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BPA and its analogues (BPS and BPF) modify the expression of genes involved in the endocrine pathway and apoptosis and a multi drug resistance gene of the aquatic midge *Chironomus riparius* (Diptera)☆

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ABSTRACT

Many countries are limiting the use of bisphenol A (BPA) because evidence shows it is dangerous to human health and wildlife. For the manufacturing of polycarbonate plastics, bisphenol S (BPS) and bisphenol F (BPF) are proposed as safer alternatives. They have already been released into the aquatic environment without previously available information about their potential adverse effects. In this study, we compared the effects of BPA, BPS and BPF exposure to the expression profile of genes involved in the endocrine pathway (*EcR* and *E74*), ecdysone metabolism (*Cyp18a1* and *Shadow*), apoptosis (*DRONC)* and the multidrug resistance-associated protein 1 gene (*MRP1*) in the midge, *Chironomus riparius* (Diptera). The three toxicants increased *Shadow* expression, which is involved in ecdysone synthesis, but only BPF significantly altered *Cyp18a1*, which is implicated in ecdysone degradation. BPS and BPF modified *EcR* and *E74* expression; BPF upregulated the effector caspase *DRONC*. Furthermore, BPA significantly increased *MRP1* expression. This study provides insights into the action of bisphenols at the molecular level and highlights the potential risks of BPS and BPF as BPA alternatives.

1. Introduction

Bisphenol A (BPA, 2, 2-bis [4-hydroxyphenyl] propane) is commonly used as a monomer in polycarbonate synthesis in the production of epoxy resins and an additive for the removal of excess of hydrochloric acid during polyvinyl chloride (PVC) production (Michałowicz, 2014). BPA is present in plastic food containers, kitchen utensils, dental materials, healthcare equipment, thermal paper, toys and articles for children and babies and in the internal coatings of cans and caps (Chen et al., 2002; Vandenberg et al., 2012). This toxicant can migrate to the food and beverages stored in the containers made from polycarbonates. Due to its widespread use in the chemical industry for more than 50 years, BPA can be detected in wastewaters, surface and drinking waters, terrestrial and marine environments and humans (urine and blood) (Fromme et al., 2002; European Union Risk Asses, 2010; Vandenberg et al., 2010). Exposure of humans to this compound can occur through different routes, including oral, inhalation and transdermal. BPA is metabolised in the liver to form BPA glucuronide and it is excreted in the urine (Yokota et al., 1999). Since it has a phenolic structure, BPA can interact with estrogen receptors and act as an antagonist or hormonal agonist (Ma and Sassoon, 2006). Numerous studies demonstrated the endocrine disruptive potential of BPA (Vandenberg et al., 2012) and its adverse effects on aquatic organisms (Alexander et al., 1988; Mihaich et al., 2009; Planelló et al., 2008; Martínez-Paz et al., 2012; Martínez-Paz et al., 2013). This chemical compound is involved in the pathogenesis of multiple endocrine disorders, including female and male infertility, precocious puberty and hormone-dependent tumors such as breast and prostate cancer (Diamanti-Kandarakis et al., 2009). Previous studies with *Echinometra lucunter* showed that BPA affected significantly the embryonic development of this sea urchin, indicating the risk that this pollutant may present to marine biota (Da Silva and de Souza Abessa, 2019). Recent research suggest that this compound may interrupt molting and reproduction in *Daphnia magna* (Kim et al., 2019a). BPA use is currently subject to strict regulation; many countries are limiting its use (Ji and Choi, 2013). Thus, the industry is replacing it with other analogues, such as the structurally similar bisphenol S (BPS; bis [4-hydroxyphenyl] sulfone) and bisphenol F (BPF; bis [4-hydroxyphenyl] methane), presumably safer alternatives to BPA (Table 1).

Currently, BPS and BPF are used for a wide variety of consumer and industrial products (Michałowicz, 2014; Liao et al., 2012; Office of Environmental Health Hazard Assessment, 2012). Thus, humans are increasingly exposed to these structural BPA analogues. According to environmental monitoring studies, BPA remains the major bisphenol

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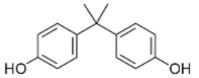
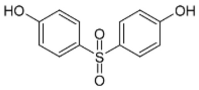
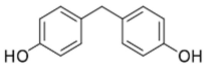
Table 1
BPA and its structural analogues.

