

# Obesogen effects after perinatal exposure of 4,4'-sulfonyldiphenol (Bisphenol S) in C57BL/6 mice

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# OBESOGEN EFFECTS AFTER PERINATAL EXPOSURE OF 4,4'-SULFONYLDIPHENOL (BISPHENOL S) IN C57BL/6 MICE

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**Abstract**

Bisphenol A were removed from consumer products and replaced by chemical substitutes such as Bisphenol S (BPS). Based on their structural similarity, BPS may be obesogen like Bisphenol A in mice. Our objective was to determine the impact of BPS on lipid homeostasis in C57Bl/6 mice after perinatal and chronic exposure. Pregnant mice were exposed to BPS via the drinking water (0.2; 1.5; 50 µg/kg bw/d). Treatment began at gestational day 0 and continued in offspring up to 23-weeks old. Then, offspring mice were fed with a standard or high fat diet. The body weight, food consumption, fat mass and energy expenditure were measured. A lipid load test was performed to check the postprandial triglyceridemia. Plasma parameters and mRNA gene expression in adipose tissues were also analysed. BPS induced overweight in male mice offspring fed with a HFD at the two highest doses. There was no change in food intake and energy expenditure. The overweight was correlated to the fat mass, hyperinsulinemia and hyperleptinemia. The plasma triglyceride clearance was significantly increased with BPS and tyloxapol® (triglyceride clearance inhibitor) reversed this phenomenon. BPS induced alteration in mRNA expression of marker genes involved in adipose tissue homeostasis: hormone sensitive lipase, PPARγ, insulin receptor, SOCS3 and adiponectin. This is the first time that BPS is described as obesogenic at low doses and after perinatal and chronic exposure in male mice. BPS potentiated the obesity induced by a HFD by inducing the lipid storage linked to faster lipid plasma clearance.

Keywords: Bisphenol S; food contaminant; perinatal exposure; low dose; obesogen.

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## 1. Introduction

Regulatory agencies including Health Canada (2009), the US Food and Drug Administration (2010) and the European Union (EC regulation 321/2011) have decided to ban the use of Bisphenol A (BPA) for the manufacture of polycarbonate infant feeding bottles. As a direct consequence, plastic substitutes have appeared on the European Union's market. Bisphenol S (4,4′-sulfonyldiphenol ; BPS), a structural analogue of BPA is often used in epoxy glues, baby bottles, canned foodstuffs, paper currencies, food paperboard and as additives in thermal paper for receipts (Gallart-Ayala et al., 2011, Liao and Kannan, 2014, Liao et al., 2012) . Currently, BPS is authorized for food contact materials with some restrictions (EU 10/2011) such as the specific migration limit (SML) of 50 µg/kg. In Europe, BPS is gradually replacing BPA with increasing production between 1,000 and 10,000 tons per year according to European Chemicals Agency (2014). BPS has also been detected in urine from the citizens of 8 countries and within the same concentration ranges as BPA (Liao and Kannan, 2012, Liao et al., 2012) . BPS has been detected in surface water, sediment and sewage effluents in the same order of magnitude as BPA (Song et al., 2014, Yang et al., 2014) . Furthermore, BPS appears to be more resistant to environmental degradation than BPA and with a better level of dermal penetration (Zalko et al., 2011) . These facts taken together may lead to BPS having a longer or higher body burden or bioavailability than BPA (Danzl et al., 2009) . Many studies describe a range of adverse effects that BPA can have on animals including fertility reproduction, breast and prostate cancer, metabolic syndrome and obesity (Le Corre et al., 2015, Rezg et al., 2014, Srivastava et al., 2015) . In contrast, there is more limited information available on the toxicological effects of BPS. However, like BPA, BPS is reported to act as an estrogenic and anti-androgenic compound (Grignard et al., 2012, Kitamura et al., 2005, Kuruto-Niwa et al., 2005, Rosenmai et al., 2014) . Consequently, it is likely that BPS affects the endocrine system in organisms leading to adverse outcomes. Many studies in rodents



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report that exposures during critical periods of differentiation and at low environmentally-relevant doses of endocrine disruptor compounds (EDC) such as xenoestrogens (BPA, Diethylstilbestrol), metals (lead, cadmium) alter developmental programming resulting in obesity (Baillie-Hamilton, 2002, Faulk et al., 2014, Newbold et al., 2007) . In human, few epidemiological studies were published but recent article reported an increased body weight in humans after perinatal exposure to endocrine disrupting mixtures (Agay-Shay et al., 2015) . More generally, according to the "environmental obesogen" hypothesis, some environmental pollutants could initiate or exacerbate obesity through alteration of critical pathways involved in adipogenesis, lipid metabolism or energy balance (Grun and Blumberg, 2009) . To our knowledge, only one *in vitro* study has investigated the potential effect of BPS in lipid homeostasis and found that BPS at low concentrations induced an increase in the lipid content in the 3T3-L1 mouse pre-adipocyte and in HepG2 human hepatoma cell lines with a decrease in lipolysis (Helies-Toussaint et al., 2014) . Then, there are few toxicological data on BPS from *in vivo* experiments on physiological functions like energetic metabolism. Therefore, in this study based on the similarity of the BPS structure to that of BPA and on its endocrine disruption activities, we investigated the impact of BPS during perinatal exposure on energy metabolism in offspring from BPS-treated dams with the aim of obtaining original data. To study the obesogenic property of BPS, we chose a high fat diet with saturated lipids as the most efficient high fat diet (HFD) to stimulate mouse obesity development (de Wit et al., 2008) and corresponding to the phenomenon of obesity observed in western countries. The small intestine, the first interface between the body and diet, is also essential for the bioavailability of dietary lipids because it secretes triglyceride-rich lipoproteins and can thus contribute to the development of metabolic diseases especially in diet induced disorders (de Wit et al., 2008) . We therefore explored the impact of BPS on body weight and fat mass increase with the potential contribution of the small intestine by evaluating its capacity to



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secrete TG-rich lipoproteins in the blood after oil gavage. In total, 131 males and 117 females divided into 8 groups were analysed. Gene expression changes of apoCII and apoCIII in the jejunum (main site of lipid absorption) were analyzed and subsequently the ratio apoCII/apoCIII was studied as an indicator of the efficiency of blood TRL hydrolysis by LPL (Jong and Havekes, 2000, Shachter, 2001) . The mRNA expression of adipose tissue homeostasis marker genes were also studied in mice exposed to BPS.

