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Multigenerational study of the obesogen effects of bisphenol S after a perinatal exposure in C57BL6/J mice fed a high fat diet.

Axelle BRULPORT^{1,2,3}, Ludovic LE CORRE^{1,2,3*}, Guillaume MAQUART^{1,2,3}, Virginie BARBET^{1,2,3}, Aurélie DASTUGUE^{1,2,3}, Isabelle SEVERIN^{1,2,3}, Daniel VAIMAN^{4,5,6}, Marie-Christine CHAGNON^{1,2,3}.

¹ Université de Bourgogne Franche-Comté, LNC UMR1231, F-21000 Dijon, France ;

² AgroSupdijon, LNC UMR1231, F-21000 Dijon, France

³ Nutrition Physiology and Toxicology Team (NUTox), INSERM, LNC UMR1231, F-21000 Dijon, France

⁴ From Gametes to Birth Team (FGTB), INSERM, U1016, Institut Cochin, F-75014 Paris, France.

⁵ CNRS UMR8104, F-75014 Paris, France.

⁶ Université Sorbonne Paris Cité, F-75014 Paris, France.

* Corresponding author: Dr. LE CORRE Ludovic, PhD, NUTox laboratory, UMR U1231 INSERM, AgroSup Dijon, 1 Esplanade Erasme, 21000 Dijon, France.

e-mail: ludovic.le-corre@agrosupdijon.fr

tel: (+33)380774035

fax: (+33)380774033

Abstract

Background: Bisphenol S is an endocrine disruptor exhibiting metabolic disturbances, especially following perinatal exposures. To date, no data are available on the ~~transgenerational~~ obesogen effects of BPS in a multigenerational issue ~~on energy metabolism~~.

Objectives: We investigated obesogen effects of BPS in a multigenerational study by focusing on body weight, adipose tissue and plasma parameters ~~related to energy metabolism~~ in male and female mice.

Methods: Pregnant C57BL6/J mice were exposed to BPS (1.5 µg/kg bw/day *ie* a human equivalent dose of 0.12 µg/kg bw/day) by drinking water from gestational day 0 to post natal day 21. All offsprings were fed with a high fat diet during 15 weeks. Body weight was monitored weekly and fat mass was measured before euthanasia. At euthanasia, blood glucose, insuline, triglyceride, cholesterol and no esterified fatty acid plasma levels were determined and gene expressions in visceral adipose tissue were assessed. F1 males and females were mated to obtain the F2 generation. Likewise, the F2 mice were cross-bred to obtain F3. The same analyses were performed.

Results: In F1 BPS induced an overweight in male mice associated to lipolysis gene expressions upregulation. In F1 females, dyslipidemia was observed. In F2, BPS exposure was associated to an increase in body weight, fat and VAT masses in males and females. Several plasma parameters were increased but with a sex related pattern (blood glucose, triglycerides and cholesterol in males and NEFA in females). We observed a down-regulation in mRNA expression of gene involved in lipogenesis and in lipolysis for females but only in the lipogenesis for males. In F3, a decrease in VAT mass and an upregulation of lipogenesis gene expression occurred only in females.

Conclusions: BPS perinatal exposure induced sex-dependent obesogen ~~and metabolic~~ multigenerational effects, the F2 generation being the most impacted. Transgenerational disturbances persisted only in females.

Bisphenol S perinatal exposure induces sex-dependant transgenerational effects in association with a potentiation of the obesity development.

Keywords: transgenerational effects, obesogen, low dose exposure, peri-natal exposure

1. Introduction

Bisphenol S (BPS) is a chemical used as monomer in the manufacture of plastics and resins by nearly sixty industrial sectors (Qiu et al., 2019). This compound is ubiquitously present in the environment: indoor air, surface water, sediment, sewage sludge, paper, paints, varnishes, inks, glues, electronic and electrical components, cosmetics, medical equipment, food and food contact materials with a specific migration limit (SML) of 50 µg/kg (EU 10/2011) (Qiu et al., 2019). Observational study highlighted that BPS appears to be one of the bisphenols that is the most resistant to biodegradability (Danzl et al., 2009; Frankowski et al., 2020), present an important dermal penetration level (Yang et al., 2019), and is one with the highest estimated daily intakes *via* indoor dust ingestion (Zhang et al., 2020). Although the food is the main route of BPS contamination, these data show that non-dietary exposure may also be significant to human exposure (Karrer et al., 2020).

The use of BPS is becoming of increasing concern since the restrictions on the use of bisphenol A (BPA) in relation to its reprotoxic and endocrine disruptor properties, but also due to its greater thermal stability compared to BPA (Karrer et al., 2020). To support this recent increase in usage of BPS, the detection frequency of BPS in American adults urine samples was about 20 % for the period 2000-2007 and about 70 % in years 2009-2014 (Ye et al., 2015). Population-based cohort of BPS biomonitoring studies in urine samples have

shown that BPS was detected at concentrations ranging from 0.03 ng/ml (mean value of 33 samples, Korea, 2010-2011) to 1.18 ng/ml (mean value of 36 samples, Japan, 2010-2011) (Qiu et al., 2019). Larger-scale studies show mean concentrations ranging from 0.1 ng/ml (1800 samples, U.S., 2013-2014) to 0.36 ng/ml (1400 samples, Netherlands, 2004-2005) (Karrer et al., 2020). A recent study measured urinary levels of BPS at 0.33 ng/ml and estimated an exposure between 0.7 and 7.5 ng/kg bw/day (mean values of 144 samples, Norway, 2016-2017) (Karrer et al., 2020). But, unlike BPA, the modelling of BPS exposure underestimated measured amounts due to patchy exposure characterization (e.g. food and textiles) and lack of data on the absorption and metabolism of BPS (Karrer et al., 2020).

From the early 2000s, the role of environmental pollution in the physiopathology of obesity has been highlighted (Baillie-Hamilton, 2002; Heindel, 2003). This awareness has led to the definition of the concept of obesogen, *i.e.* exogenous chemical substances disrupting lipid metabolism and adipogenesis leading to potentiating a pre-existing imbalance in the energy homeostasis (Grun et al., 2006; Le Corre et al., 2015; Papalou et al., 2019). In 2015, the obesogen concept was requalified as a notion of metabolic disruptor considering the globality of metabolic alterations associated with obesity (Heindel et al., 2017a; Papalou et al., 2019). Most metabolic disruptors are known to be endocrine disruptors (EDs). BPS is a structural analogue of BPA with similar ED properties (Rochester and Bolden, 2015). BPA is the most studied metabolic disruptor and BPS appears to induce similar effects in rodents and sheep (Ivry Del Moral et al., 2016; Meng et al., 2019, 2018; Thoene et al., 2020).

