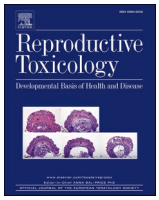
Reproductive Toxicology 132 (2025) 108854

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox



Developmental exposure to an environmentally relevant dose of Bisphenol S impairs postnatal growth and disrupts placental transcriptional profile in female rat

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ARTICLE INFO

Handling Editor: Dr. Bal-Price Anna

Keywords:
Bisphenol S
Placenta
Transcription
DNA methylation
Steroid receptor coactivator 2

ABSTRACT

Because of its possible adverse effects on human health and its ubiquitous nature, Bisphenol A (BPA) is gradually being replaced by presumably safer alternatives like Bisphenol S (BPS). However, data regarding the effects of developmental exposure to BPS on pregnancy and fetal outcomes are very scarce. Here we show that perinatal exposure to BPS at a very low dose significantly impairs postnatal growth and affects the placental transcriptome in rats. Oral exposure one week before mating and during gestation and lactation to a very low dose of BPS (25 ng/kg/day) is associated with impaired postnatal growth without significant difference in fetal weight on gestational day 18 in females. In contrast, in males, exposure to BPS 25 decreased fetal weight on gestational day 18 but growth restriction did not persist into adulthood. In female, exposure to this very low dose of BPS decreased the placental mRNA expression of fucosyltransferase2 (*Fut2*), pregnancy-specific glycoprotein 22 (*Psg22*), Wnt family member 7b (*Wnt7b*) which are involved in early placental development. Placental DNA methylation of steroid receptor coactivator 2 (*src2*), a key mediator of steroid induced decidualization, was significantly reduced, while placental *src2* mRNA expression was unaffected. These results suggest that early exposure to a very low dose of BPS has long term consequences on growth trajectory and is associated with placental dysregulation.

1. Introduction

Endocrine disrupting chemicals (EDCs) widely impact individuals and populations given their ubiquity in our environment [29]. Developmental exposure to EDCs is associated with increased risk of adult reproductive failure, cancer, obesity, metabolic syndrome, and neurodevelopmental disorders [16]. This led the World Health Organization, the United Nations, and the International Council on Chemical Management to list them as an emerging public-health concern [21,65]. Among EDCs, Bisphenol A (BPA) is used for manufacturing polycarbonate plastics and epoxy resins, and is thus present in food cans and plastic containers [51]. It is extremely ubiquitous as reflected by the presence of detectable amounts of urinary BPA in more than 90 % of the US [11,10] and European populations [14]. BPA is detected in pregnant women [61] as well as human placenta and amniotic fluid [19,20,31,54,67] indicating fetal exposure. Epidemiological studies as well as experimental models have shown that BPA exposure can impact placental development and function and consequently alter fetal growth trajectory [1]. The possible adverse effects of BPA on human health and its ubiquitous nature have prompted the industry to remove BPA from

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https://doi.org/10.1016/j.reprotox.2025.108854
Received 24 September 2024; Received in revised form 6 February 2025; Accepted 7 February 2025
Available online 9 February 2025

consumer products and develop alternative chemicals, such as Bisphenol S (BPS). BPS has a chemical structure similar to BPA. Its physical properties make it attractive to progressively replace BPA in a variety of consumer products such as thermal paper and food containers [66]. Therefore, environmental levels of BPS are continuously increasing as reflected by its presence in 81 % of urinary samples collected in the United States and seven Asian countries [41] and in almost all urine samples of the French population [4]. BPS has also been detected in human cord serum [36,39,68] suggesting placental transfer. However, the safety and lower toxicity of BPS compared to BPA remains to be proven [5,12,66]. Recent studies indicate that developmental exposure to BPS could have similar or other adverse effects than BPA on fetal growth outcomes through disruption of placental development and function [2]. However, data regarding its effects on fetal development and mechanisms of action is still scarce.

The placenta, located at the interface between the mother and the fetus, plays a critical role in fetal homeostasis. Changes in its physiology trigger an adaptive response from the fetus and affect the programming of its future health. Its vulnerability to EDCs is at least partially explained by its high content in steroid hormone receptors [26]. Exposure to BPA leads to changes both in placental morphology and function [55,63]. In rats, recent data indicates that BPS (200 μg/kg/d) also affects placental transcriptome and histology [43]. In sheep, gestational exposure to BPS was associated with impaired placental endocrine function while BPA exposure did not show any effect [25] suggesting the necessity to explore potential differential effects of both bisphenols in the placenta.

Epigenetic mechanisms have been recently proposed as one of the missing links between early life insults and long-term health outcomes [7]. Establishment of the placental methylome during post-implantation development is crucial for the regulation of placental development that support embryo and fetal development [62]. Several studies have identified an association between maternal environmental factors (maternal nutrition, gestational diabetes), specific changes in placental DNA methylation and subsequent neonatal and early childhood growth and development [58,59]. Here, we hypothesized that exposure to BPA or BPS might lead to placental transcriptome and epigenome changes that could impact placental function and fetal growth. To explore this, we characterized the effects of gestational and lactational exposure to an environmentally relevant dose of BPS (25 ng/kg/day) on pregnancy outcome and fetal growth in rats. We chose a low dose of 25 ng/kg/day which is consistent with existing data regarding current human exposure to BPA [24] and BPS [66]. Such exposure was compared to the same dose of BPA and to the tolerable daily intake of BPA as recommended by *the European Food Safety Authority* (EFSA) (4 μg/kg/d) until recently.

2. Material and methods

2.1. Animal care and exposure

All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège in accordance with Directive 2010/63/EU for animal experiments.

Female Wistar rats were purchased from the University of Liège. They were housed in standardized conditions (22.8°C, lights on from 6.30 am to 6.30 pm). The animals were raised in BPA-free cages (Polypropylene cages, Ref 1291H006, Tecnilab, Netherlands) and fed EDC- and phytoestrogen-free chow (V135 R/Z low phytoestrogen pellets, SSNIFF Diet, Netherlands). Water was supplied in glass bottles.

Female rats were exposed orally either to corn oil (n = 25) or BPA 25 ng/kg/day (n = 15), BPA 4 μg/kg/day (n = 15) (Ref: 23,9658; Sigma–Aldrich, Saint Louis, USA) or to BPS 25 ng/kg/day (n = 25) or BPS 4 μg/kg/day (n = 15). The first experiment (corn oil n = 10; BPA 25 ng/kg/day n = 10; BPA 4 μg/kg/day n = 10; BPS 25 ng/kg/day n = 10; BPS 4 μg/kg/day n = 10) was led to collect placentas for methylation and transcriptomic analysis. The second experiment (corn oil n = 15; BPA 25 ng/kg/day n = 5; BPA 4 μg/kg/day n = 5; BPS 25 ng/kg/day n = 15; BPS 4 μg/kg/day n = 5) was led to study fetal and postnatal growth. Conditions of exposure, season and food supply were strictly identical for the two experiments. Daily exposure was done by injecting 50 μl of BPA or BPS or corn oil in a waffle given to each female. Cages were systematically verified after 10 minutes to check for complete ingestion. Females were randomly assigned to treatment. Females were weighed weekly, and volume of corn oil, BPA, or BPS injected into the wafer was adjusted accordingly. Females were exposed during 1 week before being paired with one male during 48 h. That day was considered as gestational day 1. Dams continued to be exposed daily to corn oil, BPA or BPS until gestational day 18 (GD18).