## 2. Materials and methods

### *2.1. Animals and materials*

Pregnant C57Bl/6J mice were purchased from Charles Rivers (L'Arbresle, France). BPS and Tyloxapol® were provided by Sigma-Aldrich (Saint Quentin Fallavier, France). The 4RF21 standard diet was purchased from Mucedola (Milano, Italia). The high fat diet (60% kcal from lipids) was based on the 4RF25 reproduction diet (Mucedola, Milano, Italia) with the addition of 30% palm oil (La Vie Saine, Dijon, France) which is rich in saturated fatty acid. Isio4™ oil (Lesieur) was purchased from Carrefour (Dijon, France) while the triglycerides PAP 150™ and Glucose RTU™ kits were purchased from Biomérieux (Marcy l'Etoile, France). The plasma insulin level was measured by Ultrasensitive™ Mouse Insulin ELISA kits from Mercodia France SAS (Paris, France), the plasma leptin by an Elisa kit from Phoenix France SAS (Strasbourg, France) and finally plasma total cholesterol was measured with a Cholesterol FS™ kit DiaSys (Condom, France).

### *2.2. Experimental Strategy*

Pregnant C57Bl/6J mice were exposed to BPS from the first day of gestation. The treatment was continued during lactation and in pups after weaning until they were 22 weeks old. Cages



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and bottles were made of polypropylene (BPS free). Forty-five pregnant mice were individually housed in standard polypropylene mouse cages and allowed free access to food and water. They were divided into four treatment groups and exposed to BPS in their drinking water at concentration of either 0; 0.85; 6.38; 212.50 ng/ml beginning on gestation day 0. BPS concentration was based on mean daily unadjusted water intake in C57Bl/6J (about 7ml/30g mice) (Bachmanov et al., 2002) and in order to obtained expected BPS exposure of 0; 0.2; 1.5; 50 µg/kg bw/day. BPS was dissolved in absolute ethanol and the final concentration of ethanol was 0.1%. Control group drinking water contained only 0.1% ethanol. 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, and the levels of BPS consumed weekly were estimated. The offspring were then divided into two groups for each gender (male and female) at the weaning and then into two groups for each diet (standard and high fat diet) four weeks after weaning and until sacrifice (table 1). A maximum of five mice were housed in each cage. The litters were mixed randomly after weaning in order to minimize a possible litter effect. Then, in a cage, each mouse came from a different dam. In males, the average BPS intake was 0.18 (± 0.018); 1.13 (± 0.084); 42.79 (± 4.956) µg/kg bw/d in the standard diet and 0.14 (±0.016); 0.99 (±0.057); 40.30 (±3.519) µg/kg bw/d in the high fat diet for respective expected doses of 0.2; 1.5; 50 µg/kg bw/d of BPS. Likewise in females, the BPS intake was 0.19 (±0.076); 1.27 (±0.156); 41.55 (±5.867) µg/kg bw/d in the standard diet and 0.2 (±0.015); 1.33 (±0.015); 43.26 (±2.119) µg/kg bw/d in the high fat diet. The high fat diet induced a decrease in water consumption (about 50%). Consequently, the BPS concentration in the water was two times more concentrated than in standard diet condition. Furthermore, no significant difference in water uptake were observed in BPS-treated mice (whatever the dose) in comparison with control (data not shown). Mice were fasted from food four hours before sacrifice. Animals were treated in the respect of ethic and deontology and

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the experimental protocol was approved by the ministry and the University of Burgundy's ethics committee.

*2.3. Body weight and corporal composition measures*

At the end of protocol, bodyweight, fat and lean masses of 22 week old mice were measured (number of each group is reported in table 1). The fat and lean masses of each mice were determined individually using a quantitative NMR EchoMRI 500T™ (EchoMRI, Houston, USA). Before each measurement, calibration was performed in compliance with the manufacturer's guidelines.

*2.4. Food intake measure*

The experiment was conducted in all mice (20-week old). The number of each group is reported in table 1. The mice were acclimated to individual cages for 48h and daily food intake was determined by measuring the difference in the amount of food placed and the amount remaining the following 24h.

*2.5. Indirect calorimetry*

Indirect calorimetry was performed in six mice of each group using a computer-controlled, open-circuit system (Oxymax Comprehensive Lab Animal Monitoring System; Columbus Instruments). For each group, mice were selected as a representative sample of all the mice regards to the body weight mean. Likewise, analysed mice are from different cage. Sixteen-week old mice were individually tested during 24 hours in clear chambers (20 × 10 × 12.5 cm) with a plastic elevated wire floor after being acclimated over 2 days. Room air was passed through the chamber at 0.6 L/min. The chamber exhaust was sampled for 45 sec after an idle time of 90 sec at 20-min intervals and was passed through $O_2$ and $CO_2$ sensors for estimation of oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$). Outdoor air reference values were sampled every 8 measurements. Gas sensors were calibrated before



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experiments with gas standards containing known concentrations of $O_2$, $CO_2$, and $N_2$ (Air Liquide – Certified accuracy ±1%). Respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide production ($VCO_2$) to oxygen consumption ($VO_2$). At the end of the calorimetric measurements, lean body mass (LBM) and fat mass (FM) were measured by quantitative NMR (EchoMRI 500T) to adjust energy expenditure (calculated as heat production = (3.815 + 1.232×RER) x$VO_2$ )) to metabolic body size (LBM + 0.2xFM) according to the procedure reported by Even and Nadkarni (Even and Nadkarni, 2012) .

*2.6. Lipid load test*

The protocol has previously been described by Petit et al. (2007). After a 16 hour fast, 15 week old mice were weighed and received an intragastric bolus of lipids (0.5 ml of isio 4™ oil). Blood samples were collected from the tail vein before gavage and at 1, 2, 3 and 4 hours after gavage. The plasma triglyceride (TG) concentration was assayed using a commercial kit (Biomerieux, Marcy l'Etoile, France). The same experiment was performed in 18 week old mice previously subjected to a retro-orbital plexus injection of tyloxapol™ (Sigma) in saline buffer (500 mg/kg body weight) to block plasma TG clearance. Results were expressed as an area under curve (AUC). Lipid load test were performed in all mice (number of each group is reported in table 1).

*2.7. Biochemical analysis*

Blood samples of each mice (number of each group is reported in table 1) were collected from the retro-orbital plexus of each mice on heparinized propylene tubes just before sacrifice (i.e. fasted 4 hours before). After centrifugation of 10 minutes at 2,000g and 4°C in order to obtain plasma, the levels of total plasma cholesterol (TC), triacylglycerol (TG) and glucose were measured using the respective kits. Plasma leptin and insulin levels were measured using ELISA kits. Assays were performed according to manufacturers' instructions.



