Cu/Zn* and the hypoxia-related gene *HIF1α*.

5.2.3.3. Effects in detoxification mechanisms related genes

The expression profile of genes that code for enzymes involved in the three phases of the detoxification process was analyzed to evaluate the detoxification mechanisms.

As previously mentioned in 2.8.3. section, phase I involves the compound oxidation, reduction, and hydrolysis through, among others, enzymes belonging to the cytochrome P450 superfamily. Four genes coding for members of this family were assayed in this work: *cytochrome P450 2u1* (*Cyp2u1*), *cytochrome P450 3a7* (*Cyp3a7*), *cytochrome P450 4f22* (*Cyp4f22*), and *cytochrome P450 72a15* (*Cyp72a15*). Phase II involves conjugation reactions, obtaining more easily excretable forms of the compounds. Four genes coding for members belonging to one of the major phase II detoxification enzymes groups, the glutathione-S-transferases (GSTs), were studied: *Glutathione -S- transferase omega 1* (*GSTo1*), *Glutathione -S- transferase theta 2* (*GSTt2*), *Glutathione -S- transferase kappa 1* (*GSTk1*) and *Glutathione -S- transferase mu 1* (*GSTm1*). Finally, *Multidrug Resistance Protein 1* (*MRP1*), also referred to as *ABCC1*, was selected as phase III representative, where unwanted molecules are transported outside the cell (Trujillo et al., 2020).

The transcription of phase I detoxification genes *Cyp2u1*, *Cyp3a7*, and *Cyp72a15* was significantly increased at all tested concentrations for BBP, while *Cyp4f22* was altered just at 10 µg/L (figure 14C). Regarding the phase II detoxification genes, the transcription of *GSTk1* increased significantly at the three concentrations, while *GSTo1* and *GSTt2* were significantly upregulated just at the lowest concentration (0.01 µg/L) (figure 15). At last, *GSTm1* did not show any significant alterations, although there was a trend for a higher mean compared with the control (figure 15B). For the phase III representative, *MRP1*, mRNA levels were increased for 1 and 100 µg/L of BBP. These results suggest that BBP could activate the three detoxification phases in *P. acuta* after one-week exposure in the presence of BBP.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1000
100
10
1
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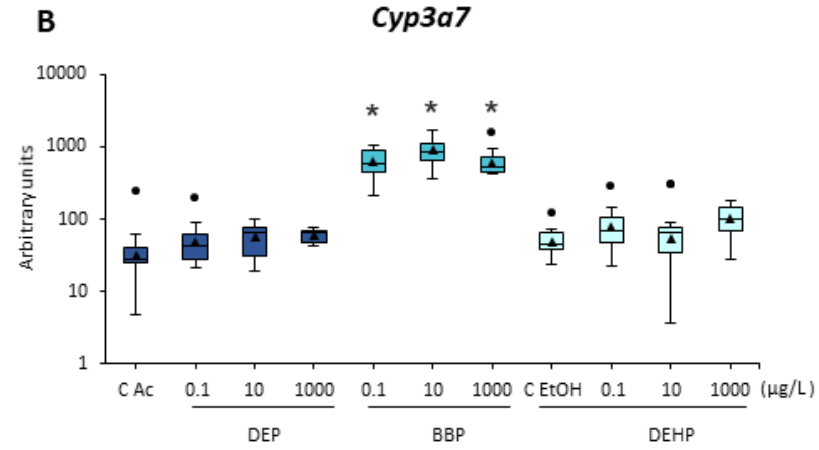
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP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100
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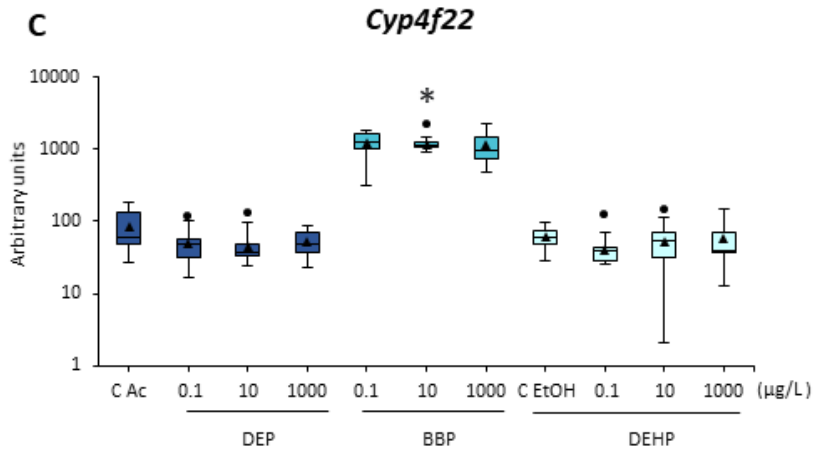
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP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Cyp4f22
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1000
100
10
1
C Ac
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10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP

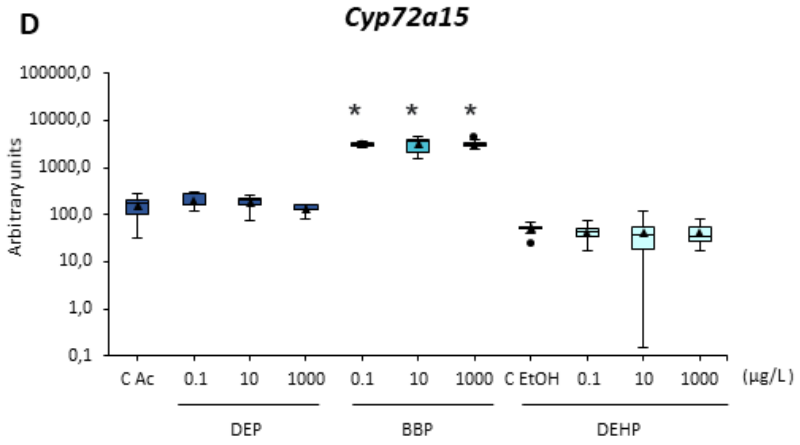
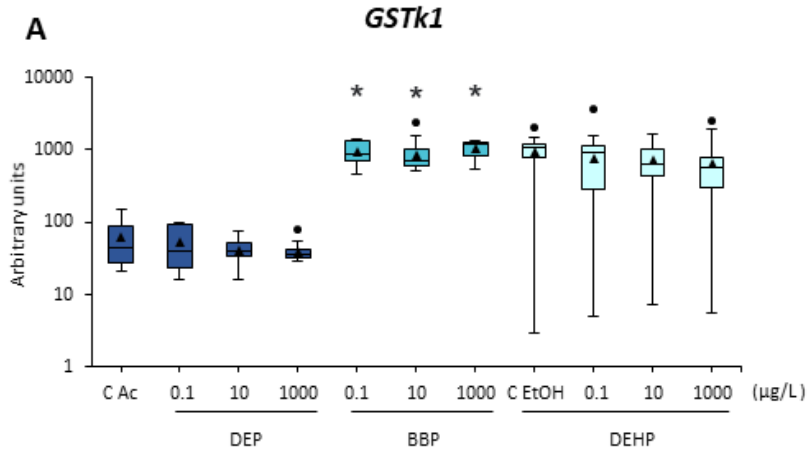
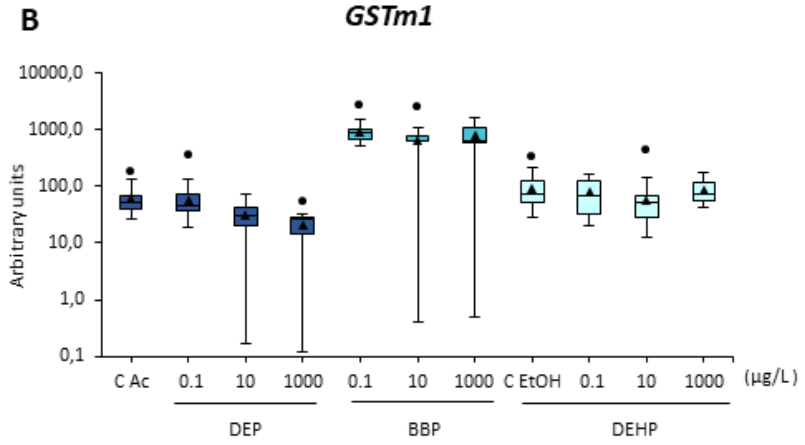


Figure 14. Expression of genes related to phase I (*Cyp2u1*, *Cyp3a7*, *Cyp4f22*, and *Cyp72a15*) detoxification in *P. acuta* adults after in vivo exposure to DEP, BBP and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

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10000,0
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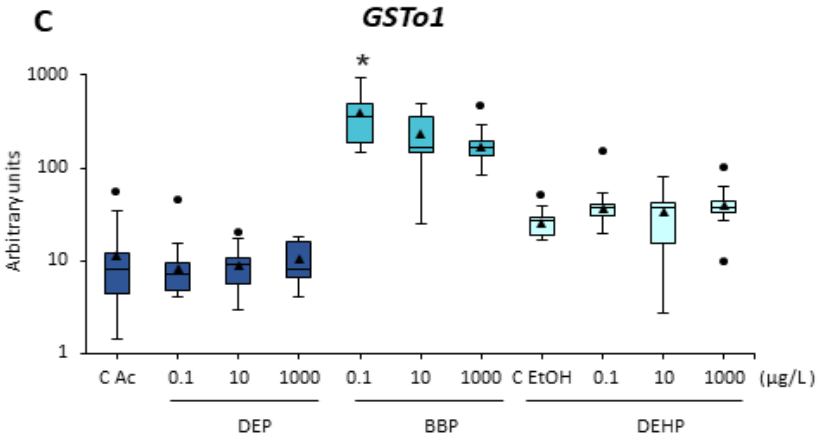
100,0
10,0
1,0
0,1
Arbitrary units
C Ac
0.1
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP


C
GSTo1
*
1000
100
10
1
Arbitrary units
C Ac
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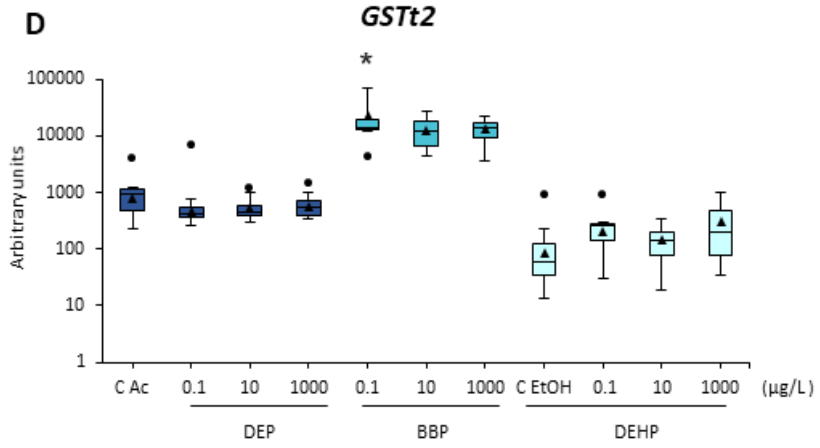
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP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10000
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100
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1
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1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP

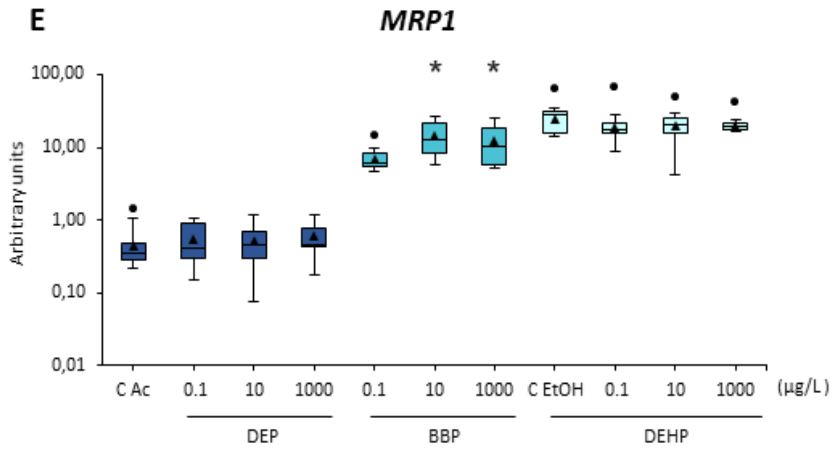
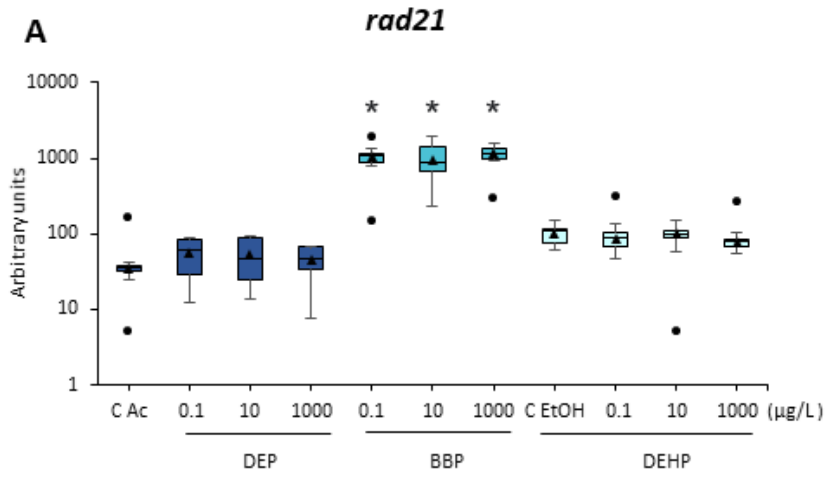


Figure 15. Expression of genes related phase II (*GSTk1*, *GSTm1*, *GSTo1*, and *GSTt2*), and phase III (*MRP1*) detoxification in *P. acuta* adults after in vivo exposure to DEP, BBP and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.2.3.4. Effects in DNA repair-related genes

To study the impact of phthalates in DNA repair mechanisms, the transcriptional levels of two genes involved in DNA repair were analyzed: *rad21* and *rad50*. *Rad21* codes a DNA double-strand break repair protein, and *rad50* belongs to the MRN protein complex (including Mre11, Rad50, and Nbs1) that participates in double-strand break repair (Watrin & Peters, 2006; Cheng et al., 2020). In addition, two apoptosis-related genes were included in the study, caspase 3-like (*Casp3*) and apoptosis-inducing factor 3 (*AIF3*). Casp3 is an executioner caspase that can cleave different proteins during apoptosis (Aquilino et al., 2019), and AIF3 is a mitochondrial protein released early in the apoptotic process translocated to the nucleus where it triggers chromatin condensation (Elvitigala et al., 2015).

As shown in figure 16, *rad21*, *Casp3*, and *AIF3* transcription increased at all tested concentrations of BBP, while *rad50* was induced at the two lowest concentrations (0.1 and 10 µg/L). According to the results, DNA repair and apoptosis seem to be modulated in *P. acuta* by altering the *rad21*, *rad50*, *AIF3*, and *Casp3* genes.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rad21
10000
1000
100
10
1
Arbitrary units
*
*
*
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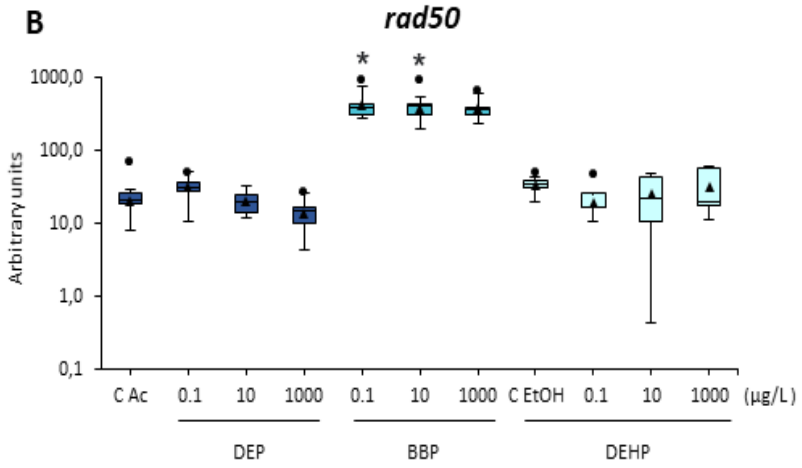
0.1
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP


B
rad50
1000,0
100,0
10,0
1,0
0,1
Arbitrary units
*
*
C Ac
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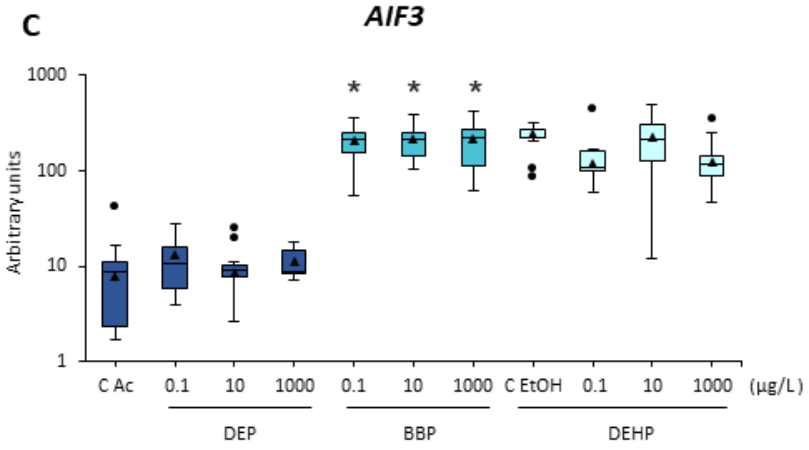
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP


C
AIF3
1000
100
10
1
Arbitrary units
*
*
*
C Ac
0.1
10
1000
0.1
10
1000
C EtOH
0.1
10
1000
(µg/L)
DEP
BBP
DEHP

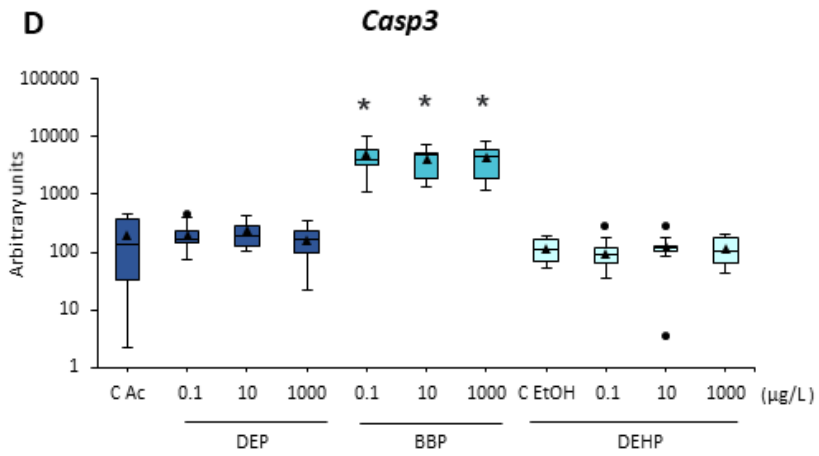


Figure 16. Expression of genes related to DNA repair (*rad21* and *rad50*) and apoptosis (*AIF3* and *Casp3*) in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.2.3.5. Effects in epigenetic regulation related genes

The alteration of three genes involved in histone and DNA modification (*KAT6B*, *HDAC1*, and *DNMT1*) related to epigenetic mechanisms was analyzed, as epigenetic regulation is considered one of the long-lasting effects of xenobiotics. Lysine acetyltransferase 6B (KATB6) is a histone acetyltransferase involved in histone acetylation, which can alter chromatin structure (Wiesel-Motiuk & Assaraf, 2020), while histone deacetylase 1 (HDAC1) promotes histone deacetylation. On the other hand, DNA methyltransferase 1 (DNMT1), involved in DNA methylation, maintains previously established methylation patterns across cell generations (Glastad et al., 2011).

As observed in figure 17, *KAT6B* and *DNMT1* were upregulated at the two lowest concentrations ((0.1 and 10 µg/L), while *HDAC1* transcription increased at all tested concentrations of BBP. Under the tested conditions, BBP seems to alter epigenetic-related genes.