They mainly act following perinatal exposures (De Coster and van Larebeke, 2012). The BPS crosses the placental barrier (Grandin et al., 2019). In women, BPS has been detected in placenta (60 samples, China, 24.4 pg/g), in cord placenta (60 samples, China, 7.2 pg/g) (Pan et al., 2020) and in breast milk (1 sample, France, 0.23 µg/kg) (Deceuninck et al., 2015). EDs are highly studied factors in the DOHaD concept (Developmental Origins of

95 Health and Disease) through potential effects of the gestational microenvironment during
96 early stages of development on the occurrence of obesity later in life (Heindel et al., 2017b;
97 Mochizuki et al., 2017). Usually, the DOHaD concept is applied to directly exposed
98 individuals, but the effects of ED can also impact the health of their children and of
99 subsequent generations (Diamanti-Kandarakis et al., 2009; Gore et al., 2015). For the
100 maternal exposure (F0), the F1 and F2 generations are exposed *in utero* and *via* the F1
101 gametes, respectively. These are considered multigenerational effects. From the F3
102 generation, the first unexposed generation, the observed effects are described as
103 transgenerational. If the exposure is paternal, we consider that transgenerational effects occur
104 in F2 (first unexposed generation) (Heard and Martienssen, 2014; Skvortsova et al., 2018).
105 BPA and BPS metabolic disruptor effects are well documented and highlighted disparities
106 according to the maternal exposure window, the diet, the doses, route of exposure and sex of
107 rodents in F1 generation (Le Corre et al., 2015; van Esterik et al., 2014). Only one study
108 showed carbohydrate metabolism alterations in F2 following perinatal exposure to BPA at 40
109 $\mu\text{g/kg bw/day}$ by gavage in male wistar rats (Li et al., 2014). Interestingly, a recent study
110 showed that another ED (Tributyltin) could induce a transgenerational obesogenic effect after
111 perinatal exposure at 50 nM in drinking water in C57BL6/J male mice (Chamorro-Garcia et
112 al., 2017). It has already been shown that other EDs (hydrocarbons,
113 dichlorodiphenyltrichloroethane (DDT), BPA and phthalates mixture, methoxychlor) can
114 induce a transgenerational obesogenic effect following perinatal exposure to non-
115 environmental doses (amounts to 25 milligrams or more per kilogram of body weight per day)
116 by intraperitoneal injection (Manikkam et al., 2014, 2013; Skinner et al., 2013; Tracey et al.,
117 2013). ~~To our knowledge, no studies on BPS induced multi- or transgenerational energy~~
118 ~~metabolism disturbances are available.~~ Here, we analyzed the obesogen effects induced by
119 BPS after perinatal exposure of the F1 generation to 1.5 $\mu\text{g / kg bw / day}$. We also

investigated these disturbances on three generations (F1, F2 and F3) of male and female C57BL6 / J mice fed with a high fat diet in the adult stage.

2. Methods

2.1. Animals and materials

Pregnant C57Bl/6J mice were purchased from Charles Rivers. Bisphenol S (BPS) was provided by Sigma-Aldrich. The high fat diet (HFD) was based on the 4RF25 reproduction diet (Mucedola) with the addition of palm oil (Huilerie de Vigan) and cholesterol from Sigma-Aldrich (Table S3). The standard diet (SD) was based on the 4RF21 diet (Mucedola). Mucedola 4RF25 and 4RF21 diets are certified as estrogen free and accurately tested for the detection of estrogenic activities. In the diet, the percentage of phytoestrogens is certified to be less than 4 ppb (parts per billion) according to international standards (U.S. Food and Drug Administration National Center for Toxicological Research Standard No. 2, September 5, 1973). In the high fat diet, 0.042% cholesterol has been added (because it is missing in palm oil and it is required by Research diet Inc. in diet-induced obesity). Triglyceride FSTTM, Cholesterol FSTTM and No Esterified Fatty Acids (NEFA) FSTTM kits were purchased from DiaSys. Blood glucose test strips (Accu-Check®, Roche Diagnostics) and glucometers (Accu-Check® Aviva, Roche Diagnostics) were purchased from the pharmacy of the University (Dijon, France).

2.2. Experimental design

Mice were housed in a 12h light-dark cycle at a temperature of 22°C in one conventional animal house and allowed free access to food and water. Cages and bottles were made of polypropylene (bisphenol-free). Thirty-seven F0 pregnant C57Bl/6J mice were individually housed. They were divided into two groups and exposed to BPS (20 females) or

not (17 females) in their drinking water at concentration of either 0 or 8.5 ng/ml from the first day of gestation (GD0) to post-natal day 21 (PND21) of F1 offsprings. The determination of GD0 was done in the morning by vaginal plug detection. Water intake was determined by measuring the difference in the amount of water placed in the water bottle each week and the amount remaining the following week. Consequently, the levels of BPS consumed weekly were estimated. Under our conventional animal facility conditions, BPS is stable for more than seven days in drinking water (verified by a SPE extraction and HPLC analysis; data not shown). The average BPS intake was $1.78 (\pm 0.03) \mu\text{g/kg bw/d}$. BPS was dissolved in absolute ethanol (0.1%). Control group drinking water contained only 0.1% ethanol. As food is the major route of BPS exposure, in animal experiments, an exposure *via* drinking water allows i) a continuous administration to be as close as possible to BPS detoxification metabolism involved in environmental exposure ii) the homogeneity of the BPS dosage and iii) minimize the degradation of BPS by microorganisms (filtered water and hermetic bottles) (Kang et al., 2006; Pivnenko et al., 2015; Pottenger et al., 2000).

To generate the mice of F2 generation, multiple couples of mice were mated at the age of 10 weeks. Each litter came from a unique couple mice made up of male and female mice derived from different litters and dams). ~~One male and one female mice (only fed with the standard diet) of the F1 generation were mated at the age of 10 weeks to obtain the F2 generation.~~ Under the same conditions, the F2 mice were cross-bred to obtain the individuals of the F3 generation (Graphical abstract). At weaning, a maximum of six mice were housed in each cage according to the BPS exposure status of their mother, grandmother or great-grandmother. The litters were mixed randomly after weaning to minimize a possible litter effect. For each generation, 12 male and female mice were analysed. These mice fed a standard diet from weaning to 8-weeks of age. C57BL/6 strain is an established model for diet induced obesity. C57BL/6J mice will develop severe obesity, hyperglycemia, and insulin

resistance if weaned onto a high-fat diet (Rossmeisl et al., 2003; Surwit et al., 1995). For each generation, at 8 weeks (sexual maturity), 12 male and 12 female puppies from the control and BPS groups were fed a diet rich in fat (60% Kcal of lipids; composed of 4RF25 diet breeding added with 30% palm oil and 0.42 g / kg cholesterol) or with a standard diet. At 8-weeks old (sexual maturity), all pups were fed with a high fat diet (60% Kcal from lipids; composed by 4RF25 reproduction diet added with 30% palm oil and 0.42 g/kg of cholesterol. All mice (fasted 4 h before) were euthanased at 23-weeks old. Mice were anesthetized with isoflurane, blood was sampled by cardiac puncture and then the mice were euthanized by cervical dislocation. Before euthanasia, the body weight was measured. At the euthanasia, adipose tissue was weighed, then immediately frozen in liquid nitrogen and stored at -80°C. Experimental protocol (Accreditation Number: #11422) was approved by the ministry and the University of Burgundy's ethic committee. Animal experiments have been carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.3. Body weight monitoring and corporal composition measurement

Body weight was monitored weekly. Just before (4h fasted) euthanasia, body weight, fat and lean masses of 23-weeks old mice were measured. The fat and lean masses of each mouse were determined individually using a quantitative EchoMRI 500T™ (EchoMRI, Houston, USA). Before each measurement, calibration was performed in compliance with the manufacturer's guidelines.

2.4. Food intake measurement

For each generation (F1; F2; F3), these experiments were performed six weeks after the beginning of the high fat diet to allow an acclimation time of diet change. Measurements were done by cage and then normalized to the number of mice per cage. The mice were not

individualized so as not generate stress which could modify the feeding behavior of the animal. The amount of food was weighed daily during four consecutive days. Daily food intake was determined by weighing out the difference in the amount of food placed and the remaining amount after 24 h. Thus, the daily consumption per mouse was calculated from the average of the data obtained for three consecutive days. Particular attention was paid to spillage and/or hoarding. Spilled food was weighed and subtracted from the measurements when necessary.