Evaluation of mating success rate: Mating success rate was defined as the percentage of females in each group getting pregnant after being paired with the male. Males were trained and were the same age to limit variability of mating success related to males. Caesarian section was done on GD18. The position of each gestational sac was recorded. Fetal tissue was collected for PCR determination of sex. Whole placenta (maternal and fetal) was frozen in liquid nitrogen before DNA and RNA extraction.

Evaluation of fetal growth and puberty timing: In order to study the effects of BPS on early development, one group of dams continued to be orally exposed to BPS 25 ng/kg/day (n = 4) or corn oil (n = 4) until postnatal day 21 (PND21). Litters were homogenized for size and sex ratio on the first postnatal day of life to have 10–12 pups per litter and a 1:1 male/female ratio. Cross-fostering of a maximum of two pups per litter was used when homogenization was required. Day of birth was considered as PND 0. Pups were weaned at PND 21. Female and male pups were followed for weight gain. From PND 25, female rats were followed for vaginal opening as described previously [23,60]. Briefly, females were examined daily by two experimenters for imperforation of the vaginal membrane to determine age at vaginal opening. Male rats were observed daily to determine the age for balano-preputial separation.

2.2. Sex determination

To determine the sex of each conceptus, DNA was isolated from fetal tissue using the DNeasy Blood & Tissue Kit (Catalogue #69504; Qiagen, Gaithersburg, MD). Y-chromosome specific gene (forward primer: 5'GGAGAGAGGCGCAAGTTGGCT3'; reverse primer: 5'GCTATGGTGCAGGGTCGCTCA3') expression was studied using Polymerase Chain Reaction (PCR) amplification. Once the sex of the fetus was established, female and male placenta systematically surrounded by two female and two male placentas respectively were selected from each litter for further analyses in order to limit variability in local uterine environment.

2.3. RNA sequencing (RNA seq)

RNAseq analysis was carried out on total RNA extracted from a pool of placental tissues of female fetuses after prenatal exposure to vehicle, BPA (25 ng/kw/day and 4 μg/kg/day) or BPS (25 ng/kg/day and 4 μg/kg/day) (n = 3/group). For each sample, we pooled 2 or 3 placentas from the same dam to better represent the whole litter. The pooled placentas were all surrounded by two female fetuses to have the similar hormonal environment. We used 3 pooled placenta samples per condition for RNA seq analysis.

Library preparation and sequencing were performed at the GIGA Genomics facility (University of Liège, Belgium). RNA integrity was verified on the Agilent Bioanalyser with RNA 6000 Nano chips, RIN scores were > 7.5 for all samples. Illumina Truseq stranded mRNA Sample Preparation kit was used to prepare libraries from 1 microgram of total RNA. Libraries were quantified by qPCR with the KAPA Library Quantification Kits for Illumina® platforms. Sequencing was performed on Illumina® NovaSeq™6000 Sequencing System. The average read-

depth of the data is ~25–30 M reads. The data were processed through the nf-core "rnaseq" pipeline version 3.0.0 (https://nf-co.re/rnaseq/1.4.2). Quality control of the samples was assessed with FastQC software v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned on the Rattus norvegicus genome, using Rnor_6.0 genome build and annotations from the Ensembl release v102 (ensembl.org) using STAR software v2.6.1d (https://github.com/alexdobin/STAR).

Following alignment, expression count data was normalized and fitted based on the binomial distribution and differential gene expression (DGE) analysis was performed using the R package DESeq2 (v1.32.0) [42]. A Principal Component Analysis (PCA) was carried out using the first 500 more variable features using the DESeq2 function "*varianceStabilizingTransformation*". Using a p value threshold of.05, differentially expressed genes per each comparison (i.e. BPA25ng vs Ctl) were used as input for a Gene Ontology (GO) analysis using DAVID.

2.4. RNA extraction, reverse transcription and real-time PCR

Real-time Quantitative PCR (qPCR) analysis was used to assess mRNA levels of specific genes in placenta (n = 6/group): *Src2*, *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Tet1*, *Tet2* (see primer sequence at Table S1). Total RNA was extracted from female placenta using the RNeasy Mini kit (Qiagen, Netherlands) following manufacturer's instructions. Five hundred nanograms of bulk mRNA for each sample were reverse transcribed using the Transcriptor first strand cDNA synthesis kit (Roche). For real-time quantitative PCR reactions, each sample cDNA was diluted 10-fold, and 4 μL were added to a mix of 5 μL FastStart Universal SYBR® Green Master (Roche), 0.4 μL of nuclease-free water and 0.3 μL of forward and reverse primer. Primers were synthesized by Integrated DNA Technologies, Inc. qPCR was performed using a Quant Studio 12 K Flex (Applied Biosystems) under the following conditions: initialization: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of denaturation: 15 sec at 95°C and elongation and data collection: 60 s at 60°C. Cycle threshold (Ct) values were obtained from each individual amplification curve, and the average Ct was calculated for each target gene in each sample. Quantification of relative gene expression was performed using the ΔΔCt method implemented with the Pfaffl equation, which takes into account reaction efficiency depending on primers [49]. All assays had efficiencies between 1.9 and 2.1. Actin was used as housekeeping gene.

2.5. DNA methylation and bisulfite sequencing

DNA from frozen female placenta was isolated using a Puregene DNA purification kit (QIAGEN, Hilden, Germany) according to the supplier's recommendation. DNA concentration was determined with a spectrophotometer (NanoDrop Technologies Inc, Wilmington, Delaware), and quality was assessed using an agarose gel electrophoresis.

Bisulfite modification-based genomic sequencing was used to establish a detailed mapping of the DNA methylation pattern of specific genes. Genomic bulk DNA from control and BPA/BPS placenta was bisulfite converted (BC), using the EZ DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's instructions. BC DNA was used as input material for PCR amplification followed by library preparation and deep sequencing.