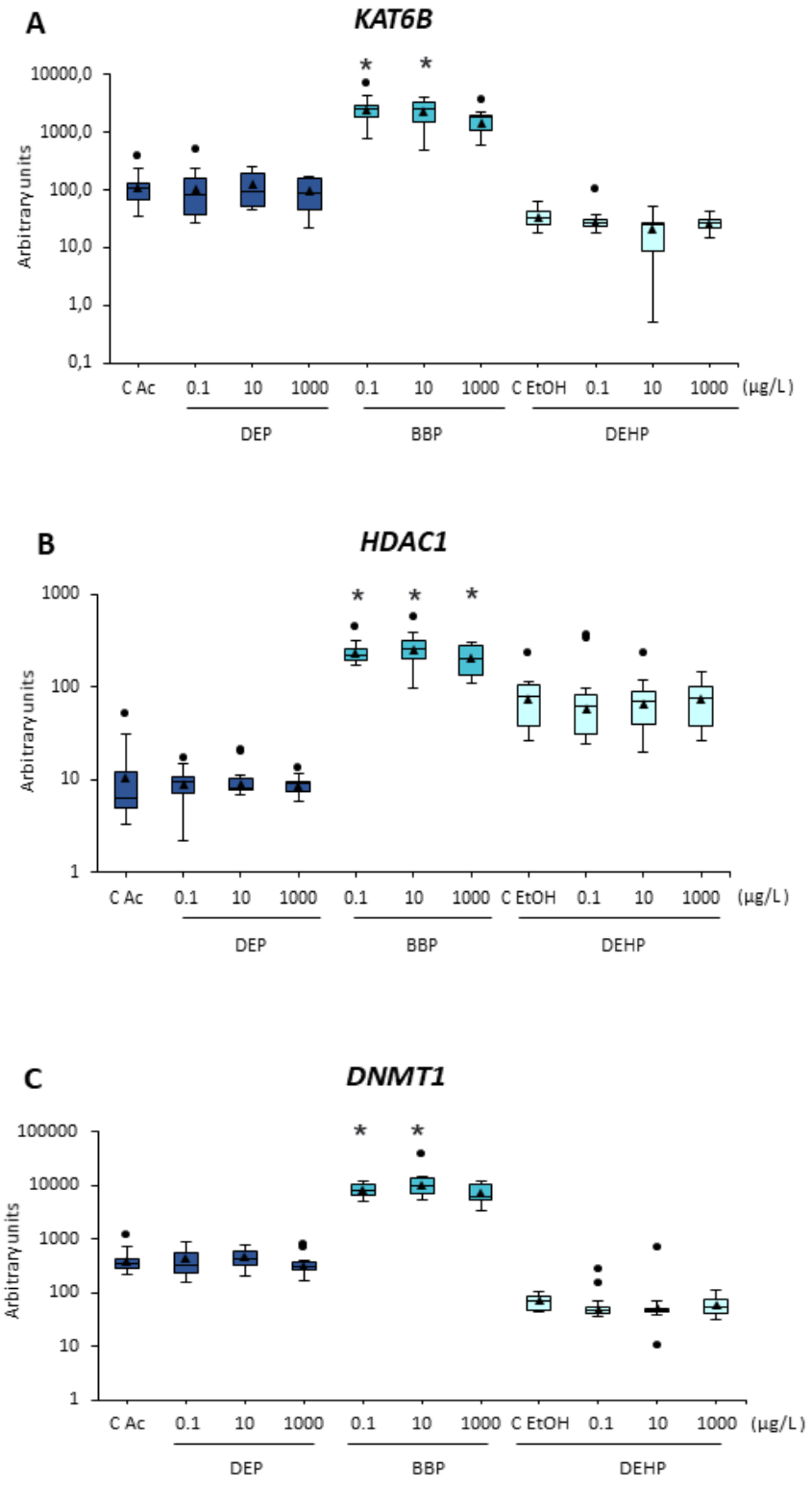
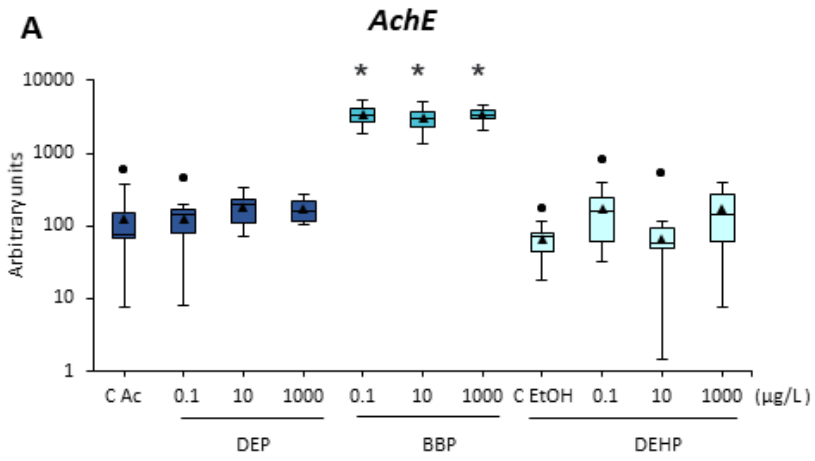


Figure 17. Expression of epigenetic-related genes (*KAT6B*, *HDAC1*, and *DNMT1*) in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.2.3.6. Effects on the nervous and immune system, energy metabolism, and lipid transport related genes

To test the ability of the phthalates to affect the nervous system, *AChE* gene expression was analyzed. The sequence codes for acetylcholinesterase (AChE), an enzyme involved in nerve impulse transmission. The remaining identified genes allow the analysis of several pathways that can be altered by toxicants, such as the immune system (*ApA*), energy reserves (*PYGL*), and lipid transport (*ORP8*).

The L-amino acid oxidase Aplysianin-A (ApA) is a glycoprotein involved in the immune response by acting as an antibacterial (Caballero et al., submitted), glycogen phosphorylase (PYGL) is involved in the regulation of glycogen metabolism and oxysterol-binding protein-related protein 8 (ORP8) belongs to the OSBP/ORP family, involved in lipid transport. As detailed in figure 18, *AChE*, *ApA*, *PYGL*, and *ORP8* transcription increased significantly at all tested concentrations of BBP, suggesting that BBP could alter the nervous system, the immune system, energy metabolism and lipid transport at tested conditions.



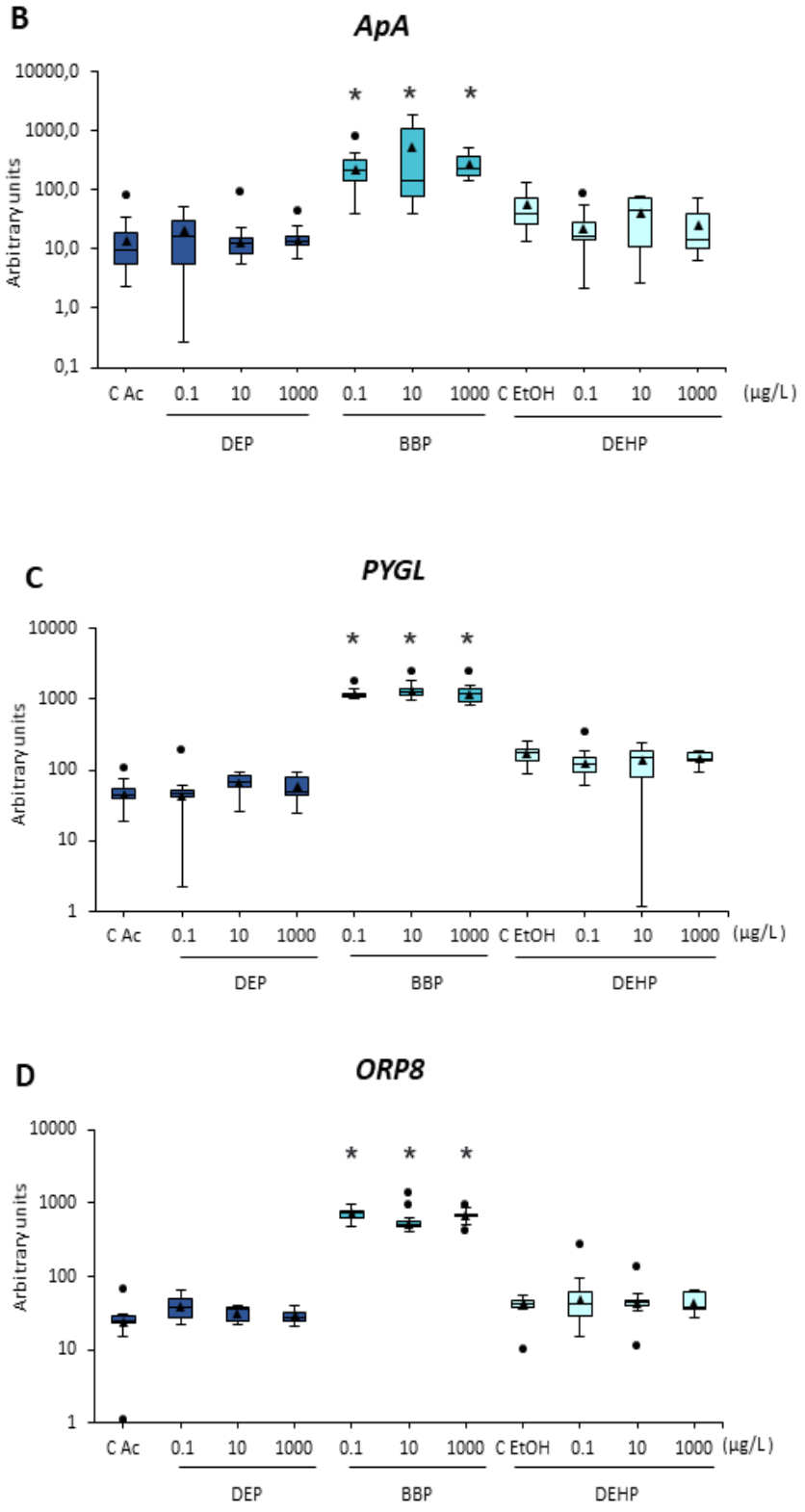


Figure 18. Expression of genes related to the nervous system (*AChE*) immune system (*ApA*), energy metabolism (*PYGL*), and lipid transport (*ORP8*) in *P. acuta* adults after in vivo exposure to DEP, BBP, and DEHP for one week. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.3. Effects of BBP on *Physa acuta* juveniles

5.3.1. Survival

As for the adults, juvenile individuals of *Physa acuta* were exposed to 10, 100, 1000, and 10000 µg/L of BBP to assess its potential toxicity. The possible lethal effects were observed every 24 hours for 96 hours. No significant survival reductions were observed after exposing the juvenile individuals at any of the concentrations tested (figure 19).

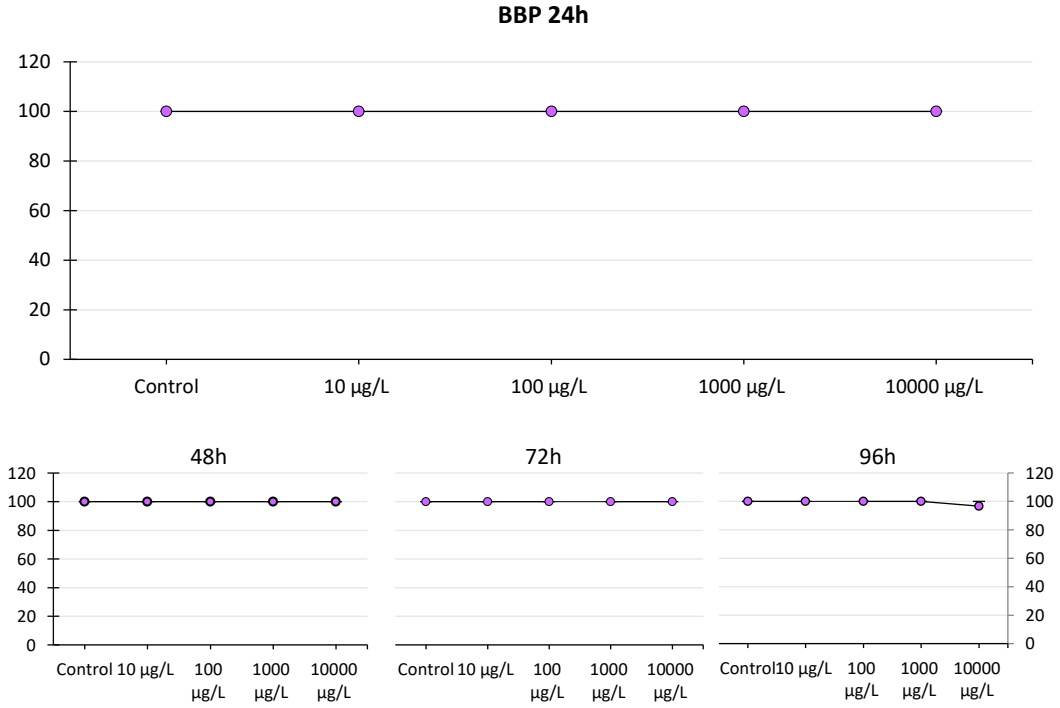
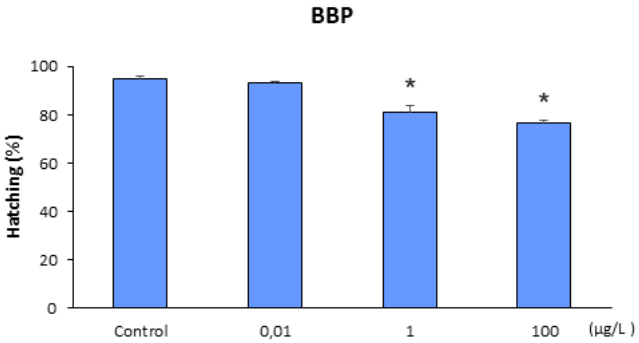


Figure 19. Survival analysis of *P. acuta* juveniles exposed to 10, 100, 1000, and 10000 µg/L of BBP after 24, 48, 72, and 96 hours.

5.3.2. Embryotoxicity

Embryos are generally found to be the most sensitive phase in the life cycle of a living organism. Egg masses were divided into two halves as the eggs belonging to the same clutch develop synchronically. Embryotoxic effects were analyzed through the hatching percentages during a 21-day embryonic development test. Embryos were exposed to 0.01, 1, and 100 µg/L of BBP from oviposition until hatching, and the number of hatched eggs was counted. As observed in figure 20, the exposure to 1 and 100 µg/L of BBP significantly affects the hatching success, while for 0.01 µg/L treatment, there are no significant differences compared to control. The hatching success was affected in a dose-dependent manner, reaching its minimum at 100 µg/L with a percentage of 76.6% hatched eggs.



Treatment (µg/L)	Hatching success (%)
Control	94.8 ± 1.7
0,01	92.9 ± 1.7
1	81.2 ± 4.2*
100	76.6 ± 1.8*

Figure 20. Hatching percentage of *P. acuta* embryos after exposure to 0.01, 1, and 100 µg/L of BBP. (* p ≤ 0.05).

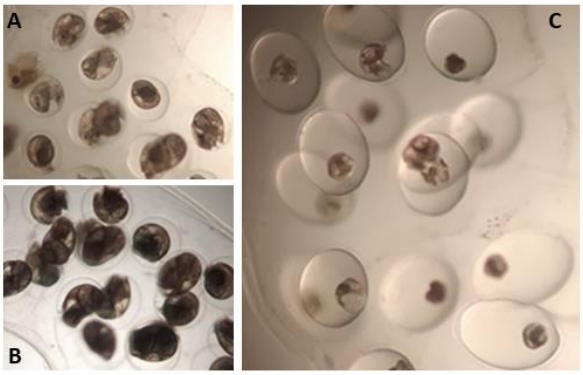


Figure 21. (A and B) Normal embryos at the 14-day stage. (C) Malformed and dead embryos at the 14-day stage.

5.3.3. Gene expression effects

Considering the lack of alterations after the exposure of DEHP and DEP and the response to BBP treatments, a second experiment was conducted solely with BBP. The gene expression analysis was performed exposing juvenile individuals of *P. acuta* for 15 days and 30 days to three concentrations of BBP; 0.01, 1, and 100 µg/L, which did not have any lethal effects. The genes analyzed were the same as those in section 5.2.3. The analysis revealed that BBP exposure for 15 days modified the mRNA levels of just three genes (*Hsp90*, *Hsc70-4*, and *GSTt2*), while it did not modify any of the genes after one month of exposure to BBP.

5.3.3.1. Effects in endocrine system related genes

As in section 5.2.3.1, with the aim to evaluate the potential alterations of BBP in the endocrine system, *Hsp90* expression was analyzed. As observed in figure 22, after 15 days of exposure, *Hsp90* was downregulated at 0.01 µg/L, while after one-month exposure, *Hsp90* was not altered at the tested concentrations (figure 22).

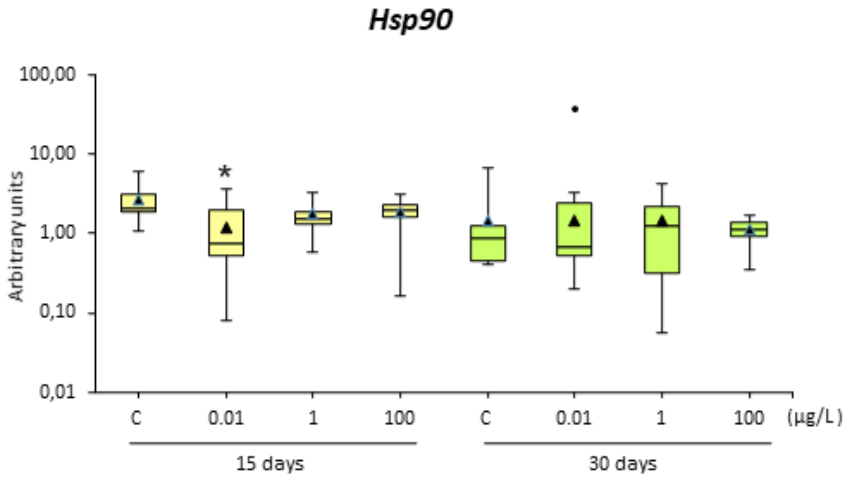
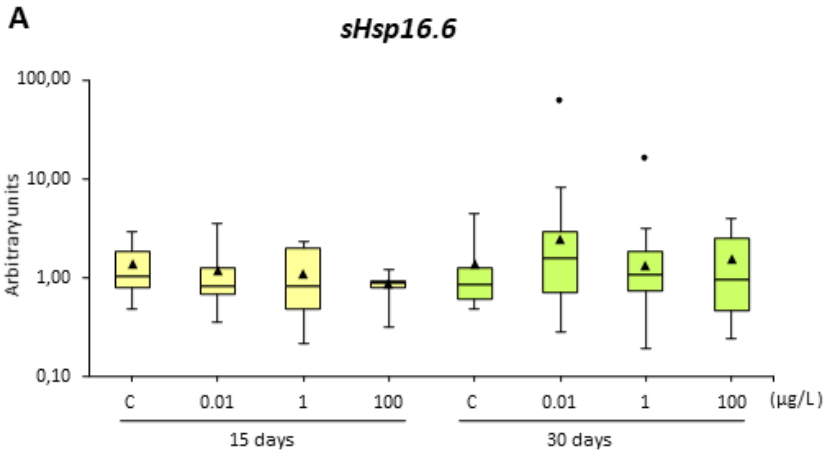


Figure 22. Expression of *Hsp90* in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.3.3.2. Effects in cellular stress response-related genes

To evaluate the stress response after exposing juveniles to BBP, the gene expression profile of the following genes was analyzed: s*Hsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4*, and *Grp78*.

After one month of exposure, the analysis of the stress-related genes showed similar levels compared to controls for all the tested concentrations of BBP. However, after 15 days of exposure, there was a significant inhibition in the mRNA levels for the lowest concentration (0.01 µg/L) of *Hsc70-4* (see figure 23C). Due to technical problems, the *Hsp60* samples after one month of exposure did not amplify. According to this data, BBP could alter the cellular stress response in a non-dose-dependent manner.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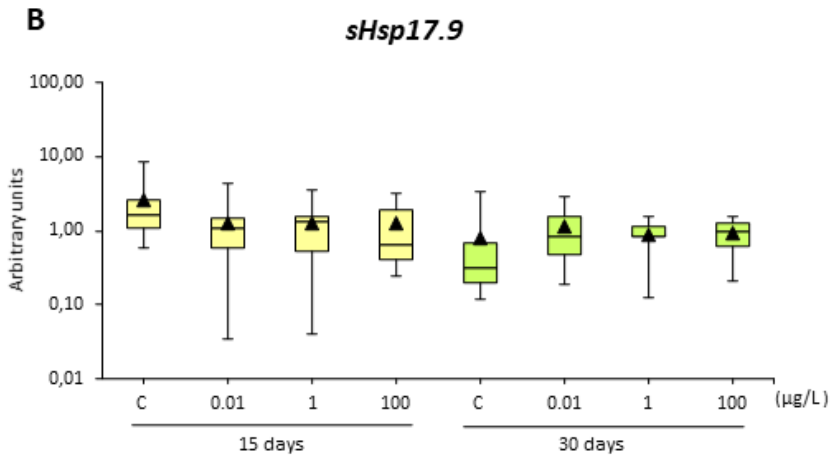
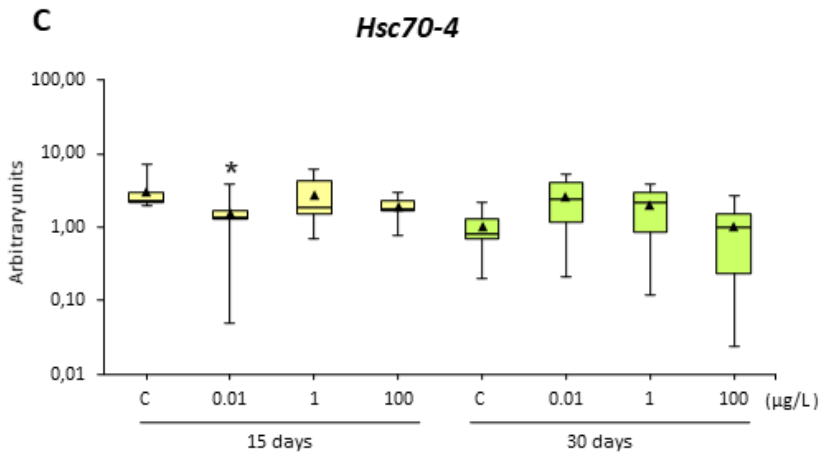
100
C
0.01
1
100
(µg/L)
15 days
30 days


B
sHsp17.9
100,00
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days


C
Hsc70-4
100,00
10,00
1,00
0,10
0,01
Arbitrary units
*
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days