	Abbreviation	Synonym	Chemical structure	MW(g-mol $^{-1}$)	Formula
Bisphenol A	BPA	2,2-bis(4-hydroxyphenyl) propane		228.291	$C_{15}H_{16}O_2$
Bisphenol S	BPS	4,4′-Sulfonyldiphenol		250.27	$C_{12}H_{10}O_4S$
Bisphenol F	BPF	bis(4-hydroxyphenyl) methane		200.237	$C_{13}H_{12}O_2$

found in surface waters in China, the United States, Japan, South Korea, Germany and Italy (Fromme et al., 2002; Chen et al., 2016). BPF and BPS are frequently detected as the second- and third-most abundant analogues in the environment. However, this scenario is currently changing, some recent studies reported similar orders of magnitude for BPS, BPF and BPA in surface waters, sediments and sewage effluents and human urine (Thomas et al., 2014; Yang et al., 2014). The BPA concentration detected in the Kogawauchi and Kiyotake Rivers in Japan was 900 ng/L (Kang and Kondo, 2006). BPF concentrations exceeded 1000 ng/L in river seawater collected from Japan, Korea and China and became up to 2850 ng/L in the Tamagawa River in Tokyo (Chen et al., 2016; Yamazaki et al., 2015; Yan et al., 2017). In aquatic environments, the reported BPS concentration was 1600 ng/L in Lake Taihu (China), located in an industrial zone, and 7200 ng/L in the Adyar River in India (Yamazaki et al., 2015). Multiple locations have median bisphenol concentrations that exceed the predicted no-effect concentration (PNEC) of 1500 ng/L for BPA recommended by the European Union (Yamazaki et al., 2015). Xenobiotics are not continuously discharged into the environment beyond this basal level. However, acute concentration peaks can often occur as a result of spills. Very little is known about the consequences of such exposures, including the cumulative effect or the pulse recovery (Ashauer and Escher, 2010). In addition, there is only scarce information with respect to the bioaccumulation of emerging bisphenols in aquatic organisms. The bioaccumulation and biomagnification of BPS, along with other analogues, have been evaluated in biota samples collected at different trophic levels in Lake Taihu, China (Wang et al., 2017). This previous study showed the detection frequency of BPS in the biota samples analysed was 70.6% and the mean proportion of this compound in the total bisphenols was 0.34%.

Compounds that replace BPA should be inert or less toxic than the original product, but many of the new synthesis chemicals have not been tested before being introduced into industries and the environment. The toxicological properties and adverse health impacts of BPA are widely known (Alexander et al., 1988; Mihaich et al., 2009; Planelló et al., 2008; Martínez-Paz et al., 2012; Martínez-Paz et al., 2013; Diamanti-Kandarakis et al., 2009; Da Silva and de Souza Abessa, 2019; Kim et al., 2019a). Recent research works have shown that bisphenol A analogues exhibit similar toxicity to BPA (Tišler et al., 2016). Comparative toxicity studies between BPA and BPF in aquatic organisms have shown that in most cases, BPA is a more toxic compound, however, BPF affects more the pigmentation of zebrafish embryos and the *Daphnia magna* reproduction after 21 days of exposure (Vincent et al., 2017). BPS and BPF have a similar potency to BPA in terms of their androgenic, antiandrogenic, estrogenic and antiestrogenic activities (Liao et al., 2012). Previous studies carried out *in vitro* and *in vivo* have demonstrated the estrogenic capacity of these three BPs in different cells and tissues and at different stages of development in zebrafish (Tišler et al., 2016). Furthermore, a recent study reported the endocrine disruptive potential of BPS in invertebrates: it enhances transcriptional activity of some genes that encode nuclear receptors, genes involved in the hormonal pathway mediated by ecdysone or genes involved in the cellular stress response and detoxification mechanisms (Feng et al., 2015; Herrero et al., 2018). Therefore, *in vivo* studies that compare the effects, mechanisms of action and putative interaction routes of these three compounds are necessary to evaluate the physiological effects and endocrine activities of BPA substitutes.

The midge, *Chironomus riparius* (Meigen, 1804), is a widely used organism in ecotoxicology studies. The larvae have a characteristic intense red color due to haemoglobin and they are used in standardised tests because of their ecological relevance in freshwater ecosystems. This species belongs to the order Diptera and presents four developmental stages: embryo, larva (with four different instars), pupa and adult. All stages, with exception of the adult, are aquatic and the life cycle takes approximately one month (Armitage et al., 1995). Several standard tests are used to analyse different physiological endpoints (EPA, 1996; OECD, 2004). Alterations in gene expression by different toxicants are described in this species. Existing data suggest that it is appropriate for molecular analysis (Lencioni et al., 2016; Martín-Folgar and Martínez-Guitarte, 2017a; Nair et al., 2011; Park and Kwak, 2012).