2.5. Biochemical analysis

Just before euthanasia (*i.e.* fasted 4h before), mouse blood samples were collected by an intracardiac puncture using heparinized syringe. After centrifugation of 10 min at 2000g and 4°C, the level of total plasma cholesterol, triacylglycerol and NEFA were measured using respective kits mentioned above. The plasma insulin was determined using ELISA kit as mentioned previously. Assays were performed according to the manufacturer's instructions. The day of euthanasia, blood glucose levels were measured, using a glucometer, from a blood sample collected at the animal's tail and before anaesthesia. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated by a formula adapted to a previous method in human (Matthews et al., 1985). For mice, reference values were calculated using average fasting glucose (4.16 mmol/l) and plasma insulin (26.11 mU/l) concentrations from the control mice fed with standard diet (van Dijk et al., 2013). The homeostatic model assessment (HOMA) adapted to mice was calculated as $([\text{glucose (mmol/l)}] \times [\text{insulin (mU/l)}])/108.6$ and used as a surrogate measure of whole-body insulin sensitivity.

2.6. Real-time PCR analysis

Lysis of visceral adipose tissue samples of each mouse were performed using Lysing Matrix D™ tubes (MP Biomedical) and a tissue lyser Precellys® (Ozyme). Total RNA was extracted using Tri-reagent™ (Sigma-Aldrich). cDNA was reverse-transcribed on 3 µg of total RNA samples using the High Capacity cDNA reverse transcription kit™ (Life Technologies). Real time PCR was done with 30 ng of cDNA, 5 µl of Taqman™ Universal Master Mix (Life Technologies), 0.5 µl of Taqman™ gene expression assay (20X; Life Technologies) for a final volume of 10 µl (qsp H2O). Taqman™ gene expression assay references were: Mm01184322_m1 (*Ppary*), Mm00503040_m1 (*Pnpla2*), Mm00495359_m1 (*Lipe*), Mm00662319_m1 (*Fasn*), Mm00449274_m1 (*Mgll*), Mm01304257_m1 (*Acaca*) and Mm99999915-g1 (*Gapdh*, reference gene). PCR was run on the Step-One plus™ system (Applied Biosystems) using following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 30s. Gene expression was determined relative to the control gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008).

2.7. Statistical analysis

All data were expressed as mean \pm standard error of mean (SEM). To determine the statistically significant difference between two groups, a Student's t test was used (two-tailed, paired samples for means and equal variance). Statistical tests were performed with GraphPad Prism7® software. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Multigenerational effects of BPS on body weight and adiposity in male mice

In mice fed with a HFD for 15 weeks, BPS induced a significant overweight in F1 (a 9 % increase, $p = 0.002$) and F2 males (a 14 % increase, $p = 0.03$) (Figure 1A and 1B). In F3,

this shift did not reach statistical significance (2 % higher) (Figure 1C). Interestingly, for both F1 and F2, overweight induced by BPS occurred after six weeks of HFD, but the body weight gain at euthanasia was higher in F2 than F1 (29 % and 18 % higher, respectively) (Figure 1D,E). To investigate whether this overweight is related to an obesogenic effect of BPS, we evaluated the mouse adiposity. Mouse fat mass and VAT mass significantly increased only in F2 (a 21 % increase and a 27 % increase, $p = 0.05$ and $p = 0.008$, respectively) (Figure 1H and 1K).

3.2. Multigenerational effects of BPS on body weight and adiposity in female mice

Under the same experimental conditions BPS induced a significant body weight increase in females, but only in F2 (a 17 % increase, $p = 0.03$) (Figure 2B). In F1 and F3, BPS exposure was associated with a weak, but not significant body weight decrease (3 % and 8 % lower, respectively) (Figure 2A and 2C). Like for F2 males, overweight induced by BPS occurred after six weeks of HFD in F2 females, but the body weight gain at euthanasia was higher in females (35 % higher) (Figure 2E). Regards to BPS obesogenic effect assessment, in F2, fat and VAT masses were significantly increased to a greater extent compared to males (a 39 % increase and a 44 % increase, $p = 0.004$ and $p = 0.04$, respectively) (Figure 2H and 2K). Unlike in F1, in F3, the small decrease in body weight was associated with a significant decrease in VAT mass (a 40 % decrease, $p = 0.03$) (Figure 2L).

3.3. BPS multigenerational effect on food intake in male and female mice

The obesogenic effect of BPS observed in F2 males and females mice was associated with a decrease in food intake which was significant only in male mice (a 15 % decrease and a 6 % decrease, $p = 0.0007$ and $p = 0.52$, respectively) (Figure 3B and 3E). For F1 males, the food intake of exposed mice with overweight was increased, but not significant (8 % higher).

3.4. Transgenerational effects of BPS on plasma parameters related to blood glucose and lipid metabolism in male and female mice

In males exposed to BPS, overweight observed in F1 was associated with a hypocholesterolemia (a 20 % decrease, $p = 0.007$) (Table 1). In F2, the obesogenic effect of BPS induced a significant increase in triglyceride and total cholesterol plasma levels (a 12 % increase and a 50 % increase, $p = 0.0003$ and $p = 0.01$, respectively) (Table 1). Regardless of the generation, no significant changes were observed in HOMA-IR and glucose, insulin and NEFA plasma level (Table 1).

In females, despite the absence of a significant effect on body weight, the metabolic disruptor effects of BPS were manifested by a hypercholesterolemia and a hypertriglyceridemia in F1 (a 39 % increase and a 44 % increase, $p = 0.0002$ and $p = 0.02$, respectively) and a decrease in blood glucose level in F3 (an 11 % decrease, $p = 0.04$) (Table 2). In F2, the obesogenic effect of BPS was associated with a significant increase in NEFA plasma level (a 116 % increase, $p = 0.02$) (Table 2). As in male and regardless the generation, no significant changes were observed in HOMA-IR and insulin plasma level (Table 2).

~~All no significant results for male and female mice F1, F2 and F3 fed with HFD (glucose, insulin and NEFA plasma level and HOMA-IR) are reported in supplementary data (Table S1).~~

3.5. BPS transgenerational effects on adipose tissue lipid metabolism marker genes expression in male and female mice

In males exposed to BPS, overweight shown in F1 was associated with a significant downregulation of the mRNA expression of Patatin Like Phospholipase Domain Containing 2 (*Pnpla2*) and Monoglyceride Lipase (*Mgl1*), two enzymes involved in the first and last stage

of lipolysis, respectively (a 30 % decrease and a 22 % decrease, $p = 0.005$ and $p = 0.03$, respectively) (Figure 4). In F2, the BPS obesogenic effect was associated with a significant decrease of *Acetyl-CoA Carboxylase Alpha (Acaca)* mRNA level, an enzyme involved in the first step of lipogenesis (a 22 % decrease, $p = 0.04$) (Figure 4).

In females, the BPS obesogenic effect highlighted in F2 was associated with a downregulation of *Pnpla2* and *Acaca* mRNA expressions (a 33 % decrease and a 39 % decrease, $p = 0.04$ and $p = 0.008$, respectively) (Figure 5). For F3 females exposed to BPS, the decrease in VAT mass was associated with an upregulation of *Acaca* and *Fatty Acid Synthase (Fasn)* mRNA levels, two enzymes involved in lipogenesis (a 65 % increase and a 69 % increase, $p = 0.01$ and $p = 0.009$, respectively) (Figure 5).

All other marker genes mRNA expressions were not modified (Figure S1 and S2).