Primers were designed to amplify a region of the CpG island of the rat *src2* (primer sequence at Table S1). Amplification was carried out on a C1000 Thermal Cycler (Bio-Rad) with 20 ng of BC DNA per reaction. The amplification conditions were: 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Sequencing libraries were prepared using the NETFLEX® DNA Sequencing Kit (BIOO Scientific) according to manufacturer's instructions. The libraries were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies) and were normalized to 2 nM with 10 mM Tris-HCl. Libraries were then pooled and sequenced on a MiSeq (Illumina, Inc.) by the GIGA Genomic platform to generate 250 base-long, paired-end reads. The reads were trimmed using Trim Galore (http://www.Bioinformatics.Babraham.Ac.Uk/Projects/Trim_galore/) and aligned to the rat reference genome (Ensemble Rat Rnor_6) using Bismark [38]. Alignment data was converted to CpG methylation rate using the Bismark methylation extractor. Only reads with a > 98 % cytosine conversion and > 98 % alignment were used; no sample was excluded from the analysis. The CpG methylation rates were calculated as the ratio of methylated reads over the total number of reads. Methylation rates for CpGs with fewer than 10 reads were excluded from further analysis; samples had an average of 1750 × read coverage.

2.6. Statistics

Data were summarized using mean ± SEM. Quantitative variable were subjected to normality test. Equal variance and parametric tests were considered when conditions were fulfilled. Quantitative variables reaching normal distribution were analyzed using one-way or two-way analysis of variance (ANOVA) or Student's *t*-test to compare more than two groups and two groups, respectively. Mann-Whitney test was applied otherwise. When comparing percentages between groups, an arcsine transformation was applied before statistical analysis to convert the data from binomial to normal distribution. To analyze the age at vaginal opening, a Cox regression model was applied. This model is an adaptation of probit analyses commonly used to study pubertal onset in humans. Probit analysis is a type of regression used with binomial response variable (in this study: vaginal opening, yes/no). The two-sided significance level was 5 %. However, when making multiple comparisons, the significance level was adjusted using the Bonferroni correction. All statistical analyzes were performed using Prism (version 7.0, GraphPad). The investigator was blinded for all physiological and molecular determination.

3. Results

3.1. Developmental exposure to environmentally relevant dose of BPS impairs growth trajectory

Dams were orally exposed to environmentally relevant doses of BPA or BPS (25 ng/kg/day) one week prior mating and during gestation (Fig. 1). For comparison purpose, 2 groups were also exposed to doses of BPA or BPS corresponding to the tolerable daily intake of BPA (4 μg/kg/d) as originally defined by EFSA.

Exposure to BPS 25 ng/kg/day and BPA 4 μg/kg/day led to a mating success rate of respectively 52 and 53 % while female rats exposed to corn oil had a mating success rate of 76 % (Fig. 2A). Mating success rate after exposure to BPS 4 μg/kg/d and BPA 25 ng/kg/d was similar to the control group (73.33 % and 80 % respectively) (Fig. 2A).

Dam weight gain during pregnancy was comparable in all exposed and control groups (Fig. 2B). Neither BPA nor BPS affected litter size (Fig. 2C) or male-to-female ratio (Fig. 2D). Female fetal growth was affected by gestational exposure to the lowest but not the highest doses of BPA as fetal weight (GD18) was significantly lower in animals developmentally exposed to BPA 25 ng (85,13 % of average control weight) compared to controls (Fig. 3A). Placental weight was not affected by exposure to BPA 25 ng (Fig. 3B). Developmental exposure to BPS led to a different phenotype as average fetal weight was higher in females developmentally exposed to BPS 4 μg/kg/day (127,85 % of average control weight) (Fig. 3A) but not BPS 25 ng compared to controls. Placental weight was also higher after exposure to BPS 4 μg but not BPS 25 ng compared to controls (Fig. 3A and B).

In male, fetal weight (GD18) was significantly lower in animals exposed to BPS 25 ng and BPA 25 ng compared to controls and average male fetal weight was significantly higher in males exposed to BPS 4 μg/kg/day (Fig. S1A). Male placental weight was higher in males developmentally exposed to BPS 4 μg/kg/day (Fig. S1B).

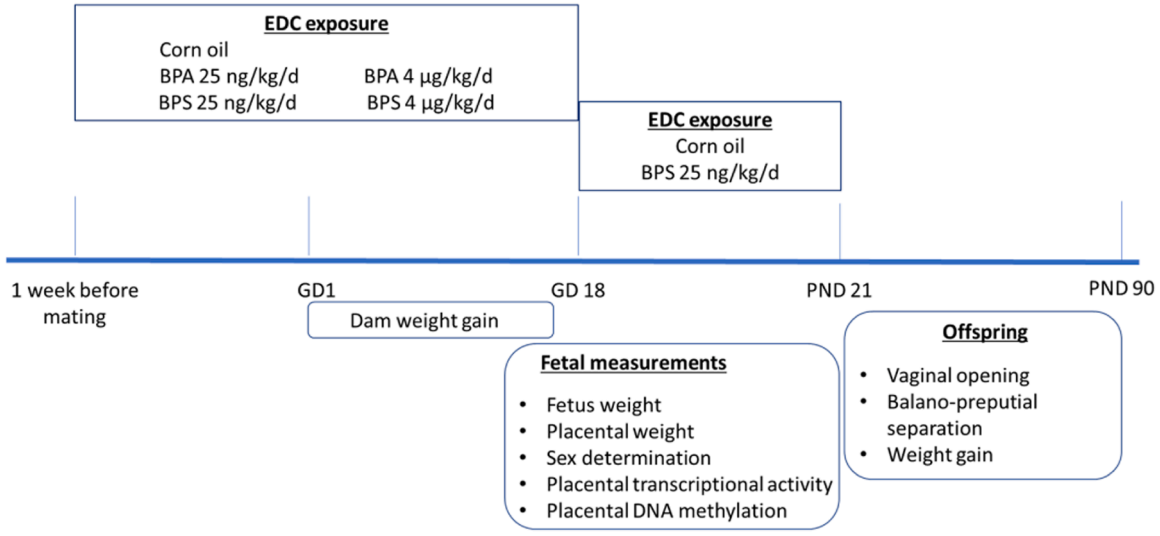

Fig. 1. Experimental timeline. Adult female rats were orally exposed one week before mating and during gestation until gestational day 18 to corn oil (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 µg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 µg/kg/day (n = 15). Mating was carried out for a period of 48 h. Caesarian section was done on gestational day 18. Dams exposed to CTRL and BPS 25 ng/kg/day continued to be exposed to corn oil (n = 20) or to BPS 25 ng/kg/d (n = 23) during lactation until weaning on postnatal day 21.