The endocrine system is essential for insect development and metamorphosis and it involves interactions among different hormonal pathways. In the transition from the larval stage to the adult, molecular and cellular alterations occur, as well as morphological and physiological changes that cause the loss of embryonic tissues and the formation of others. There are two hormones, 20-hydroxyecdisone (20E) and juvenile hormone (JH), responsible for coordinating the growth and development of insects (Dubrovsky, 2005). The genes *EcR* and *E74* encode proteins involved in the ecdysone response cascade which are essential for the activation of factors involved in development signaling pathways and cell cycle regulation during metamorphosis. *Shadow* is involved in the synthesis pathway of the ecdysone hormone while *Cyp18a1* is responsible for ecdysone degradation (Guittard et al., 2011). On the other hand, death regulator Nedd2-like caspase (*DRONC*), which encodes a protein that acts as an upstream regulator of the DNA damage response (DDR), can intervene or initiate the DDR or apoptosis in damaged cells (Khan et al., 2017). Finally, different ATP-binding cassette (ABC) transporters participate in the cellular detoxification process. Multidrug resistance-associated protein 1 gene (*MRP1*) codes for a protein involved in this process with the excretion of the conjugated compound to the extracellular medium. MRP1 is related to cellular resistance to different toxicants. Martinez-Guitarte et al. (2018) (Martínez-Guitarte, 2018) previously identified a partial sequence for this ABC transporter.

In this study, we compared how BPA and its analogues, BPS and BPF, altered the expression of genes involved in several cellular

processes using quantitative real-time polymerase chain reaction (RT-PCR). Acute exposures for a short time period (24 h) were used to simulate what would happen in the case of a spill. Concentrations were selected based on the results from previous studies in *C. riparius* with BPA (Planelló et al., 2008; Martínez-Paz et al., 2012; Martínez-Paz et al., 2013) and BPS (Herrero et al., 2018). The effects of BPA, BPS and BPF exposure to the expression profile of genes involved in the endocrine pathway (*EcR, E74*), ecdysone metabolism (*Cyp18a1* and *Shadow*), a gene related to apoptosis (*DRONC*), and *MRP1* were evaluated in *C. riparius* larvae to compare the putative mechanisms involved and the molecular interactions of these three BPs.

The aim of this study was to unravel the putative effects of BPS and BPF in relation to BPA to assess their safety in strongly polluted aquatic environment. The obtained results will allow to compare the effects and to analyse the similarities and differences in the activity of these three bisphenols. Moreover, this study will provide new evidence at the molecular level of the activity of BPA and its analogues.

2. Materials and methods

2.1. Animals

Fourth instar *C. riparius* larvae were used as the experimental model organism. They were maintained in a controlled-climate room at 20 °C for several generations according to toxicity testing guidelines (EPA, 1996; OECD, 2010). The culture conditions were defined previously (Martín-Folgar and Martínez-Guitarte, 2017b).

2.2. Treatments

Three individual fourth instar larvae were exposed to different concentrations of BPA, BPS and BPF separately in glass vessels. Each treatment consisted of three replicates (n = 9) and three independent experiments performed for each analysis (n = 27). Individuals with the same age and size were collected from different egg masses. A starting stock solution (2.5 g/L) of each compound was prepared in ethanol. Larvae were exposed to 0.05, 0.5 or 1 mg/L BPA, BPS or BPF diluted in 50 mL of culture medium for 24 h. Control larvae were maintained for 24 h in culture medium with the same concentration of ethanol (0.00016%) as the bisphenol-exposed larvae and were also measured in triplicate. Larvae did not receive food during the whole experiment. No mortality was observed during the BPs exposures. After the treatments, exposed and control larvae were stored at −80 °C.

2.3. RNA isolation

Each control and treated larvae were processed individually for the extraction of their total RNA following the methods of Martín-Folgar and Martinez-Guitarte (2017) (Martín-Folgar and Martínez-Guitarte, 2017b). Purified RNA was stored at −80 °C.

2.4. Complementary DNA (cDNA) synthesis and qRT-PCR

Reverse transcription was performed using MMLV (Invitrogen, Germany), according to the manufacturer's instructions. cDNA was used as the template for qRT-PCR to analyse the messenger RNA (mRNA) expression profile of four genes related to the endocrine system (*EcR, E74, Cyp18a1* and *Shadow*), one gene related to apoptosis (*DRONC*) and *MRP1* from control and bisphenol-treated samples. The sequences of all gene-specific primers used in this study are indicated in Table 2. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *ribosomal protein L13* (*rpL13*) genes, with a coefficient of variation < 0.25 and an M-value < 0.5, were used as endogenous reference controls (Martínez-Paz et al., 2012; Martínez-Guitarte et al., 2007; Martínez-Guitarte et al., 2012; Martínez-Paz et al., 2014; Morales et al., 2011; Morales et al., 2012). qRT-PCR conditions were defined previously (Martín-Folgar and Martínez-Guitarte, 2017b).

Table 2
Primers used for qRT-PCR amplification from *Chironomus riparius*.