4. Discussion

In our previous one-generation study in C57BL/6J mice fed with a HFD, we demonstrated obesogenic and metabolic effects resulting from perinatal and chronic exposure (up to 23 weeks-old mice) to BPS at 1.5 $\mu\text{g/kg bw/day}$ in F1 male mice only (Ivry Del Moral et al., 2016). Here, using the same experimental model, but a limiting window exposure (gestation and lactation), we investigated transgenerational metabolic disturbances of BPS. Thus, in this study, we carried out a similar analysis focused on the principal physiological parameters associated with an obesogen effect (body weight gain associated with an alteration of adipose tissue lipid metabolism).

Experimental strategy:

To conduct a study on the obesogen effect of an endocrine disruptor such as bisphenol S, choices had to be made on important parameters of this type of investigation: composition of diets, dose of exposure, method of administration and exposure windows. These choices

were based on the data available on bisphenol A, the most studied obesogen endocrine disruptor (Le Corre et al., 2015).

The use of an HFD is necessary because the BPS only potentiate the development of obesity by exacerbating an HFD-induced energy imbalance. Indeed, BPS does not induce any obesogen effect in mice having undergone the same exposure protocol but fed with a standard diet (Ivry Del Moral et al., 2016). Here, we confirm that whatever the sex and the generation, BPS did not alter body weight, corporal composition, food intake and plasma parameters (supplementary data, table S1 and S2 for male and female mice, respectively).

~~Concerning the HFD used, we previously described that it was very efficient to induce the obesity development in the same mouse strain (Ivry Del Moral et al. 2016), and also to alter physiological parameters involved in the obesity physiopathology (de Wit et al. 2008) in accordance with the emerging concept of metabolic disruptors (Heindel et al. 2017a; Papalou et al. 2019). giving rise to a metabolic disruptor effect in male mice as reported in our previous investigation (Ivry Del Moral et al. 2016). BPS concentration was based on a preliminary study evaluating the water consumption of mice during the gestation to obtain expected BPS exposure of 1.5 µg/kg body weight/day. To our knowledge, no studies have assessed human BPS exposure related to country-specific bisphenol use regulations. The choice was made to use the available data for BPA, the most studied metabolic disruptor, which is also a structural analogue of BPS. The dose of 1.5 µg/kg bw/d corresponds to the BPA exposure of adults (EFSA 2006). In our study, we used BPS at a dose of 1.5 µg/kg bw/d. This is the lowest dose of BPS known to have an obesogen effect after perinatal exposure in mice as we reported in our previous study using three different doses 0.2, 1.5 and 50 µg/kg bw/d (Ivry Del Moral et al., 2016).~~

~~In the present study, in order to better understand the role of BPS in the DOHaD concept, we have reduced the duration of exposure to the strict perinatal period and removed the notion of chronic exposure.~~

In this study, the window of exposure was limited to pregnancy and lactation to target key stages of tissue development of interest i) germline as a vector of multi- and transgenerational heritability, subjected to two reprogramming waves of DNA methylation during gametogenesis (GD0.5-GD3.5 and GD8.5-GD16.5) (Heard and Martienssen, 2014) ii) and adipose tissue that appears from the twelfth day of embryonic development in C57Bl/6J mice (Mayeuf-Louchart et al., 2019). Moreover, there are likely distinct pathways for developmental (in utero) and non-developmental exposures of obesogens, as well as persistent versus non-persistent exposures (Griffin et al., 2020). In this context, we have focused only on the early stages of development.

BPS induced obesogen effect in F1 generation in sex-dependent manner

Interestingly, in comparison with the present study, Ivry del Moral *et al* research showed a higher body weight gain (12 %) and adiposity (13 %), a hypercholesterolemia and hyperinsulinemia only in male mice (Ivry Del Moral et al., 2016). These more marked metabolic disturbances may be related to changes in the BPS window exposure. However, ~~which nevertheless suggest that BPS exposure during gestation and lactation is sufficient to potentiate the obesity in adulthood.~~ as reported with BPA, it seems important and sufficient to have an impregnation of BPS during the gestation period to observe significant obesogen effects (Le Corre et al., 2015; Nikaido et al., 2004; Rubin et al., 2001). More generally, EDs act on the same basis as endogenous hormones and consequently can interfere with development processes (Gore et al., 2015).

In accordance with our data about BPS, one other study showed an increase in body weight and adiposity in ICR F1 male mice, with or without nutritional stress (HFD),

369 following perinatal exposure to a higher dose of BPS (100 µg/kg bw/day) by gavage (Meng et
370 al., 2019). An increase in body weight was also observed in Swiss F1 male mice fed a
371 standard diet and following a BPS exposure in adulthood at doses ranging from 25 to 100
372 µg/kg bw/day by drinking water (Rezg et al., 2018). With the two experimental protocols
373 (BPS perinatal or perinatal and chronic exposure) used in the lab, we failed to induce an
374 obesogenic effect in F1 female mice (Ivry Del Moral et al., 2016). In F1, only one study
375 shows a body weight increase in ICR adolescent female mice (from 3 to 5 weeks of age) fed a
376 standard diet and following a BPS perinatal exposure (100 µg/kg bw/day) by gavage (Meng et
377 al., 2018). These mice also show an increase in triglyceride and cholesterol hepatic levels
378 (Meng et al., 2018). This is consistent with the dislipidemia observed without change in
379 adiposity in our F1 females, in which, under a HFD and at an older age, BPS appears to
380 induce a potential deregulation of energy metabolism in the liver (Titchenell et al., 2017).

381 The sex-dependent effect suggests an impact on sex hormones which are powerful regulators
382 of energy metabolism (Mauvais-Jarvis et al., 2013; Navarro et al., 2015). For example,
383 suboptimal or insufficient estrogen levels have been reported to be linked to fat accumulation
384 and obesity in women (Litwak et al., 2014). Likewise, in men, obesity is strongly associated
385 with low testosterone levels (Navarro et al., 2015). The action of endogenous hormones is
386 highly dose dependent. Thus, endocrine disruptors could reverse their effects on energy
387 metabolism. In addition, BPS is described both as a weak estrogenic compound in vitro
388 (Kuruto-Niwa et al., 2005; Molina-Molina et al., 2013) and as an anti-androgen receptor
389 antagonist. It is also reported as an inhibitor of the steroidogenesis in MA-10 Leydig murine
390 cells (Kitamura et al., 2005; Roelofs et al., 2015) and to decrease the basal secretion of
391 testosterone by the testes of mice and human fetuses (Eladak et al., 2015). Thus, a
392 combination of estrogenic and antiandrogenic properties of BPS could cause an imbalance of
393 sex hormones in humans leading to deregulation of lipid homeostasis. Conversely, in women,

the potential BPS-induced disruption of estrogen homeostasis might be negligible regards to endogenous estrogen levels.

The obesogen effect of BPS is multigenerational (F2) but not transgenerational (F3)

Our results showed difference between generations (Figures 1 and 2). However, it is not relevant to compare the generations between them. Indeed, the variation in measurements can be explained by the high fat diet. The diet-induced obesity (DIO) in mice shows significant variability (Burcelin et al., 2002; Vaanholt et al., 2015). Likewise, the range values of the plasma parameters are consistent with the data provided by Charles River in C57Bl / 6J mice. (Jax Phenom 3 study, in 2006). In 16 week-old and 4h-fasted mice, it reported that the glucose plasma levels are 159 ± 25.1 mg/dl in females and 152 ± 29.2 mg/dl in males; triglyceride levels are 0.667 ± 0.219 g/l in females and 0.707 ± 0.16 mg/dl in males; total cholesterol levels are 0.698 ± 0.605 g/l in females and 0.933 ± 0.102 g/l in males.

In male and female mice, BPS induced multigenerationnal (*i.e.* only in exposed animals) obesogen effects under our experimental conditions. In both sexes, F2 appears to be the most impacted generation regarding analyses focused on body weight, adipose tissue, blood glucose and insulinemia. No transgenerational effect is observed because the F3 mice did not show any gain in body weight.