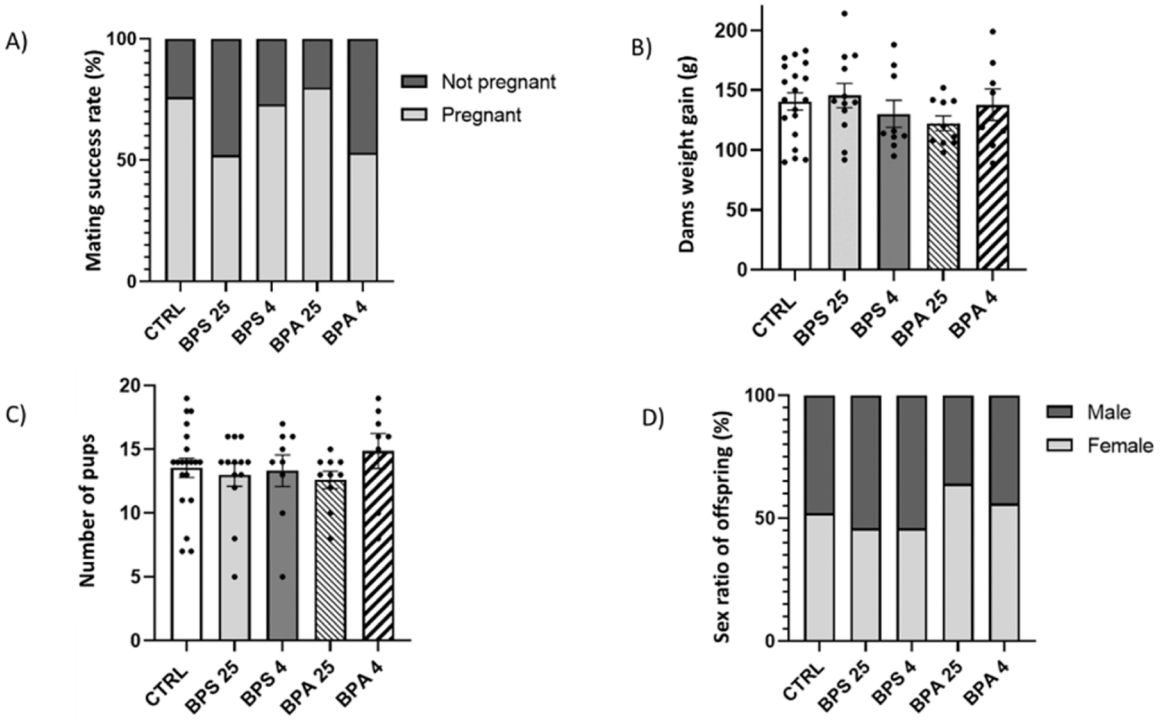

Fig. 2. Pregnancy outcomes following exposure to vehicle, Bisphenol A or Bisphenol S. (**A**) Mating success rate (%) in female rat exposed to corn oil (control) (n = 25); BPS 25 ng/kg/day (n = 25); BPS 4 µg/kg/day (n = 15); BPA 25 ng/kg/day (n = 15) or BPA 4 µg/kg/day (n = 15). (**B**) Average gestational weight gain until GD18 in dams exposed to corn oil (control) (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 µg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 µg/kg/day (n = 15). Data are presented as mean ± SEM. (**C**) Average litter size at gestational day 18 following exposure to corn oil (control) (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 µg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 µg/kg/day (n = 15). Data are presented as mean ± SEM. (**D**) Offspring sex ratio (%) following exposure to corn oil (control) (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 µg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 µg/kg/day (n = 15).

3.2. Developmental exposure to environmentally relevant dose of BPS impairs female postnatal growth

Two subgroups of lactating dams were exposed to corn oil or BPS 25 ng/kg/day until weaning to study the effects of the lowest dose of BPS on postnatal development.

Postnatal female growth was affected by exposure to BPS 25 ng/kg/day as weight was lower in BPS-exposed females compared to controls on postnatal day 90 (Fig. 3C). Food intake did not significantly differ between control and BPS-exposed females (Fig. S2A). Average age at vaginal opening did not differ between BPS 25 exposed and control females (Fig. 3D). Time-curve analysis did not show any difference in time at vaginal opening in females exposed to vehicle or BPS 25 ng (Fig. 3E).