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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 ([glucose (mmol/L)] × [insulin (mUI/l)])/108.6 and used as a surrogate measure of whole-body insulin sensitivity.

*2.8. Real-time PCR analysis*

Lysis of jejuna or abdominal adipose tissues of each mice (number of each group is reported in table 1) were performed using Lysing Matrix D™ tubes (MP Biomedical) and total RNA were extracted using Tri-reagent™ (Sigma-Aldrich). cDNA was reverse-transcripted from 3 µg of total RNA using a High Capacity cDNA reverse transcription kit™ (Life Technologies). Real time PCR was done in duplicate with 30ng 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 reaction volume of 10 µl (qsp $H_2O$). Taqman™ gene expression assay references were Mm00495359_m1 (HSL), Mm00503040_m1 (ATGL), Mm00434764_m1 (LPL), Mm00440940_m1 (PPARγ), Mm00456425_m1 (Adiponectin), Mm00545913_s1 (SOCS3), Mm01211875_m1 (INSR), MmMm00437571-m1 (apoC-II), Mm00445670_m1 (apoC-III) and Mm99999915-g1 (GAPDH, reference gene). PCR was run on the Step-One plus system (Applied Biosystems) using the following conditions - 50°C for 2min, 95°C for 10 min and 40 cycles of 95°C for 15s and 60°C for 30s. Data analysis was performed using the comparative ΔCt method (15).

*2.9. Statistical Analysis*



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All data were expressed as mean ± standard error of mean (SEM). Food intake, body weight and triglyceride, glucose, insulin, leptin and cholesterol plasma levels were compared using one-way ANOVA and Tukey post-test. Pearson r was used to calculate correlations between fat mass versus body weight. TG clearance and intestinal secretion were compared using the Kruskal-Wallis non-parametric test and Dunn's post-test. Results were considered statistically significant at $p<0.05$. All these statistical analyses were performed with Graph Pad Prism®. Data from indirect calorimetry assays were analyzed with the non-parametric Wilcoxon test using R software.

## 3. Results

### *3.1 BPS effects on body weight of mice*

As expected, the high fat diet (60% kcal from lipids) enriched with saturated lipids induced an increase in the body weight in male and female mice. At the end of the protocol, the male and female mice fed with the high fat diet were found to exhibit a significant fold overweight of 1.39± 0.06 and 1.59± 0.05 respectively in comparison with standard diet (Figure 1). In mice fed to high fat diet, the chronic exposure to BPS of the male mice induced a significant increase in body weight compared to the control group (fold increase of 1.15±0.28 and 1.19±0.31 for 1.5 and 50 µg/kg bw/d respectively; $p<0.001$) at the end of diet treatment protocol (figure 1). There was no significant difference between these both doses. BPS exposure was found to have no effect on male mice fed with a standard diet (figure 1). For the lowest dose (0.2 µg/kg bw/d), a body weight increase of 1.10±0.29 was observed which was not statistically significant with the one-way ANOVA and Tukey post-test. Interestingly, females were not affected by BPS whatever the diet (figure 1).

### *3.2. BPS effect on fat mass weight*



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Male mice fed with a HFD and exposed to BPS 1.5 and 50 µg/kg bw/d exhibited a significant increase in fat mass ($p<0.05$) of 1.26±0.54 and 1.33±0.67, respectively (figure 2, left) but no effect was observed on lean mass (data not shown). In contrast, when female mice fed with HFD were exposed to BPS, no effect was observed on the fat mass (figure 2, right). Likewise, with standard diet, no changes were obtained whatever the gender (supplementary figure S1).

*3.3. BPS effect on the food intake and energy expenditure*

To understand the origins of the BPS effect on fat mass, the food intake was analysed. The food intake of exposed mice was not found to be statistically different whatever the sex and the exposure dose of BPS (table 2). Likewise, there is no change in these parameters between BPS-exposed mice and control mice (figure 3).

*3.4. BPS effect on plasma parameters*

In 4 hours fasted mice, we measured insulin, leptin, glucose, triglyceride and total cholesterol plasma levels. In comparison with male mice fed with standard diet (figure S2), the HFD diet induced hyperinsulinemia, hyperleptinemia, hypercholesterolemia in male mice while the TG or glucose levels remained unchanged (data not shown).

The male mice fed with the HFD and exposed to BPS (1.5 and 50 µg/kg bw/d) exhibited a weak but significant hypercholesterolemia with a fold increase of 1.21± 0.08 and 1.29 ± 0.06, respectively (figure 4E). Insulin and leptin plasma levels were significantly increased ($p<0.05$) after BPS exposure at 1.5 and 50µg/kg bw/d in male mice fed with a HFD at the end of the protocol (22-week old mice). The insulin plasma level exhibited a fold increase of 4.98 ± 1.13 and 4.86 ±1.15 for each dose respectively (figure 4A). However, the glucose and TG plasma levels were not modified (figure 4B and D). Leptin plasma levels increased by 2.34±0.13 and 2.41±0.13, at 1.5 and 50µg/kg bw/d respectively (Figure 4C). In



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male mice fed with standard diet, no effect was observed in these plasma parameters (supplementary figure S2).

*3.5. BPS effect on the postprandial blood TG clearance*

To examine whether BPS affects dietary fat handling by the intestine, mice were challenged with a lipid load. In male mice fed with HFD, the post-prandial triglyceridemia which results from both TG-rich lipoproteins secreted (mainly by the intestine) and their subsequent clearance in the blood was significantly modified ($p<0.05$) when mice were exposed to BPS at 0.2 and 1.5 µg/kg bw/d. Indeed, figure 5A shows a significant decrease of the AUC (increase of blood TG clearance) in treated mice of 1.46 ± 1.11; 1.48 ± 0.92 for 0.2 and 1.5 µg/kg bw/d doses, respectively. In contrast, the highest dose (50 µg/kg bw/d) of BPS failed to alter the post-prandial triglyceridemia. To determine whether these modifications were due to alterations in TG secretion in blood or their clearance by LPL, the experiment was carried out using an inhibitor of blood TG clearance (Tyloxapol™). As described in figure 5B, in male mice fed with the HFD, the intestine secretion was not changed by BPS exposure whatever the dose and in the presence of Tyloxapol™. In contrast, no effect was observed in male mice fed with standard diet (supplementary figure S3).