The worsening obesity status in F2 male and female mice suggests different mechanisms of action compared to the F1 generation. Only germ cells of F2 mice were exposed to BPS. In this case, hereditary transmission vectors are believed to be primarily epigenetic changes such as DNA methylation, histone acetylation, or non-coding RNA (Bird, 2007; Desai et al., 2015). Interestingly, BPS has been described as inducing an obesogenic effect in male F1 mice in association with a change in hepatic DNA methylation profile (Brulport et al., 2020).

Obesogen phenotype induced by the BPS in male mice is generation-dependent

In males, individuals showed different phenotypes following the F1 and F2 generations. Unlike in F2, in F1 males, adiposity increases insignificantly and has been associated with a downregulation of genes involved in lipolysis - *Plnpla2* and *Mgll* -. The storage capacity of adipose tissue does not seem to be affected. ~~With the same insulin sensitivity than control group, the liver of these animals can normally still store fatty acids in the form of triglycerides (Titchenell et al. 2017). It seems consistent not to observe dyslipidemia in F1 males. The explanation of the lower plasma cholesterol level requires further investigation. It would be interesting to study fluctuations in bile acid levels, which are cholesterol dependent for their biosynthesis, and whose role in the physiopathology of obesity and its associated comorbidities has been the aim of many recent studies (Chavez-Talavera et al. 2017).~~

In F2 males, the significant increase in fat mass and visceral adipose tissue weight and the downregulation of *Acaca* mRNA level, a gene involved in lipogenesis, may suggest that the adipose tissue storage capacity was reaching saturation. Indeed, it has already been shown that in C57BL6/J mice fed a high fat high sucrose diet, fat accumulation reaches a plateau after 17 weeks (Karunakaran and Clee, 2018). Without sucrose, but with BPS, our animals probably reached this plateau as early as the 15th week of diet. All these data may explain dyslipidemia observed in these animals. Although our control and treated groups have the same insulin levels, as all animals fed a HFD, we cannot exclude that they have an organ specific insulin resistance preventing inhibition of hepatic glucose production resulting in hyperglycemia and a worsening of dyslipidemia in F2 males (Titchenell et al., 2017).

In this context of metabolic disturbances associated with an obesity phenotype, it's unexpected to observe a decrease in food intake in F2 males. Although not investigated in this study, it appears unlikely that this observation was related to a decrease in energy expenditure or locomotor activity. In the same model, following perinatal and chronic exposure, BPS does not lead to changes in food intake and energy expenditure in F1 males (Ivry Del Moral et al.,

2016). Interestingly, perinatal exposures to BPS (10 or 50 µg/kg bw/day) induced a decrease in food intake with no change in locomotor activity in F1 wistar male rats (da Silva et al., 2019). As highlighted by Rezg *et al.* (2018) under other experimental conditions (adulthood exposure), the deregulation of orexigenic and anorexigenic hypothalamic neuropeptides appears to be an area of investigation (Rezg et al., 2018). Likewise, it cannot be excluded that at an early stage, before the appearance of resistance to leptin and insulin, high plasma levels of leptin may inhibit food intake (Thon et al., 2016).

Multigenerational obesogen effect of BPS in female mice

Interestingly, the obesogen effect of BPS appears only in F2 female mice. ~~In F2, like for males, BPS leads to an obesogenic phenotype in females. However,~~ Compared to male mice, the storage capacity of adipose tissue seems to be greater in females. Indeed, BPS induced the downregulation of *Pnpla2* -a key gene for lipolysis- and of *Acaca* mRNA levels -first enzyme involved in lipogenesis-. Likewise, the alteration of *Acaca* mRNA expression could be related to the increase in NEFA plasma level ~~could be related to the downregulation of *Acaca* mRNA level, the first enzyme involved in lipogenesis.~~

Transgenerational effect of BPS in female, an adaption mechanism?

Only F3 female from BPS-exposed F0 dams exhibited a phenotype different of control group. ~~Concerning the F3 females, Mice of the F3 generation are the first to be unexposed. generation.~~ F3 female mice ~~they~~ presented lower body weight and adiposity without metabolic disturbances, despite an upregulation of the expression of genes involved in lipogenesis (*Acaca* and *Fasn*). Thus, the hypothesis of a transgenerational adaptation mechanism is not excluded in females and could result in a shift from a thrifty phenotype to an energy over-consumption phenotype. However, while this hypothesis requires further

investigation, it's interesting to see that this particularity appears to be present only in females while in F3 males no metabolic disruption appears to persist.

Is the multigenerational obesogen effect of BPS of concern in humans?

To our knowledge, in humans, no epidemiology studies have shown an association between maternal BPS exposure ~~during the pregnancy and lactation~~ and the occurrence of metabolic disease in subsequent generations. A cross-sectional epidemiology study (U.S. National Health and Nutrition Examination Surveys: 2013-2016) described an association between urinary BPS concentrations and prevalence of general and abdominal obesity (Jacobson et al., 2019). These observations performed on the first generation highlight the critical points already known to modulate the actions of EDs (window exposure, sex, doses, route of exposure, diet) (Le Corre et al., 2015) and we will now address the question of transgenerational effects, for which this study is innovative on the consequences of exposure to BPS.

The toxicokinetics of BPS are also different between mice and human. Little data is available on this point. A physiologically based pharmacokinetic modeling highlighted that BPS is the BPA substitute which has the most important enterohepatic recirculation leading to poorer urinary excretion and it's also the BPA analog leading to the highest internal concentrations of unconjugated bisphenols (Karrer et al., 2018). It is also important to note that the metabolism of BPS differs between human and mice. In female mice, 50% of the administered BPS is excreted in the urine in 6 hours in metabolized form (detoxification mainly in glucuro-conjugated form) (Song et al., 2017). In humans after oral absorption, Khmiri et al. (2020) reported limited amounts of BPS and its glucuronide conjugate were recovered in the urine and maximum excretion rates were reached between 5 and 11 h after administration with a poor recovery rate in the urine for BPS and its conjugate - approximately 0.004 ± 0.003 and $0.09 \pm 0.07\%$, respectively -. The authors shown higher oral

bioavailability and subsequent higher systemic levels of active BPS than BPA (Khmiri et al., 2020). Taken together, these data suggest that BPS toxicological impacts might be more critical in Humans than in mice.

At molecular level, *in vitro* were performed. It has been shown that low concentrations of BPS (at fM to μ M levels) can induce lipid accumulation in human HepG2 cell line (Helies-Toussaint et al., 2014) as in murine 3T3L1 preadipocyte cell line (Ahmed and Atlas, 2016; Ramskov Tetzlaff et al., 2020). *Ex vivo*, a recent study reported that BPS at low doses (0.1 and 25 μ M) promotes adipogenesis in visceral and subcutaneous human preadipocytes (Peshdary et al., 2020).

In conclusion, our study shows that BPS is of concern for its ability to induce obesogenic effects in the offspring (F2 or even F3) of mice exposed perinatally at low doses. These effects were also sex-dependent. These phenotypes were observed in relatively young adult mice (23 weeks of age) and in perspective, it would be interesting to see the impact of aging on the phenotypes described as obesity contributes to the aging process and *vice versa*. It would be necessary to investigate the disorders associated with obesity such as the inflammatory state, the gut microbiota and lipid homeostasis (lipid metabolism and associated protein activities), notably in adipose tissue or the liver. Likewise, the processes related to the heredity of the observed effects should be clarified by studying epigenetic modifications such as DNA methylation or histone acetylation.