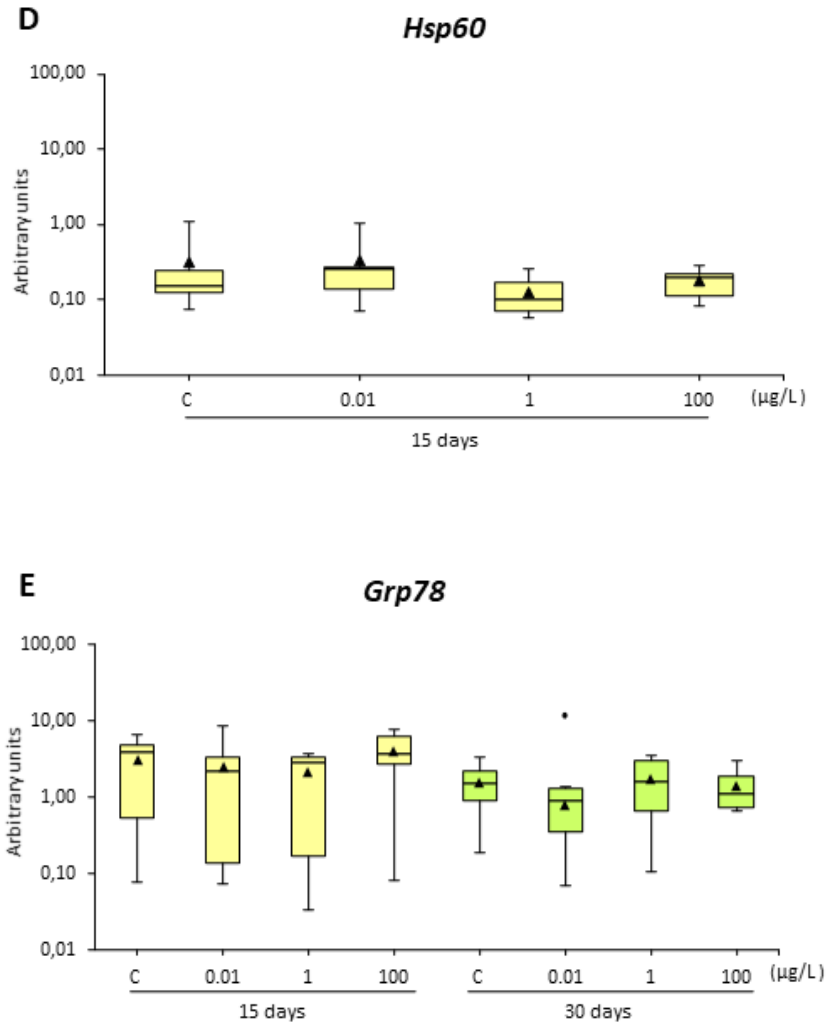
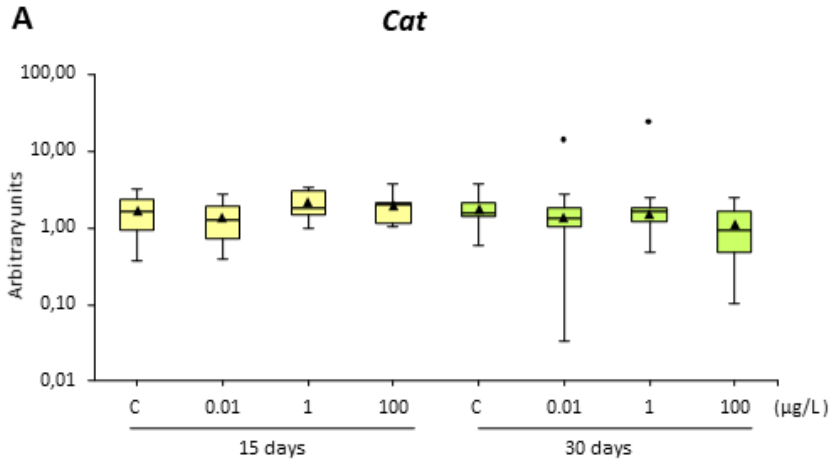
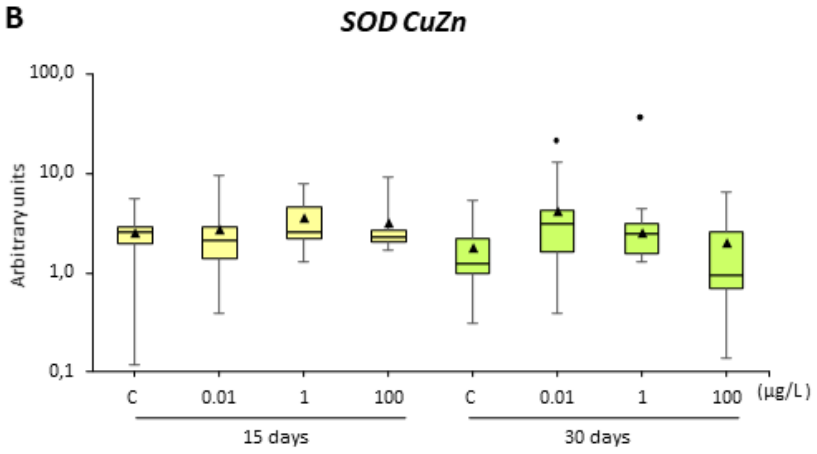
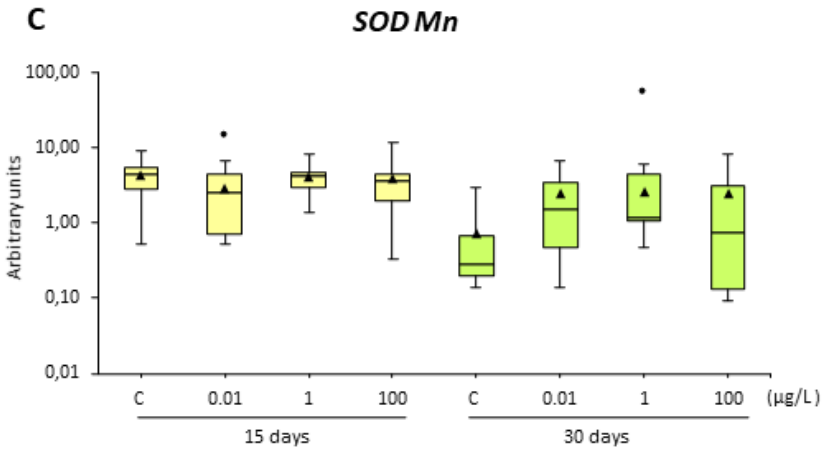


Figure 23. Expression of genes related to stress (*sHsp16.6*, *sHsp17.9*, *Hsc70-4*, *Hsp60*, and *Grp78*) in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

The expression of three oxidative stress-related genes (*Cat*, *SOD Mn*, and *SOD Cu/Zn*) and the hypoxia-related gene *HIF1α* were analyzed. As shown in figure 24, the transcription levels of the studied genes were not altered after the exposure to 0.01, 1, and 100 µg/L of BBP, suggesting that exposure to BBP during 15 and 30 days does not seem to affect the oxidative stress-related genes analyzed.


A
Cat
100,00
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days


B
SOD CuZn
100,0
10,0
1,0
0,1
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days


C
SOD Mn
100,00
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days

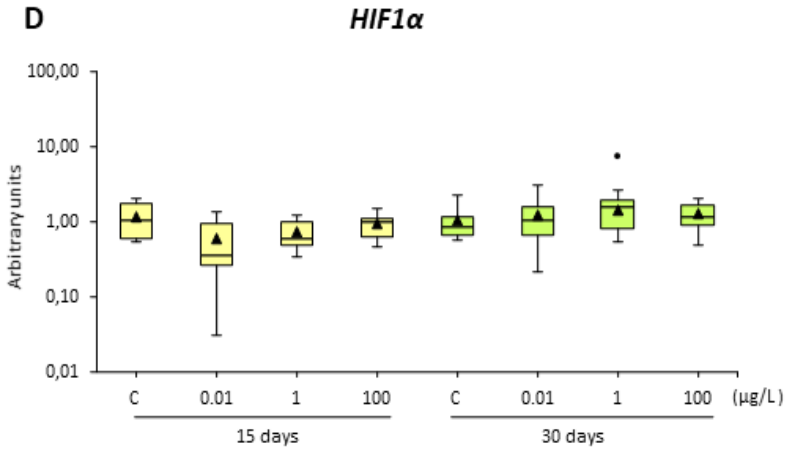
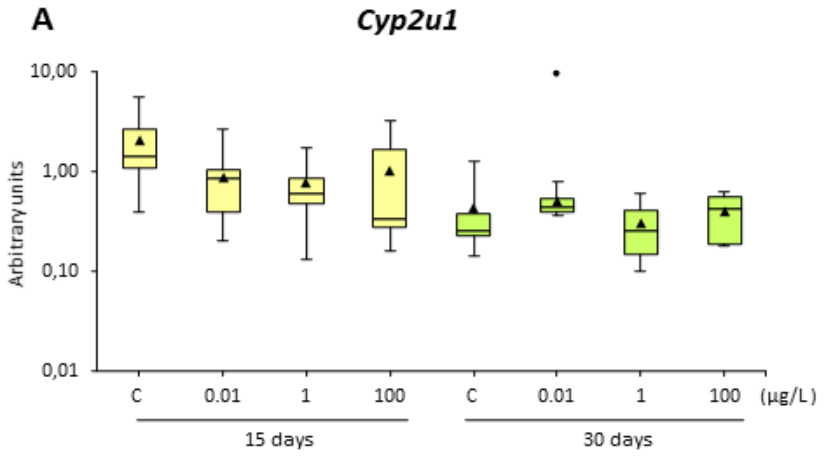


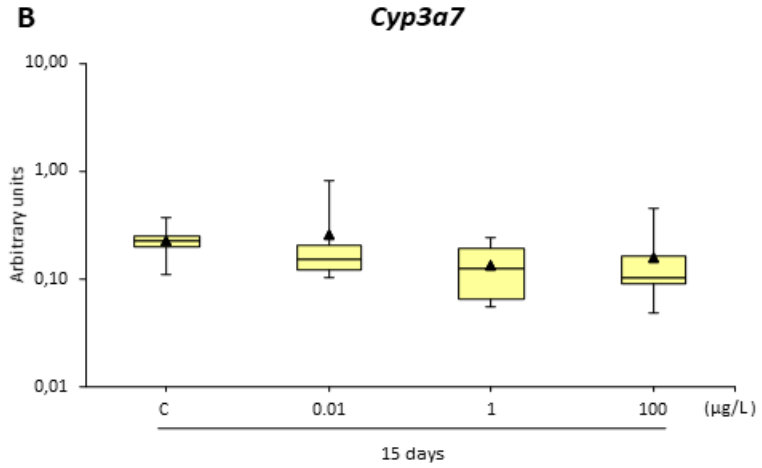
Figure 24. Expression of genes related to oxidative stress (*Cat*, *SOD Mn*, and *SOD Cu/Zn*) and hypoxia (*HIF1α*) in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

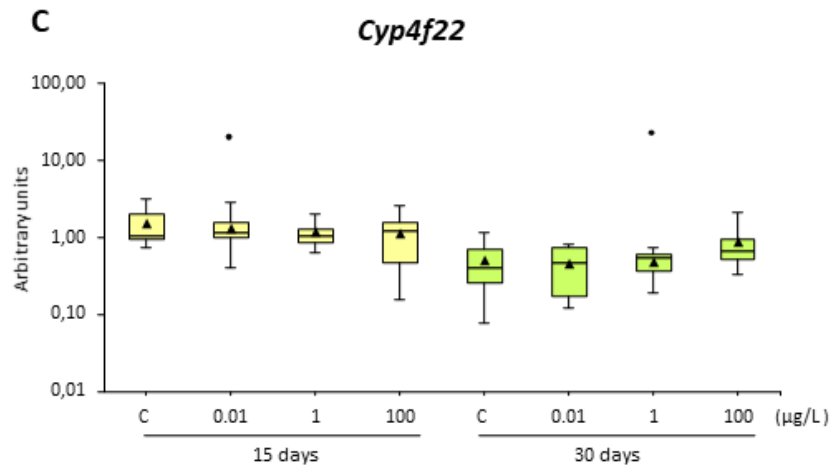
5.3.3.3. Effects in detoxification mechanisms related genes

The detoxification mechanisms were studied through the expression profile of genes that code for enzymes involved in the three detoxification phases: *Cyp2u1*, *Cyp3a7*, *Cyp4f22,* and *Cyp72a15* (phase I), *GSTo1*, *GSTt2*, *GSTk1*, and *GSTm1* (phase II) and *MRP1* (phase III). Figures 25 and 26 show the transcription levels of the studied genes.

Regarding phase I, the exposure for 15 and 30 days at 0.01, 1, and 100 µg/L did not produce statistically significant differences in any of the genes analyzed. For phase II representatives, after 15 days of exposure, only *GSTt2* showed significant inhibition at the lowest concentration (0.01 µg/L), while for the other two concentrations, the levels were similar to control (figure 26D). The same trend can be observed when analyzing the rest of phase II-related genes and the phase III representative (*MRP1*) after a 15 and 30 days exposure to BBP, showing similar mRNA levels compared to control. Thus, under the tested conditions, only phase II seems to activate in the presence of BBP after 15 days of exposure. For both, *Cyp3a7* and *GSTt2* the samples after one month of exposure did not amplify, due to technical problems.


A
Cyp2u1
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days


B
Cyp3a7
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
(µg/L)
15 days


C
Cyp4f22
100,00
10,00
1,00
0,10
0,01
Arbitrary units
C
0.01
1
100
C
0.01
1
100
(µg/L)
15 days
30 days

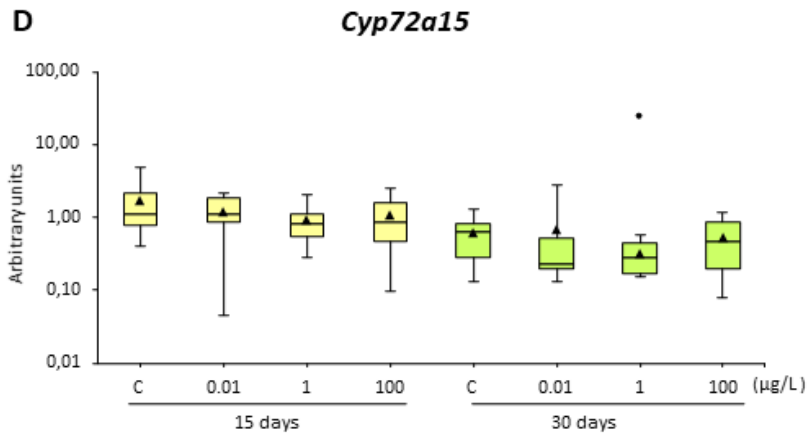
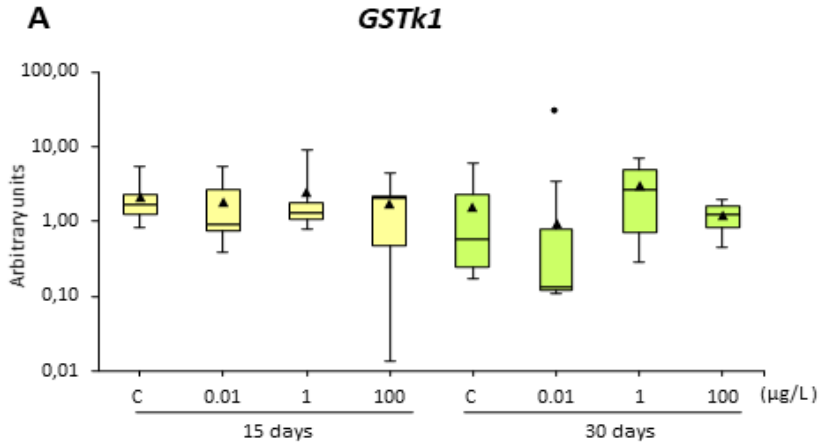
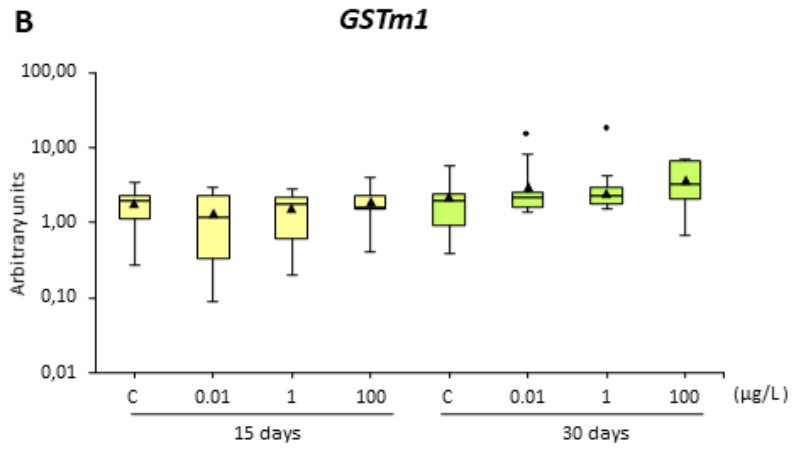


Figure 25. Expression of genes related to phase I (*Cyp2U1*, *Cyp3a7*, *Cyp4f22*, and *Cyp72a15*) detoxification in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).





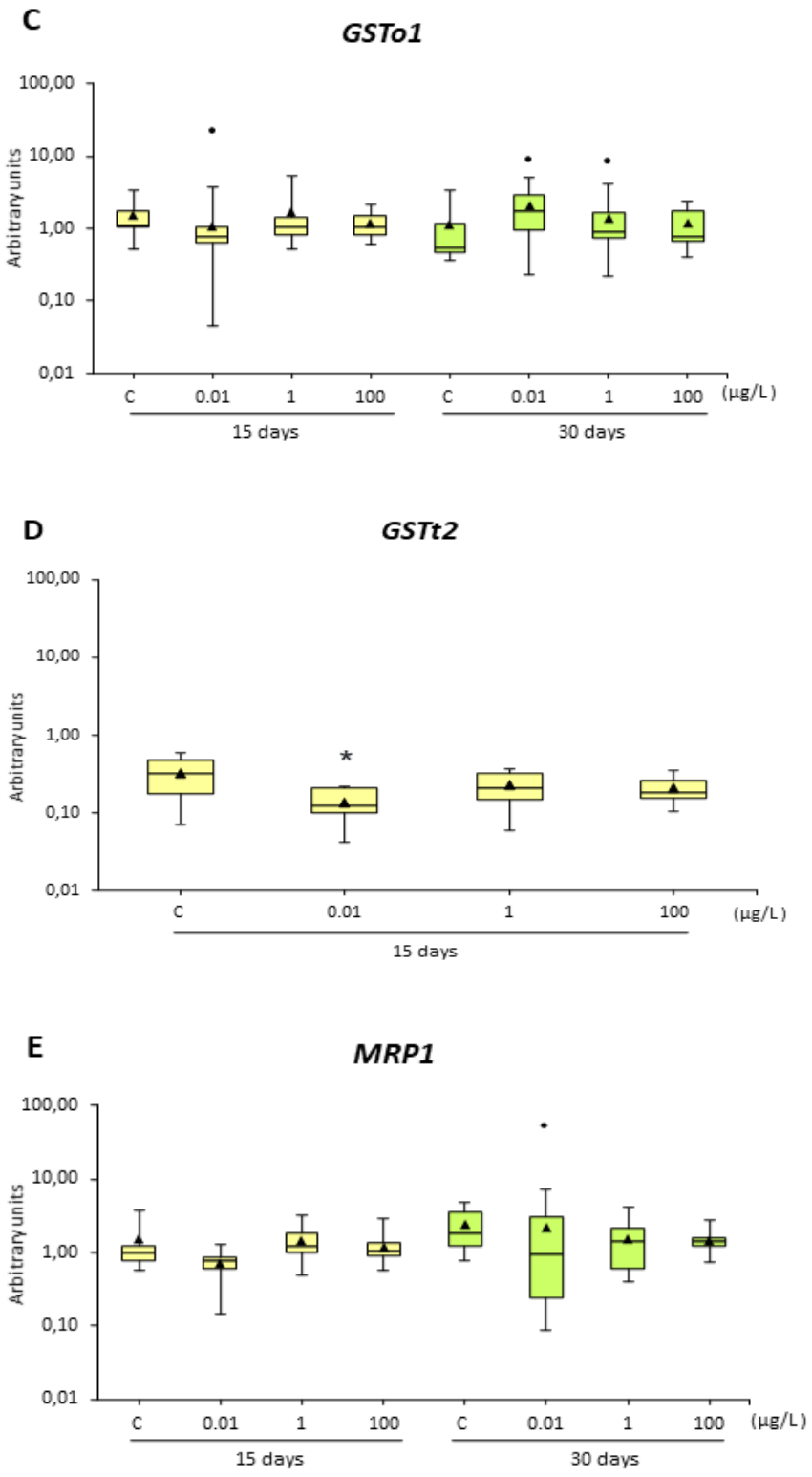


Figure 26. Expression of genes related to phase II (*GSTk1*, *GSTm1*, *GSTo1*, and *GSTt2*), and phase III (*MRP1*) detoxification in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

The alteration of the GST could be reflected in the activity of the enzyme, so GST enzymatic activity related to the detoxification process was evaluated. No significant differences were observed after neither 15 days nor after 30 days of exposure to BBP (figure 27).

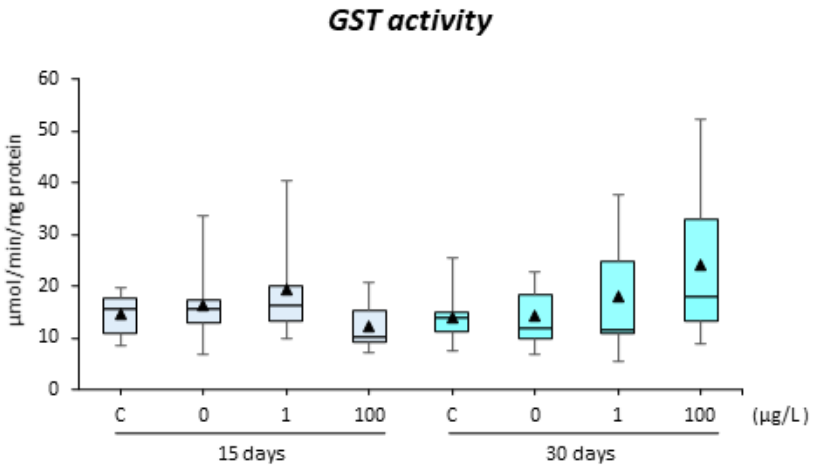
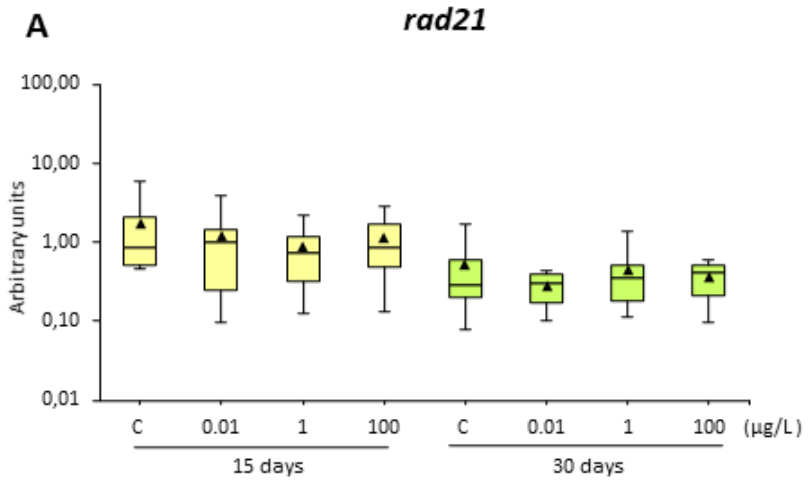


Figure 27. GST enzymatic activity in *P. acuta* juveniles after *in vivo* exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean. Significant differences (* $p < 0.05$).

5.3.3.4. Effects in DNA repair-related genes

As previously mentioned, the activity of four genes related to DNA repair mechanisms (*rad21* and *rad50*) and apoptosis (*Casp3* and *AIF3*) was assessed. Unlike the results obtained with adult individuals (see section 5.2.3.4.), the transcription levels at all tested concentrations were not altered for any studied genes. According to the results, DNA repair and apoptosis do not seem to modulate after 15 and 30 days of exposure to BBP.