As the postprandial triglyceridemia could have been be affected by LPL activity, we checked the ratio of ApoCII/CIII mRNA in jejunum. Figure 6 shows that BPS at 0.2 and 1.5 µg/kg bw/d induces a significant decrease in ApoC-III mRNA expression (2.64±0.07 and 2.35±0.07 respectively) and consequently a significant increase in the ApoCII/CIII ratio in the HFD male mice jejunum.

*3.6. Homeostasis model assessment of insulin resistance*

The calculated HOMA-IR index adjusted for mice was significantly increased ($p>0.05$) in mice exposed to 1.5 and 50 µg/kg bw/d, suggesting systemic insulin resistance. The fold



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increases were respectively of 11.7 ± 2.8 and 14.3 ± 3.7 compared to control mice fed with standard diet normalized to 1±0.2 (Figur 4F).

*3.7. Insulin-sensitivity marker genes expression*

We assessed the mRNA expression of SOCS3, insulin receptor (INSR) and adiponectin in abdominal adipose tissue of male mice fed with a HFD. BPS (at the three doses) induced a significant overexpression of SOCS3 (1.93±0.16; 2.78±0.43; 2.19±0.54 for 0.2; 1.5; 50 µg/kg bw/d, respectively) mRNA and a significant mRNA down-regulation of INSR (1.66±0.11; 1.43±0.10; 1.58±0.10) and adiponectin (1.70±0.15; 1.65±0.15; 2.13±0.19) (figure 7).

*3.8. Adipose tissue lipid metabolism marker genes expression*

We analysed the mRNA expression of hormone-sensitive lipase (HSL), adipose trygliceride lipase (ATGL) and PPARgamma in abdominal adipose tissue of male mice fed with a HFD. BPS (at the three doses) induced a weak but significant decrease in mRNA expression of HSL (1.79±0.17; 1.51±0.14; 1.98±0.19 for 0.2; 1.5; 50 µg/kg bw/d, respectively) and PPARγ (1.46±0.12; 1.44±0.11 for 0.2 and 50 µg/kg bw/d, respectively) genes in adipose tissue of male mice fed with a HFD (figure 8). ATGL and LPL mRNA expressions were not modified.

## 4. Discussion

In response to the European Commission restriction on BPA, structural analogues such as BPS are used. To date, few studies have focused on the toxicological effects of BPS except its endocrine disruption activities. These studies were all based on the assumption of an activity relationship between BPA and BPS due to their structural analogy. Thus, the aim of this



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present study was to determine whether BPS could be involved in the development of obesity in rodents as described for BPA (Alonso-Magdalena et al., 2010, Garcia-Arevalo et al., 2014, Le Corre et al., 2015, Miyawaki et al., 2007, Somm et al., 2009) . Pre- and post-natal exposure was performed with chronic exposure 0; 0.2; 1.5; 50 µg/kg bw/d of BPS *via* drinking water and use of a high fat diet rich in saturated fatty acid (60% kcal from fat).

We first checked that the high fat diet induced alterations in physiological parameters related to obesity development for male and female mice offspring. These data are in accordance with study of Wit et al. (2008). Furthermore and for the first time, our results demonstrated that perinatal exposure to the two highest doses of BPS induced a significant overweight in male mice fed with a HFD only. We failed to observe any significant overweight in male mice exposed to (0.2µg/kg bw/d) of BPS. However, intriguingly these mice tended to gain weight at the end of the protocol suggesting that the lowest dose of BPS may induce overweight if mice were exposed for a longer period. All these data suggest that BPS can potentiate a diet-induced obesity which is in accordance with studies performed with endocrine disruptors (BPA, DES, genistein…) in rodents demonstrating gender- and dose-dependent effects (Miyawaki et al., 2007, Newbold et al., 2009, Strakovsky et al., 2015, Wei et al., 2011) .

The sex-dependent effect tends to suggest an alteration at the level of sex-hormone homeostasis. It is well established that sex-hormones regulate energy metabolism. Estrogen and androgen promote energy homeostasis, reduce fat body mass and ameliorate insulin resistance (*ie* enhance insulin sensitivity) (Mauvais-Jarvis et al., 2013, Navarro et al., 2015) . However, suboptimal or deficient levels of estrogens have been linked to the fat accumulation and obesity in female (Litwak et al., 2014) . Likewise, low testosterone levels are strongly correlated with obesity in men (Navarro et al., 2015) . Then, sex-hormones exhibit a dose-dependent effect in energy metabolism which could switch in one direction or the other according to the presence and activities of endocrine disruptors. Now, BPS is described as a



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weak estrogenic compound *in vitro* (Chen et al., 2002, Kitamura et al., 2005, Kuruto-Niwa et al., 2005, Molina-Molina et al., 2013) and it has also been reported to be an antagonist of androgen receptor and to alter steroidogenesis in murine MA-10 Leydig cell models (Kitamura et al., 2005, Roelofs et al., 2015) . BPS is also reported to decrease basal testosterone secretion by mouse and human fetal testes (Eladak et al., 2015) . Thus, a combination of the estrogenic and anti-androgenic properties of BPS could provoke a sex-hormone disequilibrium in males leading to dysregulation of lipid homeostasis. Conversely, in females, the potential BPS-induced disruption of estrogen homeostasis could be artefactual with regard to the activities of endogen estrogens.

The body weight increase is classically related to a corporal composition alteration (fat, lean and water masses) and we demonstrated that the observed overweight was only correlated to the fat mass. Lipid uptake by adipose tissues is notably dependent of the hydrolysis of triglycerides from circulating TG rich lipoprotein (chylomicrons, VLDL…) which is a rate-limiting step catalyzed by lipoprotein lipase (LPL) (Beigneux et al., 2007) . Results of the lipid load test show that the plasma TG clearance significantly increased with BPS could be related to a better LPL activity as suggested by the raised apoCII/apoCIII ratio, since these apolipoproteins are known to respectively activate and inhibit LPL activity (Jong and Havekes, 2000) . However, further studies are necessary to clearly demonstrate that BPS effects on TG clearance are LPL dependent, notably by investigating the activity of LPL, for example. Intriguingly, at the highest dose 50µg/kg bw/d, post-prandial triglyceridemia and apoCII/apoCIII ratio were not changed. Now, at T0 time (*ie* after 16h fast) the plasma TG level was slightly but significantly higher than the control and other BPS treatments (supplementary figure S4). In this fasted condition, the source of TG in plasma is essentially the liver thus suggesting that BPS at the highest dose could rather dysregulate the lipid liver metabolism than intestine. Further studies would be necessary to define the BPS effect



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exposure on the mice liver. These data suggest that the overweight induced by BPS could be due to a more efficient lipid storage but not to an energy balance disequilibrium.