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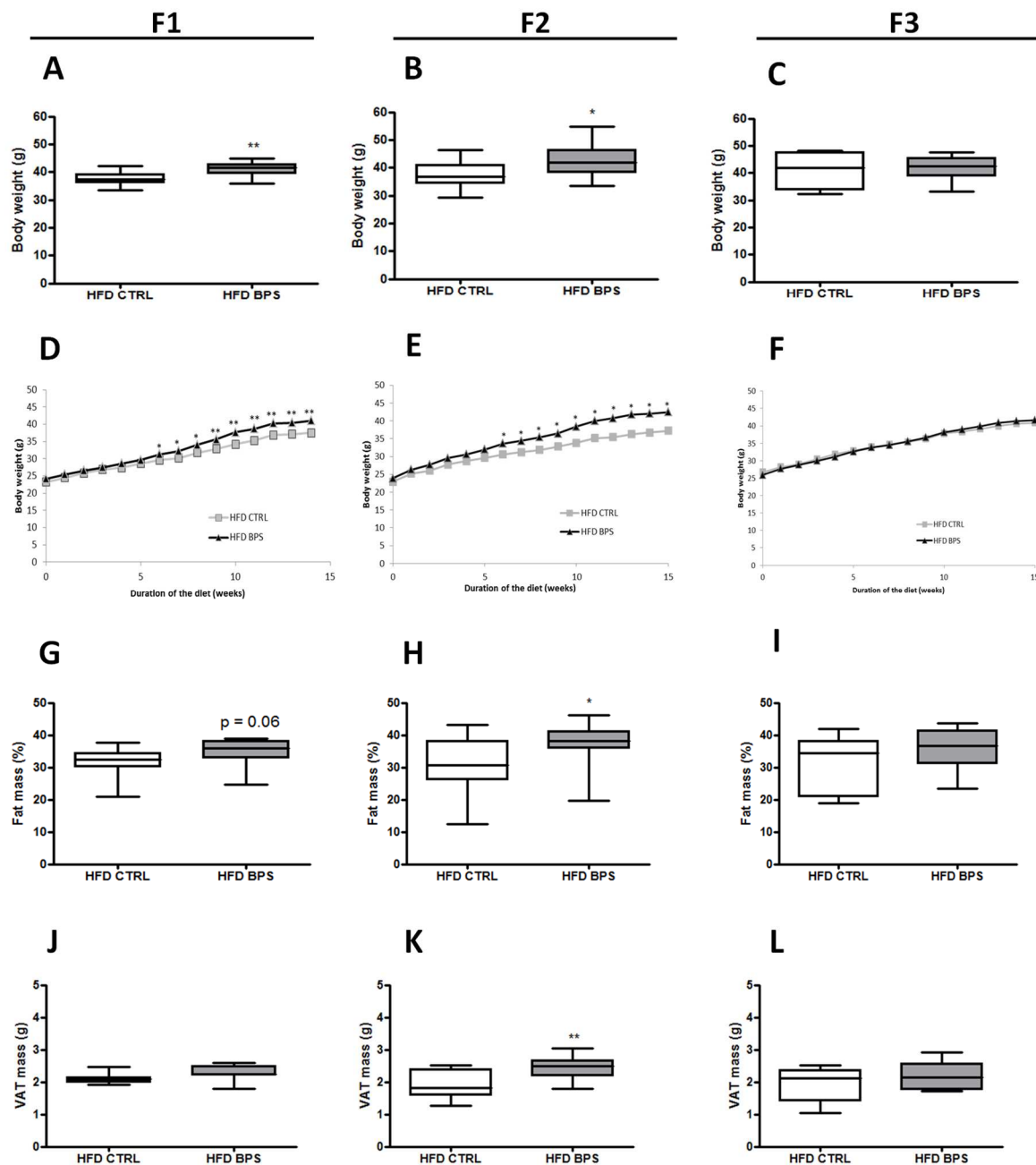
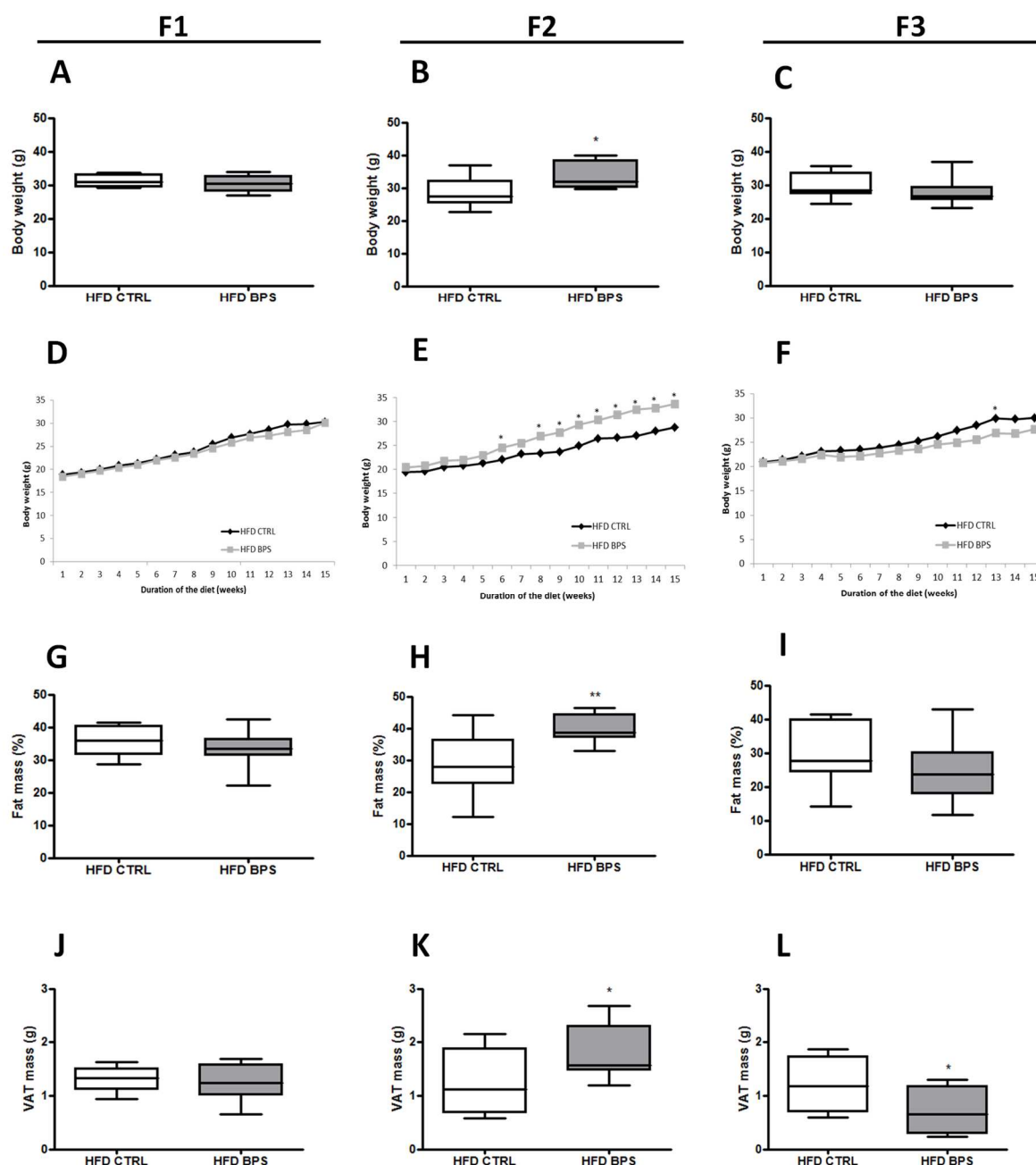