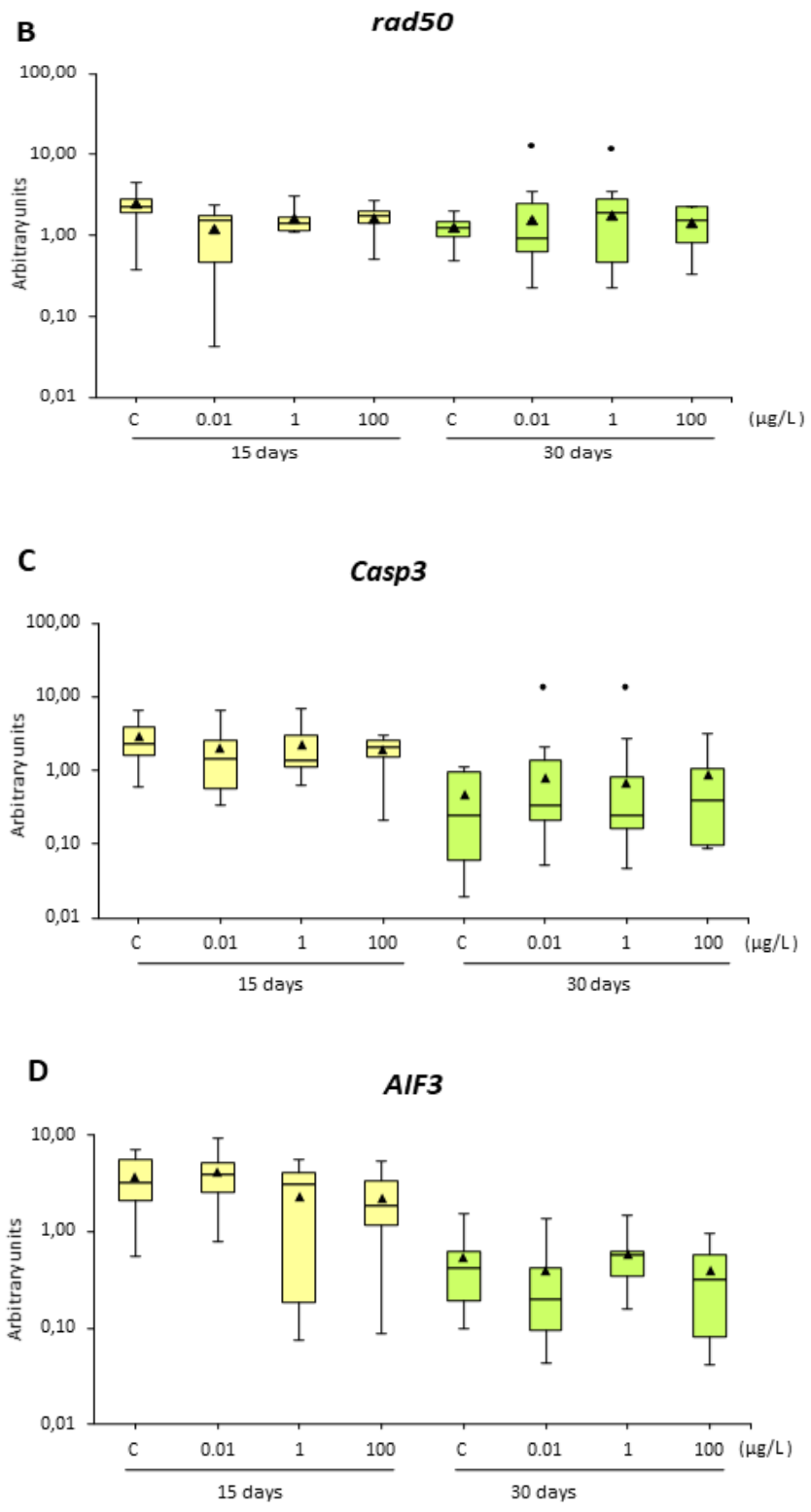
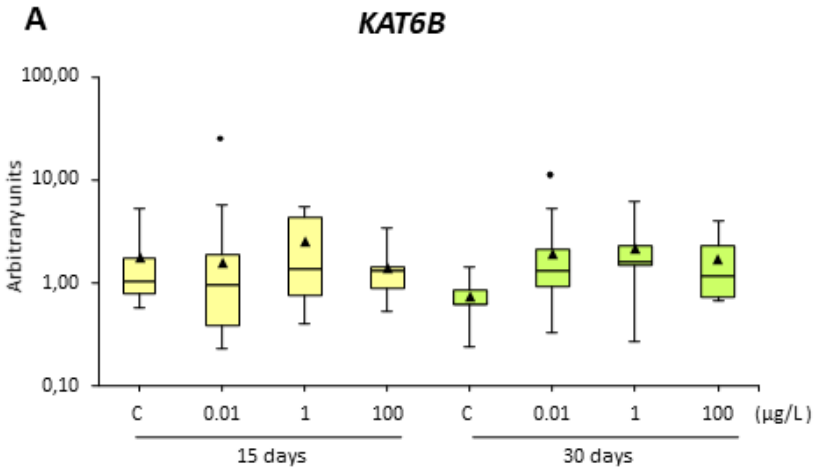
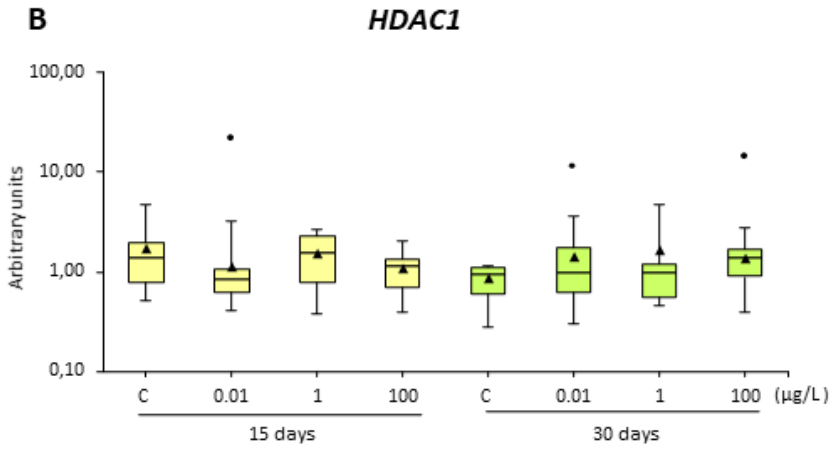


Figure 28. Expression of genes related to DNA repair (*rad21* and *rad50*) and apoptosis (*AIF3* and *Casp3*) in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.3.3.5. Effects in epigenetic regulation related genes

The mRNA expression levels of genes involved in histone and DNA modification (*KAT6B*, *HDAC1*, and *DNMT1*) did not show any changes compared to controls' expression (see figure 29). The data suggest that exposure to 0.01, 1, and 100 µg/L of BBP during 15 and 30 days does not significantly affect the studied epigenetic-related genes.





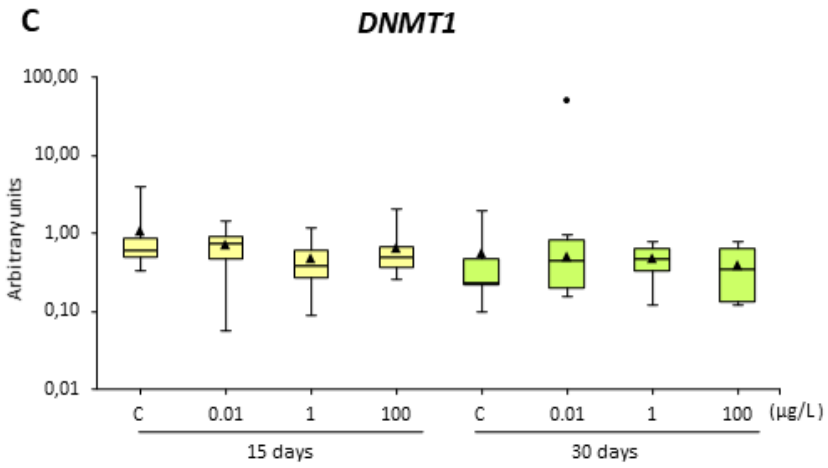
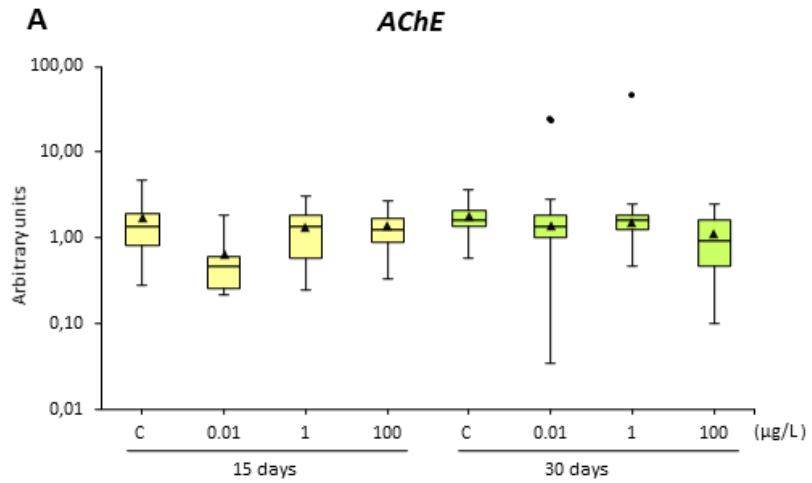


Figure 29. Expression of epigenetic-related genes (*KAT6B*, *HDAC1*, and *DNMT1*) in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

5.3.3.6. Effects on the nervous and immune system, energy metabolism, and lipid transport related genes

The activity of the immune system (*ApA*), energy reserves (*PYGL*), and lipid transport (*ORP8*) related genes were analyzed. As detailed in figure 30, the transcription of *ApA*, *PYGL*, and *ORP8* was not altered at any of the tested concentrations of BBP. To evaluate the potential of BBP to affect the nervous system, *AChE* gene expression was analyzed. As observed in figure 30A, *AChE* transcription was not modulated at any tested concentrations.



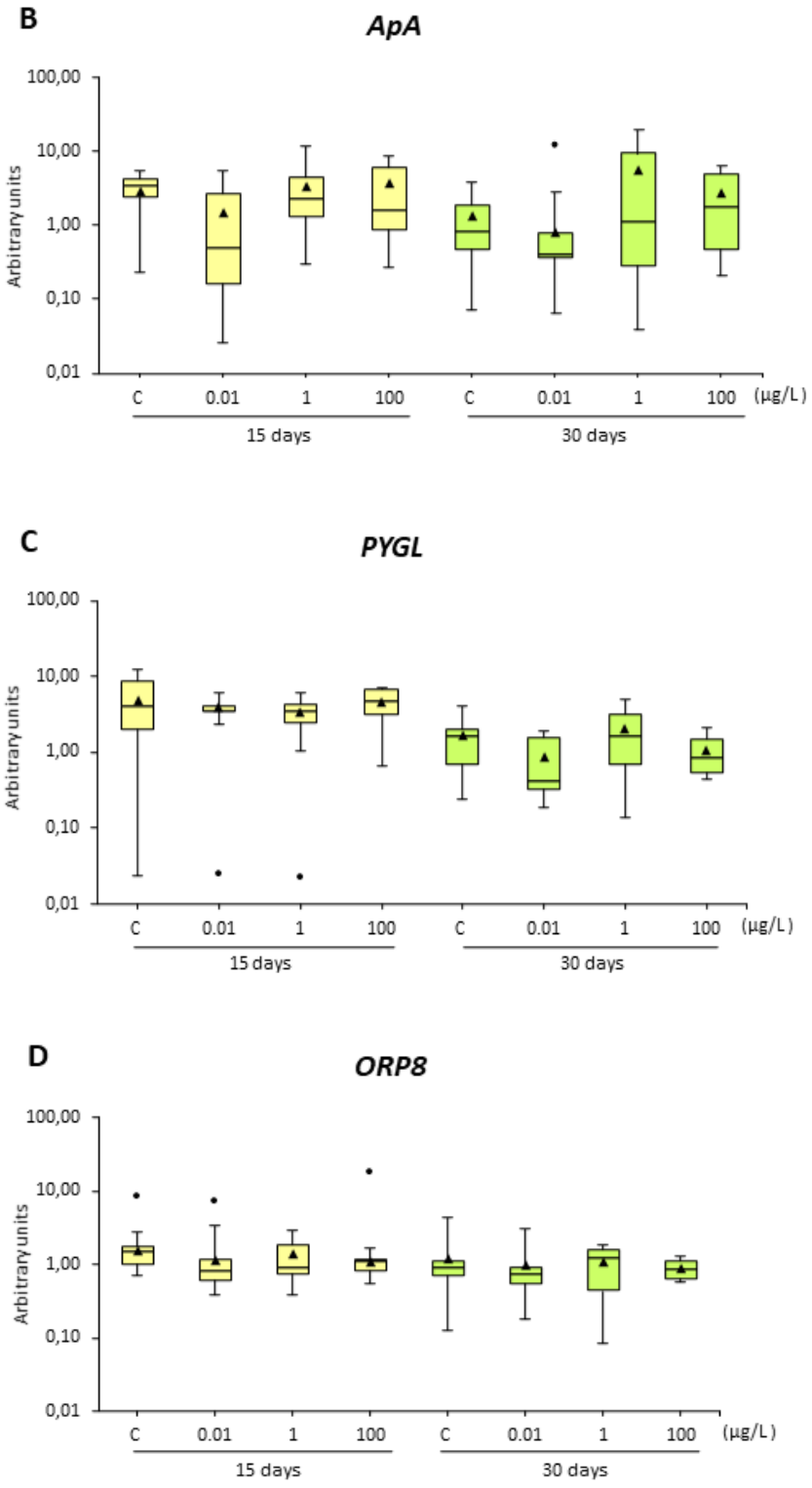


Figure 30. Expression of genes related to the nervous system (*AChE*) immune system (*ApA*), energy metabolism (*PYGL*), and lipid transport (*ORP8*) in *P. acuta* juveniles after in vivo exposure to BBP for 15 and 30 days. Whisker boxes are shown (n=9 individuals per box). The horizontal line within the box indicates the median, and 25th and 75th percentiles are indicated by the box's boundaries. The highest and lowest results are represented by the whiskers. The triangle inside the box denotes the mean, and the outliers are shown (circles). Significant differences (* $p < 0.05$).

DISCUSSION

6. DISCUSSION

Plastics are incredibly versatile materials, inexpensive, and durable with a vast range of applications. However, because of its excessive use and ineffective waste management, its occurrence in the environment is a growing problem. The polymers themselves and the compounds used as additives, like phthalates, are released during the degradation process. Hence, the presence of these plastic additives is increasing in the environment. The impact of three phthalates (BBP, DEHP, and DEP) in two aquatic invertebrates, *Physa acuta* and *Paracentrotus lividus*, was assessed in this work.

6.1. Effects on the sea urchin *Paracentrotus lividus*

It is commonly accepted that toxicants can show different effects depending on the developmental stage. In this sense, aquatic organisms' early stages are usually especially sensitive to environmental stressors. Several studies have demonstrated the reliability of *P. lividus* for toxicology studies, mainly with the early stages of development such as embryos and plutei (Bellas et al., 2005; Gambardella et al., 2013; Paredes et al., 2014; Morgana et al., 2016; Macedo et al., 2017).

The present work has taken advantage of it to analyze the impact that phthalates have on the early developmental stages of *P. lividus*. The developmental anomalies on the plutei larvae stage have been selected as an acute endpoint that provides information about deformities caused by toxicants. Furthermore, the swimming speed alteration (SSA) was selected as an endpoint since it is a behavioral endpoint that can provide information about the physiological impact of the toxicant. Both endpoints have been analyzed by exposing *P. lividus* embryos for 72 hours to six different concentrations of BBP, DEHP, and DEP (0.01, 0.1, 1, 10, 100, and 1000 µg/L).

Swimming Speed Alteration (SSA)

In echinoderms, the swimming behavior of larvae plays a vital role in their survival and dispersal by enabling them to avoid predators or locate food sources (Wheeler et al., 2016). Besides, the swimming activity can be affected by morphological characteristics such as size, shape, or anomalies, and different environmental parameters. Thus, alteration in swimming speed is a valuable endpoint that can assess the impact of environmental pollutants (Morgana et al., 2016).

Using the SBR system, the swimming speed of *P. lividus* plutei was recorded. The mean swimming speed measured at 72h after exposure to phthalates was significantly different from controls**.** The swimming speed value for controls matched with those reported for plutei (Morgana et al., 2016; Mogami et al., 1988). In this regard, the SBR system proved to be a sensitive tool for quantifying swimming behavior of the early developmental stages of *P. lividus* in response to phthalates**.**

Contrarily to the morphological anomalies resulting after phthalates exposure, where DEP displayed the weakest effects, for the SSA, the effects on *P. lividus* plutei were more potent than those observed after BBP and DEHP exposure. The highest swimming speed inhibition at the maximum concentration tested (100 µg/L) was almost identical to DEP and DEHP, 40.34 and 40.28%, respectively. Both, DEP and DEHP, showed a dose-dependent response, but the DEP reached a plateau at 10 µg/L. Swimming speed alteration evaluated in this work provides a sensitive assessment of sub-lethal effects exerted by BBP, DEHP, and DEP. However, the sensitivity of this behavioral endpoint in *P. lividus* needs to be further investigated in the presence of other stressors to elucidate the potential in environmental risk assessment.

To date, no studies have addressed the effects of phthalates in the swimming behavior of echinoderms. Therefore, this is the first study evaluating the swimming speed alteration in the purple sea urchin *P. lividus* after BBP, DEHP, and DEP exposure. Nevertheless, alterations in the swimming behavior of *P. lividus* after exposure to different contaminants like cadmium chloride or polystyrene MPs have been reported (Morgana et al., 2016; Gambardella et al., 2018), showing alterations due to the presence of toxicants.

In other aquatic organisms, some studies are addressing the effects of phthalates. For example, in zebrafish, the swimming behavior was more lethargic on larvae after maternal exposure to DEP (Tseng et al., 2012), while a recent study showed that DEHP affected the swimming speed of zebrafish larvae only co-exposed with overfeeding but not alone (Buerger et al., 2022). These data agree with the obtained results and support that the phthalates can modify the swimming behavior, which can affect the ability of the animal to feed and move in the environment, risking the future of the population viability.

Developmental anomalies

Morphological anomalies of the early developmental stages of the sea urchin *P. lividus* can be used to evaluate the potential toxicity of different xenobiotics. A correct skeleton formation is essential for larval survival and, thus, for the continuity of the population. Altered skeletogenesis has been reported after exposure of *P. lividus* gametes (Bellas et al., 2005; Gambardella et al.,

2013; Mesarič et al., 2015) and early embryos (Carballeira et al., 2012; Siller et al., 2013) to different environmental contaminants like antifouling biocides, graphene oxide, engineered nanoparticles or aquaculture effluents.

An embryotoxicity test was performed in the present work by exposing *P. lividus* embryos to several BBP, DEHP, and DEP concentrations. The three phthalates significantly affected *P. lividus* development, resulting in abnormal arrangements of the skeletal rods in 72h plutei. However, the impact of each compound varies. BBP is the most toxic, which shows a statistically significant increase of abnormalities from the lowest concentration. The increase of the abnormalities is dose-dependent, being maximum at the highest concentration with 90% of the plutei affected. DEHP and DEP show a weaker effect, being more toxic DEHP than DEP, with the lower concentrations not showing significant increases in the abnormalities.

There is no previous data in *P. lividus* analyzing developmental anomalies concerning phthalates. However, some alterations have been observed in the sea snail *Haliotis diversicolor supertexta.* BBP and DEP caused specific malformations at the blastula stage, reducing the metamorphosed larvae, being more toxic BBP than DEP, while DEHP did not affect embryonic development (Liu et al., 2009). These data suggest that BBP is probably the most active phthalate affecting development, but the results in DEHP indicate that the impact of each phthalate could depend on the species. Conversely, phthalates also caused malformations and developmental retardation in the abalone (Zhou et al., 2012), but at higher concentrations than those tested in this study (over 2 mg/mL). In the polychaete *Galeolaria caespitosa*, the exposure of spermatozoa to the phthalate DBP caused embryonic arrest and malformations (Lu et al., 2017).

Embryotoxicity has also been observed for BBP in *Daphnia magna*, showing abnormal neonates and a shortened embryonic development cycle (Li et al., 2021). These authors performed RNA-Seq of early embryo treated with 0.1 mg/L BBP, observing that the pathways involved in signal transduction, cell communication, and embryonic development were significantly down-regulated (Li et al., 2021). Further research is needed to elucidate the mechanisms involved in the effect. Still, it can be assumed that, probably, abnormalities are a consequence of previously effects observed in other organisms in the presence of phthalates, such as oxidative stress, genotoxicity, and down-regulation of critical genes involved in development (dos Santos Morais et al., 2020; Li et al., 2021).

This is the first study that shows the developmental and behavioral alterations in the sea urchin *P. lividus* after exposure to BBP, DEHP, and DEP. Swimming speed alteration was analyzed with the development anomalies as an acute endpoint.

6.2. Effects on the freshwater gastropod *Physa acuta*

Ecological relevant endpoints

The first approach to evaluate the potential toxicity of a compound is to analyze its effect on survival in the model organism used. In this way, it is possible to know the sensitivity of the individual organism and select the proper concentrations to perform further ecotoxicological studies. Survival analysis for juvenile and adult individuals of *P. acuta* was performed by exposure to four different concentrations of BBP, DEHP, and DEP (10, 100, 1000, and 10000 µg/L). For the three compounds, none of the concentrations tested significantly affected the survival, neither for juveniles nor for adults, reaching survival rates of 100% or very close to 100% in all experiments.

As in this work, DEHP did not significantly affect the survival of other invertebrates like the larvae of *C. riparius*, which contrasts with the significant mortality caused by BBP. It is worth mentioning that the highest concentration tested for *C. riparius* was higher than the one tested in this study (100 mg/L) (Planelló et al., 2011). On the other hand, in *Daphnia magna*, survival was significantly reduced at 811 µg/L DEHP after 7 days exposure (Knowles et al., 1987). From these data, the survival to phthalate exposure depends on different variables. Still, in the conditions tested, with concentrations similar to those found in the environment, the three phthalates tested are not lethal for *P. acuta*.

Although embryos that develop in an aquatic medium are usually exposed to adverse conditions and often possess protecting structures, the presence of xenobiotics in the aquatic medium can alter their development, affecting their hatching success. Besides, aquatic organisms' embryos are usually more sensitive to toxicants than adults. In the present study, the hatching success of *P. acuta* exposed to BBP was assessed, showing a significant effect on the hatching rate in a dose-dependent manner, which reached its minimum at 100 µg/L with a percentage of 76.7% hatched eggs. The effects of different xenobiotics in the hatching success of *P. acuta* have been reported. For example, the exposure to 1 mg/L of BPA severely reduced hatchability in *P. acuta* eggs. Similarly, the number of hatched snails decreased as copper concentration increased (Gao et al., 2017). In any case, the BBP affects the embryo's development and reduces the number of individuals who reach the juvenile stage. Therefore, it can have consequences in the population by reducing the number of individuals compromising its viability. Furthermore, as *P. acuta* is an essential link of the food web, it could also have consequences in the rest of the freshwater ecosystem, reducing food availability.

Gene expression

In the present work, a previously obtained transcriptome of *P. acuta* was used to identify 18 genes related to different pathways of interest in ecotoxicology and then examine how the exposure to phthalates changed the transcription of these genes. The processes of interest include the stress response, detoxification, DNA repair, apoptosis, immunity, energy reserves, and lipid transport. There is a growing interest in combining ecologically relevant endpoints with biochemical and molecular parameters to seek a more integrative analysis. An increase in the number of described genes will allow the design of standard arrays that could be combined with toxicity tests. Moreover, increasing the knowledge at the molecular level in *P. acuta* supports its use as a freshwater gastropod representative in toxicity studies.