Obesity is associated with adipose tissue dysfunction. During the postprandial phase, free fatty acids (FFAs) are taken up from the blood in adipose tissue after the hydrolysis of TG from triglyceride-rich lipoproteins by LPL. In adipose tissues, mobilization of TG reserve occurs by their hydrolysis by adipose triglycerides lipase (ATGL) and hormone sensitive lipase (HSL) (Hajer et al., 2008) . In our study, we observed no alteration of LPL mRNA expression in abdominal adipose tissue. Then, this result supports the hypothesis that improved LPL activity could be principally a consequence of a modification in chylomicron composition (Jong and Havekes, 2000) . HSL activities is regulated by several hormones such as catecholamine, natriuretic peptide and insulin. Low HSL expression is associated with obesity. In contrast, ATGL mediates the hydrolysis of triglycerides during basal lipolysis (Langin et al., 2005) . Of course, these results do not determine the protein expression or activity but they indicate a disruption of gene expression involved in adipocyte metabolism. One of the key transcription factors in the development and metabolism of adipocytes is the PPARγ expressed at high levels in adipose tissue (Janani and Ranjitha Kumari, 2015) and BPS exposure was found to induce a light but significant decrease of its mRNA expression. Regulation of PPARγ activity by obesogenic compounds was previously described as complex. It can act by activating PPARγ directly or indirectly, by increasing the levels of PPARγ protein, or by enhancing its recruitment to promoters of key genes involved in adipocyte metabolism (Janesick and Blumberg, 2011) . Furthermore, PPARγ expression and activity is inversely correlated with insulin resistance (Janani and Ranjitha Kumari, 2015) which suggests that in our study the mice may have exhibited a reduced insulin sensitivity. This hypothesis is supported by the BPS-induced hyperinsulinemia and BPS-induced expression alteration of marker genes SOCS3, INSR and adiponectin which correlates with an



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impairment of insulin sensitivities. In fact, in adipocytes, SOCS3 is a critical regulator of insulin action and its increased expression mediates insulin resistance in obesity (Shi et al., 2006) . Concerning insulin receptors, decreased expression has been observed in a variety of tissues in obese animals and humans and the causal relationship between decreased insulin receptors and insulin resistance is obvious (Kolterman et al., 1980) . Likewise, adiponectin plasma levels are low in obesity and it is well-known that adiponectin improves insulin sensitivity in animal models (Diez and Iglesias, 2003, Kubota et al., 2002) . All these data suggest that BPS aggravated the reduced insulin sensitivity triggered by HFD in male mice. BPS treatment induced an increase in insulin plasma level but without a change in glycaemia. The strong increase of HOMA-IR at the two highest doses suggest that these mice exhibited a systemic insulin resistance (van Dijk et al., 2013) . Although we failed to observe a change of glucose and TG level, BPS exposure (1.5 and 50 µg/kg bw/d) was found to weakly increase the total cholesterol in male mice fed with a HFD. These data are in correlation with a previous study showing a hypercholesterolemia in male mice fed with HFD and exposed to BPA (Miyawaki et al., 2007) .

Lipid accumulation in adipocytes is also regulated by the leptin produced by adipocytes and it plays a key role in regulating energy intake and energy expenditure including appetite and metabolism (Ceddia et al., 2002, Farooqi and O'Rahilly, 2009) . We demonstrated that BPS exposure increased leptin plasma level in male mice and positively related to the fat mass, which is in agreement with current knowledge since leptin is secreted in direct proportion to body adiposity (Woods and D'Alessio, 2008) . Hyperleptinemia is a characteristic manifestation of obesity in humans and rodents where a resistance to the action of leptin has been suggested. In this case, hyperleptinemia is commonly related to an increase of food intake. But in our study, we did not to observe this phenomenon. However, it was demonstrated that high circulating concentrations of leptin may be necessary to maintain the



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sensitivity to the hormone and the energy homeostasis (Sainz et al., 2015) . Our results suggest that BPS-exposed male mice exhibited a reduced leptin sensitivity.

In conclusion, we observed for the first time, that BPS could be an obesogen at low doses and after perinatal and chronic exposure for the male mice. BPS potentiated the impact of a high fat diet and induced some changes at different physiological levels such as the postprandial lipid metabolism, which favors the lipid storage in the adipose tissue.

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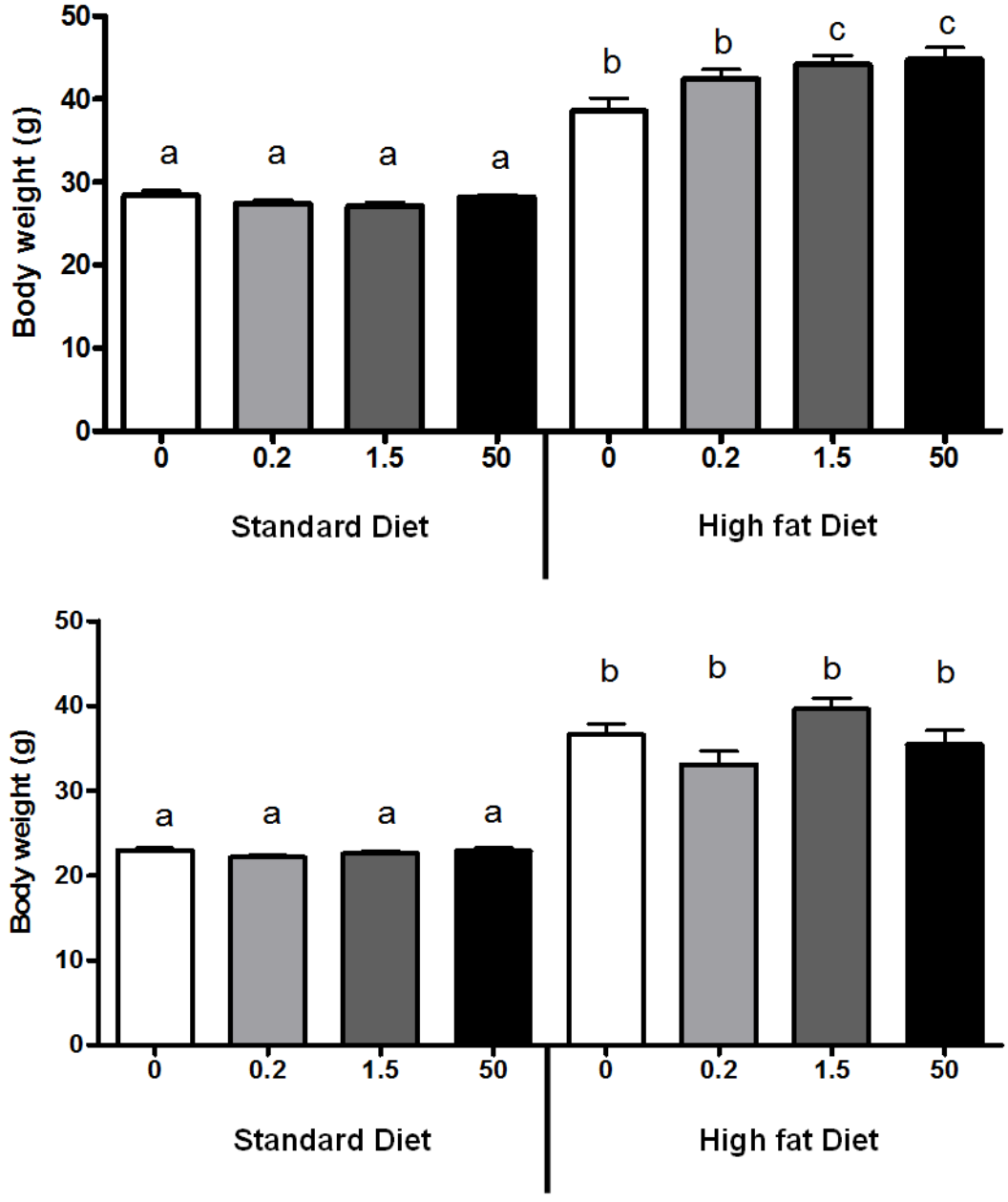
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Figure 1: Body weight of male (upper) and female (bottom) mice fed with standard or high fat diets (refer to table 1 for the number of mice by group). Values represent means ± SEM of each group. Statistical analysis was performed using one-way ANOVA with Tukey post-test (a different letter means that significant difference; p<0,001).