Figure 1: Transgenerational study of the BPS effects on body weight and adiposity in C57Bl/6J male mice: box plot displaying the 1st (lower line of the box), 2nd (horizontal line inside the box), 3rd quartiles (upper line of the box) and the interquartile (used as dispersion indicator) for long term follow-up of body weights (A - C), kinetics of weight gains (D – F), adiposity (G – I) and visceral adipose tissue (VAT) mass (J – L) in C57Bl/6J male mice after 15 weeks of high-fat diet and which are derived from F0 females exposed or not to BPS at 1.5 µg/kg body weight/day. Measurements were

776 performed at 23 week-old. Values represent means \pm SEM. Unpaired *t*-test were performed; * $p \leq$
777 0.05, ** $p \leq 0.01$. n = 12/group



778 Figure 2: Transgenerational study of the BPS effects on body weight and adiposity in C57Bl/6J female
779 mice: box plot displaying the 1st (lower line of the box), 2nd (horizontal line inside the box), 3rd
780 quartiles (upper line of the box) and the interquartile (used as dispersion indicator) for long term
781 follow-up of body weights (A - C), kinetics of weight gains (D – F), adiposity (G – I) and visceral
782 adipose tissue (VAT) mass (J – L) in C57Bl/6J female mice after 15 weeks of high-fat diet and which
783 are derived from F0 females exposed or not to BPS at 1.5 μ g/kg body weight/day. Measurements were

performed at 23 week-old. Values represent means \pm SEM. Unpaired *t*-test were performed; * $p \leq 0.05$, ** $p \leq 0.01$. n = 12/group

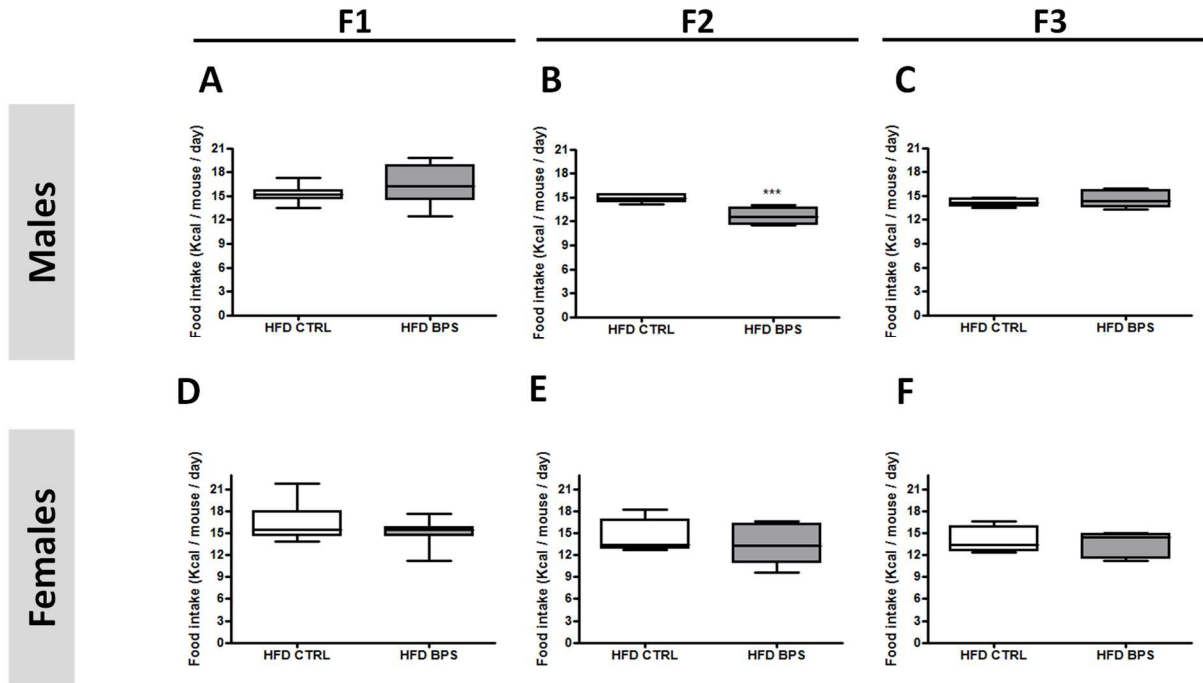


Figure 3: Transgenerational study of the BPS effects on food intake in C57Bl/6J female and male mice: box plot displaying the 1st (lower line of the box), 2nd (horizontal line inside the box), 3rd quartiles (upper line of the box) and the interquartile (used as dispersion indicator) in C57Bl/6J male (A – C) or female (D – F) mice after 8 weeks of high-fat diet and which are derived from F0 females exposed or not to BPS at 1.5 μ g/kg body weight/day. Measurements were performed at 14 week-old. Values represent means \pm SEM. Unpaired *t*-test were performed; *** $p \leq 0.001$. n = 12/group

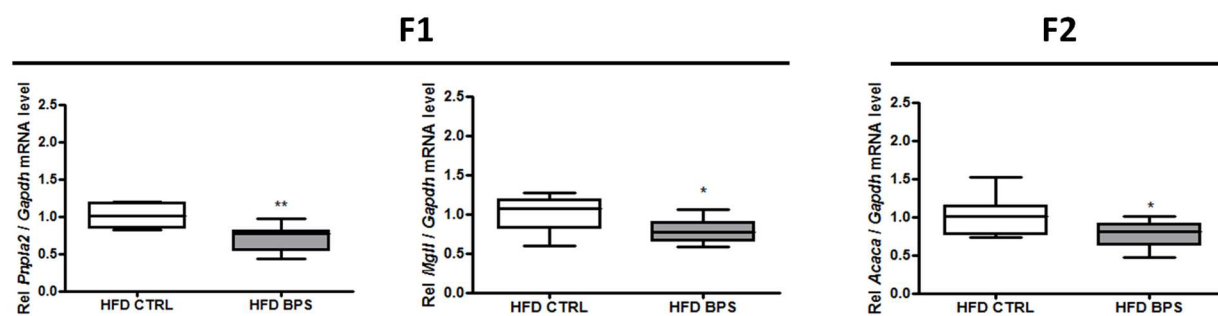
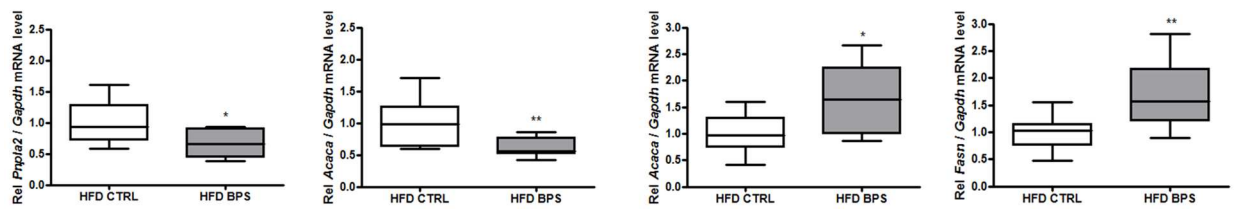


Figure 4: Transgenerational study of the BPS effects on adipose tissue lipid metabolism marker genes expression in C57Bl/6J male mice: box plot displaying the 1st (lower line of the box), 2nd (horizontal line inside the box), 3rd quartiles (upper line of the box) and the interquartile (used as dispersion indicator) in C57Bl/6J male mice after 15 weeks of high-fat diet and which are derived from F0 females exposed or not to BPS at 1.5 µg/kg body weight/day. Measurements were performed at 23 week-old. Values represent means ± SEM. Unpaired *t*-test were performed; * $p \leq 0.05$, ** $p \leq 0.01$. $n = 12/\text{group}$.