Average weight throughout development did not differ between

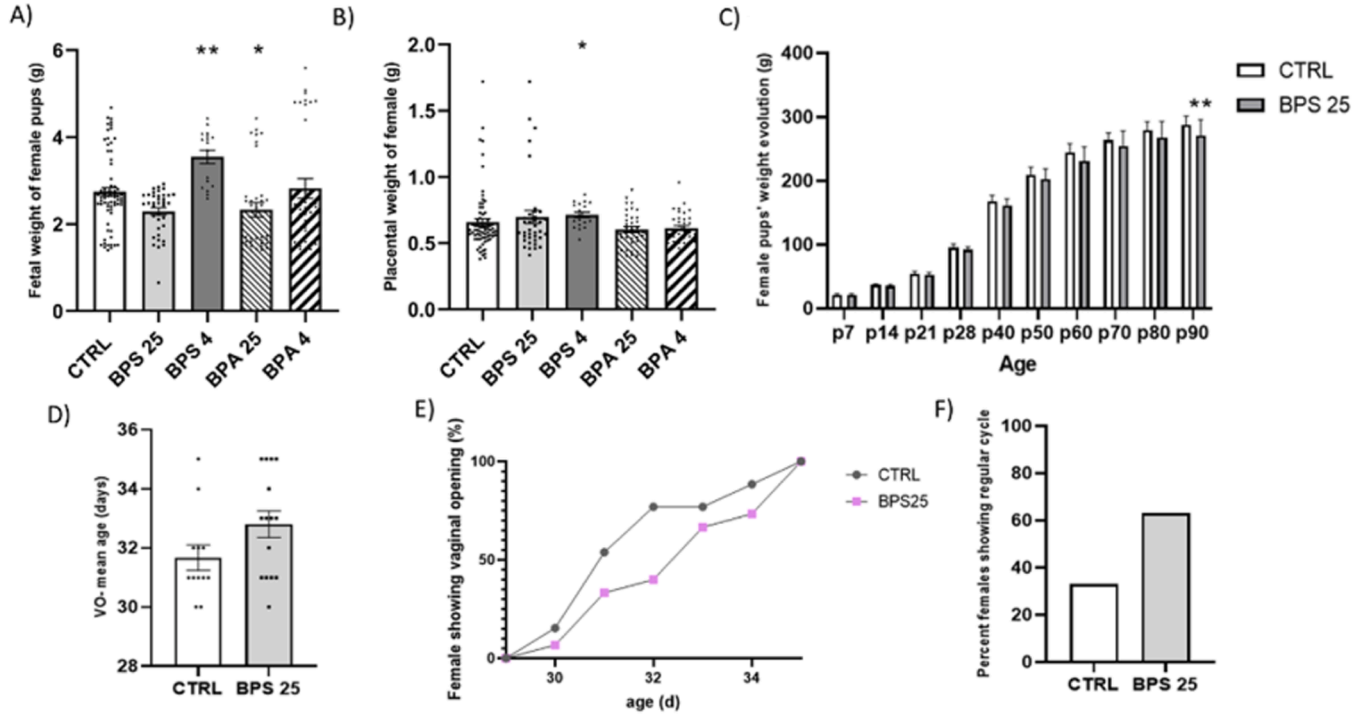

Fig. 3. Female growth and pubertal outcomes following gestational and lactational exposure to BPS 25 ng/kg/day. (**A**) Average female offspring weight at GD 18 following exposure to corn oil (control) (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 μg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 μg/kg/day (n = 15). Data are presented as mean ± SEM. * p-value < 0.05, * * p value < 0.005. Kruskal-Wallis test. (**B**) Average female placental weight at GD18 following exposure to corn oil (control) (n = 25), BPS 25 ng/kg/day (n = 25), BPS 4 μg/kg/day (n = 15), BPA 25 ng/kg/day (n = 15) or BPA 4 μg/kg/day (n = 15). Data are presented as mean ± SEM. * p-value < 0.0005. Kruskal-Wallis test. (**C**) Average weight evolution until postnatal day 90 in females exposed to corn oil (control) (n = 6) or BPS (25 ng/kg/day) (n = 8) during gestation and lactation. Data are presented as mean ± SEM. * * p-value < 0.01. Multiple unpaired *t*-test. (**D**) Average age at vaginal opening in female rats exposed during gestation and lactation to corn oil (control) (n = 12) or BPS (25 ng/kg/day) (n = 15). Data are presented as mean ± SEM. p-value = 0.08. t test. (**E**) Cumulative percentage of female rats reaching vaginal opening in relation to age in females exposed to corn oil (control) (n = 12) or BPS (25 ng/kg/day) (n = 15) during gestation and lactation. p-value = 0.17. Cox regression.

control and BPS-exposed males (Fig. S1C). Food intake was not different between control and BPS-exposed males (Fig. S2B). In males, age at balano-preputial separation was not affected by exposure to BPS 25 ng (Fig. S1D).

3.3. Effects of developmental exposure to environmentally relevant dose of BPS on placental transcriptome

Placental development is a highly regulated process that is essential for fetal programming and thereby has long term consequences on postnatal development. In order to identify BPS 25 ng placental transcriptional targets that could mediate its effects on postnatal growth, RNA was extracted from placenta exposed to corn oil or BPA or BPS (25 ng/kg/day or 4 μg/kg/day) collected on GD 18 and analyzed using RNA sequencing.

Based on a log fold change threshold > +/- 0.5 and an *adjusted p value* < 0.05, exposure to BPS 25 ng significantly affected the placental expression of a limited number of genes (Fig. 4A). Among the genes that were significantly down-regulated after exposure to BPS 25 ng, three are known to play a role in implantation and early placentation (*Wnt7b, AABR07002659.1* and *Fut2)* (Fig. 4B). Exposure to the higher dose of BPS (4 μg/kg/day) or BPA (25 ng/kg/day or 4 μg/kg/day) affected a limited number of placental genes (Fig. 4A). Among them, several genes were involved in transcription regulation (*Sap30l*, *Rmnd5b*, *Zmiz2*) (Fig. 4B).

A closer investigation showed no overlap between differentially expressed genes associated with exposure to the low dose of BPA and BPS (Fig. 4B). Exposure to the higher dose of BPA and BPS (4 μg/kg/day) affected the expression of only 2 genes in common *(LOC 103689945, Fut2)* (Fig. 4B), suggesting different transcriptional alterations caused by BPA and BPS in the placenta.

Because exposure to BPS 25 led postnatal growth restriction in female offspring, we performed a functional enrichment analysis of differentially expressed genes after exposure to this very low dose of BPS using a less restrictive enrichment analysis to find potential functions that may be slightly affected by exposure, based on a non adjusted *p value* < 0.05. This analysis revealed enrichment of GO terms (p-value < 0.05) related to fetal development (*hindlimb morphogenesis, forelimb morphogenesis, myoblast differentiation, skeletal system morphogenesis, mammary gland development, heart morphogenesis, cartilage development, palate development, lung development*) (Fig. S2). Among the enriched GO terms, 7 were involved in placental-brain axis (*neuron cellular homeostasis, adult walking behavior, learning, regulation of synaptic vesicles exocytosis, positive regulation of neuronal apoptotic process, cerebral cortex development, memory*) (Fig. S3). Genes involved in pregnancy were also found to be enriched (Fig. S3).

3.4. mRNA expression of enzymes regulating DNA methylation is not affected by exposure to environmentally relevant dose of BPS

The epigenetic state of the placenta is crucial to allow an appropriate and coordinated development of this organ and subsequently support fetal growth and development [3,62]. In order to determine whether postnatal growth restriction could be related to alterations of the placental methylome, we first studied the placental mRNA expression of enzymes involved in regulation of DNA methylation. Neither BPA nor BPS affected *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Tet1* or *Tet2* mRNA expression (Fig. 5).