Gene	Sequence	Efficiency (%)
DRONC	5′- GAAATGTCACAGATTTCAGTGCC-3′ 5′- GTGAATATCGTAAGCATGTTCTGC-3′	99.3
MRP1	5′-CGAGACAACTTCAAGTTCCACA-3′ 5′-CCTTATTATCTCCATCATCTTGG-3′	101.4
Cyp18a1	5′- GTTTCACTCGAGACGATCCA-3′ 5′- TTTAGCGGCTTGAAATGTTG-3′	104.5
E74	5′- TCTTACTGAAACTTCTTCAAGATCG-3′ 5′- GCTTTGAGACAGCTTTGGAATCG-3′	103.2
EcR	5′- CCATCGTCATCTTCTCAG-3′ 5′- TGCCCATTGTTCGTAG-3′	106.6
rpL13	5′- ACCAGCTAGAAAGCACCGTC-3′ 5′- ATGGGCATCTGACGATTGGG-3′	101.6
GAPDH	5′- GGTATTTCATTGAATGATCACTTTG-3′ 5′- TAATCCTTGGATTGCATGTACTTG-3′	107.7

2.5. Statistical analysis

mRNA levels for *EcR, E74, Cyp18a1, Shadow, DRONC and MRP1* were normalised against the expression of *GAPDH* and *rpL13* in the same samples. Statistical analyses were performed using SPSS 19 software (IBM). Data normality and variance homogeneity was assessed with the Levene test. For normally distributed data, significant differences among control and treated larvae were compared using analysis of variance (ANOVA) followed by Games-Howell's and Dunnett's multiple comparison tests. Two levels of significance are reported: $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

2.6. Bioinformatics analyses

Sequence alignments were performed with ClustalW by using MEGA 7.0.14 with default options (Kumar et al., 2016). Structures for binding site analysis were obtained from the Protein Data Bank (PDB) (Berman et al., 2000). Binding sides were established from the annotated structural features (Golovin et al., 2005) and by visual inspection of poseview diagrams in RCSB-PDB (Stierand and Rarey, 2010) and of environmental details in PDBe (see Supplementary material).

Chemical and Physical Properties of estradiol, 20-hydroxyecdysone (20E), BPA, BPS and BPF were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2019b), and ChEMBL (https://www.ebi.ac.uk/chembl/) (Davies et al., 2015; Gaulton et al., 2017).

3. Results

3.1. Effects of BPA, BPS and BPF on endocrine-related genes

The effects of BPA, BPS and BPF exposure on the expression of the endocrine-related genes *E74, EcR, Cyp18a1* and *Shadow* were examined with qRT-PCR. At 0.5 mg/L, BPA, BPS and BPF significantly elevated *Shadow* expression compared to control treatment. Thus, the three compounds affect the ecdysone synthetic pathway by altering *Shadow* expression (Fig. 1). Regarding ecdysone degradation, only the highest BPF concentration (1 mg/L) significantly increased *Cyp18a1* mRNA. However, there was a trend for increased *Cyp18a1* expression with BPS at 1 mg/L and BPA at 0.5 and 1 mg/L with respect to the control larvae (Fig. 1).