F2

F3



809 Table 1: Transgenerational study (F1; F2; F3) of BPS effects on plasma parameters related to
810 blood glucose and lipid metabolism in male mice. NEFA: No Esterified Fatty Acids.
811 Measurements were performed at 23 week-old. Values represent means \pm SEM. Paired
812 student *t* test was performed; ***p* < 0.01; ****p* < 0.001. n = 12/group

	F1		F2		F3	
Plasma level at euthanasia	HFD CTRL	HFD BPS	HFD CTRL	HFD BPS	HFD CTRL	HFD BPS
Blood glucose (mg/dl)	181.92 \pm 11.44	185.58 \pm 8.66	159.5 \pm 6.32	183 \pm 5.72**	175.9 \pm 9.34	176.25 \pm 8.33
Insulin (μ g/l)	2.23 \pm 0.50	3.03 \pm 0.43	1.39 \pm 0.28	1.39 \pm 0.30	1.69 \pm 0.46	1.15 \pm 0.23
HOMA-IR	2.36 \pm 0.73	3.69 \pm 0.53	0.91 \pm 0.2	1.05 \pm 0.2	2.77 \pm 0.89	2.15 \pm 0.55
NEFA (mmol/l)	0.35 \pm 0.20	0.29 \pm 0.07	0.75 \pm 0.18	0.99 \pm 0.23	0.49 \pm 0.03	0.51 \pm 0.10
Cholesterol (g/l)	1.31 \pm 0.05	1.05 \pm 0.07**	1.07 \pm 0.04	1.2 \pm 0.04**	1.25 \pm 0.07	1.35 \pm 0.07
Triglycerides (g/l)	0.7 \pm 0.06	0.64 \pm 0.04	0.5 \pm 0.04	0.75 \pm 0.06***	0.57 \pm 0.06	0.7 \pm 0.05

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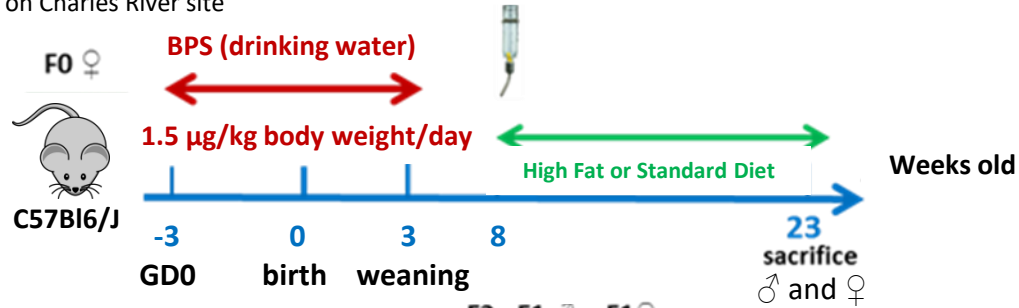
814 Table 2: Transgenerational study (F1; F2; F3) of BPS effects on plasma parameters related to
815 blood glucose and lipid metabolism in female mice. NEFA: No Esterified Fatty Acids.
816 Measurements were performed at 23 week-old. Values represent means \pm SEM. Paired
817 student *t* test was performed; **p* < 0.05; ****p* < 0.001. n = 12/group

	F1		F2		F3	
Plasma level at euthanasia	HFD CTRL	HFD BPS	HFD CTRL	HFD BPS	HFD CTRL	HFD BPS
Blood glucose (mg/dl)	159.36 \pm 6.12	156.5 \pm 7.34	156 \pm 6.65	158.11 \pm 6.16	146.92 \pm 6.13	131.5 \pm 3.38*
Insulin (μ g/l)	0.94 \pm 0.18	0.88 \pm 0.15	1.58 \pm 0.29	1.42 \pm 0.26	0.83 \pm 0.23	0.78 \pm 0.21
HOMA-IR	1.29 \pm 0.22	1.27 \pm 0.21	1.49 \pm 0.34	1.48 \pm 0.27	1.43 \pm 0.75	1.47 \pm 0.39
NEFA (mmol/l)	0.47 \pm 0.12	0.47 \pm 0.18	0.44 \pm 0.13	0.95 \pm 0.12*	0.63 \pm 0.11	0.67 \pm 0.22
Cholesterol (g/l)	0.62 \pm 0.04	0.86 \pm 0.03***	0.75 \pm 0.06	0.9 \pm 0.05	0.92 \pm 0.03	0.92 \pm 0.04
Triglycerides (g/l)	0.34 \pm 0.04	0.49 \pm 0.05*	0.4 \pm 0.03	0.45 \pm 0.02	0.5 \pm 0.03	0.46 \pm 0.04

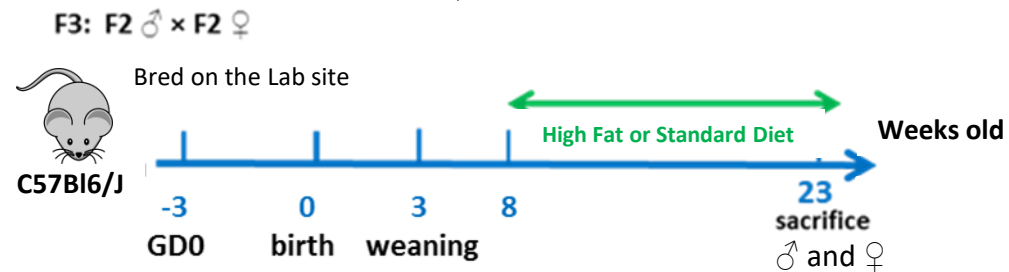
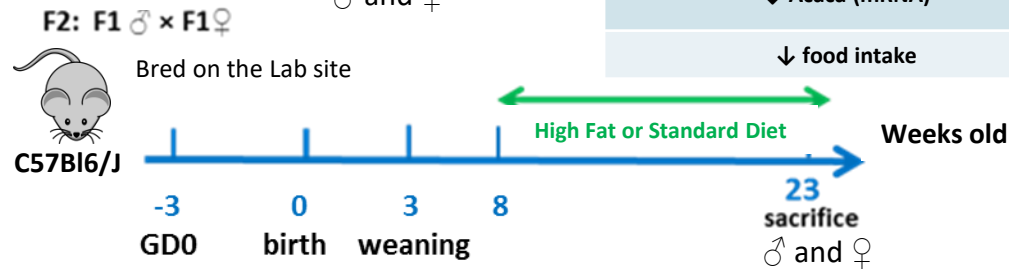
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F1 Males	F1 Females
↑ bw	↑ cholesterol / TG (plasma)
↓ cholesterol (plasma)	↓ estradiol (plasma)
↓ Pnpla2 and MglI (mRNA)	

Bred on Charles River site



F2 Males	F2 Females
↑ bw, fat mass, VAT mass	↑ bw, fat mass, VAT mass
↑ blood glucose, TG, cholesterol (plasma)	↑ NEFA, estradiol (plasma)
↓ Acaca (mRNA)	↓ Pnpla2 and Acaca (mRNA)
↓ food intake	



F3 Females
↓ VAT mass
↑ Acaca and Fasn (mRNA)

bw : body weight, VAT : Visceral Adipose Tissue, NEFA : No Esterified Fatty Acid, TG : Triglyceride, Pnpla2 : Patatin Like Phospholipase Domain Containing 2, MglI : Monoglyceride Lipase, Acaca : Acetyl-CoA Carboxylase Alpha, Fasn : Fatty Acid Synthase, GD0 : Gestational Day 0