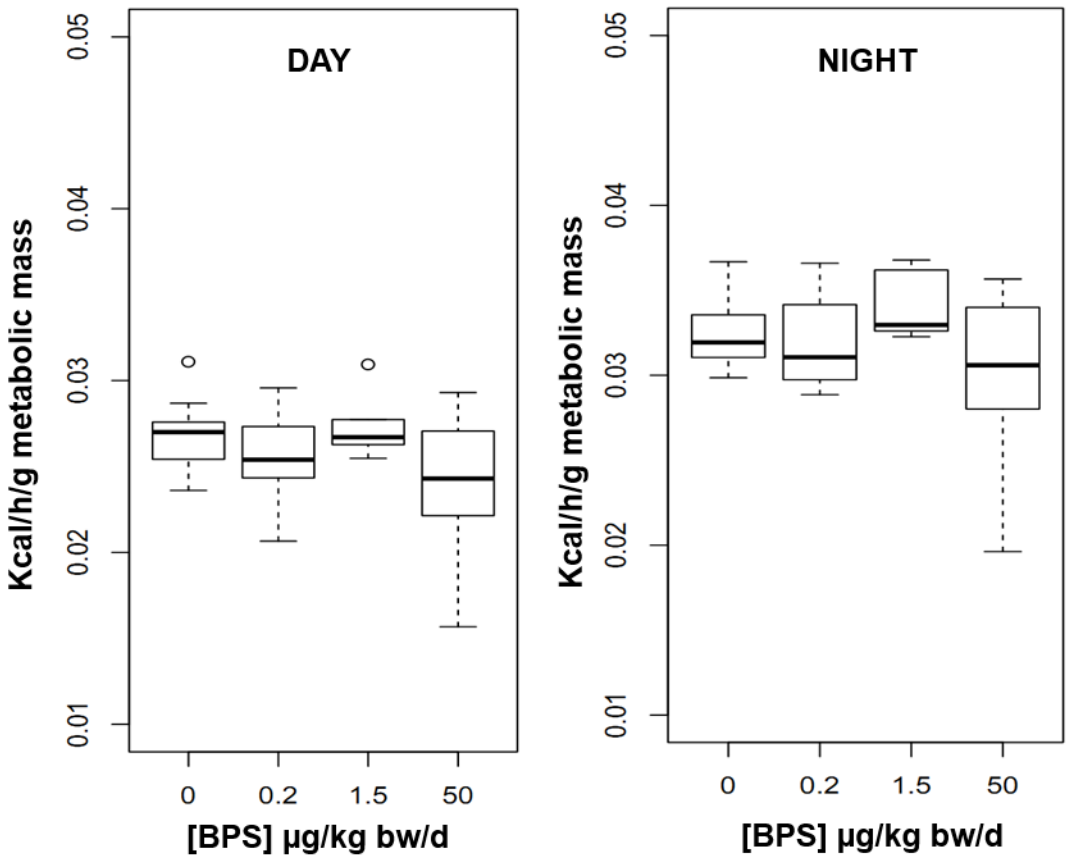



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Figure 2: Energy expenditure on male mice fed with high fat diet during the night (left) and during the day (right). Data represents energy expenditure normalized to the metabolic mass (lean mass+ 0.2 fat mass) for six mice by group (Even and Nadkarni, 2012) .



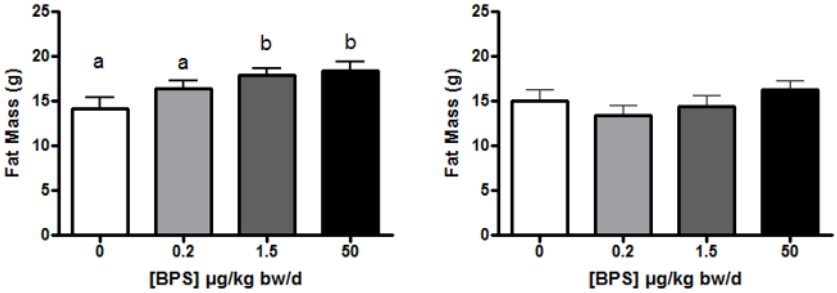



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Figure 3: Before sacrifice, all **male** (left) and **female** (right) offspring were weighed and their body fat mass measured by Echo-MRI®. All mice were fed with high fat diet (refer to table 1 for the number of mice by group). Values given are the mean ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey post-test (a different letter means that significant difference; p<0,05). Table represents value of Pearson correlation (Pearson r) between body weight and fat mass in control and BPS-treated male mice (*p<0.001).