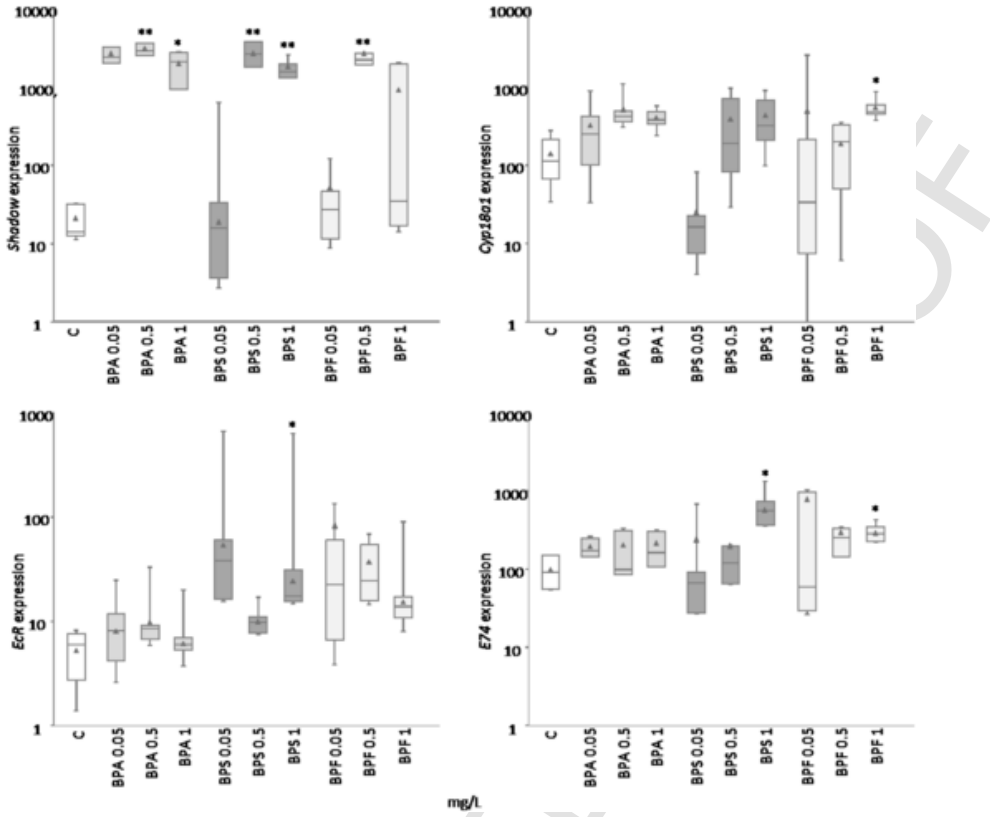


Fig. 1. Effects of BPA, BPS and BPF exposure on the expression of genes related to the endocrine system in *C. riparius* larvae. mRNA levels were quantified using qRT-PCR. Transcript levels were normalised using *GAPDH* and *rpL13* as reference genes. Distribution of the data is represented by box plot analysis. The transcript levels in the exposed groups were compared with controls using ANOVA followed by Dunnett's and Games-Howell's multiple comparison tests. Significant differences are denoted by *p ≤ 0.05 and **p ≤ 0.01.

For genes involved in the ecdysone response pathway, BPA did not alter the *EcR* mRNA level. However, BPS and BPF increased the expression of this gene. This increase was significant for 1 mg/L BPS compared to control levels. Finally, 1 mg/L BPS and BPF treatment significantly increased *E74* gene expression (Fig. 1).

3.2. DRONC and MRP1 gene expression in response to BPA and analogues

Although there is a lack of apoptosis-related genes described in *C. riparius*, we analysed one caspase gene related to apoptosis in an attempt to compare the effects produced by BPA, BPS and BPF in programmed cell death in this species. *DRONC* is an initiator caspase described in *Drosophila*; it includes a caspase recruitment domain (CARD) (Kumar and Doumanis, 2000). The exposure of the larvae for BPF for 24 h (1 mg/L) significantly upregulated *DRONC* mRNA compared to control (Fig. 2). Additionally, there was a trend for an increase in this gene expression for all the analysed compounds and concentrations (Fig. 2).

Different ABC transporters participate in the cellular detoxification process. *MRP1* codes for a protein involved in this process with the excretion of the conjugated compound to the extracellular medium. MRP1 participates in cellular resistance to different toxicants. The BPA treatment for 24 h (0.5 or 1 mg/L) significantly upregulated *MRP1* ex-

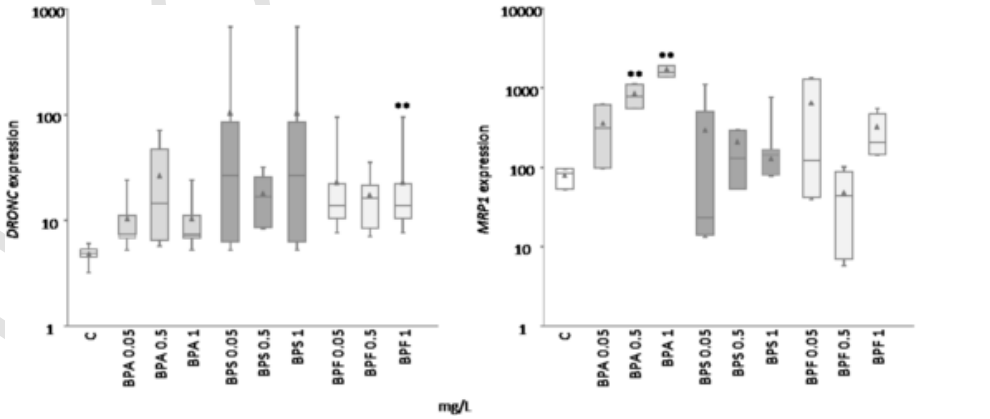


Fig. 2. mRNA levels of *DRONC* and *MRP1* in *C. riparius* larvae at 24 h after BPA, BPS and BPF treatments. The mRNA levels were quantified by qRT-PCR. Values were normalised to reference genes and compared with the controls solvent-exposed larvae. Data from three independent experiments are shown (n = 27) in the whisker boxes. Differences between BPA, BPS and BPF-treated and the corresponding solvent control samples were considered significant at **p ≤ 0.01.

pression compared to control. Notably, BPS and BPF did not alter this gene expression (Fig. 2).

3.3. Bioinformatics analyses: comparative study of binding sites in nuclear receptors (NRs) and physicochemical properties of the different ligands

It is well known that bisphenols can act on estrogen receptors (ER). This action ranges from weak agonist (BPA) to antagonism (e.g., bisphenol C) (Delfosse et al., 2012; Levy et al., 2004). The ecdysone receptor (EcR) has the characteristic protein architecture of the NR superfamily, with two highly conserved domains associated with DNA and ligand binding (Pawlak et al., 2012). In order to verify the conservation of key structural features for ligand binding in chironomid EcR, their sequences were compared with those of several NRs from insects and humans with known structures. The binding sites in the different analysed NRs (Supplementary Material 1) contain a conserved internal structure with a number of conserved binding sites regions, most of them common to all NRs and one of them unique to the studied EcRs (Fig. 3). Thus, the interaction of BPA and its analogues with EcR would be expected to be similar to the interaction of these ligands with the other previously studied NRs.