On the other hand, there is a lack of model freshwater mollusks for standardized toxicity tests (which currently is one of the animal groups whose response to environmental stress is less known. Moreover, there only is a *Lymnaea stagnalis* reproduction test (No. 243: *Lymnaea stagnalis* Reproduction Test, OECD). In this sense, *P. acuta* has also been proposed as an alternative because it is easy to culture. However, more work is still necessary to design an appropriate standardized toxicity test to analyze different molecular and ecological relevant endpoints.

The 18 newly identified genes evaluated in this work showed homology with those previously described in other species, mainly with the freshwater snail *Biomphalaria glabrata*, which belongs to the Planorbidae family. The relevance of this work is to increase the putative genes that can be analyzed, increasing at the same time the number of cellular pathways that can be studied. On the other hand, the genes identified complemented those previously described, so an array was designed to perform the analysis. It can help other researchers analyze the impact of other toxicants, increasing the data about the response and sensitivity of *P. acuta* to environmental pollution.

Endocrine system related genes

The mollusk endocrine system is poorly understood, mainly in the gastropods. Although the presence of sex steroids like those found in vertebrates has been detected in mollusks, there is a debate regarding their biological role as the aromatase, which is a key enzyme involved in the last step of steroid biosynthesis, has not been identified in mollusks. This means that if mollusks synthesize sex steroids, they will have to do so via an independently evolved set of enzymes (Scott, 2012, 2013). However, genes that code for estrogen receptors have been identified,

including *P. acuta* (Martínez-Paz et al., 2019). Furthermore, it has been identified Hsp90, which is a heat shock protein involved in cellular stabilization, regulation, and activation of a range of proteins. It participates in the maturation of steroid hormone receptors (Eckl & Richter, 2013). In this work, the response of *Hsp90* to BBP, DEHP, and DEP in *P. acuta* adults was analyzed. *Hsp90* was only significantly upregulated after one-week exposure to BBP, showing similar results as those reported after exposure of *P. acuta* to a heat shock (39 °C). In contrast to these results, the exposure to cadmium and vinclozolin did not induced *Hsp90* activation in *P. acuta* (Martínez-Paz et al., 2019; Aquilino et al., 2019). The differential response suggests a specific response and suggests some kind of effect related to the endocrine disruption of BBP. Furthermore, it confirms previous results in other organisms, suggesting that BBP is the most toxic compound of the three tested and produces a more potent response (Liu et al., 2009; Planelló et al., 2011; Chen et al., 2014).

On the other hand, juvenile individuals exposed to BBP did show an *Hsp90* downregulation at the lowest concentration after 15 days of exposure, while similar levels to those found in controls were observed after one month. The response can reflect the differences between both stages of development, adult, and juvenile. However, it cannot be discarded that an earlier response occurs. The fact that the lowest concentrations showed significant downregulation suggests that the concentration is essential to trigger the response and that a threshold should be reached before altering the *Hsp90* transcription. In that case, a combination of time and concentration would be necessary to reach the threshold. So, the two higher concentrations would reach the threshold before the fifteen days and, at this time, returned to normal levels while at 0.01 µg/L, the necessary levels of pollutant inside the organism would be reached at fifteen days. In all cases, the return to normal could be due to either acclimation to the compound or the activation of the detoxification mechanisms that can neutralize the toxicant. Another possibility is that the response follows the dynamic of a hormetic response. However, the fact that higher concentrations also showed a slightly, but not statistically significant, decrease of transcripts levels supports the previous possibility. It is also supported by the similar levels observed at 30 days, almost the same for all the concentrations and the control.

There is a lack of information regarding this gene in invertebrates and phthalates. Only a couple of reports for DEHP exist. In *Chironomus riparius*, *Hsp90* showed upregulation in response to short and long-term exposure of DEHP at concentrations of 1, 10, and 30 µg/L (Park & Kwak, 2008). On the other hand, in hemocytes of the clam *Venerupis philippinarum*, the gene *Hsp90* was upregulated after exposure to different doses of DEHP (0.4 and 4 mg/L), showing response after 12 hours and a maximum at 36h and lower levels at 96h in return to normal levels (Lu et

al., 2013). These results suggest that the response of *Hsp90* could also be related to stress response, although it is also involved with the endocrine system. In any case, additional research will help elucidate the mechanisms involved in the effects of phthalates on this gene.

The poor knowledge of the mollusk endocrine system at the molecular level does not elucidate the mechanisms involved in the endocrine disruptor activity of the three phthalates, being necessary to extend the study to additional endocrine-related genes to different hormonal response and biosynthesis pathways.

Stress and oxidative stress-related genes

Cells can respond to stress, triggering different processes to activate genes involved in cell survival. Heat shock proteins (HSPs) are a group of highly conserved proteins which play an essential role in cell survival, acting as protein chaperones, and many other roles such as modulation of apoptosis, immunity, and development (Garrido et al., 2001; Srivasta 2002; Joly et al., 2010; Garrido et al., 2012; Kurop et al., 2021).

To evaluate the stress response after the exposure of juveniles and adults to phthalates, the gene expression profile of the following genes was analyzed: *Hsp16.6*, *sHsp17.9*, *Hsp60*, *Hsc70-4*, *Grp78*, and *HIF1α*.

Surprisingly DEHP and DEP did not alter the values of any of the genes after one-week exposure of adults. Contrarily, BBP altered all tested genes, inducing its activity significantly. The upregulation of *Hsp60* suggests possible damage at the mitochondrial level, while the induction of *Grp78* could reflect some problem in the protein folding in the endoplasmic reticulum. The *sHSP17.9* and *Hsc70-4* genes extend the battery of genes available to assess the stress response of *P. acuta*. *sHSP17.9* is difficult to match with other species' genes because while all HSPs have a characteristic highly conserved alpha-crystallin domain, no other sequence presently allows for homology to be established. It is worth mentioning that *HIF1α* offers a new aspect of stress related to hypoxia. The stress response mainly focuses on the heat shock proteins, so other mechanisms involved in specific stress, like hypoxia, are usually neglected. With the identification of *HIF1α* in *P. acuta*, the effect of a toxicant on oxygen intake in this species can be assessed.

This increase in the heat shock proteins activity would correlate with more remarkable protein synthesis to reach cellular homeostasis. Although there was a positive regulation for the stress-related proteins after exposure to BBP in *P. acuta* adults, no changes at transcriptional levels were observed in juveniles, except for *Hsc70-4*, where transcriptional activity was reduced.

Besides, after one month of exposure to BBP, *Hsp60* did not amplify. It could be explained by a technical issue, such as the low amount of RNA in the extraction that maintained the level of this gene below the detection level. However, as in control, it was not detected, it would be possible the involvement of *Hsp60* in some developmental process, so there is a downregulation. Additional research with juveniles is required to define the pattern of expression of these genes during the development to adult and to define putative alterations due to environmental stressors because of the different roles that HSPs could have in the cell metabolism. In this sense, it has been reviewed recently the role of the HSP60 showing that it is involved in many cell processes, including embryonic development (Malik & Lone, 2021). Furthermore, *Hsp60* gene expression was observed differentially during *Drosophila* development (Kozlova et al., 1997), so it cannot be discarded that it also happens in *P. acuta*.

This lack of response in most heat shock protein genes could reflect that the homeostasis was recovered. However, it is essential to note that some HSPs have early responses to toxicants, as happens in *Chironomus riparius* to cadmium with the sHSP genes (Martín-Folgar & Martinez-Guitarte, 2016). Thus, additional research is needed at shorter times to determine whether an early activation occurs and to define the pattern of expression along the development to describe the behavior of these genes in *P. acuta*.

In invertebrates, knowledge of the effects of phthalates on cellular stress is still scarce, and to date, the study of these compounds in the gene expression of *P. acuta* is non-existent. The gene expression profile after exposure to these compounds has been studied in the midge *C. riparius* (Planelló et al., 2011; Herrero et al., 2015), where both, BBP and DEHP, induced the *hsp70* gene in a concentration-dependent manner, while they did not induce any changes in the constitutive *hsc70* gene. In the worm *Caenorhabditis elegans*, both DEHP and DEP altered the expression of *Hsp16* genes that were downregulated (Pradhan et al., 2018). Alterations of stress-related genes in *P. acuta* have been reported after exposure to other xenobiotics, such as bisphenol-A or cadmium. For BPA, *hsp70* transcriptional activity increased first, followed by a decrease, while after exposure to cadmium, *hsp70* activity was significantly induced (Martínez-Paz et al., 2017; Morales et al., 2018). The response observed in this thesis agrees with some of the previous reports and highlights that the species, the development stage, and the HSP studied have relevance to extract conclusions.

Cat, *SOD Mn*, and *SOD CuZn* genes evaluate oxidative stress status. Oxidative stress analysis is usually focused on biochemical parameters, like enzymatic activity. However, a transcriptional activity study can provide additional information regarding the mid- and long-term responses.

In *P. acuta* adults, *SOD Mn* and *SOD CuZn* were upregulated at all tested concentrations of BBP. *SOD Mn* encodes a mitochondrial protein that protects the cell from oxidative stress. Therefore, it is plausible that BBP caused oxidative stress, stimulating the *SOD Mn* and *SOD CuZn* upregulation to alleviate its effects. *Cat* increased its activity only at 10 µg/L. The reason why the highest concentration of BBP did not alter *Cat* expression could be a disruption of the antioxidant systems that the cell is not able to compensate for, caused by excessive levels of reactive oxygen species (ROS). Supporting this statement, signs of oxidative stress in response to phthalates have been found in aquatic organisms. For example, in zebrafish embryos, DEP induced oxidative stress causing an increase in the antioxidant enzyme activity in a concentration-dependent manner (Xu et al., 2013). In the mussel *Mytilus coruscus*, SOD activity was considerably activated after exposure to DEHP (Gu et al., 2021).

Unlike the results obtained with the adults' exposure, for the juvenile individuals, the transcription levels of *Cat*, *SOD Mn*, and *SOD CuZn* after a longer-term exposure (15 and 30 days) to BBP remained very similar to those found in untreated organisms. It could reflect the efficiency of the detoxification mechanisms, suggesting that any alteration happens in an acute response more than in a chronic response. It is worth mentioning that in *Chironomus sancticaroli* a reduction in superoxide dismutase (SOD) activity was observed at all evaluated concentrations, ranging from 0.1 to 2000 µg/L, suggesting oxidative stress in acute (48h) and subchronic (8 days) exposures (dos Santos Morais et al., 2020). Similarly, there was a reduction in CAT activity at acute exposure at the highest concentration (2000 µg/L), while in subchronic exposure, activity increased only at 1 µg/L (dos Santos Morais et al., 2020). This reduction with SOD is also observed in an experiment with *Eisenia fetida* at 28 days where the authors detected that SOD activity of each group treated with BBP was inhibited and lower than that of the control on 7 days but not at 14, 21, and 28 days (Song et al., 2019). However, in the same study, the response of CAT activity displayed a trend of activation-inhibition-activation with time. At 7 d, CAT activity was remarkably higher than control, but at 14 d, CAT activity was lower than that on 7 d. Finally, CAT activity gradually decreased with the increase of BBP dose and finally increased to the control level on 28 d (Song et al., 2019). The authors propose that the dynamic could respond to the modulation of ROS content, which weakened the synthesis and releasing ability of CAT, resulting in the decline of CAT activity.

Further research, including the analysis of enzymatic activities, could provide a better picture of the response in *P. acuta*. However, the results again indicate that the stage of development analyzed is a crucial factor to consider analyzing the response.

Detoxification mechanisms related genes

When an external compound is detected, it is common for all cells to activate biotransformation. Different enzymes are activated to reduce xenobiotic toxicity and increase its solubility to ease its elimination (Aquilino et al., 2019). Thus, detoxification mechanisms are essential to assess the response to toxicants. Three steps are distinguished in the process: phase I, phase II, and phase III. GST activity is one of the most used methods to assess detoxification, but it does not differentiate between members. The situation is similar regarding cytochrome P450s, which show high diversity with many roles in the cell. Identifying the *Cyp72a15* gene in *P. acuta* allows extending the number of families of cytochrome P450 that can be analyzed.

To date, there is no previous data on the effects of BBP, DEHP, and DEP regarding detoxification phases in *P. acuta.* For this study, phase I (*Cyp2u1*, *Cyp3a7*, *Cyp4f22*, and *Cyp72a15*), phase II (*GSTk1*, *GSTm1*, *GSTo1*, and *GSTt2*), and phase III (*MRP1*) genes were selected. For adults, all phase I, II, and III representatives were remarkably upregulated upon BBP exposure, except for *GSTm1*, which was not activated, although it showed a trend for upregulation compared to control. These results suggest activation of the detoxification mechanisms in the presence of BBP, proving that the cytochrome and GST families are enzymes that intervene in the biotransformation of these compounds. Furthermore, the absence of changes in *GSTm1* supports a differential role for each GST family member in response to toxicants. The absence of response for the other two phthalates could reflect that they are processed by another phase I enzymes or that the compound intake was not enough to trigger the response so that *P. acuta* would be less sensitive to those phthalates. Finally, it cannot be discarded that DEP and DEHP would be processed by other cytochromes P450 not analyzed here.

On the contrary, after the 15 days exposure of *P. acuta* juveniles, only inhibition of *GSTt2* at the lowest concentration of BBP was observed. It is worth noting that *GSTt2* and *Cyp3a7* did not amplify at 30 days, similarly to *Hsp60*. It could be due to a technical problem since the amount of RNA obtained from a juvenile is lower than from an adult. So, it is possible that the genes expressed below the detection level. Nevertheless, it cannot be discarded that the lack of detection, even in control, could be related to a development-related regulation. Additional research could help elucidate the response to the no detection observed by analyzing different times.

The rest of the genes studied, both after 15 days and one month, did not show any significant differences compared to the control. The strong induction observed in the adults compared to the juveniles could be due to early activation of the detoxification mechanisms, which does not

last over time. Activation of the studied genes in juveniles could be expected after a shorter exposure time. This may be because the cell effectively acts on the compound, allowing the individual to withstand the concentrations used without significantly altering cell metabolism. Therefore, the differences regarding the exposure time must be considered. It could mean that once the organisms have acclimatized to the presence of the xenobiotic, they can return to a control situation. An additional analysis, broadening the exposure times for adults and shortening them for juveniles, could help define the effects of the phthalates in the detoxification process.

As mentioned, there are no previous studies in *P. acuta* analyzing the activation of the detoxification mechanism by phthalates. However, after exposure to the pesticides endosulfan and vinclozolin, it has been evaluated. The study involved phase I (*Cyp2u1*, *Cyp3a7*, *Cyp4f22*), phase II (*GSTk1*, *GSTm1*, *GSTo1*, *GSTt2*), and phase III (*MRP1*) genes. Only *Cyp2u1* was altered after endosulfan exposure, increasing its activity compared to control, while for vinclozolin, no significant differences in gene expression were observed. Comparing the results, the differential expression of phase I, phase II, and phase III shows that analyzing these genes provides a picture concerning the ability of the animal to manage the toxicant. Additional genes could improve the approach and offer a better picture of the members of each family involved in response to different toxicants.

Although in invertebrates, the detoxification processes in relation to phthalates have been poorly studied, some alterations have been observed. In *C. riparius*, BBP exposure resulted in an evident inhibition of phase I detoxification reactions, downregulating the expression of *Cyp4G* (Herrero et al., 2015). Like for BBP, DEHP also repressed the *Cyp4G* activity in this species (Herrero et al., 2017). On the other hand, downregulation was also observed for a GST gene in the clam *Venerupis philippinarum* exposed to DEHP for 36h to 0.4 mg/L (Lu et al., 2013). No additional reports exist about the impact at the transcriptional level on invertebrates of the phthalates studied in this work. The data suggest that the response is very variable and depends on the species and the conditions of exposure, but it seems that the response is in the short-term and tends to stabilize in the long term.

There are more previous studies on GST activity on invertebrates, and to complement the analysis, the GST activity was analyzed in the juvenile individuals exposed to BBP. The results showed no activity alteration, neither after 15 days exposure nor after a month. However, there tends to increase the levels at 30 days, but it is not statistically significant.

In *C. riparius*, the GST enzyme activity was reduced after exposure to BBP for 48 hours (Herrero et al., 2015). Similarly, in *Chironomus sancticaroli*, the GST activity showed a reduction in acute (48h) and subchronic (8 days) exposures to BBP at all evaluated concentrations (range 1 to 2000 µg/L), suggesting oxidative stress (dos Santos Morais et al., 2020). These results contrast with those obtained. However, a report in the earthworm *Eisenia fetida* showed that GST activity enhanced with increasing doses of BBP in an experiment that analyzed the activity at 7, 14, 21, and 28 days. At the late exposure stage, GST activity returned to similar levels as control (Song et al., 2019). The authors propose that it was probably due to the interaction of various enzymes, which decreased the impacts of external pollutants, thus weakening GST induction. The global picture suggests that the alteration of GST activity usually happens at the start of the exposure and extend some time, but it can recover the normal levels later. It could be possible that there is no alteration in the juveniles of *P. acuta* because the normal levels have been recovered at 15 days of exposure by compensating the damage at the oxidative stress produced by BBP.

In relation to other phthalates, the literature also provides a complex situation. In *Chironomus riparius*, DEHP reduced the GST activity in acute exposure (Herrero et al., 2017). Similarly, the GST activity was blocked in *Folsomia candida*, a collembola, exposed for 2, 4, 7, and 14 days to DEHP (Zheng et al., 2022). However, in *Gammarus pulex*, exposed to different doses of DEHP for 24 and 96 h, GST activity increased at 96 h compared to the control group (Yildirim et al., 2021). Another phthalate, Di-n-butyl phthalate (DBP), also induces the GST activity on earthworms exposed for 28 days. After 21 days of treatment, GST activity in treatment groups was markedly stimulated compared to the control group (Du et al., 2015). Another study confirmed the result that showed that earthworms exposed for 7, 14, 21, and 28 days showed a GST activity increased from 7 days (Wang et al., 2018). The literature shows a complex situation where the species and the exposure conditions have a relevant role in the response. Additional research will help elucidate the response mechanisms and why there are such variable responses to this group of toxicants.

Finally, it is worth mentioning that a study compares the response of juveniles and adults in *Daphnia magna* (Wang et al., 2018). In this case, DEHP was the phthalate selected, and the authors suggest, differentially to the results of this work, that there are no differences in response to DEHP between juveniles and adults. The levels of GST activity were analyzed and were significantly higher upon initial exposure for 24 h, then diminished at high concentrations and prolonged exposure for 48 h. Concerning gene expression levels, *gst* was notably reduced for juveniles but increased for adults. The authors conclude that, on the whole, juveniles and

adults both responded similarly to DEHP. However, the different expression of *gst* suggests some differences that depend on the development stage.

The results obtained here provide a first step in analyzing detoxification mechanisms working in the presence of phthalates. Extending the number of phase I and phase II enzymes will provide new information on how the organisms can metabolize these toxicants and help define the putative degradation sequences to identify the byproducts of biotransformation.

Effects in DNA repair-related genes

Genotoxicity is a critical toxicity parameter in xenobiotic testing and environmental risk assessment. Breaks produced in DNA due to different stressors exposures can become non-repaired lesions and have been employed as biomarkers of genotoxicity. However, little is known about the genotoxic effects of phthalates in aquatic invertebrates, although reports indicate that the production of reactive oxygen species can affect DNA. The analysis of genes involved in DNA repairing can be combined with other methods, like the comet assay, to perform an integrated study to determine whether a compound is genotoxic and whether the organism can compensate for the damage.

This study evaluated the transcriptional activity of four genes, two related to DNA repairing (*rad21*, *rad50*) and two to apoptosis (*Casp3*, *AIF3*). *Rad21* and *rad50* code for proteins that participate in double-strand break repair while *Casp3* codes for a death executioner caspase and *AIF3* is a mitochondrial protein involved in apoptosis. The analysis of apoptosis genes is related to this process's activation when the DNA cannot be repaired, so the cell prevents further damage by entering apoptosis.

After exposure of *P. acuta* adults to the three phthalates, only BBP altered the mRNA levels of these genes. Both *rad21* and *rad50* showed increased activity, suggesting activation of DNA repairing mechanisms. Therefore, it suggests that BBP could work as a genotoxic compound producing double DNA breaks and activating the DNA repair. Further, the apoptosis-related genes *Casp3* and *AIF3* were also upregulated. It could reflect that the DNA repair is insufficient to compensate for the damage, and apoptotic processes are activated. On the contrary, neither DEHP nor DEP can activate these genes. It suggests a differential response due to differences in the mechanisms of action or the concentrations needed to trigger the effect.

The analysis of the impact of BBP in juvenile organisms did not show alterations after 15- and 30-days exposure. The difference could be because juvenile individuals are still in development and can compensate, in some way, for the damage produced by BBP. On the other hand, it is

also possible that the response could be earlier. Then shorter treatment times could help elucidate if BBP can alter the activity of these genes in juvenile individuals of *P. acuta*. In this sense, it is worthy to note that *Daphnia magna* showed a higher LC_{50} for DEHP in juveniles than in adults, suggesting less sensitivity to phthalates (Wang et al., 2018).

Concerning phthalates, the analysis of genotoxicity shows a complex picture. DEHP has shown contradictory results, being most of the data negative. Besides, it is not clear enough if the positive results are associated with a direct interaction of DEHP with DNA or rather with secondary oxidative stress or other events (Benedict et al., 2022). Like DEHP, there have been some indications of a carcinogenic effect of BBP, but no genotoxic effects were evident. Thus, presently BBP is considered a non-genotoxic substance (ECB, 2007).

To date, the studies of the genotoxic effects of phthalates in invertebrates and aquatic organisms are very scarce. In *Chironomus sancticaroli*, DNA damage was observed after 48h exposure of BBP, but at higher concentrations than the ones tested in this work (2000 µg/L), while after eight days exposure, DNA damage was observed at concentrations from 10 µg/L (dos Santos Morais et al., 2020). Likewise, a concentration-related increase in DNA damage in zebrafish larvae cells was observed after 96h exposure to DEHP (Boran & Terzi., 2019). After exposing gilthead seabream to diisononyl phthalate (DiNP), the expression levels of apoptosis-related genes increased, thus suggesting activation of apoptosis (Carnevali et al., 2019). On the other hand, in the freshwater prawn *Macrobrachium rosenbergii*, the annexin assay showed that hemocytes exposed to either dihexyl phthalate (DHP) or dipropyl phthalate (DPrP) primarily die via apoptosis. In contrast, BBP, dicyclohexyl phthalate (DCP), DEP, or diphenyl phthalate (DPP) would primarily die via necrosis (Sung et al., 2003). Another study in *Caenorhabditis elegans* showed that exposure to environmentally relevant levels of DEHP and its metabolites results in increased meiotic double-strand breaks (DSBs), altered DSB repair progression, and cell apoptosis (Cuenca et al., 2020), showing the genotoxicity of this compound and the effect on the repairing mechanisms. Confirming the activation of apoptosis by DEHP, the analysis performed in apoptosis-related genes in *C. elegans* by Yin et al., 2018 showed an increase in mRNA levels according to the data obtained for BBP but not for DEHP in this thesis. Again, the conclusion from all this data is that the phthalates have a very variable response that depends on the conditions of experimentation, making it difficult to obtain general conclusions. However, it seems that genotoxicity is an effect that can be included in the effects that these toxicants produce on invertebrates.