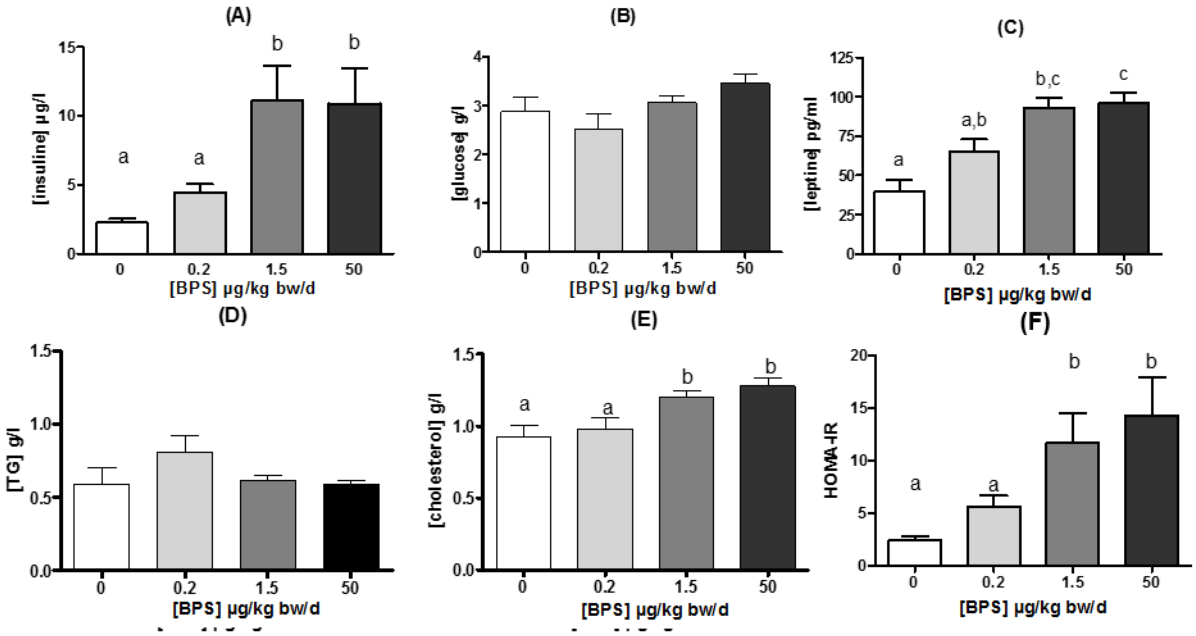

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Figure 4: Insulin (A), glucose (B), leptin (C), triglycerides (D) and total cholesterol (E) plasma levels in male mice fed with a high fat diet (refer to table 1 for the number of mice by group). HOMA-IR (F) was determined by the equation ([glucose (mmol/L)] × [insulin (mUI/l)])/108.6. Values represent means ± SEM. One-way ANOVA with Tukey post test was performed (a different letter means a significant difference $p<0.05$).



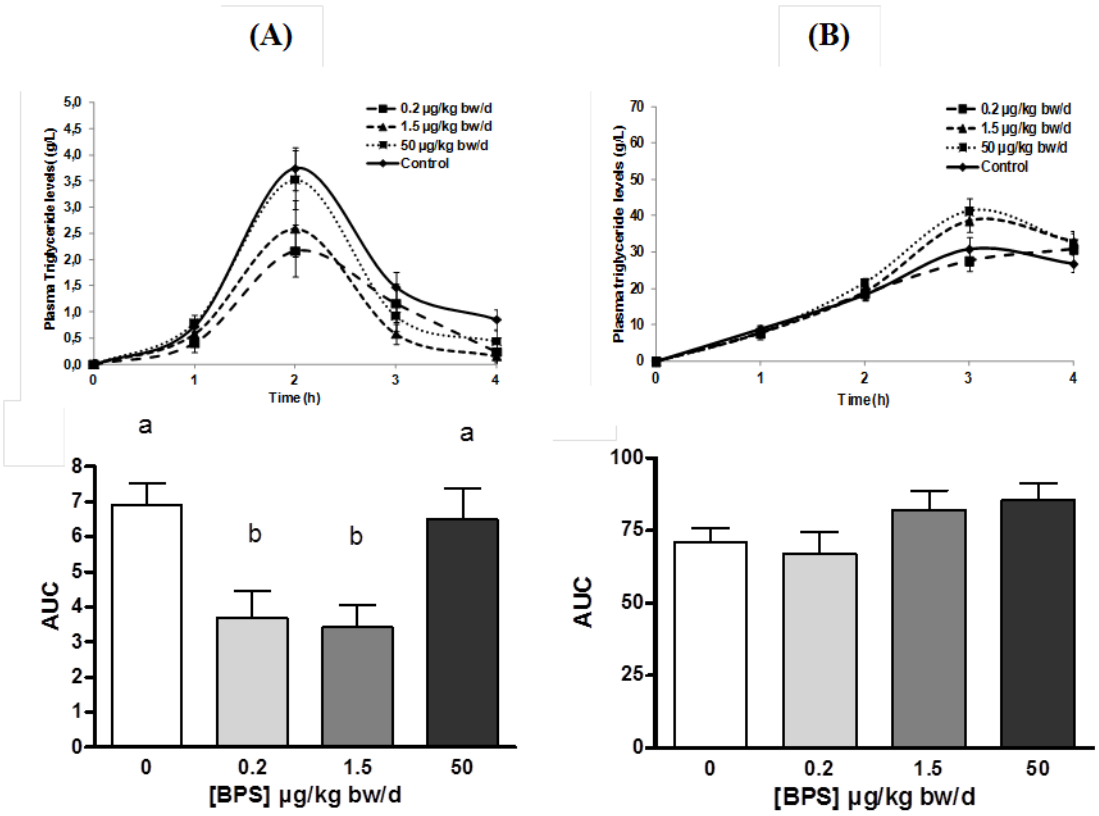



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Figure 5: Postprandial triglyceridemia (A) were determined after an oral lipid bolus (0.5 ml) in male mice fed a high fat diet and exposed to BPS (refer to table 1 for the number of mice by group). The same protocol was performed in mice previously subjected to an intraperitoneal injection of the tyloxapol® in order to check the intestine TG secretion (B). The data represent the kinetics of plasma triglyceride levels (upper) and the corresponding area under curve (bottom) between 0 and 4h after gavage (a different letter means a significant difference, p <0.05; Kruskal-Wallis with Dunns post test).