Furthermore, different physicochemical properties of the ligands of interest were examined (Table 3). Notably, while the octanol-water partition coefficient (P), a quantitative descriptor of lipophilicity, and the topological polar surface area (TPSA) parameters of BPA are comparable to those of estradiol, these properties for BPS are more similar to 20E.

4. Discussion

Humans are frequently exposed to emergent pollutants for which, in most cases, no information is available on their potential effects and their implications for the environment. The industry is continuously evolving and the synthesis of new compounds that theoretically improve our quality of life, are placed at our disposal. The negative effects

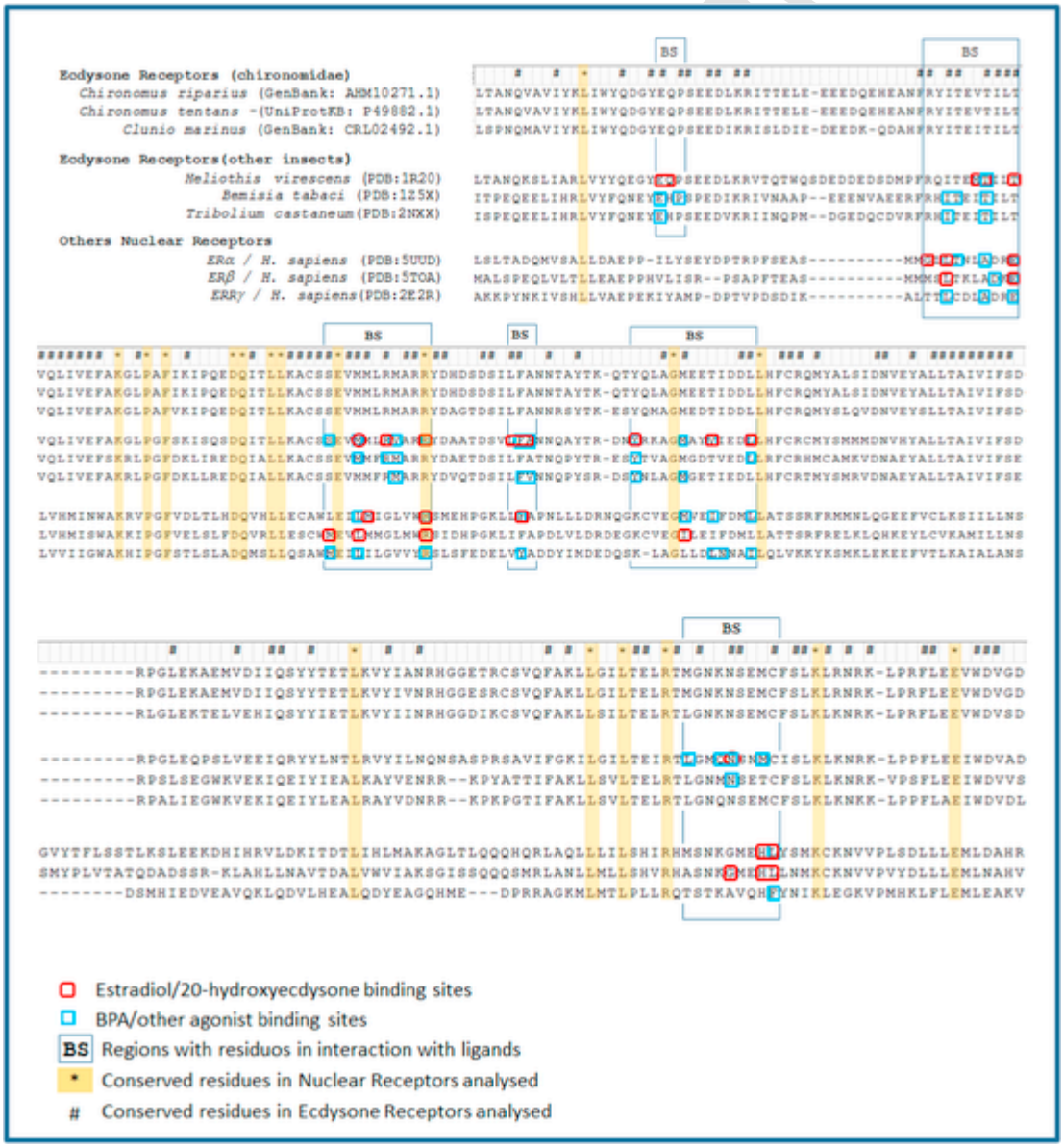


Fig. 3. Comparison of the ligand binding domain sequence for different nuclear receptors and protein-ligand binding sites. The protein-ligand binding sites for different complexes from Protein Data Bank were analysed (Supplementary Material 1). Sequence alignment of six nuclear receptors, with known structures, and the ecdysone receptors for three *Chironomidae* (*Chironomus riparius*, *Chironomus tentans* and *Clunio marinus*) was performed. The alignment revealed highly conserved amino acid residues and different well-defined regions that involve the ligand binding sites. Alignments were performed with ClustalW using MEGA 7.0.14 (default options).