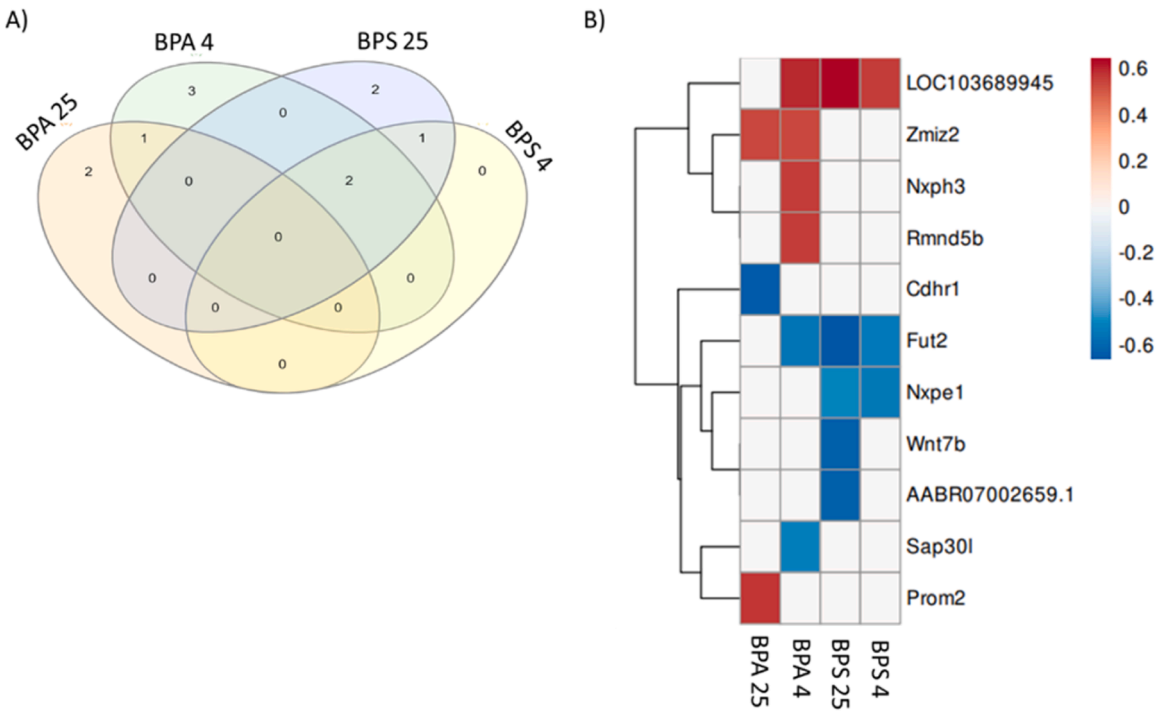

Fig. 4. Placental transcriptome modifications following developmental exposure to BPA and BPS. (**A**) Venn diagrams representing the number of differentially expressed transcripts based on an adjusted p-value < 0.05 for 2 doses of BPA (25 ng/kg/day or 4 μg/kg/day) or BPS (25 ng/kg/day or 4 μg/kg/day) compared to controls (n = 3/group). BPA25: BPA 25 ng/kg/d, BPA4: BPA 4 μg/kg/d, BPS 25: BPS 25 ng/kg/d, BPS4: BPS 4 μg/kg/d. (**B**) Heatmap representing the Log2 Fold change of the most affected up- or down-regulated genes in the placenta exposed to BPS or BPA 25 ng/kg/day or 4 mg/kg/day compared to controls. Red color indicates a significant increase in expression and blue color indicates a significant decrease in expression (adjusted p-value < 0.05). Grey indicates non-significant changes.

3.5. Developmental exposure to environmentally relevant dose of BPS affects placental steroid receptor coactivator 2 (Src2) DNA methylation

Sex steroids are essential for the development and the function of the placenta. The major coregulators of estradiol responsiveness are SRCs. Because of the role of Src2 in early placentation [56] and its potential involvement in fetal growth restriction [50], we assessed Src2 DNA methylation pattern of the *Src2* CpG island in female placenta exposed to BPA and BPS using bisulfite sequencing. The percentage of methylation was significantly lower at 6 out of 24 CpG sites of the *Src2* promoter after exposure to BPS 25 ng compared to controls but was not significantly affected by the higher dose of BPS (Fig. 6). BPA 4 μg significantly decreased the percentage of methylation at 1 CpG site of the *Src2* CpG island (Fig. 6). Placental Src2 mRNA expression was unaffected by BPA or BPS (Fig. 7).

4. Discussion

Numerous studies have raised concerns regarding the adverse health effects of developmental exposure to BPA [27,51]. Therefore, regulations regarding BPA in consumer products have been tightened in order to limit prenatal and early postnatal exposure. These regulations led the industry to develop BPA substitutes such as Bisphenol S (BPS; 4,4′-sulfonyldiphenol). However, despite its chemical similarities with BPA, data evaluating BPS effects *in vivo* is still limited.

In the present study, we provide evidence that prenatal exposure to environmentally relevant doses of BPS impairs postnatal female growth in rats. Female fetal growth was not affected by exposure to this low dose of BPS but was impaired by the same dose of BPA, indicating differential effects and mechanisms of action. The decrease in postnatal growth caused by BPS might be associated with early alterations of placental function as indicated by limited but significant changes in the transcription and DNA methylation pattern of specific genes involved in placental development.

To our knowledge, very few studies have investigated the effects of early exposure to low dose BPS on pregnancy outcomes and fetal and postnatal growth trajectory. Because sex influences response to environmental chemicals [13,52], we analyzed male and female placentas and pups separately.

Based on our results, it seems that exposure to BPS leads to sex specific effects on growth trajectory as suggested by the different growth trajectories of female and male offspring that were perinatally exposed to BPS. Our results show that average fetal weight was significantly decreased by developmental exposure to the lower dose of BPS (25 ng/kg/day) in males but not in females. In contrast, female postnatal growth was reduced after perinatal exposure to the lower dose of BPS (25 ng/kg/day) while adult male weight was not affected.

Our study is the first to identify an association between prenatal exposure to BPS and reduced fetal growth in males. This result differs from previous studies showing no effect of developmental exposure to BPS on average fetal weight at birth in rats [33]. In our model, exposure to BPS started one week before gestation while other studies started exposure later during gestation (GD6 to GD9). This suggests that specific susceptibility windows of BPS exposure could differentially impact fetal growth trajectory. Moreover, other studies used higher dosing (1 or 5 μg/kg/day) [33]. This suggests more detrimental effects with very low and environmentally relevant doses of BPS. In humans, few studies regarding BPS impact on fetal growth have been published [5]. Some authors reported an association between high maternal BPS urinary concentrations and low birth weight [28,30,34] while others did not report any effects of BPS on birth weight [22,40,47,64,69]) Lastly, very few studies have taken sex difference into account.

Implantation and early placental development are key factors supporting a successful pregnancy and optimal fetal growth trajectory. RNA sequencing analysis highlighted the decrease in expression of genes involved in embryo implantation and early placenta development.