Finally, it is worthy to say that the results obtained suggest for the first time in the gastropod *P. acuta* the genotoxic effects induced by BBP. The analysis of mRNA levels in adults exposed to BBP for one week revealed that BBP could differentially modulate the expression of DNA repair and apoptosis-related genes. However, it is necessary to perform additional studies with juveniles to define the response and determine if there is any DNA damage in earlier stages of exposure and the individuals can compensate for it.

Epigenetic regulation related genes

Three genes involved in epigenetic mechanisms were identified in this study: *DNMT1*, *KATB6*, and *HDAC1*. There is increasing evidence that epigenetic regulation is one of the long-term effects of toxicants. However, in toxicity analysis, the genes involved in this process in invertebrates are still poorly represented. Therefore, the description of these three genes opens the possibility to analyze their role in the epigenetic response and its relevance in the transgenerational effects that have started to be described with different toxicants (Vandegehucht et al., 2010; Xi et al., 2015).

In the case of *P. acuta* adults, the three epigenetic-related genes were overexpressed after exposure to BBP. At the same time, no changes were observed after DEHP and DEP exposure, which could reflect differential sensitivity to the compound. Thus, BBP seems to alter epigenetic related genes clearly. On the contrary, after the 15 days and one-month exposure of *P. acuta* juveniles, none of the BBP tested concentrations showed any significant differences compared to control. Again, the different times and the stage of development would be variables that could justify the different results obtained.

The alterations in epigenetic regulation mechanisms after exposure to phthalates have been reported. In mice, DEHP exposure significantly increased *DNMT1* expression (Wu et al., 2010), while in *Rana chensinensis* tadpoles, *HDAC1* expression increased after exposure to a cadmium-DEHP mixture (Shen et al., 2022). Aberrations in histone deacetylase gene expression have been implicated in a wide range of cancers. For example, *HDAC1* and *HDAC3* genes are over-expressed in ovarian cancer tissues, and *HDAC1* overexpression has been reported in gastric cancers (Bassett & Barnett, 2014). Like *HDAC1*, *KAT6B* has also been found to be overexpressed in ovarian cancer (Wiesel-Motiuk & Assaraf, 2020). The epigenetic regulation concerning phthalates should be considered since they can have long-term effects that could be even transgenerational.

The results obtained are the first report concerning phthalates and epigenetic regulation mechanisms in invertebrates. Furthermore, the results obtained justify the extension of this kind of study since the long-term effects could compromise the population's future.

Nervous system, immune system, energy reserves, and lipid transport-related genes

Acetylcholinesterase is widely distributed in vertebrates and invertebrates, being a key enzyme for nerve conduction (Zhang et al., 2014). Phthalates have been proved to affect AChE activity in other aquatic organisms. In zebrafish embryos, the gene expression of AChE was upregulated by the exposure of DiNP and DEHP (Tran et al., 2021), while after DBP and DEP exposure, AChE enzymatic activity was significantly inhibited (Xu et al., 2013). In adult zebrafish, AChE activity also decreased upon exposure to BBP in a dose-dependent manner. AChE activity was inhibited in *P. acuta* after exposure to the biocide abamectin (Ma et al., 2014). However, the impacts of BBP, DEHP, and DEP on AChE expression on *P. acuta* have not been studied.

To test the ability of the phthalates to affect the nervous system, *AChE* gene expression was analyzed. While DEHP and DEP did not affect its levels, BBP increased its expression in *P. acuta* adults. The altered acetylcholinesterase mRNA levels suggest effects on the nervous system, which could affect the ability of the snail to survive. This indicates that BBP can alter the nervous system in non-target organisms. The increase observed in the transcription could reflect and attempt to compensate for the inhibition of enzyme activity, requiring additional research to elucidate the damage in the nervous system. For juveniles, no changes in *AChE* activity were observed upon exposure to BBP. Thus, further research at different time exposures could help determine whether some alteration occurs.

The remaining identified genes allow the analysis of several pathways that can be altered by toxicants, such as the immune system (*ApA*), energy reserves (*PYGL*), and lipid transport (*ORP8*). In the clam *Venerupis philippinarum*, DEHP altered the immune response (Lu et al., 2013) while DBP and DEP induced innate immune-related gene expression in zebrafish embryo (Xu et al., 2013). In this study, these genes have been analyzed concerning pollution in freshwater mollusks for the first time. The three genes were upregulated upon BBP exposure in adult individuals, while for juveniles, no significant differences were observed in any of the studied genes. For DEHP and DEP, the adults did not show any change.

There is no previous data involving these genes since it is the first time they are analyzed. However, the response analysis in other conditions and with additional phthalates would provide information about their impact on cell metabolism. In addition, since the genes are

related to critical processes such as defense and energy metabolism, they could be promising biomarkers to follow the toxicant's impact in those processes.

6.3. Concluding remarks

Several studies have already proven that phthalates can affect the development of aquatic organisms, reducing its body length (Zanotelli et al., 2010), causing developmental malformations (Zhou et al., 2011) and mouthpart deformities (Park & Kwak., 2008). Alterations in the swimming behavior upon phthalates exposure have also been reported (Tseng et al., 2012; Buerger et al., 2022), but the studies are limited. Thus, these data agree with the results obtained in this study, proving the toxicity of phthalates in the development and behavior of aquatic organisms.

Considering the results obtained by exposing sea urchin to BBP, they confirm the sensitivity of the developmental anomalies if compared to the swimming behavioral endpoint, according to the EC_{50} values. Morphological changes can be considered as structural damage, and because of those changes, functional damages can appear, like an alteration in the swimming speed. Besides, in general, the swimming performance of planktonic larvae is tightly related to larval morphology (Chan et al., 2012), so the findings on BBP confirm this hypothesis. Regarding DEP and DEHP, the behavioral endpoint (EC_{50} could not be calculated) was more sensitive in terms of LOEC values (0.1 and 0.1 µg/L) than the morphological anomalies (100 and 10 µg/L, respectively). Further investigations are required, by performing experiments based on higher concentrations of these two phthalates (> 1000 µg/L) in order to compare the two endpoints in terms of EC_{50} and to confirm the LOEC values.

In this thesis, BBP displayed the highest toxicity for the acute endpoint, while for the behavioral endpoint, DEP caused the highest swimming speed inhibition. Therefore, additional research is required trying shorter exposure times (24 and 48 h) to BBP, DEHP, and DEP, in order to confirm the differences of toxicity depending on the endpoint analyzed and to elucidate the putative mechanisms underlying the differences observed in the relation between the structural damage and the behavior.

The developmental anomalies and swimming speed alteration evaluated in this work provide a sensitive assessment of sub-lethal effects exerted by phthalates. However, the sensitivity of the studied endpoints in sea urchin should be further investigated in the presence of other stressors.

Even though researchers have described previously that DEHP and DEP can alter the physiology of invertebrates (Chen et al., 2019; Shaha & Pandit 2020; Aviles et al., 2020), including mollusks (Liu et al., 2009; Lu et al., 2013; Zhou et al., 2015; Xiang et al., 2017), these two compounds did not alter any mRNA levels, showing a differential impact in *P. acuta* compared to BBP. Other phthalates can also alter development and growth, which could be related to the endocrine-disrupting activity described for those chemicals. The molecular mechanisms involved are still under investigation, but some data are available. In *H. diversicolor*, DBP affects oxidative stress, lipid, energy metabolism, and osmoregulation (Zhou et al., 2015). In other invertebrates, like *Chironomus riparius* (Herrero et al., 2015; Herrero et al., 2017), phthalates alter endocrine pathways. Phthalates also affect other pathways, such as oxidative stress and detoxification routes (Llorente et al., 2020). Finally, in *C. elegans*, exposure to environmentally relevant concentrations of DEHP alters the expression of genes involved in DNA repair (Cuenca et al., 2020). Thus, it is proven that these phthalates can have a broad spectrum of actions in the cell.

A recent review of the impact of phthalates on aquatic animals summarizes the effects observed, suggesting that activation of the detoxification system (cytochrome P450s) and endocrine system receptors of aquatic animals cause oxidative stress, metabolic disorders endocrine disorders, and immunosuppression (Zhang et al., 2021). In addition, it would activate a cascade response that could cause genotoxicity and cell apoptosis, resulting in the disruption of growth and development.

Considering this, the absence of a response observed in *P. acuta* exposed to DEP and DEHP is striking. The differences observed can be assigned to the type of analysis (molecular vs. physiological), the exposure time (1 week vs. a few hours or days), the concentrations used (µg/L vs. mg/L), and obviously, the species used. Additional research will help elucidate the differential response in *P. acuta* compared with other organisms. However, it is essential to highlight that the obtained results suggest that *P. acuta* can manage the environmentally relevant doses of DEP and DEHP used in this work. 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.

Most studies on invertebrates that involve transcriptional activity analysis use arthropods and short exposure times (Planelló et al., 2011; Herrero et al., 2015; Li et al., 2021), but limited data are available on mollusks and, usually, they are marine representatives (Lu et al., 2013; Li et al., 2021). In contrast to DEP and DEHP, BBP showed a marked effect after one-week exposure of adult organisms: it increased the mRNA levels of almost all the analyzed genes. To date, this is

the first study on a freshwater snail that shows that BBP can produce a substantial effect on cell metabolism.

Several pathways were altered, like DNA repair processes, by the alteration of *rad21* and *rad50*, related to DNA damage, or epigenetic regulation, by altering the genes involved in histone and DNA modification (*KAT6B*, *HDAC1*, and *DNMT1*). Apoptosis also seems to be modulated in *P. acuta* by altering *Casp3* and *AIF3* genes. Furthermore, the three detoxification phases could be acting since the genes tested (four cytochrome P450s, three GSTs, and MRP-1) were upregulated. Genes involved in oxidative stress and the stress response were also altered, as shown by the changes in the mRNA levels of *Cat*, *SODs*, stress proteins, and the *hypoxia-inducible factor* genes. These changes support the alteration of oxidative stress, stress response, and detoxification, backing previous analysis and adding new insight into the mechanisms involved in modulating these processes. In this sense, the absence of changes in *GSTm1* supports a differential role for each GST family member in response to toxicants. The altered *acetylcholinesterase* mRNA level also suggests effects in the nervous system.

In summary, the present gene profile obtained in response to BBP in *P. acuta* supports the proposed mechanisms and cellular processes in previous studies with other animals (Zhang et al., 2021). Stress response and oxidative stress, detoxification, DNA repair and apoptosis, epigenetic modulation, immunity, lipid metabolism, and energy metabolism are modulated. In addition, the nervous system could also be affected.

The obtained results are in line with previous studies in other organisms, which have confirmed that BBP can induce different types of damage, such as apoptosis (Alam & Kurohmaru., 2016), genotoxicity (dos Santos Morais et al., 2020), oxidative stress (Song et al., 2019), stress response activation or endocrine disruption (Herrero et al., 2015). Although there are studies in invertebrates showing the impact on development and other physiological processes (Liu et al.,2009; Oehlmann et al., 2009), most of them did not focus on the putative mode of action, with just a few of them trying to delve into the response mechanisms. Here it has been shown that BBP can extensively affect the cell transcriptional activity in *P. acuta*. This scenario would mean that BBP is the most active phthalate in *P. acuta*, with a broad spectrum of action and a potential effect on many pathways. However, a more presumable picture has been recently proposed: alterations in the oxidative stress response and the endocrine system could cause a cascade of responses that affect different pathways and ultimately impact growth and development (Zhang et al., 2021).

In a recent study with *Daphnia magna*, BBP exposure significantly downregulated genes involved in signal transduction, cell communication, and embryonic development. In contrast, those related to cell homeostasis and redox homeostasis were remarkably upregulated upon BBP exposure (Li et al., 2021). Although the organism and the stage analyzed differ from this thesis, these results support that BBP can simultaneously alter multiple pathways.

As stated before, the results obtained in this work show that DEP and DEHP had no apparent effect on *P. acuta* after one-week exposure to environmentally relevant concentrations. However, BBP showed a strong effect. The difference in response could be due to several reasons that need to be explored in future analyses. One possibility is the structure of each compound. In this sense, BBP has two benzene rings while DEP and DEHP have only one. This factor could determine the biological activity of these compounds. Another possibility is that DEHP and DEP have earlier effects than the time studied, so the cell returned to the basal state, processing and removing the compounds. Finally, it cannot be dismissed that DEP and DEHP are not toxic to *P. acuta*, at least at environmentally relevant concentrations, or that they did not reach the threshold to trigger the response because *P. acuta* can tolerate them better. In any case, BBP alters the metabolism of this species and produces a broad impact on different pathways. Additional research should be done in *P. acuta* and other freshwater species to determine the impact of these compounds. 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, 18 of the analyzed genes have been described for the first time in *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.

CONCLUSIONS

7. CONCLUSIONS

- *Paracentrotus lividus* larvae exhibit different sensitivity to the three tested compounds.
- Embryos exposure to BBP, DEHP, and DEP cause a swimming speed inhibition in *P. lividus* plutei. DEP displays the highest effects on the SSA of the plutei, followed by DEHP and BBP.
- Embryos exposure to BBP, DEHP, and DEP cause developmental abnormalities in *P. lividus* plutei, affecting skeletal architecture and larval shape. Contrarily to the behavioral endpoint, BBP displays the highest toxicity, followed by DEHP and DEP. This reinforces the idea of the endocrine-disrupting character of this group of chemicals.
- Sea urchin larval development and swimming speed alteration are sensitive models for phthalates effects.
- BBP, DEHP, and DEP do not affect the survival of *Physa acuta* adults and juveniles at the concentrations and exposure times studied. However, BBP significantly inhibits the number of hatched eggs in a dose-dependent manner after embryo exposure.
- DEHP and DEP do not alter the expression profile of the 30 genes analyzed in *P. acuta* adults. However, BBP strongly modulates the expression of almost all the genes and thus, appears to have an extensive action on this snail, altering different pathways like the cellular stress response, detoxification mechanism, DNA repair and apoptosis, epigenetic regulation, immunity, and energy metabolism.
- After 15- and 30-days exposure of *P. acuta* juveniles, BBP does not alter the GST enzymatic activity nor the expression of most genes tested, except for *Hsp90*, *Hsc70-4*, and *GSTt2*.
- For *P. acuta* evaluation and *P. lividus* development, BBP was the most toxic compound, while for the SSA, its effects were the weakest.
- In this respect, additional investigations on phthalate toxicity on *P. acuta* and *P. lividus* are required to improve phthalates safe criteria, and the data herein presented may provide a suitable background for future studies.

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8. REFERENCES

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APPENDIX

APPENDIX I

Sequences used for primers´ design:

The transcriptome sequences are deposited in GenBank with the following accession numbers:

Gene	Accession number
rad21	MW456925
rad50	MW456929
AChE	MW456922
Catalase	MW456920
AIF	MW456928
Cyp72a15	MW456924
DNMT1	MW456930
KAT6B	MW456923
HDAC1	MW456919
sHSP17.9	MW456918
ApA	MW456926
ORP8	MW456921
PFKFB2	MW456927

Sequences obtained from: Romiguier, J., Gayral, P., Ballenghien, M. et al. Comparative population genomics in animals uncovers the determinants of genetic diversity. *Nature* 515, 261–263 (2014). https://doi.org/10.1038/nature13685

ACTB_G1

ATGTGCGACGATGAAGTAGCTGCCCTCGTAGTGGACAATGGCTCCGGCATGTGCAAAGCCGGGT
TCGCCGGAGACGATGCCCCCAGGGCAGTCTTCCCATCCATTGTCGGCAGACCCCGTCATCAGGG
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ATCCTGACCCTCAAGTACCCCATCGAACACGGCATCGTCACAAACTGGGACGACATGGAGAAAA
TCTGGCACCACACCTTCTACAATGAGTTGAGAGTCGCCCCCGAGGAGCACCCCGTCCTCCTCAC
TGAGGCCCCCCTCAACCCCAAAGCCAACAGAGAAAAGATGACCCAGATCATGTTCGAGACCTTC
AACGCCCCCGCCATGTACGTCGCCATCCAGGCCGTACTCTCCCTGTACGCCTCTGGCCGTACCA
CCGGTATCGTGCTCGACTCTGGTGATGGTGTCACCCACACTGTCCCCATCTACGAAGGTTACGC
CCTCCCCCACGCCATCCTCCGTCTGGACTTGGCCGGCCGTGATCTCACCGACTACCTGATGAAG
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APPENDIX II

Published articles directly related to this thesis:

- Prieto-Amador, M., Caballero, P., & Martinez-Guitarte, J. L. (2021). Analysis of the impact of three phthalates on the freshwater gastropod *Physella acuta* at the transcriptional level. *Scientific reports*, *11*(1), 1-14. https://doi.org/10.1038/s41598-021-90934-9

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Analysis of the impact of three phthalates on the freshwater gastropod *Physella acuta* at the transcriptional level

Marina Prieto-Amador[1,2], Patricia Caballero[1,2] & José-Luis Martínez-Guitarte[1]✉

Plastic pollution is one of the leading environmental problems. Phthalates are widely used plastic additives released into the environment. Although the effects of phthalates on vertebrates have been extensively studied, there is a knowledge gap regarding 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 30 genes in animals exposed for 1 week to 0.1, 10, and 1000 µg/L of each phthalate. The genes cover DNA repair, detoxification, apoptosis, oxidative and stress responses, immunity, energy reserves, and lipid transport. The results show that while DEP and DEHP did not alter the mRNA levels, BBP modulated almost all the analyzed genes. 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 the gastropod *P. acuta*.

Plastics are incredibly versatile materials and are useful for a wide range of applications. However, plastic production is under the scope of green policies to reduce the pollution of the environment. 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]. Plastics production requires specific catalysts and other additives. Phthalates are esters of phthalic acid 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 the atmosphere, foodstuffs, and other materials. Their worldwide production increased from 2.7 to nearly 6 million tons per year during the decade of 2007–2017[2], and they are now ubiquitous environmental contaminants. They are released regularly from the products that contain them[2,3], and reach almost all the environment's compartments[4]. In German rivers, phthalates have been found 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 dry weight 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, including dimethyl phthalate (DMP) from 0.03 to 0.05 µg/L; diethyl phthalate (DEP) from 0.04 to 2.14 µg/L; di-*n*–butyl phthalate (DnBP) from not detected (ND) to 2.27 µg/L; benzyl butyl phthalate (BBP) from ND to 0.13 µg/L; bis (2-ethylhexyl) adipate (DEHA) from ND to 0.19 µg/L; bis (2-ethylhexyl) phthalate (DEHP) from 0.11 to 6.3 µg/L; and di-*n*-octyl phthalate (DnOP) from ND to 0.05 µg/L[6].

The impact of phthalates on the environment has been studied in recent few years, with a focus mainly on vertebrates[7,8]. It is known that phthalates act as endocrine-disrupting chemicals (EDCs), producing severe health effects[7,9–12] and even have a long-term impact on the epigenome[13]. Phthalates can alter an animal's metabolism[8], but there is still a lack of information about their effects on invertebrates. Although the studies often include analysis at the molecular level[14–16], the diversity of invertebrates demands additional studies involving other species and groups.

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APPENDIX III

Other articles in progress or published not directly related to this thesis:

- Trophic niche segregation between the sympatric tunas *Thunnus alalunga* and *Katsuwonus pelamis* in the Gulf of Cadiz (East Atlantic). *Marine Biodiversity.* 52, 18 (2022). https://doi.org/10.1007/s12526-021-01256-y

- Caballero, P., Prieto-Amador, M., & Martinez-Guitarte, J. L. (2021). Gene Expression Response of the Non-target Gastropod *Physella Acuta* to Fenoxycarb, a Juvenile Hormone Analog Pesticide. https://doi.org/10.21203/rs.3.rs-1036012/v1

Marine Biodiversity (2022) 52:18
https://doi.org/10.1007/s12526-021-01256-y

SENCKENBERG

ORIGINAL PAPER

Trophic niche segregation between the sympatric tunas *Thunnus alalunga* and *Katsuwonus pelamis* in the Gulf of Cadiz (East Atlantic)

Marina Prieto-Amador[1,2] · Antonio Medina[1] · José Luis Varela[1]

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Abstract
This study presents information about trophic relationships between juvenile albacore (ALB, *Thunnus alalunga*) and skipjack tuna (SKJ, *Katsuwonus pelamis*) in the Gulf of Cadiz (East Atlantic Ocean). Data on stomach contents (SCA) and stable isotope ratios of carbon and nitrogen (SIA) were analyzed to ascertain foraging patterns and trophic niche breadths and overlapping. SCA showed that European anchovy (*Engraulis encrasicolus*) and Atlantic chub mackerel (*Scomber colias*) were the most important prey species in both tuna species. A PERMANOVA analysis of the SCA data revealed interspecific differences in the diet ($p < 0.05$). δ^{15}N and δ^{13}C values from ALB muscle and liver tissues were higher than those from SKJ samples. As with SCA, the PERMANOVA analysis of isotopic data indicated significant interspecific differences in both muscle and liver samples ($p < 0.01$). Isotopic trophic niche values estimated by Bayesian standard ellipse corrected areas (SEA_C) showed low (muscle) or no (liver) overlap between ALB and SKJ. Taken together, the present results suggest that, although juveniles of the two species fed for an extended period of time in the same area and shared some prey species, there is evidence of food resource partitioning, thus preventing trophic competition.

Keywords Trophic biology · Stomach content analysis · Stable isotope analysis · Scombridae · Albacore · Skipjack tuna

Introduction

The albacore (ALB, *Thunnus alalunga* Bonnaterre, 1788) and the skipjack tuna (SKJ, *Katsuwonus pelamis* Linnaeus, 1758) are commercially important tuna species that are located at high levels in food webs and play a significant ecological role within marine pelagic ecosystems. ALB is a highly migratory and widely distributed species. Adults are found in tropical and subtropical waters, while juveniles occur in temperate waters worldwide, including the Mediterranean Sea and the Atlantic Ocean (Arrizabalaga et al. 2002). For management purposes, two stocks are considered in the Atlantic Ocean and Mediterranean Sea (northern and southern stocks). SKJ is a cosmopolitan and highly migratory species widely distributed in warm and temperate waters of all oceans (Collette and Nauen 1983). For management purposes, two stocks are considered in the Atlantic Ocean: the western stock and the eastern stock (including the Mediterranean Sea) (ICCAT 2006). Although both species are heavily fished, they are listed as Least Concern in the IUCN Red List of Threatened Species (Collette et al. 2011, 2015).