Table 3
Chemical and physical properties of estradiol, 20-hydroxyecdysone (20E), BPA, BPS and BPF (Data from PubChem, https://pubchem.ncbi.nlm.nih.gov/and ChEMBL, https://www.ebi.ac.uk/chembl/).

		Estradiol (EST) PubChem CID 5757 ChEMBL ID: CHEMBL135	*20_hydroxyecdysone (20E)* PubChem CID 5459840 ChEMBL ID: CHEMBL224128	*bisphenol A, BPA (2OH)* 2,2-Bis (4-hydroxyphenyl)propane PubChem CID 6623 ChEMBL ID: CHEMBL418971	*bisphenol S, BPS* 4,4'-Sulfonyldiphenol PubChem CID 6626 ChEMBL ID: CHEMBL384441	*bisphenol F (BPF)* 4,4'-Methylenediphenol PubChem CID 12111 ChEMBL ID: CHEMBL138061
Molecular Weight		272.4	480.6	228.3	250.3	200.23
[1]*LogP (lipophilicity)*	**XLogP3** (PubChem)	4	0.5*	3.3	1.9	2.9
	ALogP (ChemBl)	3.6	1.9	3.4	1.9	2.7
	ACD LogP (ChemBl)	4.1	-0.3	3.6	2.1	2.8
Hydrogen Bond Donor Count		2	6	2	2	2
Hydrogen Bond Acceptor Count		2	7	2	4	2
[2]*Topological Polar Surface Area /Å²*		40.5	138.5	40.5	83	40.5

[1] **Calculated LogP**: P is the Octanol/Water Partition Coefficient. (*)0LogP3-AA method.
[2] **Topological Polar Surface Area (TPSA)**: surface sum over all polar atoms in a molecule.

of BPA on human health have been demonstrated in multiple studies. There is increasing evidence of the toxic effects of BPA analogues (Tišler et al., 2016; Vincent et al., 2017). In the present study, we demonstrated that exposure to BPA analogues, namely BPS and BPF, altered the expression of genes involved in several pathways (endocrine, detoxification and apoptosis) in fourth instar *C. riparius* larvae.

The obtained results indicated that BPA, BPS and BPF behave in a similar manner with respect to the ecdysone biosynthesis route: they all increased the expression of *Shadow*. This gene is a member of the ecdysone biosynthetic (Halloween) embryonic lethal genes that codes for mitochondrial cytochrome P450 (Cyp450) hydroxylases (Gilbert, 2004). Steroid hormones play crucial roles in the coordinated regulation of many developmental and physiological events in invertebrates and vertebrates. Ecdysone is the main hormone of the insect endocrine system and it regulates the processes of moulting and metamorphosis that are essential for development (Nation James, 2008). In *Drosophila*, these enzymes mediate the last hydroxylation reactions in the ecdysone biosynthetic pathway and they convert dietary sterols to ecdysteroids, such as 20E (Lavrynenko et al., 2015). *Shadow* encodes a Cyp450 hydroxylase that performs the last synthetic step for ecdysone production (Warren et al., 2002). In our study, *Shadow* was statistically significantly upregulated after BPA, BPS and BPF exposures. These results showed that both BPA and its two analogues similarly affected ecdysone synthesis. They apparently activated its synthesis at the same point of the biosynthetic route. In arthropods, steroid hormones play vital developmental roles, especially in mediating transitions between different developmental stages. An increase in *Shadow* mRNA expression implies an increase in the synthesis of the hormone ecdysone. Ecdysone and its derivative 20E were originally identified as "moulting hormones", and differences in their expression levels would induce drastic morphological and developmental changes in the natural processes of moulting and metamorphosis. Once ecdysone performs its function, it must be inactivated by Cyp18a1, a Cyp450 enzyme (Guittard et al., 2011). Thus, the *Cyp18a1* gene is related to inactivation of the active form of ecdysone. As shown in Fig. 1, BP treatments tended to increase the levels of *Cyp18a1*, but the increase was only significant for 1 mg/L BPF. Similar results were previously described with BPS in *C. riparius* at lower concentrations (Herrero et al., 2018). Taken together, these data suggest activation of ecdysone synthesis, observed as an increase *Shadow* gene expression that would subsequently activate the *Cyp18a1* gene that codes for the protein involved in its degradation.