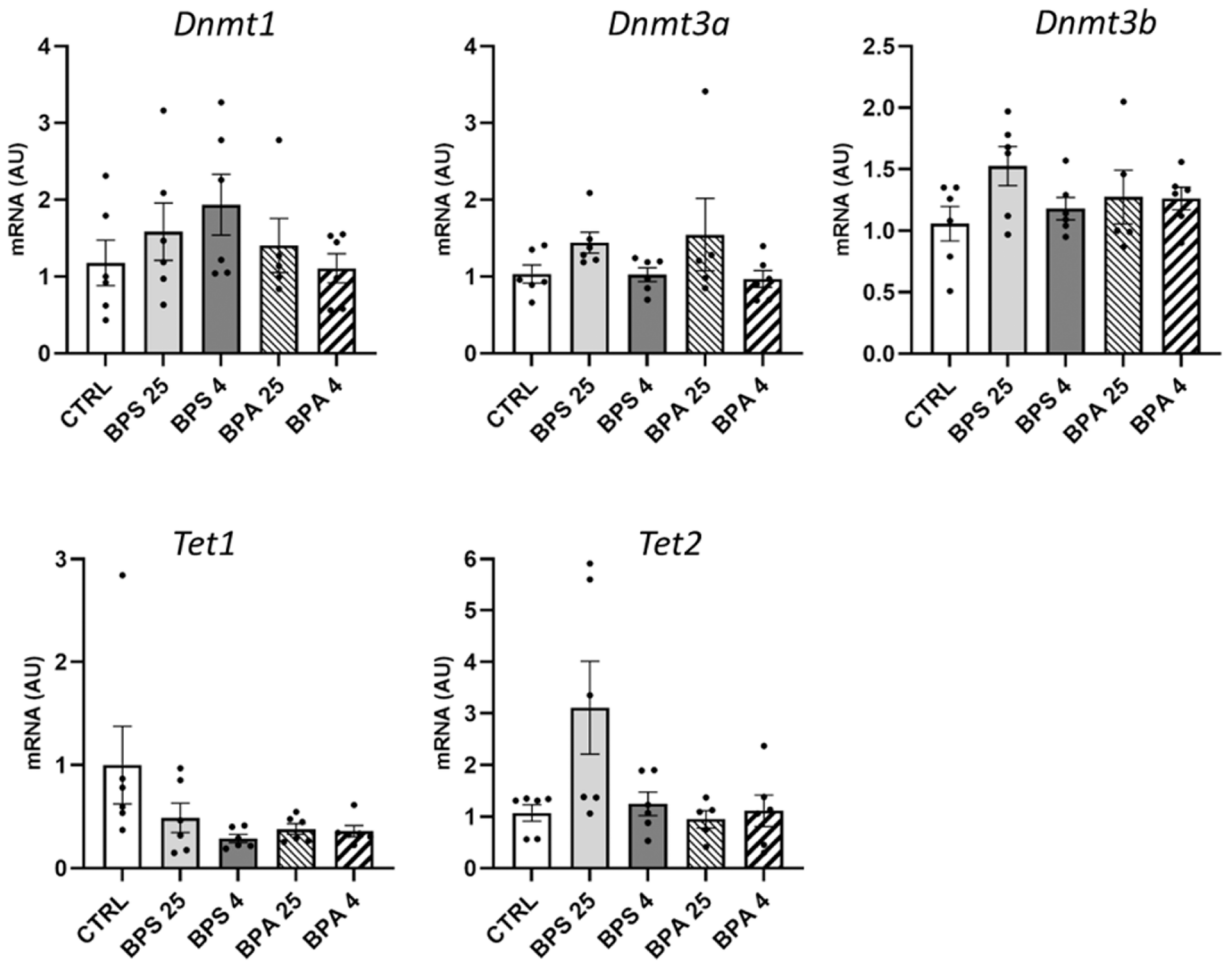

Fig. 5. mRNA expression of enzymes regulating DNA methylation in female placenta following developmental exposure to BPA and BPS. Expression of Dnmt1, Dnmt3a, Dnmt3b, Tet1 and Tet2 mRNA in female placenta exposed to corn oil, BPA 25 ng/kg/d, BPA 4 μg/kg/day, BPS 25 ng/kg/day and BPS 4 μg/kg/day as determined by QPCR (n = 6/group). Samples originates from six different litters per group. RNA expression data were normalized by dividing each individual value by the control group average value. Dnmt1: DNA methyltransferase 1, Dnmt3a: DNA methyltransferase 3a, Dnmt3b: DNA methyltransferase 3b, Tet1: ten-eleven-translocation 1, Tet2: ten-eleven-translocation 2.

Although fetal weight at E18 was not significantly affected by exposure to BPS 25 in females, those genes could be further validated as marker of exposure and risk of altered postnatal growth. *Fut2,* which is known to be regulated by estradiol [17] is involved in the synthesis of embryoadhesive fucosylated glycoconjugates. Its stage specific expression on the surface of uterine endometrial cells plays crucial roles in the intracellular interactions between the embryo and endometrium during implantation, contributing to blastocyst attachment to the uterine wall [18]. *AABR07002659.1*, also known as *pregnancy-specific glycoprotein 22-like*, is detected as early as GD 5.5 in the implantation unit from rodents [8]. It encodes a protein belonging to the pregnancy-specific glycoprotein family which supports vascular adaptations and angiogenesis involved in successful implantation and placental development [8]. *Wnt7b* expression was significantly decreased after exposure to BPS 25 ng. *Wnt7b* belongs to the family of secreted wingless ligands. In mice, homozygous *Wnt7b* mutation leads to death at mid gestation as a result of placental abnormalities [48] demonstrating the crucial role of the *Wnt* signaling pathway in early placental development in mammals [35,70]. Previous studies have already shown that *Wnt* signaling pathway appears to be a target of BPA and BPS exposure [43,57]. In our study, *Wnt7b* expression was decreased in placentas exposed to the low dose of BPS but not in placentas exposed to the higher dose of BPS or both doses of BPA, illustrating the potential role of *Wnt7b* in mediating BPS 25 ng effects on foeto-placental unit. Those 3 affected genes are commonly expressed early in the development of the foeto-placental unit but have been identified at GD18, a stage where the placenta is functionally mature. The expression of those genes should be studied earlier during placental development in order validate their functional relevance.

Additionally, GO analysis performed with a less restrictive enrichment analysis highlighted the impact of BPS on the placenta-brain-axis. This is consistent with previous study showing that exposure to BPA and BPS affects this axis through modification of serotonin concentration in the placenta [43]. In our model, only a small number of genes was affected by BPA and BPS exposure, which is consistent with previous studies [43]. Those results suggest that the sensitivity of the placental transcriptome to BPA and BPS is limited and selective. The present study used whole placentas which might explain the limited effect on transcription. Further studies will need to focus on individual cell populations.

Our study showed that exposure to a very low dose of BPS significantly decreased placental DNA methylation of *Src2,* also known as *Ncoa2*. *Src2* belongs to the steroid receptor coactivator (SRC) family, and is a key mediator of placental and endometrial response to estrogens and progesterone [15]. It prepares the endometrium for implantation [37] as illustrated by the implantation failure and partial loss of decidualization caused by uterine ablation of *Src2* [45,46]. *Src2* mediates the effects of progesterone on genes coding for developmentally important signaling molecule such as Wnt signaling pathway [32]. Notably, *Wnt7b* expression was decreased by the lower dose of BPS in our study. Moreover, *Src2* appears to play a critical role in the progression of fetal growth