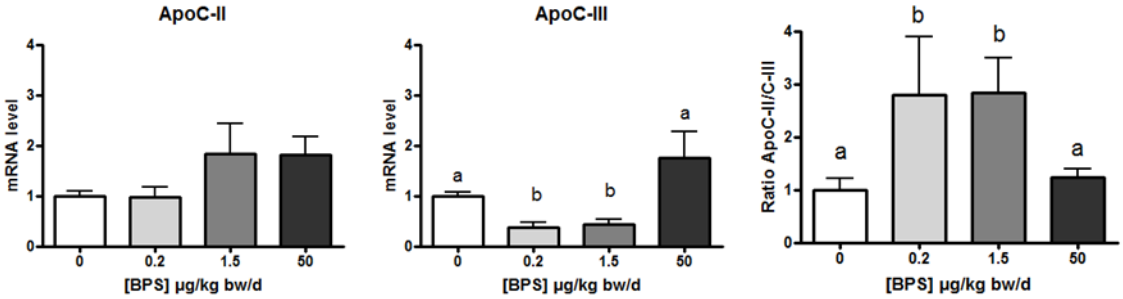



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Figure 6: mRNA expression of ApoC-II and ApoC-III and ApoC-II/III mRNA ratio in jejunal tissue of male mice fed a high fat diet (refer to table 1 for the number of mice by group). The expression of each gene was normalized to control group following $2^{-\Delta\Delta Ct}$ method. Values represent means ± SEM. One-way ANOVA and Tukey post test were performed (a different letter means a significant difference, $p < 0.05$).



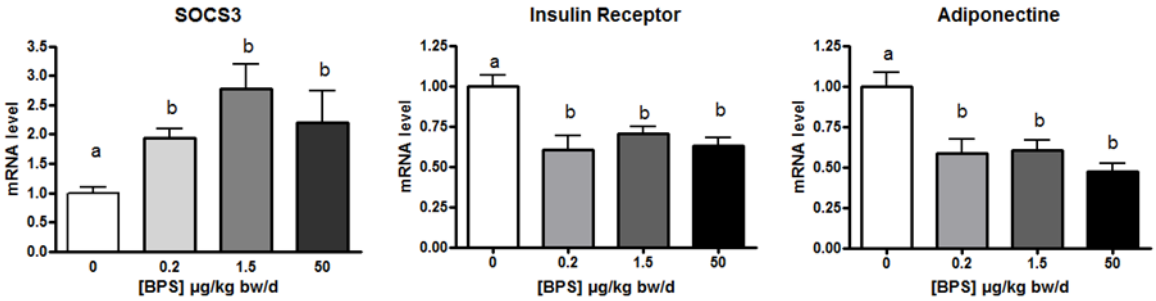



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Figure 7: mRNA expression of SOSC3, INSR and adiponectin in abdominal adipose tissue of male mice fed a high fat diet. The expression of each gene was normalized to control group following $2^{-\Delta\Delta Ct}$ method. Values represent means ± SEM. One-way ANOVA and Tukey post test were performed (a different letter means a significant difference, $p < 0.05$).



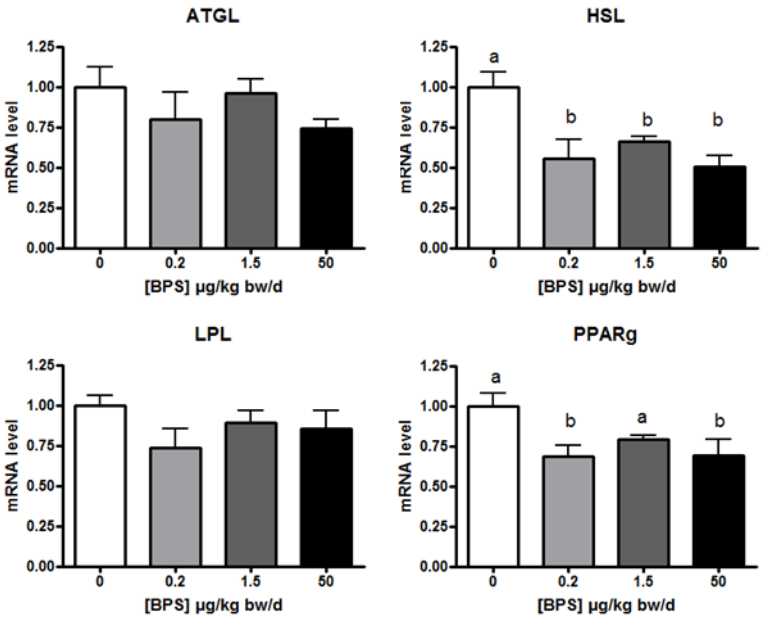

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Figure 8: mRNA expression of LPL, HSL, ATGL and PPARgamma in abdominal adipose tissue of male mice fed a high fat diet (refer to table 1 for the number of mice by group). The expression of each gene was normalized to control group following $2^{-\Delta\Delta Ct}$ method. Values represent means ± SEM. One-way ANOVA and Tukey post test were performed (a different letter means a significant difference, $p < 0.05$).




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Table 1: Number of pregnant mice for each group and repartition of offspring according to gender, diet and treatment.

| | | Standard Diet | | High Fat Diet | |
|---|---|---|---|---|---|
| BPS (µg/kg bw/pc) | Pregnant mice | ♂ | ♀ | ♂ | ♀ |
| 0 | 11 | 16 | 13 | 15 | 15 |
| 0.2 | 10 | 13 | 17 | 13 | 18 |
| 1.5 | 11 | 15 | 15 | 17 | 15 |
| 50 | 13 | 22 | 12 | 20 | 12 |

Table 2: Food intake in male and female mice fed a standard or high fat diet (refer to table 1 for the number of mice by group). Results represent means of food intake (kcal/mice) +/- SEM. One-way ANOVA with Tukey post-test; *p<0.01 in comparison with respective control (high fat versus standard diets).

| | | BPS (µg/kg bw/d) | | | |
|---|---|---|---|---|---|
| | Diet | 0 | 0.2 | 1.5 | 50 |
| ♂ | Standard | 13.34<br>0.54 | 14.13<br>0.98 | 11.69<br>0.31 | 14.09<br>1.09 |
| | High fat | 15.18*<br>0.65 | 15.95*<br>0.63 | 15.24*<br>0.92 | 16.50*<br>1.74 |
| ♀ | Standard | 12.58<br>1.02 | 12.22<br>1.82 | 10.75<br>0.90 | 12.32<br>0.45 |
| | High fat | 14.27*<br>1.22 | 15.30*<br>0.84 | 15.33*<br>0.71 | 15.00*<br>0.37 |



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