In spite of the economic importance of ALB and SKJ, essential aspects of their early life history, including trophic ecology, are not yet fully understood. Both ALB and SKJ are considered opportunist predators, often hunting aggregations of fishes and crustaceans (Dragovich and Pottohff 1972; Ankenbrandt 1985; Nikolic et al. 2017). Their wide geographical distribution suggests that these tuna species target a variety of prey and display flexible feeding behaviors (Goñi et al. 2011; Varela et al. 2019a). Goñi et al. (2011) reported that ALB shows nighttime feeding behavior in surface waters on the continental slope and daytime feeding activity in deeper waters

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Gene expression response of the non-target gastropod *Physella acuta* to Fenoxycarb, a juvenile hormone analog pesticide

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Abstract

Nowadays the pesticides are an environmental problem since they can act on non-target species. Therefore, the search for new pest control methods has focused on compounds with low or no toxic effects. Analogs of the juvenile hormone are one of those pesticides since they work by interfering in the endocrine system of arthropods. However, the effect on non-target species is frequently assumed, and it requires to be confirmed. This article analyzes the impact of Fenoxycarb, an analog of juvenile hormone, on *Physella acuta*, an aquatic gastropod. Animals exposed for one week to 0.01, 1, and 100 µg/L were used to obtain RNA and carry out retrotranscription and Real-Time PCR. Forty genes related to the endocrine system, the DNA repairing mechanisms, the different phases of detoxification, oxidative stress, stress response, nervous system, hypoxia, energy metabolism, immune system, and apoptosis were analyzed. Three of the genes, *AchE*, *Hsp17.9*, and *ApA*, showed response to the presence of Fenoxycarb at 1 µg/L with no statistically significant response in the rest of the genes and concentrations. From the results can be concluded that Fenoxycarb shows low toxicity in *Physella acuta*. However, the fact that a gene related to immunity was altered prevents in relation to the putative long-term effects that could have this juvenile hormone analog. Therefore, additional research would be necessary to confirm the harmless of Fenoxycarb in non-arthropod species.

Introduction

The pesticide Fenoxycarb (IUPAC: ethyl [2-(4-phenoxy-phenoxy)ethyl] carbamate, CAS No. 72490-01-8) is a carbamate used to control various insect pests in crops and ornamental cultures (Sullivan 2010). It regulates insect growth by mimicking the juvenile hormone, preventing reaching maturity (Dhadialla et al. 1998). It is considered not harmful for vertebrates and non-target species, affecting insects and crustaceans (Arambourou et al. 2017; Navis et al. 2018; Jindra and Bittova 2020). Furthermore, Fenoxycarb is considered environmentally safe because of its fast degradation (Schaefer et al. 1987), with a dissipation time 50 (DT_{50}) of 4.13 days in the water column and 15 days in sediment (PPDB database, (Sullivan 2010)). It has a low drift to adjacent lands from fields where it is applied (Thomas et al. 2016), although it may end up in surface waters due to spray drift, runoff, or drainage (Jungmann et al. 2009).

In vertebrates, no effect on reproduction of sheeps was observed (Barr et al. 1997) but it has been described that to cultured rat cortical neurons exposed for one week, Fenoxycarb considerably decreased ATP levels, mitochondrial membrane potential and glucose consumption (Schmuck and Mihail 2004). Furthermore, it inhibits rat brain acetylcholinesterase and nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes (Smulders et al. 2003). In the other hand, some reports show that it can negatively affect the egg production and hatching rate on the collembola *Yuukianura szeptyckii* (Lee et al. 2020), although previous studies on *Folsomia candida* did not show effect (Campiche et al. 2006). Similarly, some adverse effects were observed with *Neocaridina davidi*, a shrimp, exposed for two weeks to concentration as low as 10 µg/L with inhibition of molting and body length growth (Hu et al. 2019), while the crab *Rhithropanopeus harrisii* delayed the metamorphosis at 48 µg/L of Fenoxycarb (Cripe et al., 2003). Taking together, additional studies are required to know the impact of this pesticed on ecosystems. There is a lack of information in non-arthopod freshwater invertebrates, but it is expected to be harmless for them. However, the poor knowledge of the physiology on invertebrates, especially on endocrine system, requires to confirm this extreme. This work aims to test the toxicity of Fenoxycarb. at the transcription level, in the freshwater gastropod *Physella acuta* (Draparnaud, 1805) by exposing the animals for one week and analyzing the transcription profile with an array covering different relevant cellular pathways.

The freshwater snail *Physella acuta*, also known as *Physa acuta*, is hermaphroditic and a cosmopolitan species. It lives in lakes and ponds and lays the eggs in an egg mass that

requires around two weeks to develop. The hatched juveniles grow for two months until they reach the adult stage, mate, and lay eggs. The culture in the laboratory is easy, so it is used in toxicity studies as representative of the gastropods (Sánchez-Argüello et al. 2009, 2012). Recently, we have designed an array to study the response to toxicants at the gene expression level in this species (Prieto-Amador et al., 2021). It included thirty-four genes and four reference genes. In addition, we have extended it to forty target genes, including some of them to analyze the alterations at the transcriptional activity level in several cellular processes. The sequence of nine genes is described for the first time for this species and extends the number of genes that can be used as biomarkers. The sequences code for proteins related to the endocrine system (Galanin receptor type 2, Estrogen related receptor, membrane progestin receptor-beta, estradiol 17-beta-dehydrogenase 8, Retinoic acid receptor RXR), DNA repair (poly-ADP-ribose polymerase I, DNA repair protein XRCC3, IkBa), and stress response (Heat shock protein 70 B2-like). Overall, the array allows analyzing alterations in the endocrine system, detoxification mechanisms, DNA repairing, nervous system, apoptosis, oxidative stress, stress, epigenetics, the immune system, energy metabolism, and lipid transport. In this way, the array shows changes in main mechanisms involved in response to stress and detoxification, but also the response of processes involved in long-term effects such as the epigenetics modification mechanisms and the DNA repairing, that would be activated in case of any genotoxic effect of the compound.

As stated, the work aims to assess the putative toxicity of Fenoxycarb in *Physella acuta* exposed for one week to environmentally relevant concentrations by analyzing different processes involved in the short and long-term response, and some of them, such as the endocrine system and the immune system, involved in relevant physiological mechanisms to survive.

Material and methods

Chemicals

Fenoxycarb was purchased from Sigma-Aldrich (Germany). In addition, 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 (Germany), DNA polymerase and dNTPs were obtained from Biotools (Spain), and EvaGreen was purchased from Biotium (USA).

Animals

The populations of *Physella acuta* have been grown in the laboratory for numerous generations. They were established from animals provided by Dr. Sánchez-Argüello (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain). The gelatinous egg masses are collected and allocated in 500 ml glass vessels with 250 ml of culture medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.77 mM $NaHCO_3$, and 0.08 mM KCl). The medium is changed twice per week, and the animals are fed twice per week with a mixture of Shrimps Natural (Sera) and Micron Nature (Sera). The cultures are maintained at 18 ± 1 °C under a 16 h light and 8 h dark cycle.

Treatment

Six adult snails (0.091 ± 0.01 g and 0.79 ± 0.08 cm) were used per concentration. Fenoxycarb was diluted in acetone for a final stock of 100 mg/mL. The stock was diluted for a final concentration of 0, 0.01, 1, and 100 µg/L (0, 0.031, 3.31, and 331.8 nM respectively). The exposure was performed in glass vessels with 300 mL of culture medium. The control was performed with the solvent at the same concentration of treatments, 1:10000. The medium was changed at the three days with Fenoxycarb, and the animals were fed with 18 mg of the food mixture (3 mg/animal). In each experiment, three of the snails were recovered for mRNA analysis per concentration. Four experiments were carried out so n=12 for each concentration.

Sequence identification and primer design

As previously stated, most of the array genes have been described in another article (Prieto-Amador et al. 2021). They are summarized in table 1. Nine genes are described here for the first time. The sequences were isolated from a transcriptome obtained in the laboratory (Aquilino et al. 2019) and the sequences published by Romiguier et al. (2014), that are available on the web (http://kimura.univ-montp2.fr/PopPhyl/index.php?section=data#dataset_0). The sequences obtained from the transcriptome and Romiguier et al. were identified by blasting them to the database using the txblast tool, a non-redundant database, and an e-value threshold of 1e-3. The search was performed with OmicsBox [OmicsBox - Bioinformatics made easy (Version 2.0.29). BioBam Bioinformatics. March 3, 2019. https://www.biobam.com/omicsbox]. The identified sequences were translated with Snapgene software (GSL Biotech LLC, USA), and the protein was compared to the GenBank protein database to confirm the identity of the gene. Estrogen-related receptor, IkBa, and DNA repair protein XRCC3 were identified from Romiguier et al. sequences while Galanin

receptor type 2, membrane progestin receptor beta, estradiol 17-beta-dehydrogenase 8, Retinoic acid receptor RXR, poly-ADP-ribose polymerase I, and Heat shock protein 70 B2-like were obtained from the transcriptome.

Pathway	Gene	Pathway	Gene	Pathway	Gene	Pathway	Gene
Endocrine system	*ER* *ERR* *MPR* *Hsd17b8* *GalR2* *RXR*	Nervous system	*AChE*	Epigenetics	*DNMT1* *KAT6B* *Hda1*	Oxidative stress	*Cat* *SOD CuZn* *SOD Mn*
		Apoptosis	*Casp3* *AIF3*				
		Detoxification	*Cyp 2U1* *Cyp 3A7* *Cyp 4F22* *Cyp 72A15* *GST K1* *GST M1* *GST O1* *GST T2* *Mrp1/ABCC1*	Stress	*sHSP 16.6* *sHSP 17.9* *Hsp60* *Hsc70 4* *Hsp70 B2* *Grp78/Bip* *Hsp83*	Glycogen	*PYGL*
						Lipids	*OSBPL8*
						Antibacterial	*ApA*
Repairing DNA	*PARP I* *IkBa* *XRCC3* *rad21* *rad50*					Reference	*Act* *PHFK* *GAPDH* *rpL10*
				Hypoxia	*HIF1a*		

Primers were designed using Primer-Blast (Ye et al., 2012). The amplicon size was 100-200 bp, and the optimal melting temperature was 58 ºC. All primer pairs were tested by polymerase chain reaction (PCR) in a C1000 thermocycler (BioRad, USA) with DNA AmpliTools Green Master Mix (Biotools, Spain). The single band was confirmed by gel electrophoresis (1.5% agarose gel). The PCR program was the same used for Real-Time PCR (RT-PCR).

RNA extraction and retrotranscription

Frozen adults were used to perform the RNA extraction. Each animal was processed individually. TRIzol extraction was made by following the manufacturer's indications. Briefly, the sample was homogenated. Then, chloroform was added to the sample and incubated for three minutes at room temperature. Afterward, the sample was centrifuged for 10 minutes, and the upper phase recovered. The RNA was precipitated with 0.7 volumes of isopropanol and washed with 75% ethanol. The RNA was resuspended in diethylpyrocarbonate treated water and incubated for 45 minutes with RNAse-free DNAse (Roche, Germany). A phenol: chloroform treatment was done with Phase-Lock tubes (5prime, USA) to remove the DNAse. The RNA was precipitated again with isopropanol and resuspended in 100 microliters of DEPC-treated water.

The retrotranscription was performed with MMLV (Invitrogen, Germany) in a final volume of 40 microliters with 10 micrograms of RNA following the manufacturer's indications. The primer used was a poly T_{18}. The retrotranscribed sample was maintained at -20 °C until used.

Real-Time PCR

The Real-Time PCR was done using a 96-well with 40 target genes and 4 reference genes, so each gene has two replicas. First, the efficiency of each primer pair was established by amplifying the sequence by PCR with the same conditions as Real-Time. Then, a 1:25000 dilution was done by mixing the PCRs of array genes, mimicking the variety of sequences in an RNA extraction. Finally, a five dilution series was used to obtain the efficiency curve with the same program used for Real-Time PCR (see below).

First, the primers were added to each well (250 nM each). Then, a master mix with the cDNA (8 µL per plate), Evagreen (0.5X), dNTPs (0.2 mM), 1X buffer, and 2.5 mM Cl_2Mg was prepared, and 10 microliters were added to each well. Two technical replicates were performed for each sample. The program used was an initial denaturation at 95 °C for 2 minutes and then 95 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 15 seconds repeated 39 times. After that, a melting curve from 60 to 85 °C was done with 0.5 °C steps to confirm a single product. To establish the threshold cycle, the regression option was used in the Maestro software. The Ct was the value used for to later analysis of the data.

Statistics

The statistical analysis was done with SPSS 25 (IBM, USA). Data showed no normal distribution when the Shapiro-Wilk test was performed; the non-parametric Kruskal-Wallis test was used to analyze them. Significance was set at $p \leq 0.05$. The number of samples per condition was n=9. There were considered as alterations those statistically significant changes related to control.

Results

1. Identification of sequences

Nine sequences were identified that code for different proteins related to the endocrine system (estradiol 17-beta-dehydrogenase 8, Estrogen related receptor, Galanin receptor type 2, membrane progestin receptor-beta, and Retinoic acid receptor), the stress response (Heat shock protein 70 B2-like), and the DNA repairing mechanisms (DNA repair protein XRCC3, NF-kappa-B inhibitor alpha, and poly-ADP-ribose polymerase I). The size of the sequence and the ORF size are shown in table 2. Furthermore, it is shown the identity and the similarity at the amino acid level with the indicated protein. The comparison with the database showed homology with proteins from other mollusks, mainly gastropods, except for

membrane progestin receptor beta, which the homology was with a protein from a bivalve. The homology was high except for the galanin receptor and the membrane progestin receptor-beta, while ERR and HSP70 B2 showed more than 90% identity. In figure 1 are shown the scheme of the proteins with the different motifs that characterize them. All of them showed the characteristic domains associated with those proteins, so it can be concluded that the isolated sequences correspond to the genes coding those proteins.

	Gene	Size bp (DNA)	Size aa (prot)	Homology	identity (%)	Similarity (%)
Contig11036	DNA repair protein XRCC3	1273	345	PREDICTED: DNA repair protein XRCC3-like isoform X1 - *Biomphalaria glabrata* XP_013089306	58	72
c26085	estradiol 17-beta-dehydrogenase 8	1055	253	17 beta-hydroxysteroid dehydrogenase 8 - *Lymnaea stagnalis* QNG40045	78	90
Contig1634	Estrogen related receptor	3125	443	PREDICTED: steroid hormone receptor ERR2-like isoform X3 - *Biomphalaria glabrata* XP_013080351	91	96
c16863	Galanin receptor type 2	2066	525	PREDICTED: galanin receptor type 2-like - *Biomphalaria glabrata* XP_013071428	38	55
c31504	Heat shock protein 70 B2-like	2486	636	PREDICTED: heat shock protein 70 B2-like - *Biomphalaria glabrata* XP_013072147	91	96
Contig8155	NF-kappa-B inhibitor alpha	1611	377	PREDICTED: NF-kappa-B inhibitor alpha-like - *Biomphalaria glabrata* XP_013067082	57	74
c21665	membrane progestin receptor beta	2308	340	membrane progestin receptor beta-like - *Mizuhopecten yessoensis* XP_021341591	39	58
c30036	poly-ADP-ribose polymerase I	3162	991	poly-(ADP-ribose) polymerase I - *Aplysia californica* NP_001191521	77	87
c31104	Retinoic acid receptor RXR	3551	435	Retinoic acid receptor RXR - *Lymnaea stagnalis* Q5I7G2	96	97

2. Gene expression profile in response to Fenoxycarb exposure

Adults snails were exposed to 0.01, 1, and 100 µg/L of Fenoxycarb for seven days to assess the mid-term response of the genes analyzed (figures 2 to 8). Fenoxycarb is an analog of juvenile hormone that is expected does not to affect non- arthropods. There was no statistically significant response in those genes related with the endocrine system (figure 2), DNA repairing mechanisms (figure 3), oxidative stress (figure 4), apoptosis (figure 4), phase I (figure 5), phase II (figure 6) and phase III (figure 6) of detoxification, most of stress proteins

(figure 7), hypoxia (figure 7), and energy metabolism (figure 8). Only three genes were modified at 1 µg/L: *acetylcholinesterase* (figure 4), *HSP 17.9* (figure 7), and *aplysianin A* (figure 8). Therefore, the two genes analyzed in relation to nervous system and immunity showed some alteration, suggesting some mid-term effects in the physiology of *P. acuta*.

Discussion

The advances in toxicological studies demand to extend the toxicity tests to additional levels and not stay with the traditional endpoints such as survival, reproduction, or development. Nowadays, the molecular approach to evaluate the toxicity is frequent, and to perform it requires additional putative biomarkers that assess the modulation of different cellular processes and physiological mechanisms (Lee et al. 2015; Martins et al. 2019; Steiblen et al. 2020). In this sense, adding new genes to the battery of biomarkers allows it to accomplish it by extending the number of processes studied and the levels of response depending on the pathway analyzed. Therefore, the description new genes is a step in extending the value of *Physella acuta* in toxicological studies. Here we have described nine new sequences that code for different hormone receptors, an enzyme involved in regulating the concentration of active estrogens and androgens (estradiol 17-beta-dehydrogenase 8), one stress protein, and three proteins involved in the DNA repairing. These genes can improve the analysis of different processes related to endocrine disruption, genotoxicity, and development. Furthermore, all of them can help understand the response in the different levels of organization, from molecular to ecological, providing insights into the mechanisms of the toxicant and the responses of the organisms to maintain the homeostasis to manage the changing environment. On the other hand, these putative biomarkers open new ways to assess the toxicity before being observed at the individual level, preventing irreversible damage that affects the population. Thus, similar to clinical practice, new tools are required for a better approach to the molecular events to get an earlier diagnosis that helps detect the pollution before it causes irreversible effects damaging the ecosystems.

For a long time, the search for pesticides with either low or no impact on non-target species has been one of the agricultural aims. Analogs of the juvenile hormone have been one of such pesticides since they mimic one specific hormone of arthropods, drastically reducing the risk for other species (Wilson 2004). It is known that Fenoxycarb affects the development and different cell processes in insects, arachnids, and crustacean (Jungmann et al. 2009; Navis et al. 2018; Lee et al. 2020). However, the poor knowledge of invertebrate physiology makes it necessary to test them in non-target species to ensure the low impact. The first

element to consider is the fact that the response is observed at 1 µg/L of Fenoxycarb, which is the intermediate concentration used. It is very low compared to those that have effect on insects and crustacean (Cripe et al. 2003; Mahmoudvand and Moharramipour 2015; Hu et al. 2019). The lack of response at higher concentration can be due to an earlier response, recovering the normal condition to the time of the analysis by the action of detoxification mechanisms. For the lower concentration, the non observed response could be because the amount of toxicant it is below the threshold concentration to trigger it. Other possibility could be that it is necessary more time to reach the threshold time. Additional research at different times would allow to elucidate what it is happening and the consequences depending on the concentration.

In this work, we have tested the response at the gene expression level of the analog of juvenile hormone, Fenoxycarb, and observed a response that, although it is weak, demands additional studies to ensure the lack of toxicity in non-arthropods. As expected, no effect was observed in genes related to DNA repairing mechanisms or stress response. There is no data in literature that study these genes, even in insects. Similar consideration can be done with the energy metabolism, although there are some reports about the impact of fenoxycarb in lipids and carbodhydrates of crustanceans (Arambourou et al. 2018; Hu et al. 2019, 2020). As far we know, there is no previous report analyzing the detoxification mechanisms response in presence of Fenoxycarb. In *P. acuta* there is no change in the genes analyzed involved in phase I, phase II, and phase III of detoxification. It suggests that other proteins different from those analyzed here are responsible for the biotransformation of this chemical. Differentially to our results, with no changes in genes related to epigenenic regulation, it has been described that a exposure for the days at 50 µg/L of Fenoxycarb can upregulate histone deacetylase in the water flea *Moina macrocopa* (Hu et al. 2020). It could be reflecting the different sensitivity to the compound but also the fact that the epigenetic changes in the water flea are related with the mimicking of the juvenile hormone effects.

The effect on the acetylcholinesterase gene suggests some impact on the nervous system. As stated in introduction, it has been observed some effects on rat brain acetylcholinesterase and nicotinic receptors (Smulders et al. 2003), suggesting that Fenoxycarb can have nervous effects on non-target organisms. The studies in nicotinic receptors showed that Fenoxycarb mechanism was noncompetitive (Smulders et al. 2004). Although the Fenoxycarb has been used as an analog of juvenile hormone, it seems to have also some effect as the rest of carbamates by affecting the nervous system. The response observed in *P. acuta* support

nervous effect and suggests that it could affect the ability of the snail to survive by altering the central nervous system. Additional studies would help elucidate the putative effect on the snail's behavior or ability to respond to situations involving the nervous system. In any case, it is a fact to consider in the impact that Fenoxycarb can have in non-target species at the mid and long-term.

On the other hand, the modulation of *sHSP17.9* suggests some effect, but it is complicated to ensure the actual impact in the cell. Small heat shock proteins are diverse proteins involved in stress response and related to multiple cellular processes, including neural functions (de Los Reyes and Casas-Tintó 2022). In this sense, it is tempting to speculate that *sHSP17.9* could code some sHSP involved in neural physiology but the difficulties to establish the homology demand to be cautious. Additional research will provide more information and could help to define the role of this protein in the cell. As biomarkers, the fact that sHSPs share the alpha-crystallin domain makes it easy to identify them. However, their high diversity in N- and C-terminal regions difficult the identification of homologies between species. Consequently, a deeper study of this protein family is required to know the roles in cell metabolism that they have and to establish functional homologies between them. In any case, the alteration observed suggests that Fenoxycarb has some effect in the mid-term in *Physella acuta*, raising the possibility that it causes some reduction in the wellness of the snail.

The Aplysianin-A is a protein involved in the immune response by acting as an antibacterial. This antibacterial glycoprotein inhibited both Gram-positive and Gram-negative bacteria in *Aplysia kurodai* (Kamiya et al. 1986). The alteration in the transcriptional activity can produce a modulation of the response to bacterial infections, making *P. acuta* more sensitive to them. The impact observed in *P. acuta* suggests a putative alteration in immunity, being the first time that this possibility is suggested for Fenoxycarb. Additional studies involving more immune related genes are needed to confirm it and determine the effect in the long term survival of the population.

Safety at the environmental level is a concern of all the products used as pesticides. The search for new pesticides with a reduced impact in non-target species demands testing in these species because some of them can have low impact. However, the lack of knowledge about the physiology of invertebrates requires experimental work to confirm it. It will also provide additional information about the physiology of the invertebrates, decreasing the gap with the vertebrates and favoring the use of invertebrates as alternative methods that reduce the use of vertebrates in the test of toxicity. The present evidence suggests a low impact for

293 the environment of Fenoxycarb, but the results observed in the non-target species *Physella*
294 *acuta* requires an extended analysis because it can reflect an impact at the long-term as a
295 consequence of the exposure. To ensure the harmless of the Fenoxycarb, it should be
296 analyzed in several non-target species covering different groups of invertebrates.