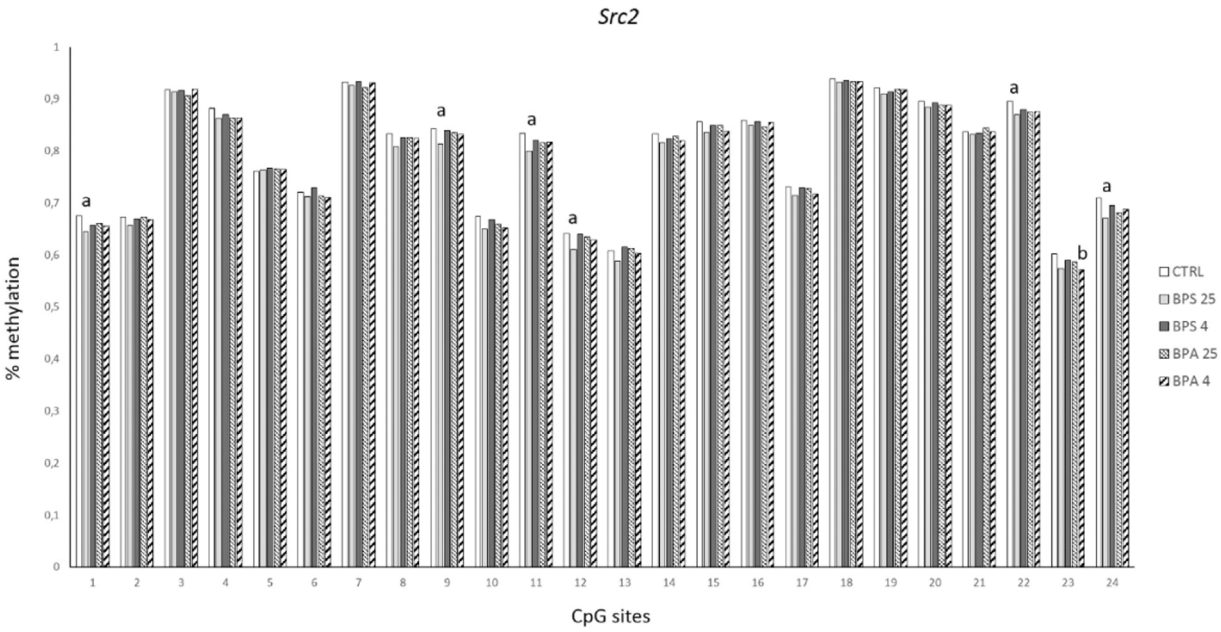

Fig. 6. DNA methylation of Src2 promoter in female placenta following developmental exposure to BPA and BPS. DNA Methylation state at 24 CpG sites of SRC2 promoter in female placenta exposed to BPS 25 ng/kg/d, BPS 4 μg/kg/day, BPA 25 ng/kg/day and BPA 4 μg/kg/day as determined by bisulfite sequencing N = 6/group a: p-value < 0.05 CTRL vs BPS 25; b: p-value < 0.05 CTRL vs BPA 4.

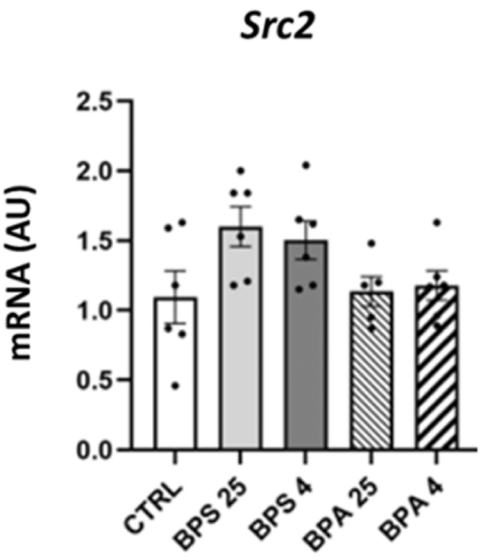

Fig. 7. Female placental mRNA expression of estrogen receptors (ERs) and steroid receptor coactivators (SRCs) following developmental exposure to BPA and BPS. mRNA expression of Steroid receptor coactivator 2 (Src2) in female placenta exposed to corn oil (control); BPS 25 ng/kg/day; BPS 4 μg/kg/day; BPA 25 ng/kg/day and BPA 4 μg/kg/day as determined by real time PCR (n = 6/group). Samples originates from six different litters per group. RNA expression data were normalized by dividing each individual value by the control group average value.

restriction in human through inducing placental inflammation [50]. One study has reported that *Src2* mRNA and protein expression in the testis and the mammary gland is sensitive to developmental exposure to BPA [6,53]. However, no study had reported an effect of developmental exposure to BPS on *Src2* expression or DNA methylation so far. In our study, epigenetic modifications were mainly present in placentas exposed to the lower dose of BPS. Those results indicate that placental DNA methylation of *Src2* is more sensitive to low doses of BPS which is consistent with a previous study showing that hepatic DNA methylation is sensitive to low doses of BPS (1.5 μg/kg/day) [9]. In our model, DNA methylation modifications of the *Src*2 promoter were not associated with transcriptional changes in *Src2* expression. This suggests that *Src2* DNA methylation is very sensitive to endocrine disruption but is not sufficient to explain the potential defect in placental function. Other epigenetic mechanisms than methylation [44] might explain that placental decrease in *Src2* promoter DNA methylation after exposure to BPS 25 ng was not associated with changes in expression of *Src2* mRNA in the placenta. Alternatively, as methylation marks are more stable than transcriptional changes, SRC2 methylation changes could reflect an earlier impact of BPS exposure.

Exposure to BPA and BPS did not affect the placental mRNA expression of enzymes involved in regulation of placental DNA methylation/demethylation, suggesting that DNA methylation changes in *Src2* promoter after BPS exposure involve other mechanisms.

In conclusion, the present study showed that developmental exposure to a very low dose of BPS impairs female postnatal growth and decreases the placental expression of key genes involved in early placental development. Our data illustrates the impact of an early environmental insult on the fetoplacental unit and indicates that transcriptional and epigenetic changes caused by endocrine disrupting chemicals at the level of the placenta may be involved in early programming of health, along the concept of developmental origin of health and disease. This study reinforces the importance of thorough risk assessment to avoid regrettable substitutions when considering replacement products like BPS, which may exhibit effects and mechanisms of action potentially distinct from BPA.

CRediT authorship contribution statement

Fudvoye J.: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Lopez-Rodriguez D.:** Conceptualization, Formal analysis, Investigation, Writing – review & editing, Visualization. **Glachet C.:** Formal analysis, Investigation, Writing – review & editing, Visualization. **Franssen D.:** Formal analysis, Investigation, Writing – review & editing. **Terwargne Q.:** Investigation. **Lavergne A.:** Formal analysis, Data curation. **Donneau A.F.:** Formal analysis. **Munaut C.:** Formal analysis.

Lomniczi A.: Formal analysis. **Dehan P.:** Methodology, Investigation. **Parent A.S.:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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.

Acknowledgements

This research was supported by the FRS-FNRS (Belgian National Foundation for Research, CDR-J013019F-33661942), the University of Liège and the University Hospital of Liège.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2025.108854.

Data Availability

Data will be made available on request.

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