The ecdysone receptor (EcR) is the first target for ecdysone action inside the cells (Lezzi et al., 1999). In our work, while BPA, at the tested concentrations and time, did not induce changes in *EcR* expression, the treatment with 1 mg/L of BPS or BPF for 24 h significantly increased *EcR* mRNA levels. Previous results obtained with BPA and *C. riparius* larvae showed that this toxicant induces *EcR* upregulation, albeit at a higher concentration (3 mg/L) than those used in our study (Planelló et al., 2008). Our results suggest that BPA and its analogues can modulate *Chironomus EcR*. Moreover, 1 mg/L BPS and BPF increased *E74* mRNA expression. The *EcR* data are consistent with the results obtained by analysing the early ecdysone-inducible *E74* gene. Once 20 OH-Ec binds to the EcR-USP heterodimer, a cascade of transcription factors is triggered, beginning with the expression of the early-response gene *E74* (Riddiford et al., 2000). Interestingly, these results demonstrated that the two BPA analogues could act as endocrine disruptors, as has been clearly demonstrated with the original compound (Kang et al., 2007), even at lower concentrations.

Some of the physicochemical properties of BPS could at least partially justify these results. As mentioned earlier, the BPA lipophilicity and TPSA parameters are comparable to those of estradiol, whereas the BPS properties are more similar to those of 20E. Accordingly, BPA and estradiol would be expected to bind their target receptor in a similar manner, a supposition that is consistent with previously reported experimental data (Delfosse et al., 2012). On the other hand, the higher TPSA and the lower lipophilicity of BPS and 20E could suggest a more analogous behaviour for these two compounds in their interaction with NRs.

Our obtained data suggest a specific effect of BPS and BPF on genes related to the endocrine pathway in *C. riparius*. These BPA alternatives, with similar chemical structures, can bind to EcR and cause the endocrine disruptive effects. The effects of these chemicals were already examined in the testes and on spermatogenesis of male rats by means of *in vivo* and *in vitro* studies (Ullah et al., 2018).

Detoxification mechanisms are activated in cells in response to toxic compounds. The detoxification process is performed in three phases that each involves different proteins. Cyp450s in phase I introduce reactive or polar groups into the compound to decrease toxicity (Schuetz, 2001) or efflux transporters (phase III), like ABC transporters (Dermauw and VanLeeuwen, 2014), that are responsible for removing the modified compounds from the cell. MRP1 is one such ABC transporter. MRP1 was previously studied because of its involvement in resistance to anticancer drugs (Lu et al., 2015) and it also has been related to the efflux of different endobiotics from the cell (Cole, 2014). The role of MRP1 is not clear in invertebrates. There are only two reports in *Drosophila melanogaster* and *Pediculus humanus* that show the involvement of this protein in the elimination of methotrexate and the pesticide ivermectin, respectively (Chahine et al., 2012; Yoon et

al., 2011). Recent results showed that two ultraviolet (UV) filters, namely benzophenone-3 (BP3) and 4-methylbenzylidenecamphor (4MBC), increase *MRP1* mRNA levels in *C. riparius* (Martínez-Guitarte, 2018). In our work, BPA exposure significantly increased *MRP1* expression, but BPS and BPF did not alter this gene. These data help to understand the role of the transport protein MRP1 in the mechanisms of detoxification in invertebrates. On the other hand, the effector caspase *DRONC* was upregulated after treatment with the highest tested BPF concentration. These data suggest activation of the apoptosis.

5. Conclusions

All BPs studied affected in a similar way to the ecdysone biosynthesis route. BPA, BPS and BPF increased the expression of *Shadow* gene involved in the last step of this metabolic pathway. An increase in *Shadow* mRNA expression implies an increase in the synthesis of the ecdysone, which would alter the stages of larval development. A tendency to increase the expression levels of the *Cyp18a1* gene, related to the inactivation of the active form of ecdysone, was observed. This result might suggest that chronic exposures, at longer times than those studied here, bisphenol A and bisphenol S also could cause a significant alteration in *Cyp18a1*, such as the significant increased caused after BPF exposure in *C. riparius*. Moreover, BPS and BPF are likely biologically active compounds that would act as endocrine disruptors at the level of the *EcR* and *E74* genes. Therefore, based on the results obtained we can conclude that the ecdysone pathway is a target of BPS and BPF. Both acute and short-term exposures could cause alterations in *C. riparius* development. Furthermore, this finding suggests a similar mechanism of action for BPA and its analogues, specifically through interaction with steroid hormone receptors. Remarkably, our data suggest that the endocrine disruptive effects of BPS and BPF in invertebrates could be more significant than the clearly established effects of BPA. Moreover, our findings imply an increase in cellular sensitivity to BPS and BPF and a pro-apoptotic effect in cells under their exposure. The pro-apoptotic effect of BPA analogous may be related to a reduced expression of *MRP1*. These results showed that BPA analogous affect the transcriptional activity and produce changes in apoptosis and stress processes in *C. riparius*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114806.

Uncited references

Michailova et al., 1996; OECD, 2011.

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