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305 **Author contributions**

306 J-L. M-G. conceived and designed the analysis, wrote the paper, and contributed to data
307 analysis. M. P-A. and P. C. collected the data and contributed to data analysis.

308 **Competing Interests Statement**

309 The authors declare no competing interests.

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400 **Captions**

401 **Figure 1.** Structure and conserved domains of the *Physella acuta* proteins code by the
402 sequences identified. The characteristic motifs of each protein are shown. The domains have
403 been defined according to the CCD functional classification of proteins. The size is indicated
404 by the numbers.

405 **Figure 2.** Transcript levels of endocrine related sequences (*estrogen receptor*, *estrogen-*
406 *related receptor*, *membrane progestin receptor beta*, *estradiol 17-beta-dehydrogenase 8*,
407 *galanin receptor*, and *Retinoic acid receptor*) in *Physella acuta* adults after *in vivo* exposure
408 to Fenoxycarb for seven days at 19 °C. Transcriptional activity was quantified by RT-PCR
409 using *rpL10*, *Act*, *PFKFB2*, and *GAPDH* as reference genes. The comparison was performed
410 with the solvent-exposed controls. Whisker boxes are shown. Each box corresponds to
411 twelve individuals. The median is indicated by the horizontal line within the box, and the 25th
412 and 75th percentiles are indicated by the boundaries of the box. The highest and lowest
413 results are represented by the whiskers. The small triangle inside the box denotes the mean,
414 and the outliers are shown (circles). No significant differences to control were observed in
415 those genes ($p < 0.05$).

416 **Figure 3.** Transcriptional activity of genes related with DNA repairing. It is shown the mRNA
417 levels of *Poly(ADP-Ribose) Polymerase*, *NFKB inhibitor Iκβ*, *X-Ray Repair Cross*
418 *Complementing 3*, *RAD21*, and *RAD50* in *Physella acuta* adults after *in vivo* exposure to
419 Fenoxycarb for seven days at 19 °C. RT-PCR was used to quantify the mRNA levels and
420 *rpL10*, *Act*, *PFKFB2*, and *GAPDH* were used as reference genes. The comparison was
421 performed with the solvent-exposed controls. Whisker boxes are shown. Each box
422 corresponds to twelve individuals. The median is indicated by the horizontal line within the
423 box, and the 25th and 75th percentiles are indicated by the boundaries of the box. The highest
424 and lowest results are represented by the whiskers. The small triangle inside the box denotes
425 the mean, and the outliers are shown (circles). No significant differences were observed to
426 control ($p < 0.05$).

427 **Figure 4.** Transcriptional activity of genes related to nervous system (*acetylcholinesterase*),
428 oxidative stress (*catalase*, Copper-Zinc superoxide dismutase, and *Manganese superoxide*
429 *dismutase*), and apoptosis (*caspase 3* and *apoptosis inducing factor 3*). Snails were exposed
430 to Fenoxycarb for seven days at 19 °C. Quantification by RT-PCR was performed using
431 *rpL10*, *Act*, *PFKFB2*, and *GAPDH* as reference genes. The comparison was performed with
432 the solvent-exposed controls. Whisker boxes are shown. Each box corresponds to twelve

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 difference to control (asterisk) is indicated ($p < 0.05$).

Figure 5. Transcript levels of cytochrome P450 genes (*Cyp2u1*, *Cyp3a7*, *Cyp4f22*, and *Cyp72a15*). Adult snails were exposed for one week to Fenoxycarb at 19 °C. The mRNA levels were analyzed 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 twelve 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). No significant differences to control were detected ($p < 0.05$).

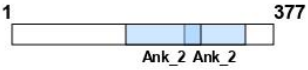
Figure 6. Transcript levels of Phase II related (*GSTk1*, *GSTm1*, *GSTo1*, and *GSTt2*) and Phase III (*multidrug resistance protein 1*) related genes in *Physella acuta* adults after *in vivo* exposure to Fenoxycarb for seven days at 19 °C. Transcriptional activity was quantified by RT-PCR using *rpL10*, *Act*, *PFKFB2*, and *GAPDH* as reference genes. The comparison was performed with the solvent-exposed controls. Whisker boxes are shown. Each box corresponds to twelve 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). No significant differences were detected to control condition ($p < 0.05$).

Figure 7. Transcriptional activity of stress (*sHSP16.6*, *sHSP17.9*, *HSP60*, *HSC70 (4)*, *HSP70B2*, *Grp78/BiP*, and *HSP90*) and hypoxia (*HIF1α*) genes in adult snails. The animals were exposed for one week to Fenoxycarb 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 twelve 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 difference to control (asterisk) is indicated ($p < 0.05$).

Figure 8. Transcriptional activity of genes related to epigenetic modulation (*DNA methylase I*, *Lysine Acetyltransferase 6B*, and *histone deacetylase 1*), immunity (*aplysianin-A*), and energy metabolism (*Glycogen Phosphorylase L* and *Oxysterol Binding Protein Like 8*) in *Physella acuta* adults after *in vivo* exposure to Fenoxycarb 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 twelve individuals. The median is indicated by the horizontal line within the box, and the 25^{th} and 75^{th} 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 difference to controls (asterisk) is indicated ($p < 0.05$).

Poly-ADP-ribose polymerase I

NF-kappa-β inhibitor alpha-like

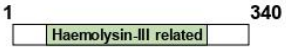


Heat shock protein 70 B2-like

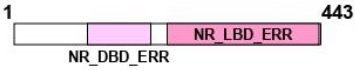
DNA repair protein XRCC3

Galanin receptor type 2

Membrane progestin receptor beta



Estrogen related receptor



Estradiol 17-beta-dehydrogenase 8

Retinoic acid receptor

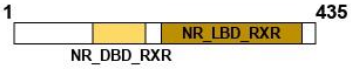
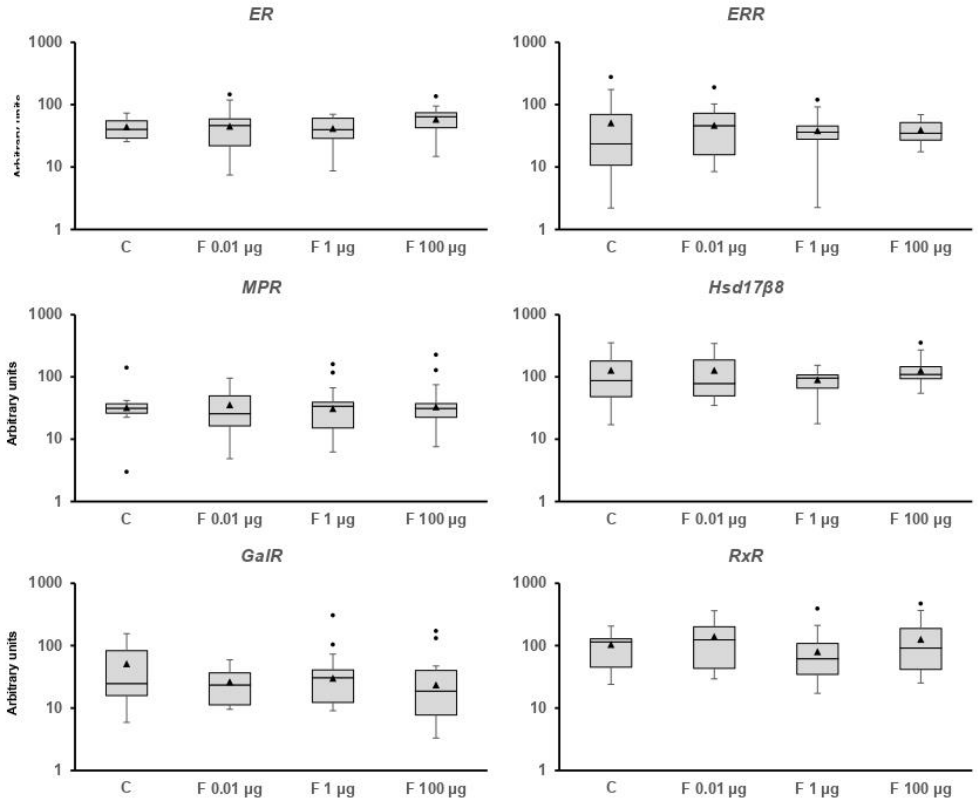


Figure 2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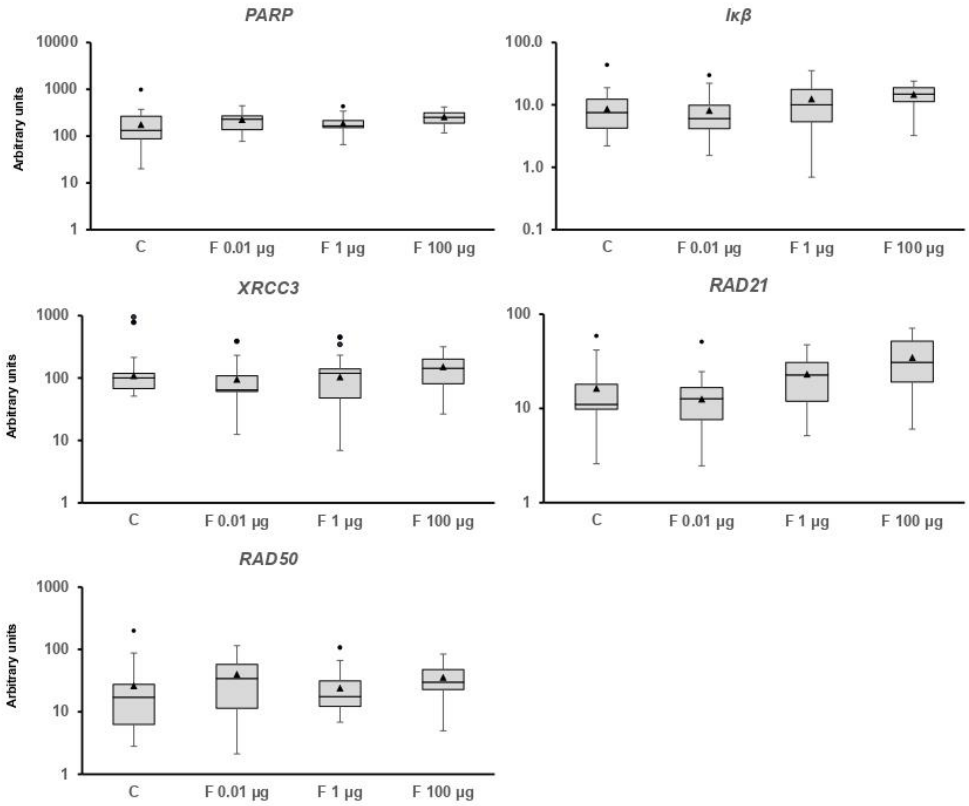
ERR
MPR
Hsd17β8
GalR
RxR
Arbitrary units
1000
100
10
1
C
F 0.01 µg
F 1 µg
F 100 µg

Figure 3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10
1
C
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10.0
1.0
0.1
XRCC3
RAD21
RAD50

Figure 4

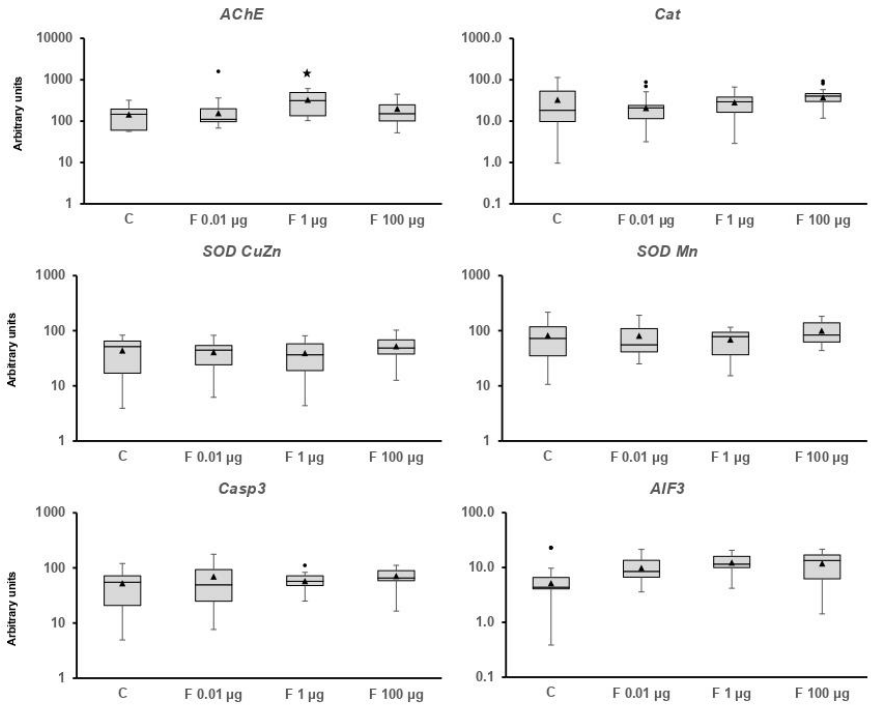
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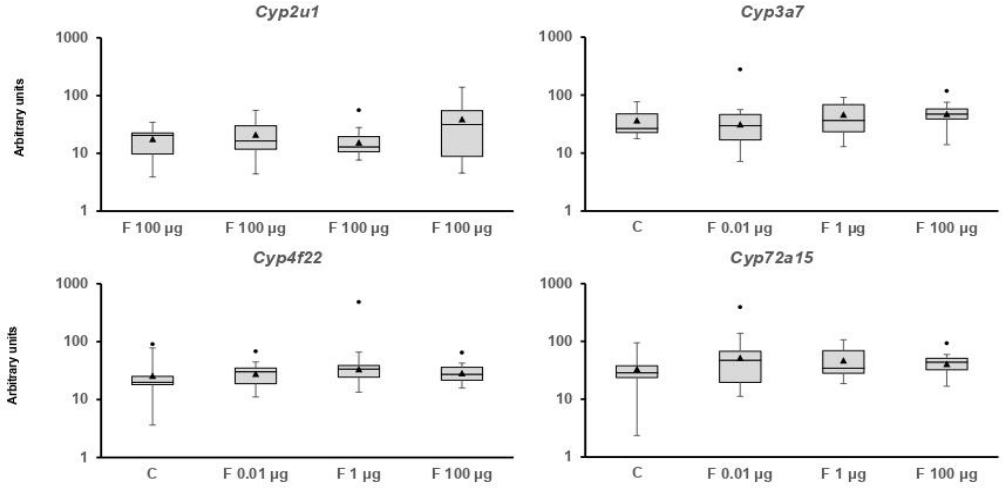
Figure 5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10
1
C
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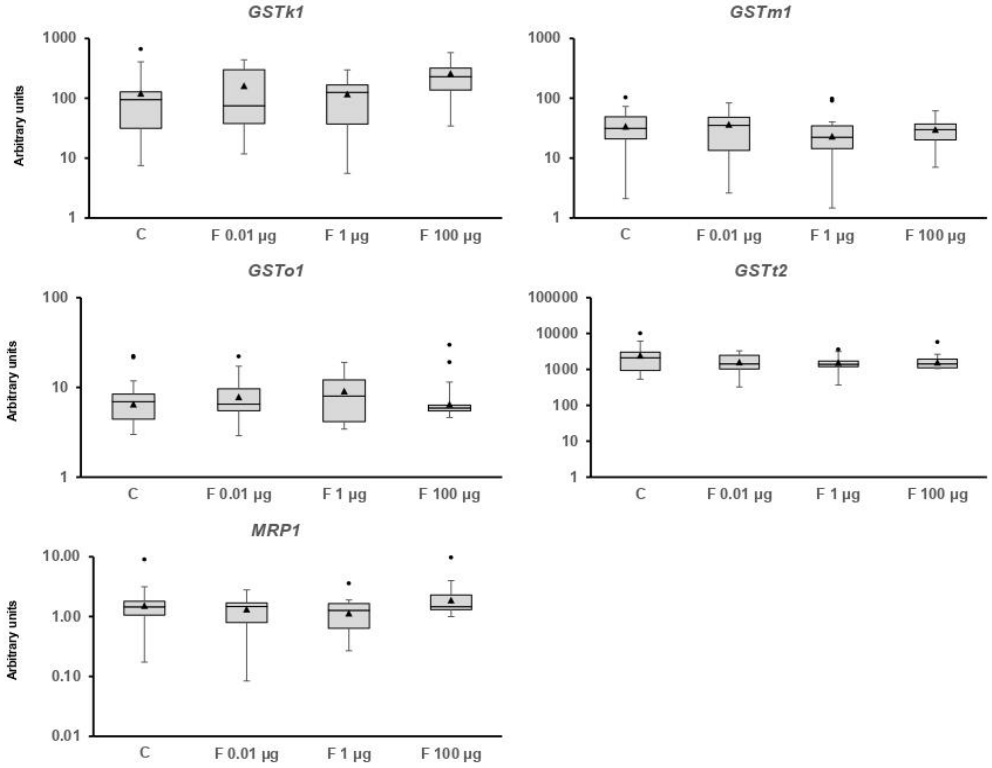
F 100 µg

Figure 6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MRP1
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C
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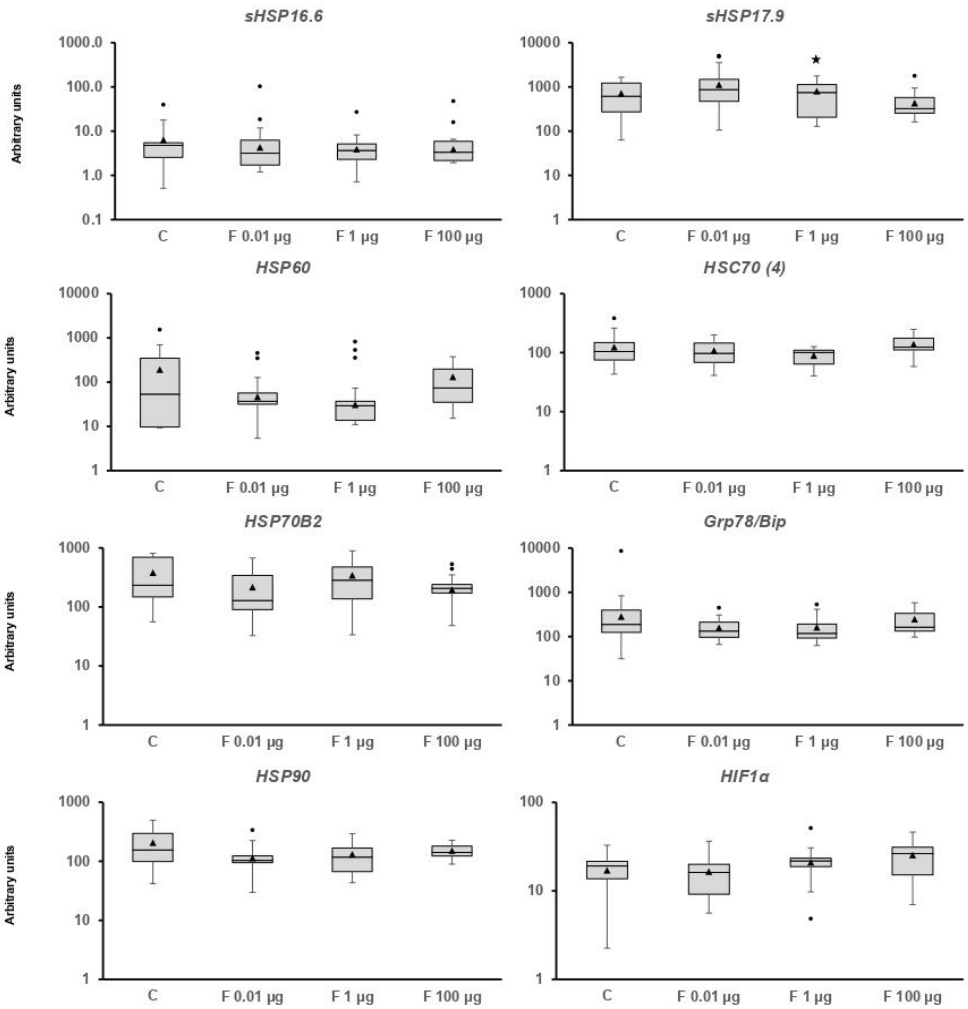
F 1 µg
F 100 µg

Figure 7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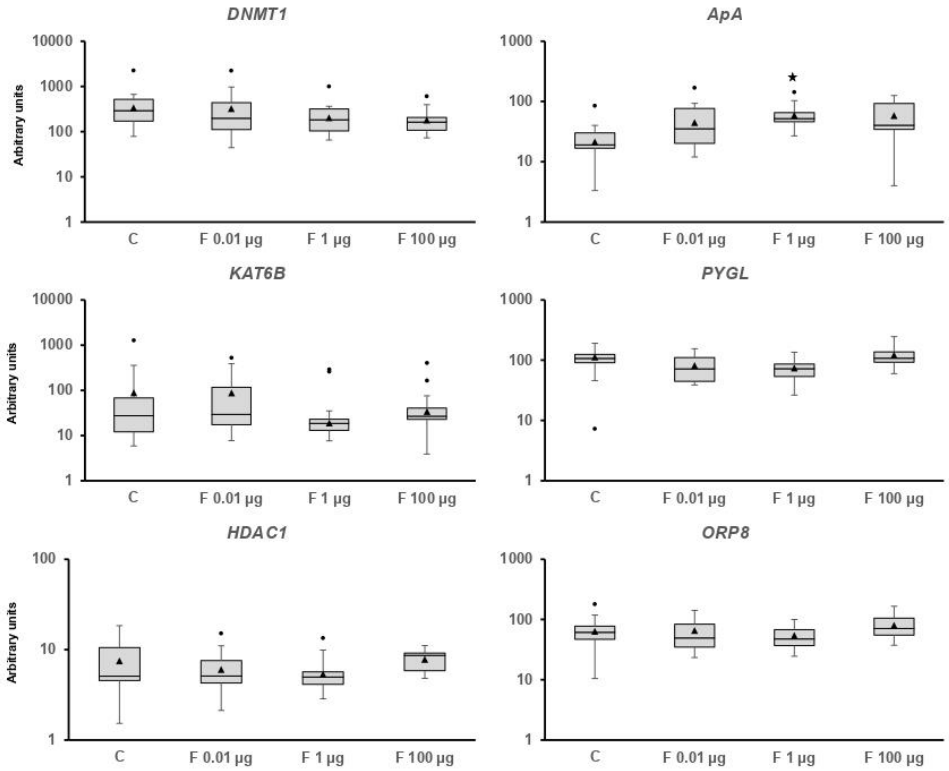
sHSP17.9
HSP60
HSC70 (4)
HSP70B2
Grp78/Bip
HSP90
HIF1α
Arbitrary units
C
F 0.01 µg
F 1 µg
F 100 µg

Figure 8


DNMT1
ApA
KAT6B
PYGL
HDAC1
ORP8
Arbitrary units
C
F 0.01 µg
F 1 µg
F 100 µg